Manmohan Singh Chauhan
Naresh Selokar *Editors*

Biotechnological Applications in Buffalo Research

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Preface

Buffalo (*Bubalus bubalis*) is a valuable farm animal species providing livelihood to millions of people in many Asian, European, African, and Latin American countries. There are two types of buffalo, (1) river buffalo firstly domesticated in the Indian subcontinent and (2) swamp buffalo domesticated in the China-India border. Since domestication (about 6300 years ago), buffaloes have been employed to produce milk, meat, and leather to satisfy human demand. Over the years, selective breeding has led to the development of commercial buffalo breeds such as Murrah (India's river breed), Nili-Ravi (Pakistan's river breed), Mediterranean buffalo (found in Italy, Bulgaria, Romania, Greece, Turkey), Shanghai (China's swamp breed), and Toraja Spotted (Indonesia's swamp breed). In tropical countries, mainly India, China, and Pakistan, the buffalo is an animal of choice for the milk and meat industry. For example, India's white (milk) and pink (meat) revolution cannot be envisioned without the contribution of buffalo. Another notable example, in the European Union, the celebrated Mozzarella cheese is prepared from buffalo milk. At present, 233 million buffaloes are housed in 45 countries across all farm animal production systems. The world's human population is 7.9 billion, which is expected to reach 9.8 billion in 2050. Thus, buffalo production systems are under an enormous burden to provide nutritional food and to secure livelihoods to millions of people. Modern biotechnologies provide unique opportunities to study domestication, physiology, and genomics for augmenting the production and health of buffalo species.

Despite enormous contributions, buffalo receives little importance as compared to cattle and pigs. A major bottleneck for the growth of the buffalo industry is a lack of scientific information and knowledge. Therefore, we thought to bring a book that has sufficient scientific information for improving the production and health of buffalo worldwide. In the last several years, biotechnological applications have been utilized to improve production (milk and meat) and health (disease resistance) of farm animals by using selective breeding plans, semen cryopreservation and artificial insemination, genomic selection, assisted reproductive technologies, manipulation of reproduction and nutrition, and artificial intelligence-based husbandry practices. We believe that the wider application of biotechnological tools could ensure the productivity, nutritional quality, safety of food products, and welfare of the buffalo.

This book presents the global status of buffalo, well-established biotechnological methods in the buffalo industry, as well as advanced biotechniques. This book is extremely valuable because each topic is written by renowned authors, who contributed significantly in their field. This book is not only intended for learning about the buffalo species and acquiring knowledge, but also for envisioning the prospect for a vibrant buffalo industry. Chapters related to recent developments such as advances in artificial breedings, genomic applications, semen cryopreservation, biomarker discovery, animal cloning, semen sexing, and stem cell biology are well covered in this book. We hope this book will be a useful guide to students, academicians, researchers, and policymakers who are interested or engaged in buffalo science and industry.

We want to express our sincere gratitude to all authors who contributed outstanding chapters to this book. We would also like to express our appreciation to Dr. Bhavik Sawhney of Springer's editorial team and Mr. Suraj Kumar of the publishing team for their constructive support and extraordinary patience from the beginning to the end.

We are truly honored to bring this book to you.

Karnal, Haryana, India

Manmohan Singh Chauhan
Naresh Selokar

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About the Editors

Manmohan Singh Chauhan is presently a Director and Vice-chancellor of the ICAR-National Dairy Research Institute (NDRI), Karnal, India. Previously, he was the Director of ICAR-Central Institute for Research on Goats (CIRG), Makhdum, India. His research group mainly focuses on reproductive techniques such as in vitro fertilization (IVF) of embryos, ovum pick-up (OPU), embryonic stem cells, animal cloning, and transgenesis in livestock, such as buffalo, goats, cattle, and yaks. He published around 150 peer-reviewed research articles and authored 5 books. Dr. Chauhan is a distinguished fellow of Indian National Science Academy (INSA), National Academy of Sciences (NASI), India's National Academy of Agricultural Sciences (NAAS) and the National Academy of Dairy Sciences (NADS). He received several research awards and recognitions which include Rafi Ahmed Kidwai award and research team award from the Indian Council of Agricultural Research, Labhaswetar award from Indian Society for the Study of Reproduction & Fertility, and certificate of appreciation from Hon'ble Minister of Agriculture, Govt. of India, for achieving success in buffalo cloning in India.

Naresh Selokar is presently working as a scientist at the ICAR-National Dairy Research Institute (NDRI), Karnal, India. Previously, he was a scientist at the ICAR-Central Institute for Research on Buffaloes (CIRB), Hisar, India. He contributed significantly to establish a simplified, economical, and efficient buffalo cloning technique called handmade cloning, which is being used to produce cloned and transgenic/edited buffaloes in India. He was instrumental in establishing

a somatic cell biobank of elite buffaloes. He also works on other assisted reproductive techniques such as in vitro embryo production (IVF), embryonic, adult, and induced pluripotent stem cells, cryopreservation of somatic cells, oocytes and embryos, artificial insemination, and embryo transfer. His current research interests are focused on CRISPR-based genome editing in buffalo.

Dr. Selokar authored more than 50 research articles in peer-reviewed journals. He received several awards, including the Associate fellowship-2020 (National Academy of Agricultural Sciences, India), Young Scientist Platinum Jubilee Awards-2019 (The National Academy of Sciences, India), Innovative Young Biotechnologist Award-2018 (Department of Biotechnology, India), Young Scientist Award-2015 (Science and Engineering Research Board, India), and Jawaharlal Nehru award for outstanding doctoral thesis research in agricultural and allied sciences-2015 (Indian Council of Agricultural Research).

Part I

Buffalo, A Black Beauty in Livestock Farming

Buffalo in the World: Situation and Perspectives

1

Antonio Borghese, Antonella Chiariotti, and Vittoria Lucia Barile

Abstract

The increasing of world population, which is expected to reach more than nine billion people in 2050, urbanization, and the growing concern about the environmental impact of animal farming, need a long-term global strategy for more sustainable ruminant production. Human requirements of high nutritional value proteins could be reached by milk and meat product coming from buffalo (*Bubalus bubalis*). Thus, this species, represented by more than 204 million head in the world, plays a strategical role for economy and society. The main features for buffalo being so utilized are its high capacity to convert fiber into energy and to adapt in areas with hot-humid climate where other ruminants cannot thrive. Moreover, it contributes to the sustenance of populations living in rural areas. The future of livestock production systems is closely related to sustainability, welfare, and quality of products. The application of new technologies will play a pivotal role in animal management, also for the buffalo. Genetic selection will have to focus on animal health and welfare improvement, increase tolerance to stressors, reduce livestock environmental impact and produce milk and meat of higher nutritional quality both selecting cosmopolitan breeds, and valorizing local breeds and their genes. Local breeds, the most adapted to low-input production systems and to a range of environments, can produce food with unique properties and can preserve cultural and social values.

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In this chapter, buffalo livestock systems and world perspectives for its exploitation will be examined.

Keywords

Buffalo · Breeds · Meat · Milk · Perspectives · Management · Market

1.1 Buffalo: A Tool for Sustainability?

The increasing of world population, which is expected to reach more than nine billion people in 2050 (UN 2015), the expected increase in both per capita income and prevalence of animal products in human diets (Bruinsma 2003; FAO 2011), urbanization, and the growing concern over the environmental impact of animal farming, need a long-term global strategy for more intensive and sustainable ruminant production (Pulina et al. 2017). It is estimated that more than 50% of the world's population now lives in cities and large towns classified as urban and is expected to rise to 68% by 2050 (UN 2018). Population density in the developing countries can be directly correlated to the dependence of farmers on livestock for their livelihood for more than a billion people in different parts of the world, although livestock husbandry is a commercial activity with fairly high capital investment. The unique feature of livestock is its easy mobility and ability to withstand the changing weather conditions, while generating year-round employment. However, most of the small farmers are scattered in remote villages, deprived of technical services, market connectivity and poor market development, and experiencing reduced income. This is due to low productivity, shortage of fodder and feed resources, outbreak of various diseases, which need to be addressed on priority. In such a situation, livestock often turns into a liability, instead of contributing to the economy.

Ruminant livestock is important to produce high-quantity and -quality animal protein (milk and meat) in human diets as well as for providing hides, fiber, manure, and animal power for farming and transportation in many countries. To produce which, they also use fibrous feeds that cannot be consumed by humans. Among ruminants, buffaloes utilize low-quality feed stuffs, which contain high levels of fiber and low levels of fermentable carbohydrates, more efficiently than cows. As such, there is increasing interest in this more sustainable ruminant species for livestock production. This is evidenced by buffalo milk production increase in worldwide production of 19.4% compared to 8.5%, and meat production increase of 6.7% compared to 5.7% of cattle, during 2014–2019 period (FAO. FAOSTAT 2019). In order to decrease the impact of both buffalo and cattle production on the environment, new approaches are also needed to improve the sustainability of feed production. In addition to meet the growing demand for animal products, animal breeding will have to ensure the economic sustainability and social acceptance of ruminant farming.

Genetic selection will have to redirect breeding objectives to improve animal health and welfare, increase tolerance to stressors, reduce livestock environmental

impact, and produce milk and meat having improved nutritional and functional properties. Two strategies could be adopted: (1) selection in cosmopolitan breeds, by integrating traditional and new breeding goals; and (2) exploitation of local breeds and their genes. Local breeds, adapted to a variety of environments, are able to produce food with unique properties and can preserve cultural and social values. Local breeds also hold a reservoir of valuable genes that, once discovered, may be reintroduced in cosmopolitan breeds by traditional or innovative methods (Pulina et al. 2017).

1.2 Buffalo Population Trend

In the world, there are 204 million head with an increase of 5.06% in the last 10 years (Table 1.1, FAO. FAOSTAT 2019). In Asia is concentrated 97.08% world's buffalo population, mainly in India and Pakistan, due to dairy breeds selection and the enhancement of milk market, partially balanced by other countries decrease due to reduction of draft animals.

In **Africa**, buffalo can be found only in Egypt, 1.71% of world entire population (FAO. FAOSTAT 2019). Egyptian buffalo is not only considered an important genetic resource with a great potential for meat and milk production, but also a main dairy animal for small Egyptian farmers (Eid 2019).

In **Europe,** buffalo represents 0.22% on world population, the majority of which is in Italy. There is a decreasing trend in many Balkan countries as Romania, Bulgaria, Macedonia, Greece, Albania, and Serbia, partially balanced by the increasing trend in Italy, due to the increase of *mozzarella* and other cheese products market.

In the **Americas** according to Minervino et al. (2020) there are 2.5 million buffaloes (1.23% of world population) and in Brazil 1.4 million head. Buffalo has good perspectives of development in South America, due either to grassland and free pasture' availableness, or to thriving capacity in lagoons and marshland, or to the breed transition from meat to dual attitude (milk and meat) livestock.

In **Oceania** (0.07% of world population), buffalo were imported from Asia, to be bred in extensive system. According to Minervino et al. (2020), the 96.5% of the population is in Australia.

The increasing trend in Asia, Italy, and America is basically due to the growing number of dairy purpose animals, related to the world markets high demand of milk, cheese, and processed products. Nowadays both Governments and people understand that milk and meat are essential for human subsistence, above all for covering children's high biological value protein nutritional requirements.

Table 1.1 Changes in world buffalo population during the last decade (FAOSTAT 2019)

Continents	2010 head	2019 head	Variation %
Africa	3,818,261	3,506,086	−8.90
Egypt	3,818,236	3,506,061	−8.90
Mauritius	25	25	
Americas	1,191,132	1,985,388	+40.01
Brazil	1,184,511	1,434,141	+17.41
Colombia		544,238	
Suriname	721	891	+19.08
Trinidad and Tobago	5900	6118	+3.56
Asia	188,634,118	198,414,255	+4.93
Armenia	455	670	+32.09
Azerbaijan	277,400	162,475	−70.73
Bangladesh	1,349,000	1,490,000	+9.46
Bhutan	928	477	−94.55
Brunei Darussalam	4124	2292	−79.93
Cambodia	702,074	605,638	−15.92
China	29,464,034	27,338,428	−7.78
Georgia	17,000	18,320	+7.21
India	107,375,000	109,851,678	+2.25
Indonesia	1,999,604	1,141,298	−75.20
Iran (Islamic Republic of)	195,000	72,434	−169.21
Iraq	295,000	225,058	−31.08
Jordan	100	98	−2.04
Kazakhstan	10,000	10,507	+4.83
Laos People's Democratic Republic	1,189,000	1,209,712	+1.71
Malaysia	129,878	107,347	−20.99
Myanmar	2,977,150	4,082,914	+27.08
Nepal	4,836,984	5,308,664	+8.89
Pakistan	29,413,000	40,002,000	+26.47
Philippines	3,270,400	2,873,561	−13.81
Sri Lanka	422,650	298,430	−41.62
Syrian Arab Republic	7000	7146	+2.04
Tajikistan	15,000	15,390	+2.53
Thailand	1,622,646	897,368	−80.82
Timor-Leste	96,484	126,066	+23.47
Turkey	87,207	178,397	+51.12
Viet Nam	2,877,000	2,387,887	−20.48
Europe	390,682	466,175	+16.19
Albania	120	107	−12.15
Bulgaria	8311	16,730	+50.32
Germany	4200	10,110	+58.46
Greece	1785	4000	+55.38
Hungary		6000	
Italy	365,086	402,290	+9.25

(continued)

Table 1.1 (continued)

Continents	2010 head	2019 head	Variation %
North Macedonia	640	633	−1.11
Romania		19,000	
Russian Federation	10,540	6335	−66.38
Spain		970	
Oceania	150	180	
Micronesia (Federated States of)	150	180	+20.00
World	194,034,343	204,372,084	+5.06

1.3 Buffalo in Central Asia

1.3.1 India

Buffalo livestock in India satisfy the nutritional requirement of animal protein for millions of people. Besides milk and meat, buffalo supplies leather, bones, pharmaceuticals, dung and manure and draft energy power. Buffalo population is 109.8 million almost 53.75% of the total world buffalo and produces 68.78% of the world buffalo milk (92.00 million tons) (FAO. FAOSTAT 2019). India is highly advanced in buffalo scientific and technological development of nutrition, management, reproduction, biotechnologies, and genetic improvement. The country holds 13 recognized breeds, plus 20 different populations of River and Swamp types; in particular, the best Asian River milk breeds such as Nili-Ravi (Fig. 1.1a), Surti, Jaffarabadi, and Murrah (Fig. 1.1b), the most widespread breed in the world. All these breeds originated from North-Western states, have high milk and fat production potential, besides being used for work and surplus stock for meat production. Indigenous animals have high biodiversity and high disease resistance and tolerance to hot and humid conditions. As reported by the Buffalo Research Institute in Hisar, the milk production for the principal breeds in 305 days of lactation length has been as follows: Murrah 2075 ± 54 kg, Nili-Ravi 1929 ± 68 kg, Surti 1566 ± 46 kg, Bhadawari 1434 kg, Pandharpuri 1841 kg, Swamp 412 kg (Sethi 2009). Singh (2018) reports on Murrah breeds 2358.7 kg on average with a maximum of 2457 kg. Most of the milk is used for direct consumption after skimming. Fat is used to produce butter, ghee, and cream. In national market as well as for export, dry milk, condensed milk, milk replacers are also commonly used. A typical buffalo cheese is *Paneer*, used for various vegetarian curry dishes both in India and in other Asian countries.

Buffalo meat is comparatively a new area of production, and it is available where other kinds of meat (bovine and pork) are not consumed because of religious reasons. India produces 1.61 million tons of buffalo meat, which represent 37.69% of the world production (FAO. FAOSTAT 2019). Although India is a dairy country, it has a high export potential for buffalo meat. Buffalo has also efficient work capacity especially for load pulling and plowing in land cultivation and its milk

Fig. 1.1 Asian River Buffalo. (**a**) Nili Ravi champion in Hisar, India (Chiariotti photo, 2018). (**b**) Murrah buffalo in intensive system at ICAR, Hisar, India (Chiariotti photo, 2018). (**c**) Nepalese family buffaloes in the village near Chitwan, Nepal (Moioli photo, 2017). (**d**) Mesopotamian buffalo in Iraqi lagoons (Borghese photo, 2019)

yield is not affected by 1–2 h of continuously moderate work (Borghese and Perrone 2019). Buffalo Research Institute in Hisar carries out many important national projects on buffalo development, while the National Dairy Research Institute in Karnal studies milk processing.

1.3.2 Pakistan

Pakistan is the second country in the world after India, with a population of 40 million head (FAO. FAOSTAT 2019), showing a 26.47% of increase in the last decade. The most common breeds are River type Kundi and Nili-Ravi. Total milk production is 54.97 million tons and 34.37 million of which (62.52%) comes from buffalo (FAO. FAOSTAT 2019), therefore buffalo role both in milk production system and food availability in Pakistan is pivotal. The demand for milk and milk products are constantly growing, due to the increasing urbanization. The milk is used for direct consumption after skimming. The fat is a richness per se and it is used to produce butter, ghee for cooking, cream, sweets. The annual buffalo meat production in Pakistan is 1.08 million tons, contributing to 25.29% of world buffalo meat

(FAO. FAOSTAT 2019). Actual rearing systems are traditional, and the meat comes from young males and cull cows. The skin is used in leather industry and the manure is used as fuel as well as fertilizer in the rural villages. Buffalo research and development are well organized. There is the Buffalo Research Institute in Pattoki, Punjab, established in October 2005. Several research are supported by UVAS (University of Veterinary and Animal Sciences) in Lahore and by the Livestock Production Research Institute in Bahadurnagar, Okara. The Semen Production Unit in Qadirabad, Sahiwal, deals with progeny testing, semen collection, and Artificial Insemination (AI).

1.3.3 China

China has 27.33 million buffaloes, the third largest population in the world (13.37%) (FAO. FAOSTAT 2019). Chinese buffalo belongs to the Swamp type, with a total of 18 local breeds. They are very strong animals mainly used for draft in the marshlands, particularly in rice fields. They are also a source of meat, but their milk production is very low (500–700 kg milk yield for lactation on average). Murrah buffalo arrived in China from India in the late 1950s, while Nili-Ravi arrived from Pakistan 20 years later. The buffalo food products availability in China increased for the widespread application of crossbreeding of River breeds with high genetic value (Italian Mediterranean, Nili-Ravi, Murrah) on local Swamp breeds, that markedly improved the milk performances in the crossbreds (Bingzuang et al. 2003). The buffalo milk production is 2.92 million tons (FAO. FAOSTAT 2019). There are few small-scale buffalo milk processing factories, distributed mostly in Guangxi Province and produce pasteurized milk, yogurt, condensed milk, milk cake, milk drink, milk bean curd and cream. Buffalo meat production reaches 0.66 million tons (FAO. FAOSTAT 2019). Meat is sold directly to consumers for the most part, the rest is processed as dried beef, sausages, and hams (Bingzuang et al. 2010). Buffalo hide production is 0.19 million tons (FAO. FAOSTAT 2019), transformed into various marketed products. Guangxi Buffalo Research Institute is the only research institute dedicated to buffalo in China and deals with technical training, technological development, management, and promotion of buffalo breed.

1.3.4 Nepal

The buffalo population in Nepal increased from 4.8 million head in 2010 to 5.3 in 2019 (FAO. FAOSTAT 2019). Among livestock species, buffalo is the main provider of animal source food to Nepalese people, with 65.2% of the total milk and 54.3% of the meat, becoming the principal source of income and nutrition (Fig. 1.1c). The indigenous buffalo River breeds in Nepal are: Terai, Gaddi, Lime, and Parkote (Devkota 2018). Lime and Parkote are living on the hills and mountains areas of the country, producing about 900 kg milk yield per lactation. Nowadays

these purebreds are going to be reduced, as crossbreeding with Murrah is applied to increase milk yield, reaching 1500 kg per lactation (Nirmal et al. 2018). Nevertheless, the Nepal Government implemented a long-term Action Plan for Animal Genetic Resources (2011–2021), aiming at the conservation of native breeds. Similarly, Buffalo Genetic Improvement Programs, AI, nutrition programs, and male fattening for meat in many districts have been implemented, to achieve the national target of 91 kg milk and 14 kg meat pro capita per year (Devkota 2018).

1.3.5 Bangladesh

In Bangladesh, the total buffalo population is 1.49 million head (FAO. FAOSTAT 2019). They are managed in household subsistence farming and extensive Bathan farming in saline coastal region. They are used as a draft animal and partially for milk and meat production (Hamid et al. 2017). The buffalo of Bangladesh are of indigenous origin for the most part and their productivity is low. Karyotyping and phylogenetic studies were carried out on 18 indigenous populations from 1984 to 2016 to characterize the type (River or Swamp) (Faruque et al. 2018). The 45% of total population belongs to the River type, Bangladeshi breed. Average milk yield is about 620 kg in a lactation period of 270 days (Borghese 2013a). The management system is semi-intensive.

To increase milk production and create a new market for buffalo dairy products, Ministry of Agriculture of Bangladesh and LAL TEER Livestock Limited founded in Jamuna, a new semi-intensive farm where a crossbreeding program introducing semen of high genetic value of Italian Mediterranean breed was started since 2011 (Borghese 2013a).

1.3.6 Sri Lanka

The buffalo population in Sri Lanka is stable, around 298,430 heads (FAO. FAOSTAT 2019). Buffalo is not a primary livestock, as only few farmers raise buffaloes purely for milk. The 70% of buffalo is bred in the dry intermediate area of the country, in extensive system and used for draft, obtaining low milk yield. The native buffalo of Sri Lanka, although phenotypically similar to the Swamp type, has 50 chromosomes and cluster strongly with River type populations (Barker et al. 1997). The local Sri Lanka breeds produce 1–2 L of milk per day in a short lactation period of 3–5 months, due also to the poor quality of fodder utilized in the diet. Intensive systems are going to be applied in peri-urban areas, where the demand for milk and dairy products is higher (Thakshala 2018). The perspectives of buffalo breeding in Sri Lanka are to obtain a multipurpose animal in rural farming systems and to improve food security (Thakshala 2018). The Government has started a vigorous program to attain self-sufficiency in milk, based on the use of semi-intensive systems with crossbreds or pure breeds as Murrah, Nili-Ravi, and Surti,

improving housing, feeding, health care, reproduction, management, recycling of wastes (Perera 2019).

1.4 Buffalo in Far East of Asia

1.4.1 Myanmar

Livestock plays a key role in the Myanmar agriculture sector. Main crop being rice, cattle and buffaloes are used for draft purpose (soil preparation, threshing, and transport) since ancient time, but livestock farming for meat production has been developed since no more than 50 years. In Myanmar, there were 2.97 million buffaloes in 2010 showing a 27.08% increase; they reached 4.08 million in 2019 (FAO. FAOSTAT 2019). Buffalo are concentrated in the delta and coastal areas, as well as some upland areas. Buffalo belong to the swamp type with no breed subdivisions, although it is reported that buffalo in upland areas are generally larger in size than those in lowland areas. They have more working capacity than oxen and are well suited to work in low-lying swampy areas. There is also a small number of Murrah breed and crossbred buffaloes in the country (FAO 2003). Meat production was 489,584 tons and buffalo meat is 11.07% of the total in 2019 (FAO. FAOSTAT 2019) although there is no differentiation between cattle and buffalo meat. Meat is retailed freshly slaughtered and sold at local markets. Smallholder animal health services are poor and reliable data on the extent of livestock mortality and morbidity are lacking.

1.4.2 Philippines

In the Philippines there were 3.27 million buffaloes in 2010, with a 13.81% of decrease in 10 years reached 2.87 million in 2019 (FAO. FAOSTAT 2019). Ninety-nine percent are owned by small farmers who have limited resources, low income, and little access to other economic opportunities. The buffalo breed, known as Carabao, is a swamp type (Fig. 1.2a) which has played a major role in small-hold land-based agriculture as draft animal, to produce primary agricultural crops, such as rice, corn, sugar cane, and coconut. To improve the local Swamp buffalo for meat, milk, and draft potentiality, the Carabao Development Program was begun in 1993. An elite herd of Riverine buffalo has been established at the Philippine Carabao Center, Science City of Muňoz, importing about 3000 Murrah buffaloes with pedigree performance records from Bulgaria. An increase in milk yield per lactation was obtained from F1 and F2 crossbred (1100 and 1350 kg, respectively) compared to the parental breed: Bulgarian Murrah (1800 kg) and local Swamp population (400 kg) (Cruz 2006). In 2018 was released the first Philippine dairy buffalo (F4), with the perspective to achieve at least 10% sufficiency in domestic milk production by 2022. Moreover, they aimed at developing markets for Halal carabao meat

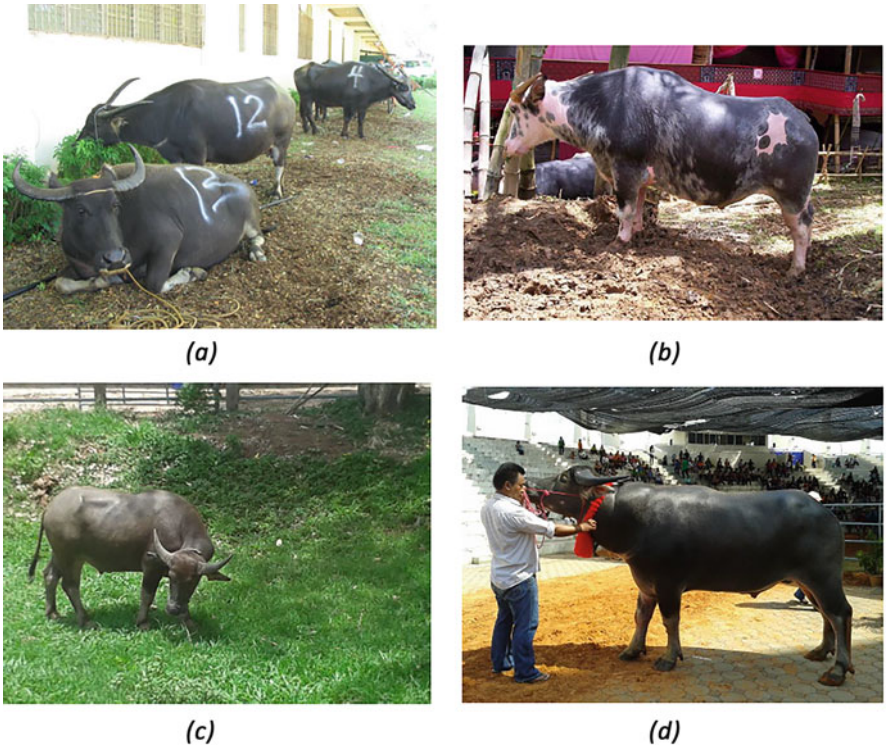

Fig. 1.2 Asian Swamp buffalo. (**a**) Carabao buffaloes at the Carabao Festival, Muñoz, Philippines (Barile photo, 2004). (**b**) Spotted buffalo, Tana Toraja, Indonesia (Borghese photo, 2008). (**c**) Thai buffalo in Buriram village, Thailand (Barile photo, 2013). (**d**) Thai buffalo bull champion, SUT competition, Thailand (Barile photo, 2013)

products, engaging more private entrepreneurs, commercializing carabao-based products (Palacpac and Aquino 2018).

1.4.3 Vietnam

In Vietnam, the buffalo population was 2.87 million in 2010 reduced to 2.38 million head in 2019 (FAO. FAOSTAT 2019). The population is almost exclusive Swamp, used mainly for draft power and plays an important role in Vietnam's rural economy. North Vietnam is hilly or mountainous and plots size are quite small, so it is difficult to apply mechanization due to orography, this is the reason why more than 52% of Swamp population is distributed there. Traditional farming practices are based on extensive systems that include daytime grazing on natural pastures, generally supplemented with rice straw and other crop residues during the dry season (Do Kim Tuyen 2009). Buffalo also provide meat which is preferred to beef by Vietnamese people. Vietnam has a relatively short tradition both in milk production

and consumption of dairy products. The actual milk production is 27,211 tons (FAO. FAOSTAT 2019). Since the end of 1990s crossbreeding program started introducing Murrah breed to increase milk production with promising results (van Thu 2000; Van Sanh 2004).

1.4.4 Laos

In Laos we find 1.21 million head (FAO. FAOSTAT 2019), the majority are of Swamp type, used for draft in rice fields as family animal, similarly to Thailand, Vietnam, and Malaysia. Milk and meat products are of small quantity and used in family and local economy. In the last few years, a private project in Luang Prabang has been developed to increase milk production and encourage the production of buffalo dairy products both for local consumption and for tourism. This breeding program is developed with the Laos Agricultural Institute and foreseen the cooperation with people from villages in and around Luang Prabang. The program aims to increase milk yield, ensure better calf survival rates and enhance the farmers income, crossbreeding local Swamp buffalo with the Indian Murrah.

1.4.5 Indonesia

Indonesia has a buffalo population of about 1.14 million head; there has been a declining trend since 2010 when buffalo were 1.99 million (FAO. FAOSTAT 2019). This decline is related to the reduction in the number of farmers (15.5%) between 2010 and 2017 (Firman et al. 2018). In the different isles of Indonesia, there are various Swamp breeds differing in size, weight, color, skin marking, and horn dimensions. Most of rural buffaloes in the villages produce less than 1000 kg of milk per lactation. Swamp buffalo are used for draft power in most areas and for meat in the Java lowland areas and the Sumatra uplands. Spotted buffaloes (Fig. 1.2b) are extremely valued, particularly in Tana Toraja, Sulawesi, to be sacrificed and consumed on special occasions such as marriages and obsequies ceremonies for their sacral value, so the selection to obtain spotted animals is highly profitable (Borghese 2013a).

Generally, milk is not widely marketed and it is only processed traditionally. Indonesia produces 3 types of traditional dairy foods, namely the butter-like product *minyaksamin*; yogurt-like product *dadih*; and cheese-like products *dali* or *bagotnihorbo*, *dangke*, *litsusu*, and *cologant* (Surono 2015). The *dadiah* or *dadih* is a typical product in Sumatra: the milk is maintained in bamboo-cane for 2 days where undergo a natural fermentation process. *Dadih* is rich of probiotics, as kefir or yogurt, but more similar to a fresh cheese cream, rich in fat and protein (Borghese and Sokidin 2008).

1.4.6 Thailand

Thai buffaloes are genetically of the Swamp type and drastically declined from 1.62 million in 2010 to 0.90 million in 2019 (FAO. FAOSTAT 2019). The growing rate of Swamp buffalo population of Thailand has been affected by various factors, such as no policy for buffalo development, local economic development, mechanization, and fast urbanization. Traditionally, buffaloes were raised by small farm holders as multipurpose animal in agriculture production: draft power, manure as fertilizer for crop fields and as saving bank. The local swamp buffalo (Fig. 1.2c, d) have low meat and milk production, so these productions were not the main focus for these small farmers. Local buffalo are adapted to poor feeding management as well as to the hot-humid tropical climate conditions. Buffalo rearing is closely integrated with paddy rice cultivation and it is important for Thailand's agricultural production (Sanghuayphrai 2011). The Buriram Livestock Research and Testing Station of the Department of Livestock Development of Ministry for Agricultural and Cooperatives are involved in developing dairy buffalo breeding in Thailand.

1.4.7 Malaysia

The buffalo population in Malaysia was 129,878 head in 2010 and 107,343 in 2019 (FAO. FAOSTAT 2019). The indigenous population is prevalently Swamp buffalo or Kerbau Sawah used for plowing, harrowing, and working in rice fields. At the end of working life, the Swamp buffalo is slaughtered for consumption, producing meat of low quality. Better meat quality is obtained by fattening buffalo calves, fed local feedstuffs, such as oil palm and rice by-products. The Malaysian occasionally milk their swamp buffaloes and the milk is used to produce *dadih*. Today the buffalo has lost its prominence due mainly to farm mechanization and urbanization. New genetic material in the form of live river buffaloes were introduced by The Department of Veterinary Services (DVS) importing 150 Murrah buffaloes from India in 2010 and 170 Nili-Ravi buffaloes from Pakistan in 2011.

1.5 Buffalo in Near East of Asia

1.5.1 Iran

In the 1930s, there were 1.5 million buffaloes in Iran; currently, this number is decreased to 72,434 head (FAO. FAOSTAT 2019). Some of the main reasons for this decline are industrialization, the increasing demand for buffalo meat but a lack of replacement of the slaughtered animals and farming diversification income together with pro-Holstein propaganda (Mohsen pour Azary et al. 2004). There are two principal breeds in Iran, Khuzestani (22%) and Azeri (70%) and an 8% of Mazandarani breed. Khuzestani, living in the south of the country (Khuzestan), produce 2107 kg milk with 6.23% fat/lactation; East Azeri 1711 kg (7.50% fat)

and West Azeri 1141 (8.12% fat), living in the north and north-west of the country (Azerbaijan Province); Mazandarani 1410 kg (6.75% fat) with an average lactation period of 210 days (Naderfard et al. 2009). The buffalo farming system is based on small holders (99%). In Khuzestan, buffaloes are raised outdoors throughout the year, living free in courtyard and are milked together with calves; in the north-west (Azerbaijan), living also in the marshes, they are housed in the autumn and winter. Buffalo living in the lagoons villages and fed water plants are milked twice a day producing 8 kg milk/day (Borghese 2013a). The main dairy buffalo products are yogurt, fresh cream, fresh cheese, butter, ice-cream, rice pudding, churned yogurt, dried whey, and ghee. In Iran, buffalo milk has a high value, and the price is double compared to cows' milk. Meat production was 4725 tons and milk production was 128,000 tons in 2019 (FAO. FAOSTAT 2019). Other products are hide used in the leather industry and manure used as a fuel in rural areas.

1.5.2 Iraq

According to data provided by Al-Fartosi and Al-Saedy (2019) in 2008, the Iraqi population was estimated 285,537 buffalo heads with the highest percentage in the Basra province (20%). The shortage of water in marshland, the increasing salinity of water, the appearance of some diseases, and the migration of buffalo breeders to other areas reduced the number to 225,058 in 2019 (FAO. FAOSTAT 2019). The breed is River Khuzestani named also Mesopotamian. Buffaloes are reared in the marshes and in the Tigris and Euphrates rivers, swim far for feed, eating papyrus, reeds, and other plants (Fig. 1.1d). They like to stay in the mud, to maintain humid and protected skin. When the flood water is high is the owner who collects plants to feed buffaloes on platforms. In other parts of the country, they are feed concentrates, green forages, straw, and agricultural by-products. Women play the pivotal role in the production, processing, and marketing of dairy products. Fat skimmed from milk is used to produce butter, ghee, sweet, and cake; skimmed milk is used for direct consumption and to produce thick cream named *gaymer*, curd, fresh cheese, yogurt, and sweet. The meat is used for direct consumption or to produce salami and ham, as these products cannot come from pork forbidden by Islamic religion. Recently, a National Plan for development of Mesopotamian Buffalo in Central Marshes, south of Iraq was prepared with the support of the Ministries of Agriculture and Ministry of Water Resources and the International Buffalo Federation. The program has the following purposes: creation of the Official Buffalo Book of Mesopotamian Breed, extension of Veterinary Service, correct animal management and feeding, applying of AI, and genetic improvement (Al-Fartosi 2019, personal communication).

1.5.3 Azerbaijan

The number of buffalo in Azerbaijan is 162,465 in 2019 represented by Azeri breed. In the last decade, buffalo population has decreased by nearly 70.73% (FAO.

FAOSTAT 2019). Buffalo milk accounts for 9% of the total milk production, with an average milk yield of 1200 kg in 210 days of lactation and milk fat of 6.6% in average (Borghese 2013a). Most of the population of Azerbaijan lives in rural areas, where 90% of dairy products is sold in local farmers' market. The system of milk collection and the retail sector for sustainable dairy development need to be improved. Nowadays, in the capital Baku is produced *mozzarella* cheese and served in five stars hotels restaurants together with the red sturgeon caviar as a luxurious food for tourists. This is an example of a local rich market, one of the strategies to improve buffalo industry.

1.5.4 Other Near East Asian Countries

In **Syria** the buffalo population is 7146 head (FAO. FAOSTAT 2019) but now no information is available, nor is food industry possible because of the war. There are two types of Syrian buffalo: Ghab and Qamishly, belonging to Mediterranean breed. Buffalo cows produce 1190 kg milk yield in 254 days of lactation (Borghese 2005). The Center for Buffalo Research and Development (CBRD) was established in 1998 to preserve a natural Syrian heritage with the participation of provincial Directorates of Agriculture (Swaid 1998). In **Armenia** after the collapse of USSR, all livestock, including buffaloes, became totally privatized. After privatization no precise livestock recording has been undertaken and the data presently available is merely indicative. Scientific research has been conducted in the past, but no research or selection has been carried out recently. There are 670 head in Armenia, according to FAO. FAOSTAT (2019). There is an urgent need for a preservation plan of Armenian buffalo.

1.6 Buffalo in Mediterranean Area

In this section are included the countries of Europe, near east of Asia and north Africa, overlooking Mediterranean Sea with similar climatic conditions and where buffaloes are River Mediterranean with comparable characteristics (Fig. 1.3).

1.6.1 Egypt

Egypt is the only African country with economy based on buffalo farming. The total number of buffaloes in Egypt is 3.5 million head (FAO. FAOSTAT 2019). Egyptian buffalo (Fig. 1.3a) is not only considered as an important genetic resource with a great potential for milk and meat production, but it is also considered as the main dairy animal raised by small Egyptian farmers. Buffalo milk represents 45–50% of the total Egyptian milk (Eid 2019). The Egyptian buffalo milk yield up to 2500 kg in 305 days of lactation (Shafik Basant et al. 2017). As indicated by Sallam et al. (2012), high levels of buffalo production are usually associated with high breeding

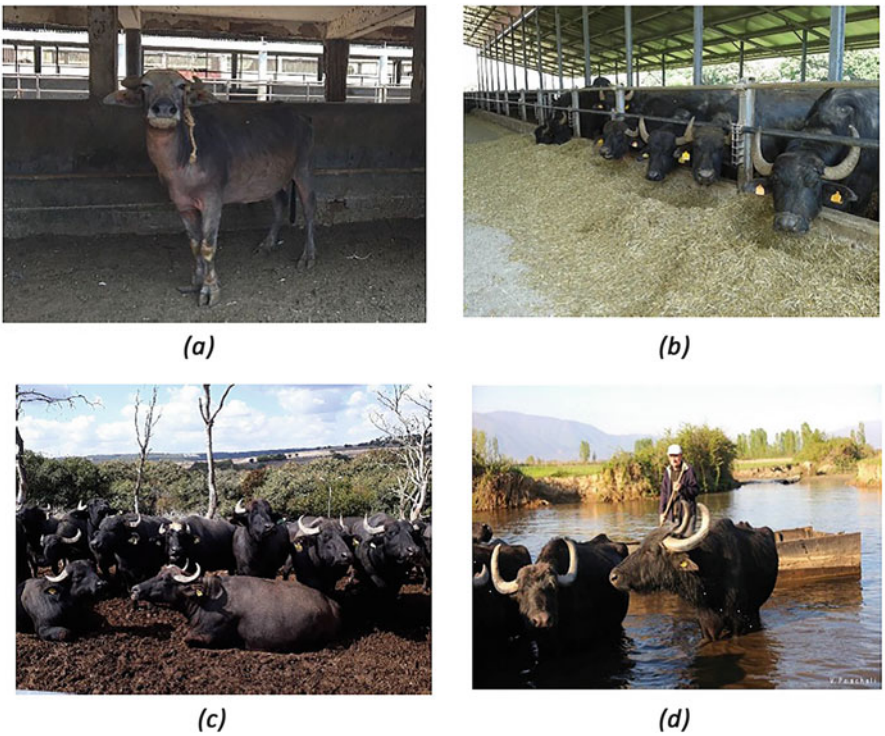

Fig. 1.3 Buffalo in Mediterranean Area. (**a**) Egyptian buffalo, Mehallet Moussa Buffalo Experimental Station, Egypt (Chiariotti photo, 2019). (**b**) Italian Mediterranean at CREA Tormancina Experimental farm, Monterotondo (Barile photo, 2019). (**c**) Anatolian and Mediterranean crossbreds Buffalo in Sarai Mandira farm, Turkey (Barile photo, 2019). (**d**) Kerkini Lake, Water buffalo farming in Greece (Pashali photo, 2017)

efficiency accompanied by good management that maintain body condition score and avoid negative energy balance. Buffalo is also accounted as an important source of meat and it supplies 35–40% of total meat production in Egypt, mainly through culled animals, and male calves (MALR 2017), which showed good performances with average daily gain of 1.22 kg/d and conversion ratio of 7.44 kg DM/kg gain (Eid 2019). Buffalo meat is considered a healthy red meat due to its lower fat and cholesterol and higher iron content.

Most buffaloes are concentrated around the Middle Nile Delta (about 32.2% of total buffalo population), where feed is more abundant and in the Nile valley (22.4%) (Fahim and Abou Hadid 2014). Eid (2019) reported body measurements significantly higher in Delta Region than in Nile Valley. There are also present some commercial farms that have used the crossing with semen of the Italian Mediterranean bulls to increase milk yield and fat and protein content to produce *mozzarella* cheese for the food service and tourist market. The typical cheeses that we found in Egypt are produced with the addition of cow milk: *Domiati, Karish, Mish, Rahss*. These cheeses as well as yogurt, creams, butter, and ghee are common in local

market. There are different research Institutes at the Ministry of Agriculture and at the University in Giza (Cairo) involved in developing project concerning buffaloes and buffalo products. Actually, the Egyptian Association of Buffalo Breeders is working to fight the gap of knowledge in small holders, promoting information, courses, workshop, and publications, as buffalo species is recognized as priority for the development of local economy and of food industry in Egypt.

1.6.2 Italy

Since 1 January 2000, the Ministry of Health has instituted the National Data Bank of the Zootechnical Registry (BDN), which guarantees: the tracking and tracing of animals and their products, the protection of public health and livestock. According to the BDN (2020) in Italy there are 412.889 buffalo head, almost the 90% of the Europe buffalo population. Since its appearance, probably in the ninth century introduced by the Arabs during the conquest of the south of the peninsula, the breed has not been influenced by any other genetic types and has been preserved as a pure breed (Zicarelli 2014) and for this officially registered as *Mediterranea Italiana* [Italian Mediterranean] (Ministry of Agriculture D.M. 201,992, July 5th, 2000). A dairy purpose animal has been selected during the years reaching genetic potential of 5000 kg milk/270 days of lactation. In Italy, where buffaloes are raised in intensive system as dairy cattle (Fig. 1.3b), the buffalo milk is not utilized for fresh consumption, but all the production is processed to make cheese, in particular *mozzarella*. The growing demands for this cheese, both on the national and international market, have favored the increment of buffalo population and milk production, the latter up to 22% in the 2014–2019 period (FAO. FAOSTAT 2019).

Significant milestones for the development and promotion of buffalo breeding in Italy were: establishment of the Herd Book; recognition of Protected Designation of Origin (PDO) of the *mozzarella*; obligation to use only milk from Italian Mediterranean buffalo for the *mozzarella* PDO; regulation for the hygienic and feed management of buffalo breeding aimed at *mozzarella* PDO production. The *mozzarella di Bufala Campana* is the most important PDO brand in Central-Southern Italy (50,677 tons—CLAL 2020) and is the third Italian PDO cheese in terms of market value (426 Mil Euros) and export (Consortium for the Protection of Mozzarella Bufala Campana PDO 2020). The *mozzarella* PDO production increased of 22% in the last 5 years. In addition to the traditional buffalo *mozzarella, ricotta, burrata, caciocavallo,* and other cheeses are also produced.

Since the establishment of the Herd Book, in the 1979, the Italian Mediterranean breed has shown increasing production both in terms of quantity and quality. The average milk yield is 2356 kg per lactation (270 days) with 8.01 fat % and 4.63 protein % (AIA 2019). The Italian Breeders Association (AIA) is currently in charge of milk recording, while the Herd Book and the selection scheme are managed by the Italian Buffalo Breeder Association (ANASB) since 2000. As a result of the new EU REGULATION (2016/1012) on breeding programs, in June 2018 the Italian Ministry of Agriculture recognized the association RISBUFALA as breed society and

approved their breeding programs for the improvement of Italian Mediterranean breed. All activities by AIA, ANASB, and RISBUFALA are performed under the supervision of the Italian Ministry of Agriculture.

The selective tool used until 2018, was the PKM index, aiming at the production in terms of kg *mozzarella* produced in a single lactation, correlating the quantity of milk with fat and proteins content. In December 2018, a new IBMI selection index was developed and applied. Different characteristics are taken into account, such as limbs and feet, mammary system, kg of milk, and percentages of fat and proteins. It should not be underestimated that the parameters of the new index drag other extremely positive ones, such as longevity and better fertility, other fundamental elements in farm economy (ANASB 2020). The animal recording together with the progeny testing performed on young bulls to select sire used in AI breeding programs, paternity DNA test for ascertaining paternity and genetic evaluation for production and type trait, have been important tools to attain the genetic improvement of Italian Mediterranean breed. For this reason, it is one of the most renowned breed and it is widely used across the world in crossbreeding programs.

Buffalo meat is not in great demand by the Italian consumer and it is limited to local consumption in areas where buffalo farming is traditionally present, marketing both fresh meat cuts and delicatessen (salami, *bresaola*, etc.). Live weight at slaughter is 400–440 kg on average, obtained at 15–16 months of age and Average Daily Gain (ADG) of 800–1000 g (Borghese 2013b). The main focal points for the research on buffalo in Italy are (1) the CREA Research Centre for Animal Production and Aquaculture, in Monterotondo, Rome, where was established the General Secretariat of the International Buffalo Federation (IBF) and the FAO Inter-Regional Cooperative Research Network on Buffalo, and (2) the Department of Veterinary Medicine and Animal Productions of University of Naples "Federico II."

1.6.3 Turkey

According to FAOSTAT in 1974 in Turkey there were one million buffalo head reduced to 178,397 head in 2019 (FAO. FAOSTAT 2019), represented by the Anatolian breed. The decrease was due to the preference for cattle over buffalo in the Aegean and Marmara regions, where most of the buffaloes were bred. Moreover, all the genetic improvement efforts were concentrated on cattle. Buffalo milk is renowned and appreciated particularly to produce the famous Turkish desserts. This is one of the main reasons why farmers rear buffaloes near big cities (Fig. 1.3c) (Soysal and Kok 2004). Typical milk buffalo products are: a semi-hard cheese called *peyazpeyneri* and *ayran* a yogurt-based beverage made with yogurt, water, and salt. Meat production is all processed into sausages. The price of buffalo meat is 10% less on average than the price of beef. A crossbreeding program in Ilikpinar village (Hatay), using semen from Italian Mediterranean bulls was carried out some years ago. In F1 crossbred (Mediterranean Italian X Anatolian), milk yield average was 1386 $\pm$ 246 kg for a lactation length of 305 days; while the milk yield for Anatolian pure breed buffaloes was 987 $\pm$ 327 kg (Sekerden 2009a, b). The Anatolian buffalo

improvement program started in 2011, is included in the Turkey Nationwide improvement program, and involves progressively 16 provinces and 27,693 buffaloes, applying selection on pedigreed buffalo population, to achieve 1200 kg milk yield in 250 days of lactation length (Kaplan et al. 2019).

1.6.4 Greece

In Greece the number of buffaloes has declined dramatically over the last decades, there were around 75,000 animals in the country during the 1960s but then developments at the socioeconomic level and the Holsteinization of the dairy sector caused a dramatic reduction. As a result, only few head remained, 6000 according to Roustemis (2021 personal communication). The animals are River buffaloes of Mediterranean breed and are spread near lakes or rivers, especially in the North of Greece, near lake Kerkini (Fig. 1.3d) (Roustemis 2017). The milk production per lactation is between 700 and 1000 kg with a lactation length from 210 to 280 days (Borghese 2013a). The young bulls are slaughtered at 15–17 months and the weight at slaughtering is 350–400 kg on average (Borghese 2013a). The riparian zones of Strimonas River and Kerkini Lake are used for grazing during the whole year, even though, during the winter, complementary feeding is given (straw of wheat, clover, corn, and maize silage). Buffaloes are used for milk and meat production. The dairy products obtained from buffaloes are yogurt, white cheese in brain, butter, *kaimaki,* and cream. Efforts for buffalo production are made by the Greek Buffalo Breeders Association, by researchers of Greek Focal Point for the Preservation and Conservation of the Animal Genetic Resources, at the Aristotle University of Thessaloniki, with the support of the Ministry of Agriculture to rehabilitate buffalo production and to let buffalo farming, at present under protection, to become an economic viable activity.

1.7 Buffalo in Europe

In Europe, there are two opposite situations: countries which had a long tradition in buffalo breeding and autochthonous breeds that are drastically declining in numbers due to Holsteinization and mechanization (Balkan countries, Hungary) and countries where buffalo has been newly introduced as sustainable alternative to dairy cow (Germany, UK).

1.7.1 Bulgaria

In Bulgaria, the buffalo situation is totally different from other Balkan countries: a new buffalo population, named Bulgarian Murrah, was created importing of Indian Murrah in 1962 and later in 1975 by crossing them with indigenous Mediterranean of Bulgaria. This activity was carried out systematically under the scientific

management of the Buffalo Research Institute in Shumen and the National Animal Selection Centre (Alexiev 1998). After 1989' changes in the political and social-economic system, the animal numbers drastically declined due to privatization of the farms, and the limited scientific and genetic activities. Recently, the number increased again to 16,730 head (FAO. FAOSTAT 2019). Milk recording, selection, AI and progeny testing are coordinated by the Buffalo Research Institute of Shumen and by the Bulgarian National Association of Buffalo Breeding. Many buffalo cows have an average production of 2500–3000 kg, others more than 4500 kg, and the champion 5349 kg with 6.64% fat, in 305 days of lactation (Peeva 2009). Main source of meat are male calves with an average daily gain between 650 and 1083 g. The most effective slaughter body weight is 400 kg (Dimov and Peeva 1994). Main Bulgarian dairy products are white brain cheese, typical yogurt, salami, sausages, and *Pastarma*.

1.7.2 Romania

The buffalo population in Romania was more than 200,000 head between 1980 and 1996, reduced now to 14,000 heads (Popa et al. 2018). The rapid decrease is due to the introduction of dairy cows, the mechanization and the contraction in the number of farmers in rural areas. According to the National Agency for Zootechnics in Romania, in 2018 there were 2892 farms included in the Genealogical Romanian Buffalo Registry with 10,095 buffalo cows; of these 1796 were dairy farms, with 5458 buffalo cows, entered in the Official Production Control, registering an average milk production of 1923 kg per lactation (Matiuti et al. 2020). The breed, classified as Mediterranean Carpathian, presents hard hooves for better moving over stones and a thick winter coat. Most of the buffalo population (97%) is reared in Transylvania. Mainly, these buffaloes are bred for ecological and traditional products. White salty cheese, butter, yogurt, cream, kefir, *Vladaesa cheese, Braila cheese* as well as meat and sausage and salami, are produced (Matiuti et al. 2020). Buffaloes are still reared in small private farms for draft and the goal of selection is to create a dual-purpose animal (milk and meat), reaching good ADG (600–800 g), in order to slaughter the males at 22 months with 460 kg of live weight. Nevertheless, young calves at 4 months of age and 100 kg live weight are also slaughtered (Borghese 2013a). Since 2011 the Transilvania Lactate (private company) is putting efforts in enhancing products quality, processing and distributing all over the country dairy products originated from buffaloes reared on pasture. Recently to increase the value of buffalo milk, a project to create a buffalo dairy organic farm was carried out at the Institute of Research and Development for Buffalo Breeding in Sercaia, Brasov. The overall objective of the project is to establish a model for organic buffalo milk farm and a guide of best practice to convert conventional farms into organic (Vidu 2015).

1.7.3 Hungary

Buffalo were introduced by the Turks in sixteenth century and the population is named Carpathian or Mediterranean Hungarian Breed. According to direct information from the Ministry of Agriculture and Rural Development, there are 6222 buffaloes, most of them living in National Parks, as gene reserves (Borghese 2020, personal communication). In recent years, according to a new project, some new modern farms are created in Mezotur area, introducing Mediterranean Italian animals of high genetic value. The purpose was creating a market for high-quality milk and meat products, such as *mozzarella* to supply restaurants and shops in Budapest.

1.7.4 Other Balkan Countries

In **Serbia,** the last data available on buffalo population was 1200 head of Mediterranean breed, Balkan type according to SAVE (2011). National Ministry of Agriculture is supporting biodiversity conservation activities, through subsidies and programs for pure breed maintenance (Stojanovic 2011). The buffalo farms in **Macedonia** are very few, 4 or 5 at all perhaps, and the total population is very limited, 633 animals of Mediterranean breed, according FAO. FAOSTAT (2019).

In **Albania,** buffalo population was 120 head in 2010 reduced to 107 head, according to FAO. FAOSTAT (2019). Albanian buffalo is an indigenous breed, classified in the group of Mediterranean. Buffalo in Balkan countries has been used mainly as draft power; however, farmers have been interested in milk and meat products, both as local fresh cheeses and typical products particularly appreciated by consumers, and to enhance touristic economy (Borghese 2011). A program to save and develop buffaloes in these Balkan countries is a priority both to preserve buffalo genetic that was introduced five centuries ago by Turkish invasion, and to control inbreeding level providing breeders for exchange in breeding stocks (Papa and Kume 2011).

1.7.5 Rest of Europe

Buffalo has been recently introduced in other European countries such as Germany, United Kingdom, Spain, Ireland, and Sweden to widen the market of buffalo products. The majority of animals belong to Italian Mediterranean breed. Germany is the country with 7614 buffaloes head, according to the German Buffalo Association (Hoeflich et al. 2020) recorded in the German livestock controlling system. Germany is an example of adaptation capacity of buffaloes to cold climates; they can live out in the snow without problem. The animals are kept in the stables during winter and on the pasture during spring and summer. The population had a fast increase due to the development of a rich market of high-quality products, such as *mozzarella* and other cheeses, cream, yogurt, sausages, meat boxes, and also beauty products.

1.8 Buffalo in America

According to Minervino et al. (2020) in America there are 2.41 million buffaloes. Buffalo is not an autochthonous species but has been imported from Europe and Asia and one of the reasons of its diffusion in this continent is the great availability of lands and the richness of pasture. Buffaloes can be reared in extensive system for meat production as export meat market is a historical priority in South American countries. More recently, the growing cheese market demand produced an increased interest in milk and dairy factory and consequently intensive breeding management also in North America Countries (USA, Canada) with a total population of about 8000 head (Minervino et al. 2020).

1.8.1 Brazil

In the American country, the majority of buffaloes can be found in Brazil with 1.43 million head (FAO. FAOSTAT 2019) where buffaloes were introduced from Asia, Europe, and the Caribbean at the end of the nineteenth century. The predominant breeds are Murrah, Mediterranean, Carabao, and Jaffarabadi.

The great adaptability to the different countries' environments resulted in a consistent annual increase of the population associated with the growing consumers' demand of buffalo dairy products. Buffalo population is spread all over the country, with the highest concentration in the Amazonian area (Fig. 1.4a). Therefore, in some Brazilian States, buffaloes have become an economic option, primarily for milk yield and, consequently, for *mozzarella* cheese production, following the Italian footsteps. Usually, the beef production is made under extensive systems having as basic foodstuff, native or cultivated tropical pastures. The males reach slaughter weight (around 430–480 kg) between 18 and 24 months (Bernardes 2007).

Matos et al. (2020) reported the results of the Program of Buffalo Genetic Improvement (PROMEBULL) on the influence of environmental factors on milk production traits in buffalo (data recorded from 1969 to 2016). The milk yield was 1963 $\pm$ 714 kg/lactation (281.7 days) from Murrah breed and its crosses. The best females were selected among PROMEBULL's partner breeders in the state, to be inseminated with the semen of superior males from India and generate offspring that are more productive in meat and milk (Brazilian Agricultural Research Corporation, EMBRAPA 2019). Higher milk yield of 2218 $\pm$ 408 kg was reported by Bezerra et al. (2014) in farm with advanced management and selection.

1.8.2 Venezuela

First buffaloes were introduced in Venezuela at the beginning of 1900 from Trinidad and Tobago. Later, in the 1970s imports from Australia, Bulgaria, and Italy contributed to buffalo genetic and recent imports from Brazil of Indian-Murrah pure breed have contributed to such improvement remarkably as well. Data

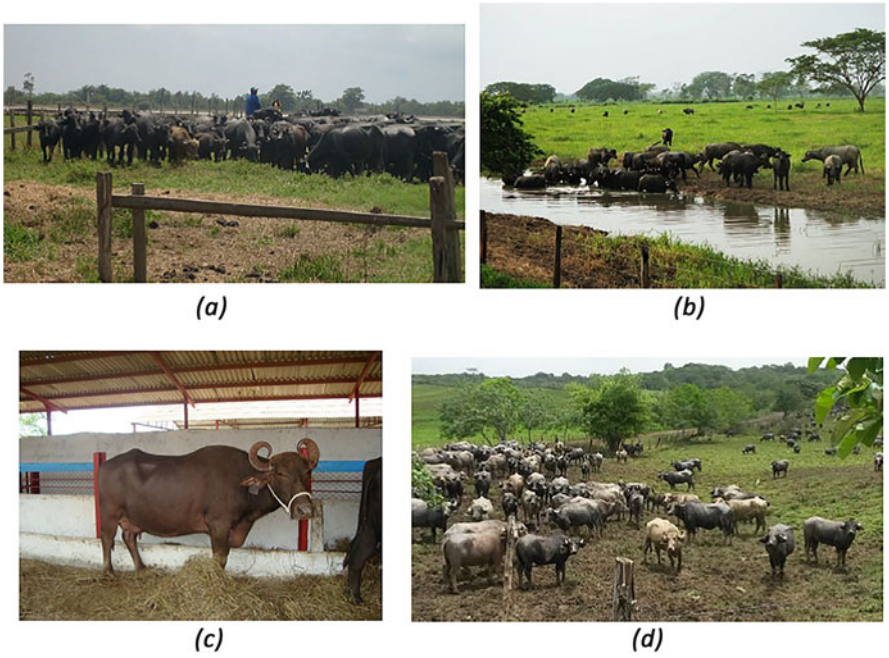

Fig. 1.4 Buffalo in the Americas. (**a**) Murrah crossbreds near Santarem, Amazonia, Brazil (Borghese photo, 2011). (**b**) Crossbreds Mediterranean x Murrah, Maracaibo, Lake, Venezuela (Gutiérrez-Añez photo, 2016). (**c**) Cuban buffalypso, Macun, Cuba (Barile photo, 2011). (**d**) Buffalypso in Mora Valley farm, Trinidad (Borghese photo, 2015)

regarding buffalo population in Venezuela reported in this chapter come from Gutiérrez-Añez (2019). According to the Venezuelan Buffalo Breeders Association (CRIABUFALOS) there are 500,000 heads recorded, but probably are many more considering unregistered independent breeders. The 15% increase rate per year of buffalo population is due to the shift in farmers' preference from cattle to buffalo production because of the superior reproductive performances and better growth rate observed under tropical conditions. Buffalo farms in Venezuela are often large properties (from 1000 to 10,000 hectares) and the management and feeding are mainly grazing-based (Fig. 1.4b). The milk yield per cow is widely diverse from one farm to another, but generally, it oscillates around 1400 kg per buffalo per lactation (range: 1200 to 3500–4000 kg/cow/lactation), that could extend to 270–310 days.

The main advantage to produce buffalo's meat under tropical conditions, is the shorter time to reach the ideal slaughtering weight (425–550 kg at the age of 18–28 months old), due to their better ability to convert forages to increase live weight compared to cattle in natural pasture feeding-based management. During the last years, the Department of Veterinary Medicine, University of Zulia, Maracaibo, has focused the research on the area of reproductive biotechnologies, mainly related to the improvement of the efficiency of estrous cycle manipulation for both fixed-

time AI and embryo transfer programs using proven semen from Italian Mediterranean and Indian-Murrah bulls. The application of reproductive biotechnologies will improve the genetic selection which will allow the increase of milk production, productivity, and economic profitability of the Venezuelan herds.

1.8.3 Colombia

The first buffaloes arrived in Colombia were Buffalypso from Trinidad and Tobago. They were used mainly for draft power in swampy areas where cattle couldn't be introduced. Buffalo in fact, is suitable for work in plantation due to its broad articulation in the hoofs, especially during the rainy period, when the soil becomes muddy causing difficult mobility for other species. For these characteristics the buffalo become the best draft power animal option for oil palm harvest in the tropic (Cortés 2006). Successively, numerous Murrah and Mediterranean buffaloes were imported from Venezuela and Brazil for meat and milk production (Zava 2012). As reported by Minervino et al. (2020), the population is 336,400. The actual trend is to use buffalo for dairy purpose, importing high-quality semen from Brazil and Italy, Murrah and Italian Mediterranean, respectively, to increase milk potential. Milk is used for direct consumption, mixed with cow milk, or it is processed to produce cheeses, yogurt, and dessert. In a study on milk control in buffalo dairy herds of the Colombian Buffalo Breeders Association, milk yield varied from 3.21 ± 1.24 to 4.84 ± 1.60 kg/day (Cerón-Muñoz et al. 2011). These average yield values seem low because they come from dual-purpose systems (meat and milk) with one daily milking and with the calves suckling from the mother during the day. The Colombian Buffalo Breeders Association is developing programs on animal identification, genetic improvement, genealogic Herd Book, milk recording, AI and embryo transfer. The goal is to increase Colombian production of milk.

1.8.4 Argentina

In Argentina, there are 121,276 buffalo head (ABUAR 2020), notably Mediterranean, Murrah, and Jaffarabadi imported from Brazil with an annual growth rate of 9.47% since 2016 (Crudeli et al. 2016). The Northeast Region of Argentine (NEA) concentrates 85% of the total buffalo population, due to characteristics and availability of pasture making buffaloes more convenient than cattle. They easily reach 220 kg in 8 months, 480 kg in 24 months, and 550 kg in 27–30 months, as slaughtering weight, 1 year earlier than cattle improving the carcass quality. The suckling (or baby) buffalo of 11 months with 250–300 kg of weight has good market niches in the medium cities of NEA. Buffalo milk production and industrial cheese processing are not yet economically significant in Argentina (Zava 2012). Some farms in Formosa and Corrientes provinces produce milk from selected groups of Murrah breed or Murrah x Mediterranean or Mediterranean. The milk production is about 5–6 L per day for a lactation period of 240 days (Zava 2011).

1.8.5 Bolivia

Bolivia imported buffalo since 1974, starting with Italian Mediterranean, and afterwards Murrah and Jaffarabadi from Brazil. Actually, there are 35,000 buffalo head (ChacónCondori 2019), in the regions of El Beni, Santa Cruz, and Cochabamba. The Bolivian Buffalo Breeders Association (ASOCRIABUF-Bolivia) was legally constituted in 2018. Buffalo milk and meat, *mozzarella* cheese, and *chorizo* meat are sold on a small scale in local market. The ASOCRIABUF is active to promote the spread out of buffalo in the country for the favorable conditions of tropical climate, where buffalo is the most competitive ruminant.

1.8.6 Center American countries

In Center American countries as Costa Rica, Trinidad and Tobago and Cuba the most representative breed is Buffalypso. It is a double purpose animal, predominantly used for draft power in sugar cane fields and meat production.

The Buffalypso is a result of crossbreeding between the Carabao and other River breeds such as the Murrah, Surti, Jaffarabadi, Nelli, and Bhadawari, began in 1949 by Dr. Bennett, at the Caroni Limited Sugar Company in Trinidad (Bennett et al. 2007). In Cuba and Costa Rica, crossing with Italian Mediterranean is applied to increase milk production.

In **Cuba** since the introduction in 1983, the species has experienced accelerated growth, up to the last 5 years when the growth has been modest. At the end of 2019, the National Office of Statistics and Information (ONEI) reported that the buffalo population in Cuba was 59,300 head, 92.3% of which in the state and business sector. The production was 5.7 million liters from 5600 milking buffalo cows with an average yield of 2.5 L with the calves suckling. The meat production in the country is not reported, because it is considered as by-product of the dairy production system (López Álvarez et al. 2020). The breed is named Cuban Buffalypso (Fig. 1.4c) but the variability is very high, with albino animals too.

Buffalypso breed in **Trinidad and Tobago** (Fig. 1.4d) is considered a dual-purpose animal (milk and meat), reaching 6118 head on total (FAO. FAOSTAT 2019). Different milk products are available in the market: yogurt, butter, ghee, *mozzarella*, *queso blanco*, soft cheese, *dahih,* and *paneer* (cottage cheese). In **Mexico,** buffalo was imported since 1996 from the Caribe countries and now there are 45,000 head (AMEXBU 2019) of mixed breeds, with double purpose, milk and meat. In **Costa Rica**, there are 20,000 head (Minervino et al. 2020) and the buffalo milk production is becoming a priority creating a dairy purpose breed and a milk and cheese industry. The animals are reared on pasture with integration if needed be. The actual perspectives for buffalo development in Costa Rica are going to be applied by a national project, led by the Foundation for the investigation and social development of buffalo (FUNDEBUFALO CR) (Quesada 2016).

1.9 Buffalo Livestock in Oceania

In Oceania Continent there are 173 buffaloes in Micronesia, 1000 in New Zealand, 94 in Guam, 3500 in Papua New Guinea, and 90,000 in Australia (Minervino et al. 2020). Australia imported buffalo from Asia in the nineteenth century and now they are bred extensively on free pasture and in lagoons areas. There were many problems in 2019 and 2020 for the dramatic fires in the country. As were imported more buffaloes of Swamp type than River type, now there are different mixed genotypes, without a genealogic book. The Australia biggest market is not based on dairy products, but on the export to Asia market of live animals: 4288 head were exported to Vietnam and 3377 to Indonesia in 2019. The perspectives are to develop buffalo meat and cheese market: the Victoria's Riverine Blue cheese from buffalo was named champion of its category at 2020 Sydney Royal Cheese and Dairy Produce Awards.

1.10 Perspectives

Livestock farming has to take into consideration various economics and social factors: food security, poverty alleviation, environmental footprint reduction, preservation of social and cultural values. This could be achieved through policy dialogue through multi stakeholder's initiative, enable discussion with all involved in livestock supply chain to build common knowledge and solutions and develop policies that support the adoption of best practice.

The importance of animal recording in selection programs is well acknowledged all over the world. The main issues to be faced in order to implement a milk recording activity in buffalo, reported by Moioli et al. (2000) but still valid today, can be concisely summarized as follows: (1) lack of finance; (2) farmers reluctance; (3) cost of animals' identification; (4) recording costs proportionally increasing with the distance between herds. These constraints explain why in countries where buffalo is a main livestock the animals recording is lacking.

Therefore, it would be desirable that each country in which the buffalo farming represents an important socioeconomic resource take action for the: (1) creation and/or implementation of National Registry Office to have animals' data bank that guarantees the tracking and tracing of animals and their products to control both animal health and product safety; (2) creation of an Herd Book for animal recording, genetic evaluation of dams and sires, and approbation of breeding plans: breeding associations could be formed to control and maintain records; (3) promotion of animal recording and AI technique to increase the pressure of genetic selection; the animal recording and the progeny test remain the basic approach for selection and genetic improvement: Italian Mediterranean, Murrah, and Nili-Ravi are the best breeds for milk production, as they have the longest history of recording, progeny testing, and selection. The use of reproductive technologies might have a rapid impact in improving productivity, but uncontrolled crossbreeding needs to be also addressed because it is mandatory to have a preservation program for local breeds. In

various European and Asian countries, the buffalo population and productions show a decreasing trend and some of the local breeds are endangered. Local breeds are well adapted to low-input production systems and to a range of environments, are able to produce food with unique properties, and can preserve cultural and social values.

Diseases and disease control, and climate change impacts will need more attention. This goal could be addressed: (1) providing veterinary services at large extent especially in rural areas, (2) promoting the adoption of environmentally friendly agricultural practices, (3) a better utilization of local feed resources and enrichment of its nutritional values in a sustainable way, considering land and water shortage. Buffalo with its genetic diversity and adaptability to different environments could help achieve some Agenda 2030 Sustainable Development Goals (https://sdgs.un.org/2030agenda). An important action for the preservation and the exploitation of the local breed would be the differentiation of local production considering the territorial potentialities, setting up manufacturing units near to the farm to create added value for fresh products as well as requirement for maintaining the cold chain and providing a legal framework for food quality and safety (label, brand, PDO, etc.). The future of farm animal production systems is closely related to sustainability, welfare, and quality of products. The new technologies will play a pivotal role in animal management, economic and industrial choices, also for the buffalo.

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Water Buffalo Genomic Diversity

2

Licia Colli, John L. Williams, and Paolo Ajmone-Marsan

Abstract

Since the advent of molecular genetics, the analysis of polymorphic loci has been used to study diversity between and within species at all levels of biological complexity, including domestic animals. Following technological improvements, different types of molecular markers have been used to characterize population variability, including allozymes, restriction fragment length polymorphisms (RFLPs), microsatellite loci, and single nucleotide polymorphisms. Recently, high-throughput sequencing technologies have made it possible to study molecular diversity at the whole genome level. This chapter summarizes the main aspects of genomic diversity of water buffalo, as revealed by the analysis of different types of molecular markers and with respect to evolutionary history of the two subspecies.

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Buffalo · Genome · Molecular genetics · Diversity · Evolution

2.1 The Water Buffalo Genome

There are two types of Asian water buffalo, the river- and the swamp-type, which are classified into distinct subspecies, *Bubalus bubalis bubalis* and *Bubalus bubalis carabanensis,* respectively, which were derived from independent domestication of different ancestral populations of wild Asian buffalo, *Bubalus arnee* (Cockrill 1981; Kumar et al. 2007a; Yindee et al. 2010; Nagarajan et al. 2015; Wang et al. 2017a; Colli et al. 2018; Sun et al. 2020). Their divergence predated domestication and was likely caused by isolation following the Pleistocenic glaciations (Wang et al. 2017a; Sun et al. 2020). Domestication of river buffaloes occurred in the western Indian subcontinent about 6000 years ago (Kyr BP) (Kumar et al. 2007b; Nagarajan et al. 2015) and in the China-Indo-China border region between 3000 and 7000 years ago for swamp buffaloes (Zhang et al. 2016; Wang et al. 2017a). Following domestication, river buffaloes migrated westwards through southwestern Asia to the Mediterranean basin, while swamp buffaloes spread to south- and north-eastern Asia (Colli et al. 2018; Zhang et al. 2020). There is extensive differentiation between the types in terms of morphology, behavior, and current geographical distribution (Macgregor 1941; Cockrill 1974; Zhang et al. 2020). The two types are genetically differentiated, as revealed by allozymes (Amano et al. 1980; Barker et al. 1997a), microsatellite markers (Barker et al. 1997b; Zhang et al. 2011; Kumar et al. 2006), single-nucleotide polymorphisms (SNPs) (Colli et al. 2018), and whole-genome sequence data (Dutta et al. 2020; Sun et al. 2020). The two types are also differentiated by mitochondrial DNA and Y-chromosome variation (Zhang et al. 2006; Yindee et al. 2010; Zhang et al. 2016; Wang et al. 2017a; Sun et al. 2020).

Both river and swamp buffaloes have five sub-metacentric autosomes, while the remaining autosomes and the sex chromosomes are acrocentric (Iannuzzi and Di Meo 2009). The number of chromosomes (2n) differ between the two types, the river karyotype (2n) being 50 while the swamp karyotype (2n) is 48 (Ulbrich and Fischer 1967; Fischer and Ulbrich 1968). The one-pair difference in chromosome number is the result of a rearrangement creating swamp buffalo chromosome 1 from a tandem fusion translocation between the telomere of river buffalo chromosome BBU4p and the centromere of BBU9 (Fig. 2.1) (Di Berardino and Iannuzzi 1981; Iannuzzi and Di Meo 2009; Luo et al. 2020). Fluorescent in situ hybridization (FISH) and C-banding studies (Di Meo et al. 1995; Tanaka et al. 1999) suggested that this fusion caused the loss of a large portion of heterochromatin and satellite DNA from BBU9, and of the nucleolus organizer region (NOR) present at the telomere of BBU4p (Fig. 2.1). In river buffalo, six nucleolus organizing regions (NOR) are found at the telomeres of chromosomes 3p, 4p, 6, 21, 23, and 24, while only five NORs are found in the swamp buffalo on chromosomes 4p (which corresponds to river buffalo chromosome 3p), 6, 20, 22, and 23 as a result of the tandem fusion translocation

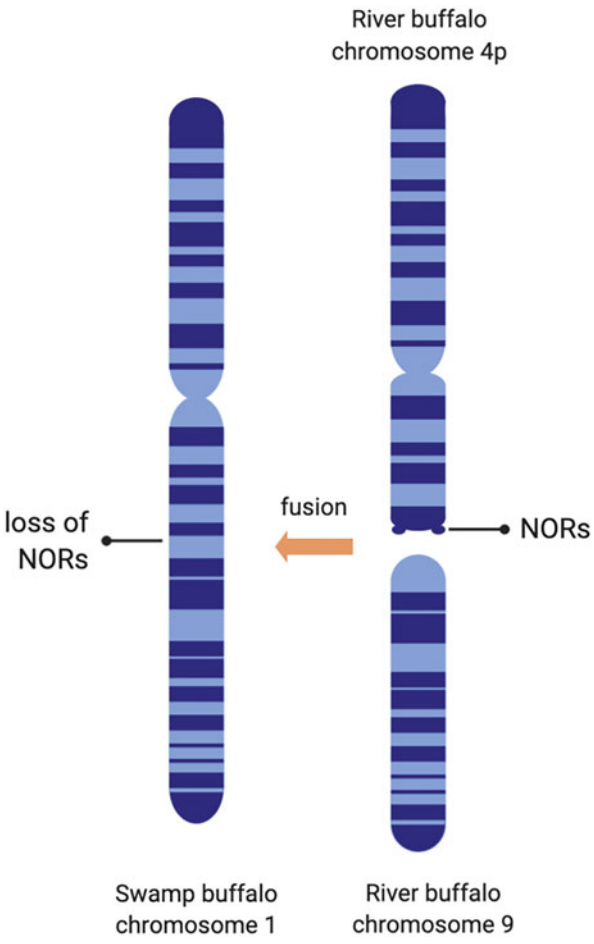

Fig. 2.1 Tandem fusion translocation between the centromere and the telomere of river buffalo chromosomes 9 and 4p, respectively, originating swamp buffalo chromosome 1. The loss of NORs following the fusion is also indicated. (Modified with BioRender.com from Iannuzzi and Di Meo (2009))

(Degrandi et al. 2014). As all chromosome arms pairs are conserved between the two species, river x swamp crosses show $2n = 49$ and are fertile, although with a possible reduced fertility due to unbalanced chromosome sorting during meiosis (Iannuzzi and Di Meo 2009).

Initial information on the general organization and gene localization on domestic buffalo chromosomes came from the comparison with cattle. Cytogenetic c-banding studies provided the arm-by-arm matching between water buffalo and cattle chromosomes, while Fluorescent In Situ Hybridization (FISH) enabled cattle-derived genes and microsatellite loci to be mapped to water buffalo chromosomes (Amaral et al. 2008; Iannuzzi and Di Meo 2009; Michelizzi et al. 2010). A first-generation whole-genome Radiation Hybrid (RH) map was obtained by mapping 2621 cattle-derived markers on to a river buffalo-hamster panel of hybrid cells (Amaral et al. 2008). This showed that there was good conservation of synteny between cattle and buffalo genomes within linkage groups (Michelizzi et al. 2010).

2.2 Reference Genome Sequences

A first draft of the domestic buffalo whole-genome sequence *Bbu_2.0-alpha* was obtained by Illumina paired-end sequencing from a female Murrah river buffalo (Tantia et al. 2011). The genome sequence, which had 17-19X read depth, was assembled by alignment with the bovine genome and was deposited in the NCBI Short Read Archive (SRA) in 2009 under Accession Numbers SRX016621 and SRX015182. The first de novo buffalo genome assembly was created by the International Buffalo Genome Consortium (Williams et al. 2017). A total of 242 Gb raw sequence data derived from Olimpia, a highly homozygous female Mediterranean buffalo, was obtained by combined high-throughput sequencing on the Illumina Genome Analyzer IIx System and Roche 454 FLX titanium platforms, with an approximate 70X genome coverage. This high coverage assembly *UMD_CASPUR_WB_2.0* (GenBank Accession Number GCF_000471725.1. Table 2.1) was 2.83 Gb in size, with a 21.94 kb contig N50 and 21,711 protein-coding genes that were annotated based on RNA-seq data from 30 tissues (NCBI SRA project PRJNA207334).

The river buffalo reference genome was further improved using PacBio long read sequence data from the DNA of the same individual used for *UMD_CASPUR_WB_2.0* which were combined with Chicago- and Hi-C-based chromatin interaction maps (Cairns et al. 2016) to scaffold a 69X de novo chromosome-level sequence (Low et al. 2019). Indels were corrected using additional Illumina paired-end sequencing. The total length of *UOA_WB_1* is 2.66 Gb and contains 509 scaffolds with an N50 of 117.2 Mb (Table 2.1). About 1.53 Gb (58%) of the sequence is haplotype-resolved and has only 383 gaps. The 29 chromosome-level scaffolds are ordered consistently with the buffalo whole-genome radiation hybrid (RH) map and show high conservation of synteny with the homologous chromosomes of *Bos taurus* genome version *UMD3.1* (Fig. 2.2). Annotation of *UOA_WB_1* was produced by incorporating information on 3462 buffalo transcripts from GenBank, 1013 buffalo GenBank protein sequences, 50,553 and 13,381 Ref Seq protein from human and cattle, respectively, and RNA sequencing data from more than 50 different buffalo tissue types (Low et al. 2019). This high contiguity assembly constitutes the current domestic buffalo reference genome (Gen Bank Ref Seq GCF_003121395.1), and the annotation can be browsed with the NCBI Genome Data Viewer tool (https://www.ncbi.nlm.nih.gov/genome/gdv/?org=bubalus-bubalis&group=bovidae).

The trio-binning approach (Koren et al. 2018) in which parental information is used to phase sequence data prior to assembly, has been recently used to create a haplotype-resolved assembly of the Murrah buffalo (Ananthasayanam et al. 2020). PacBio long reads and 10X Genomics linked reads at 166X coverage with an additional 802 Gb of optical mapping data were phased using 274 Gb paired-end data from the parents. Chromosomal level assembly for paternal and maternal genomes with 25 scaffolds and N50 of 117.48 Mb (sire haplotype) and 118.51 Mb (dam haplotype) were achieved; however, this genome is not presently annotated. Recently, a combined PacBio and Hi-C sequencing approaches were used to create

Table 2.1 Summary information on the water buffalo genome assemblies currently available in either the National Center for Biotechnology Information (NCBI) or in the National Genomics Data Center of the China National Center for Bioinformation (CNCB-NGDC)

Assembly	Date	Type	Breed	GenBank ID	Project ID	Level	Size (Gbp)	Scaffold N50 (Mbp)	Contig N50 (kbp)	Seq. technology
UOA_WB_1 (RefSeq)	Jan 2019	River	Mediterranean	GCA_003121395.1	NCBI SRA PRJNA437177	Chromosome	2.66	117.22	22,441.51	PacBio
Bubbub1.0	Apr 2019	NA	Bangladesh	GCA_004794615.1	NCBI SRA PRJNA349106	Scaffold	2.77	6.96	25.04	Illumina HiSeq 2000
ASM299383v1	Mar 2018	River	Egypt	GCA_002993835.1	NCBI SRA PRJNA267486	Scaffold	3.00	3.58	14.57	SOLiD
Bubalus_bubalis_Jaffrabadi_v3.0	Feb 2018	River	Jaffrabadi	GCA_000180995.3	NCBI SRA PRJNA40113	Scaffold	3.76	102.35	13.98	454; Illumina NextSeq 500
UMD_CASPUR_WB_2.0	Sep 2013	River	Mediterranean	GCA_000471725.1	NCBI SRA PRJNA207334	Scaffold	2.84	1.41	21.94	Illumina GAIIx; Illumina HiSeq; 454
CUSA_SWP	Feb 2019	Swamp	Fuzhong	GWHAAJZ00000000	CNCB-NGDC PRJCA001297	Chromosome	2.63	117.30	8.8 Mbp	PacBio

Data from El-Khishin et al. (2020) and Luo et al. (2020)

Fig. 2.2 Circular plot of water buffalo chromosomes mapping to *B. taurus* genome. (From Low et al. (2019))

the first reference genome sequence for swamp buffalo *CUSA_SWP* (CNCB-NGDC Accession number GWHAAJZ00000000), estimated to be 2.63 Gb in size (Luo et al. 2020). The chromosome-scale scaffolds of this assembly have an N50 of 117.3 Mb (Table 2.1), representing the 24 swamp buffalo chromosomes and covering 97.5% of the genome.

The availability of high-quality genomic sequences for both river and swamp buffaloes has made it possible to compare the genome arrangement of the two subspecies and to evaluate their relationship in an evolutionary perspective. Analysis of the sequence similarity confirmed that swamp buffalo chromosome 1 arose from the fusion of river buffalo chromosomes 4p and 9. Comparison of the sequences also facilitated the calculation of divergence times based on 6429 single copy orthologs sequences, which showed that the common ancestor of swamp and river buffaloes dates back to 1.1–3.5 million years ago (Luo et al. 2020). This suggests that glacial

events known to have occurred at that time may have played a role in separating ancestral buffalo populations on either side of the present-day India-Myanmar border, leading to geographical isolation and genetic divergence. Expanded genes were found in both subspecies, 179 in swamp and 261 in river-type, which may account for the phenotypic differences, particularly in relation to muscle growth and environmental adaptation.

2.3 Genetic Diversity

The first genetic studies on buffalo were based on variations in protein-coding loci (i.e., allozymes) and microsatellite loci (Barker et al. 1997a, b) in the nuclear genome. Direct sequencing of the control region or of the *cytochrome b* (Cytb) gene was used to assess maternally inherited mitochondrial DNA (Lau et al. 1998), and sequences from the non-recombining part of Y-chromosome have been used more recently to study the inheritance of the paternal genome (Yindee et al. 2010). High-throughput sequencing and the availability of whole-genome sequence information have made it possible to discover a large number of single-nucleotide polymorphism (SNP) markers and to devise marker panels (Iamartino et al. 2017) for cost-effective genotyping of large numbers of animals to investigate genome diversity and for genome-wide association studies (GWAS).

2.3.1 Microsatellite Markers

Being highly polymorphic, microsatellite loci that contain Short-Tandem Repeats (STRs) were among the first markers to be widely used for the characterization of the nuclear genomic diversity in both river and swamp buffaloes. Although a panel of 30 microsatellite loci was proposed by ISAG-FAO (FAO 2004), most studies have used different or partially overlapping marker sets. This, together with the difficulties in harmonizing allele scoring of STRs across laboratories, prevented a global scale characterization of water buffalo diversity.

In domestic species, diversity levels are higher close to domestication centers, and decrease as populations move away from it. This pattern of diversity is also seen for both water buffalo subspecies. At the local level, microsatellite-based studies showed that for both buffalo types the highest diversity was found close to the respective likely domestication centers, with a gradual decrease along the dispersal routes. The expected heterozygosity (HE) of river buffalo assessed using STR loci, decreases from HE = 0.71–0.78 for the Murrah, Nili-Ravi, and Kundi group from the Indian subcontinent (Kumar et al. 2006; Vijh et al. 2008) to HE = 0.58–0.68 of the Mediterranean population from Italy (Moioli et al. 2001), which is consistent with the supposed center of domestication in the Indian subcontinent. Similarly, the highest heterozygosity in swamp buffaloes is in Thailand (HE = 0.573), close to the likely domestication center across the China-Indochina border, although overall

swamp-type populations display little differentiation from each other all over southeast Asia (Zhang et al. 2011).

A recent meta-analysis made use of model-based clustering of microsatellite marker data from both river and swamp buffaloes (Zhang et al. 2020), which showed a clear difference between the two types and the occurrence of some degree of introgression from the river-type gene pool into swamp buffaloes in Far Southwest China, in Thailand, and to a lesser extent, in the Malaysian peninsula and the Philippines.

2.3.2 SNP (Single-Nucleotide Polymorphism) Diversity

SNPs represent the most frequent type of mutation occurring genome-wide. Comparison of WGS sequences in mammalian livestock has shown that ca. 30 million SNP loci can be found on average within a species (Alberto et al. 2018; Júnior et al. 2020; Luo et al. 2020). Most SNPs are biallelic, which makes them ideal for the development of array-based genotyping panels and simplifies data analysis.

Cattle SNP arrays have been evaluated for use in buffalo (Michelizzi et al. 2011; Wu et al. 2013; Borquis et al. 2014; Pérez-Pardal et al. 2018). Most loci on the cattle panel can be genotyped successfully in buffalo; however, only a small percentage of cattle SNP are polymorphic in buffalo, e.g., only 926 loci (1.71%) out of the ca. 50K markers included in the Illumina BovineSNP50 Bead Chip and only 16,580 (2.41%) out of the 777K SNP in the Illumina Bovine HD Bead Chip (Michelizzi et al. 2011; Borquis et al. 2014). This shows the low cross-species applicability of SNP arrays, which decreases proportionally to the evolutionary divergence time and that most polymorphisms targeted by SNP panels are generally of recent evolutionary origin.

A buffalo-specific SNP panel including 89,988 SNPs, 5799 probes for quality control and 1784 gender identification probes (Iamartino et al. 2017) is commercially available as the Axiom® Buffalo Genotyping Array (Thermo Fisher). The SNP loci were originally selected to have an even genome-wide distribution using the bovine UMD 3.1 genome to estimate their position but have recently been remapped to the new version of the buffalo reference genome (Low et al. 2019). SNPs were initially identified from whole-genome sequences of Mediterranean, Murrah, Jaffarabadi, and Nili-Ravi buffaloes. As these are all river-type breeds, only ca. 22.74% of the loci are polymorphic in swamp buffaloes (Colli et al. 2018). The 90K panel has also been evaluated in Lowland Anoa (*Bubalus depressicornis*) and Cape buffalo (*Syncerus caffer*), resulting in 7652 (7.8%) and 3239 (3.3%) out of 89,988 loci respectively polymorphic in these species (Iamartino et al. 2017).

A multi-species SNP panel has been developed by the European IMAGE consortium to reduce genotyping costs. The IMAGE panel incudes ca. 11K buffalo SNP markers, selected to be evenly distributed genome-wide, to be informative in river and swamp populations by including ancestral polymorphisms, and also to include functional, mtDNA, and Y-chromosome variants (Crooijmans et al. in preparation). Many loci are shared with the Axiom 90K panel to make data integration and meta-analysis possible.

The Axiom Buffalo Genotyping Array has been used to study diversity, linkage disequilibrium, effective population size, genome-wide distribution of runs of homozygosity (ROH), and GWAS have been carried out for milk production, reproduction and disease traits (de Camargo et al. 2015; Colli et al. 2018; Liu et al. 2018; Deng et al. 2019; Du et al. 2019; Guzman et al. 2019). Using genotype data from 20,463 loci that were polymorphic in both river and swamp buffaloes, Colli et al. (2018) evaluated genetic variation, population structure, and gene flow in 30 buffalo populations worldwide. Three distinct gene pools were found, corresponding to individuals of pure river, pure swamp, and admixed river × swamp ancestries. The occurrence of two independent domestication events was confirmed, and several links between populations were identified that were consistent with human migrations, importation, and crossbreeding to improve performance. Association studies have identified candidate genes, that are either trait-specific or with pleiotropic effects, which can be used by breeders to select animals carrying advantageous alleles to improve production traits, particularly milk fat yield and milk protein percentage (Liu et al. 2018). Candidate genes likely to affect milk yield, fat yield, and protein yield have been reported in two genomic regions which respectively harbored the genes *MFSD14A, SLC35A3, PALMD* and *RGS22* and *VPS13B* (Liu et al. 2018). These regions corresponded to locations on cattle chromosomes BTA3 and BTA14 where QTLs influencing milk performance have been reported in dairy cattle (Harder et al. 2006; Wibowo et al. 2008). The comparison of data from cattle and buffalo suggested that different alleles affect milk traits, for fat production in particular, in the two species (de Camargo et al. 2015; Liu et al. 2018).

Runs of homozygosity (ROHs) are contiguous genomic regions where an individual has inherited the same segment on both chromosomes from a common ancestor (Ceballos et al. 2018). Inbreeding at the genomic level can therefore be estimated from the frequency and length of ROHs. Additionally, ROHs can indicate regions impacted by selective pressure over time. The distribution of ROHs over the genome, their abundance, and length are determined by a number of factors including region-specific recombination rate, GC content, selective pressures, and demographic events (McQuillan et al. 2008; Bosse et al. 2014). The length of the ROH regions is in general inversely proportional to time passed since historic, demographical or selection events, with longer ROHs corresponding to recent inbreeding and shorter ROHs arising from ancient demographic changes as bottlenecks or founder effects (Cardoso et al. 2018). Genomic patterns of runs of homozygosity have been studied in several livestock species to find signatures of natural or human-mediated selection, and to identify advantageous genetic variants that may have become fixed or almost fixed as a consequence (Peripolli et al. 2017). The availability of the Axiom buffalo genotyping array has facilitated studies on the occurrence of ROHs genome-wide in water buffalo populations. Runs of homozygosity have been studied at the local level in Iranian Azeri and Khuzestani, and in Brazilian Murrah river buffaloes (Ghoreishifar et al. 2020; Nascimento et al. 2021), and also in a set of 15 river and 15 swamp buffalo populations worldwide (Macciotta et al. 2021). Particularly long ROHs, up to 10 Mb with mean ROH length per animal of

4.28 ± 1.85 Mb, were found in all Brazilian Murrah individuals, most likely resulting from recent strong selection for milk production traits in this population (Nascimento et al. 2021). Conversely, Iranian breeds showed ROHs about 1 Mb long in all animals, but the total number and total length varied considerably between individuals, suggesting the occurrence of both recent and past inbreeding events which have affected the populations (Ghoreishifar et al. 2020). In both studies, genes and QTLs found within ROH islands bore signatures of selection and were associated with selected traits and functions including body size, muscle and bone development, immune response, milk traits (milk yield, milk fat yield and percentage, milk protein yield and percentage), coat color and pigmentation, reproduction and morphology (Ghoreishifar et al. 2020; Nascimento et al. 2021).

ROH analysis across buffalo types identified >18K homozygous regions overall (Macciotta et al. 2021). Swamp-type buffalo populations possessed more ROHs and had higher genomic inbreeding and number of ROHs per animal. Although differences found between river and swamp populations may have been partly affected by ascertainment bias in the SNP on the array, a convergent signature of selection was found in both river and swamp buffaloes on chromosome 2 where a large ROH island spans genes involved in adaptation to the environment and reproduction (Macciotta et al. 2021).

2.4 Nuclear Genome Diversity

Huge amounts of whole-genome resequencing data are being produced using high-throughput sequencing platforms. WGS sequence data have been published for water buffaloes from many countries: Bangladesh, Bengal, China, Egypt, India (multiple breeds), Indonesia, Iran, Iraq, Italy, Laos, Myanmar, Nepal, Pakistan, the Philippines, Vietnam, and Thailand (Dutta et al. 2020; El-Khishin et al. 2020; Luo et al. 2020; Mintoo et al. 2020; Sun et al. 2020). Based on 5–10X resequencing data from swamp- and river-type animals from 14 Asian and one European country, ca. 33.5 million SNPs were identified overall for the two subspecies, while 18.7 and 23.7 million SNPs were found to be polymorphic within swamp and river buffalo populations, respectively (Luo et al. 2020). Genetic variation within subspecies has been estimated by calculating nucleotide diversity ($\theta\pi$), a statistic frequently used in population genetics, which corresponds to the average number of differences per nucleotide site found when the DNA sequences are compared pairwise among all individuals in the sampled set. Overall, swamp buffaloes have lower genetic diversity than river buffalo, with nucleotide diversity values of ca. $\theta\pi_{\text{swamp}} = 0.0018$ and $\theta\pi_{\text{river}} = 0.0027$. Among the river-type, the Italian Mediterranean population has a lower-than-average nucleotide diversity, (ca. $\theta\pi = 0.002$) compared to buffaloes from Pakistan, Iran, and Iraq (ca. $\theta\pi = 0.0028$) and to Indian breeds (ca. $\theta\pi = 0.0025$ Luo et al. 2020). This reduction of variability is consistent with the loss of diversity as populations move from their geographic origin and alleles are lost. Gene flow has been seen in the regions of Southeast Asia where the two subspecies hybridize naturally (Luo et al. 2020). Introgression from river buffalo into swamp populations

is now used widely to improve productivity, especially in Laos, Myanmar, and the Philippines. Interestingly in Yunnan Province of China, river buffaloes displayed genomic introgression from swamp buffalo (Luo et al. 2020).

Genome-wide analyses have identified hundreds of genes harboring differential selection signatures in swamp and river buffaloes (Dutta et al. 2020; Luo et al. 2020), with a set of 67 genes under selection that are common to both groups (Luo et al. 2020). Pathway and gene ontology analyses of genes under selection in the swamp buffalo identified a significant enrichment in muscle- and cardiac-related functions, nerve development, and behavior, which may account for physical strength and endurance, and docile temperament (Luo et al. 2020). Selective sweeps have also been found in two starch-digestion enzymes genes, which suggests a possible adaptation to reduce rumen acidosis induced by the starch-rich feed traditionally supplied to swamp buffaloes used for traction in several Asian countries (Luo et al. 2020). In the case of river buffaloes, selection signatures have been reported in genes related to body size, fecundity, fetal growth, birth size, and milk production. Selective sweeps common to both subspecies suggest the occurrence of convergent evolution affecting body size, immune response to pathogens, and behavioral changes related to the "domestication syndrome" (Dutta et al. 2020; Luo et al. 2020).

2.5 Functional Variation

Putative sequence variants affecting a range of phenotypes have been proposed by screening the candidate genes in the water buffalo genome. This strategy identified variations related to coat color, milk production, reproductive performance, and diseases, although the validation of these associations is difficult.

In swamp buffaloes, the most frequent coat color is solid dark gray. Solid white animals are not uncommon with a 10% frequency in some populations. White seems to be dominant over the dark variant (Rife and Buranamanas 1959; Rife 1962), but so far variants that control the white coat have not been identified. Swamp buffalo bulls with a white spotted coat, a phenotype found only in animals from Tana Toraja in Indonesia, are highly prized for ceremonial purposes. Two independent loss of function mutations in the microphthalmia-associated transcription factor (*MITF*) gene are associated with the white spotted coat of swamp buffalo (Yusnizar et al. 2015). Specific allelic variants have been reported that affect milk production and growth traits. Milk protein percentage in Mediterranean buffalo has been associated with a C > T transition in the signal transducer and activator of transcription 5A (*STAT5A*) gene and a non-synonymous point mutation in the insulin-like growth factor 2 (*IGF2*) gene which also has an effect on average daily gain (Abo-Al-Ela et al. 2014; Coizet et al. 2018). SNPs affecting milk yield, fat and protein percentage and yield have been identified within the growth hormone receptor (*GHR*) gene in Egyptian buffaloes, in which animals carrying the AA homozygous genotype at both missense mutations 380G > A and 836T > A displayed higher productive performance (El-Komy et al. 2020).

The identification of genetic variants related to reproduction and fertility is particularly important for the efficient implementation of breeding plans. In male buffaloes from China variations at two genes, luteinizing hormone beta polypeptide (*LHB*) and gonadotropin-releasing hormone receptor (*GnRHR*) were recently found to affect semen quality traits, i.e., volume of the ejaculate and quality of the sperm cells (Cheng et al. 2017; Wang et al. 2017b, 2020). In females, pregnancy rate and susceptibility to anestrus are influenced by variants occurring in the melatonin receptor 1A (*MTNR1A*) gene and the cytochrome P450 aromatase gene (*CYP19A1*), respectively (El-Bayomi et al. 2017; Pandey et al. 2019). Susceptibility to disease is a trait that potentially has a significant impact on the viability of livestock production. Susceptibility to tuberculosis and mastitis have been associated with specific mutations. In Mediterranean buffaloes, a G > A transition at nucleotide position 4467 in the 3′-UTR (untranslated region) of the interferon gamma (*IFNG*) gene disrupts the target site for micro-RNA miR-125b, which seems to be responsible for an increased susceptibility to *Mycobacterium bovis* (Iannaccone et al. 2018). In Egyptian buffaloes, a C > A transversion in exon 27 of the complement component 3 (*C3*) gene has been shown to have a significant association with milk somatic cell score, which is correlated with mastitis (El-Halawany et al. 2017).

The heritable defect transverse hemimelia (TH) causes abnormal development of the terminal portion of the limbs, which has varying degrees of severity. A case-control study based on whole-genome sequence data suggests that TH is an oligogenic trait and 13 putative candidate genes have been identified. In particular, variants in wingless-type MMTV integration site family, Member 7a (*WNT7A*) and SWI/SNF Related, Matrix Associated, Actin Dependent Regulator (*SMARCA4*) genes in the homozygous state are associated with the extreme forms of the phenotype (Whitacre et al. 2017).

2.6 Uniparental Genomes Diversity

2.6.1 Mitochondrial Genome

Mitochondrial DNA (mtDNA) is found at high number of copies per cell and is maternally inherited. The mitochondrial genome is haploid and hence does not recombine, but nevertheless has a high mutation rate because of the absence of repair mechanisms. As a result of these features, mtDNA variation has been used to investigate diversity and phylogenies of the maternal lineage in many animal species (Lenstra et al. 2012). The current water buffalo mtDNA reference sequence (Gen Bank Accession Number NC_006295) is from a swamp-type buffalo belonging to the Haikou breed from China. The swamp buffalo mitochondrial genome has a total size of 16,359 bp, and includes the displacement loop (which is non-coding but contains the origin of replication of the H-strand), two genes coding for 12s and 16s ribosomal RNAs (rRNA), 22 genes for transfer RNAs (tRNA), and 13 genes encoding proteins involved in the mitochondrial electron transport chain and ATP synthesis (Fig. 2.3). Partial copies of varying length of the mtDNA genome have

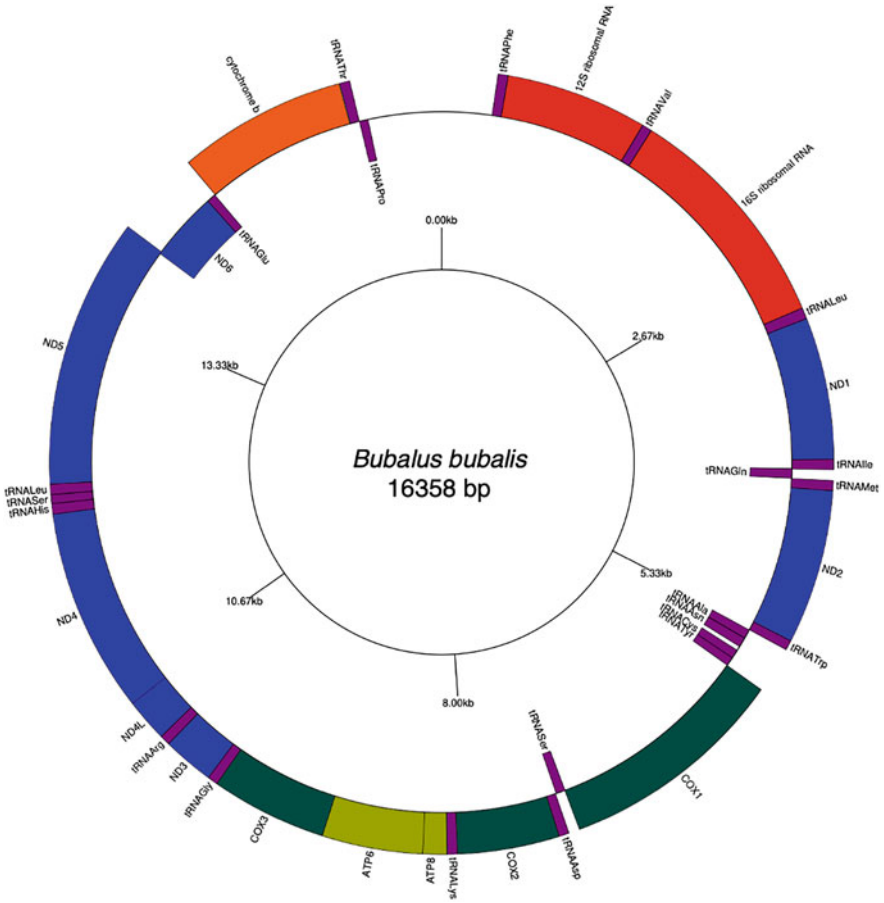

Fig. 2.3 Water buffalo mitochondrial DNA organization based on the current *B. bubalis* mtDNA Reference Sequence (Gen Bank Accession Number NC_006295). Protein, tRNA, and rRNA coding genes are grouped and color coded as follows: purple, tRNAs; red, rRNAs; blue, NADH dehydrogenase; dark green, cytochrome oxidase; light green, ATP synthase; orange, cytochrome b. (The picture was created with Genome VX tool (http://wolfe.ucd.ie/GenomeVx/))

been integrated into the nuclear genome in eukaryotic species in the course of their evolution (Richly and Leister 2004). These nuclear-mitochondrial transpositions (NUMTs) have not been investigated in detail in water buffalo; however, a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) for sequences occurring in both mtDNA and nuclear genome reference sequences returns 24 hits, with percentages of identity between 74.89–100% and length 31–5346 bp. The longest uninterrupted hit of 6430 bp is found on chromosome X, and covers positions 9991–16,359 of the mtDNA reference. The water buffalo mtDNA control region, or displacement loop (D-loop) is 909 bp long, similar to other buffalo species, e.g., the tamaraw *Bubalus mindorensis* (922–925 bp) and the lowland

anoa *B. depressicornis* (927 bp), and also cattle, *B. taurus* (909 bp) and *Bos indicus* (912 bp), but is shorter than that of pig (1175 bp), sheep (1179 bp), and goat (1212 bp) due to the lack of repetitive motifs.

The first mtDNA investigations, based on the analysis of D-loop and Cytb sequence data, highlighted a deep divergence between swamp and river buffalo female lineages and the presence of a hotspot of diversity in animals from southeast Asia, which pointed to domestication in this region (Tanaka et al. 1996; Kikkawa et al. 1997; Lau et al. 1998). The analysis of the entire control region sequence clearly suggested different maternal origin and independent domestication of river- and swamp-type buffaloes (Kumar et al. 2007a; Lei et al. 2007). Large-scale screening of control region variation (Zhang et al. 2016) identified five frequently found swamp buffalo haplogroups, SA1, SA2, SB1, SB2 and SB3, and three rare and highly divergent additional haplogroups, SC, SD, and SE (Figs. 2.4 and 2.5) (Sun et al. 2020). In river buffalo, four haplogroups were found, R1, R2, R3, and R4 (Zhang et al. 2016; Sun et al. 2020). Three are spread across the whole distribution area of river buffalo from the Asian continent to the Mediterranean basin (Figs. 2.4 and 2.5). The R1 haplogroup occurs more frequently (75.4%) compared with R2 (16.4%), and R3 is relatively rare (8.2%) (Fig. 2.4) (Kumar et al. 2007a; Zhang et al. 2016). The R4 haplogroup has only been identified in Italian animals so far (Fig. 2.5. Sun et al. 2020). For both river and swamp buffaloes, mtDNA haplogroup frequencies vary slightly across the distribution areas (Figs. 2.4 and 2.5), following the general trend of a progressive loss of diversity moving away from the domestication centers. The occurrence of rare and highly divergent variants points to the repeated post-domestication introgression of wild buffalo lineages into domestic buffalo (Nagarajan et al. 2015; Wang et al. 2017a), which has also been seen in several livestock species (Bonfiglio et al. 2010; Colli et al. 2015).

Using mtDNA data, water buffalo phylogeographic patterns of variation and demographic history have been investigated. MtDNA data show that swamp buffalo populations are strongly partitioned geographically with little gene flow, despite their phenotypic uniformity. In contrast, river buffaloes have a lower haplotypic diversity and a weaker phylogeographic structure (Zhang et al. 2016; Wang et al. 2017a), which may be partly due to the human-mediated effects, including selection for productivity and exchange of animals for the improvement of milk production. Swamp buffaloes in southern China/northern Indochina straddling the Mekong River have the highest diversity, suggesting this region as the likely center of domestication of the swamp type, which is estimated to have occurred ca. 3–7 Kya (Zhang et al. 2016; Wang et al. 2017a). Diversity of river buffaloes is highest in India and gradually decreases westward towards the Mediterranean (Nagarajan et al. 2015), supporting the hypothesis of domestication in the Indian subcontinent. The gradual decline in diversity from the Indian subcontinent suggests that after domestication migration of river buffalo westward towards southwestern Asia and the Mediterranean occurred gradually and without major bottlenecks (Zhang et al. 2016), in agreement with microsatellite- (Moioli et al. 2001; Kumar et al. 2006; Vijh et al. 2008) and SNP-based evidence (Colli et al. 2018).

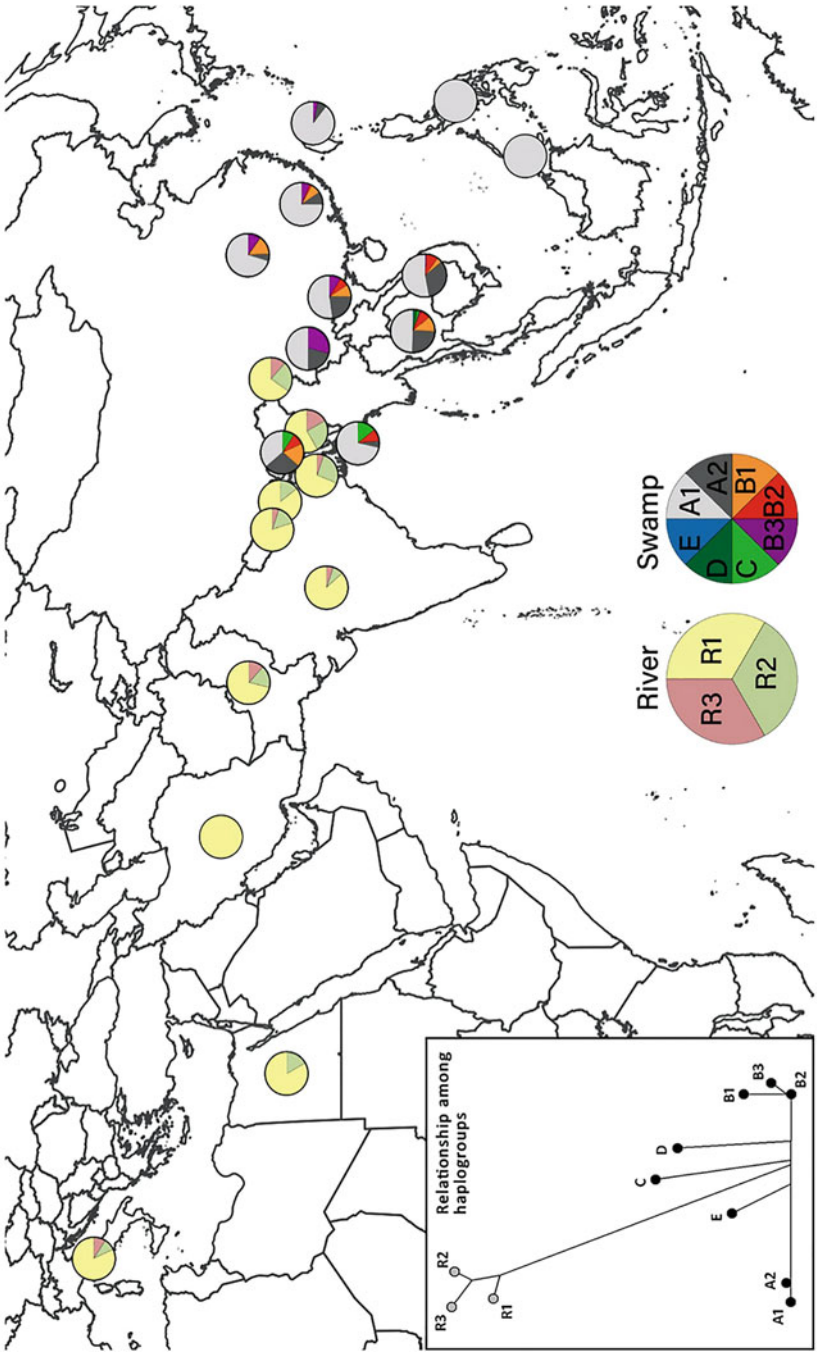

Fig. 2.4 Distribution and frequency of river and swamp buffalo mtDNA haplogroups in different areas of the world based on control region data. The inset shows the phylogenetic relationships between the haplogroups. (From Zhang et al. (2020))

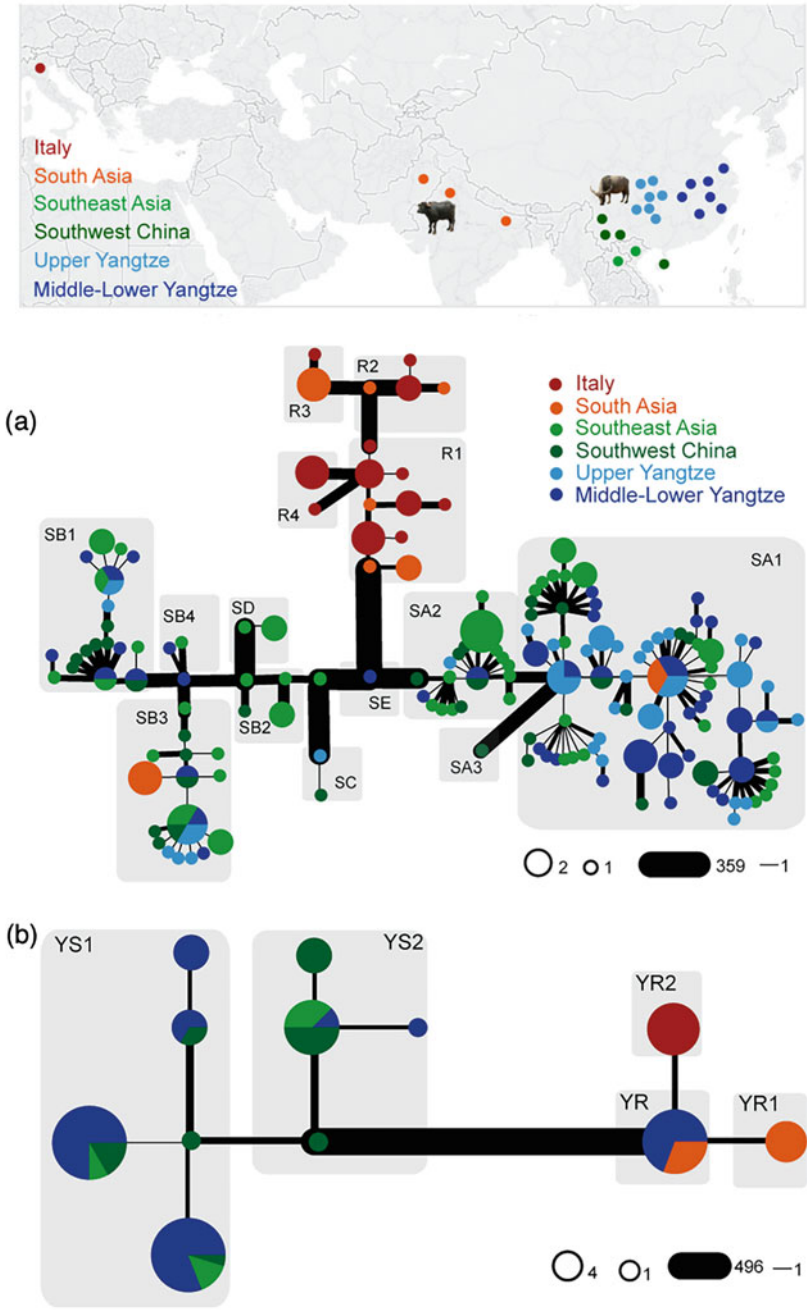

Fig. 2.5 Phylogenetic networks of (**a**) river and swamp buffalo complete mitochondrial genomes, and (**b**) Y-chromosome haplotype variation based on 520 SNPs. Gray-shadowed rectangles identify different haplogroups. Edge widths are proportional to the number of mismatches between the joined haplotypes. Geographical provenance of the samples is shown in the top panel. (Modified from Sun et al. (2020))

Whole mitogenomes of swamp buffalo show that Pleistocenic glacial periods, before 11K years ago, played a role in shaping phylogeny and demographic history of the subspecies. Divergence between river- and swamp-type mitochondrial lineages has been estimated to have occurred at the beginning of a glacial period (900–860 Kya). Within swamp buffaloes, the two major macro-lineages diverged at the time of the second glacial event 200–130 Kya (Wang et al. 2017a). Interestingly, most present-day swamp buffalo mitochondrial genome originated from two ancestors dating back to the Last Glacial Maximum 26–19 Kya and further differentiated during the Holocene warm period 11–6 Kya. A substantial bottleneck was observed at 7–3 Kya, corresponding to a reduction in effective population size that occurred at the time of domestication (Wang et al. 2017a).

2.6.2 Y-Chromosome

The non-recombining part of the Y-chromosome, i.e., the portion lacking a homologous counterpart on the X-chromosome, provides information on the paternal lineage. Low variability of the Y-chromosome has made the identification of Y-chromosome polymorphisms difficult in most livestock species (Hellborg and Ellegren 2004). As a consequence, Y-chromosome variation has been analyzed by sequencing short gene fragments or by typing a few microsatellite loci (Zhang et al. 2006, 2016; Yindee et al. 2010; Wang et al. 2018). Microsatellite-based studies of the buffalo Y-chromosome diversity initially exploited cattle STR loci with limited success. Out of 40 cattle loci tested in Asian swamp buffaloes, only 12 could be satisfactorily amplified and only seven were polymorphic (Wang et al. 2018). Together they described nine haplotypes and four haplogroups, i.e., Y1, Y2, Y3, and Y4. Haplogroup Y1 occurred more frequently (83.4%) across southeastern Asia, while Y2 and Y3 had discontinuous distributions and varying frequencies. The high frequency of Y1 around the Yangtze Valley suggested this as the domestication center. Interestingly, the rare and highly divergent Y4 was found only on Hainan Island, and was interpreted as introgression from an ancient wild Asian buffalo population native to the island (Wang et al. 2018).

Studies of the Y-chromosomes genes of water buffalo have produced sequence data from specific genes, e.g., DEAD-Box Helicase 3 (*DBY*), Y-linked Zinc finger Y-chromosomal protein (*ZFY*), and Sex-determining Region Y (*SRY*) gene segments (Zhang et al. 2006; Yindee et al. 2010; Zhang et al. 2016). Sequencing of *SRY* identified a single SNP difference between river and swamp buffalo at position 202 of the coding region. PCR genotyping of this SNP was used to reveal male-mediated introgression of river-type buffalo into swamp buffalo in Southern China (Zhang et al. 2006). A 2310-bp Y-chromosome fragment was sequenced in 495 males (450 swamp and 45 river) from 35 populations spanning most of southeastern and central Asia (Zhang et al. 2016). Among swamp buffaloes sequence analysis identified only nine SNPs originating 11 haplotypes, of which four were frequent and seven rare. Only one SNP defining two haplotypes was found in river buffalo (Zhang et al. 2016). Nevertheless, the geographical distribution of

the haplogroups and the values of haplotype diversity in swamp buffaloes corroborated the hypothesis of domestication around the border between China and Indochina (Zhang et al. 2016).

Despite the lack of a buffalo Y-chromosome reference assembly, sequencing of whole genomes of about 90 male buffaloes from different geographical areas produced Y-chromosome data (Fig. 2.5) (Sun et al. 2020). A total of 520 Y-chromosome specific SNPs were detected in these data, which identified a clear subdivision of the paternal lineages into swamp- (YS) and river-type clades (YR). Two haplogroups were identified within each clade: swamp-type haplogroups YS1 and YS2 are spread across the whole distribution area of the subspecies, although with varying frequencies, while river-type haplogroups were geographically separated, YR1 and YR2 were detected in southern Asia and Italy, respectively. The YR clade haplotypes have been identified in swamp individuals showing the male-mediated river buffalo introgression into swamp buffalo populations due to crossbreeding plans to improve productivity (Fig. 2.5). Y-chromosome data confirms the clear genetic subdivision between river and swamp paternal lineages, indicating that they originated from different wild ancestral population.

2.7 Conclusions

Y-chromosome specific loci and mitochondrial genome variation suggest that swamp and river buffaloes were domesticated from different ancestral populations that had already diverged due to prolonged isolation following glacial events during the early Pleistocene period. Likely domestication sites of river and swamp buffaloes were the northwestern Indian subcontinent and the China/Indochina border region, respectively (Kumar et al. 2007b; Yindee et al. 2010; Nagarajan et al. 2015; Zhang et al. 2016). This hypothesis has been confirmed by microsatellite loci (Kumar et al. 2006; Vijh et al. 2008; Zhang et al. 2011, 2020) and genome-wide SNP analysis (Colli et al. 2018). River buffaloes have fewer haplotypes and lower overall diversity compared with swamp buffalo for both mtDNA and Y-chromosome markers, which contrasts with the higher values of genomic diversity estimated in river buffaloes compared to swamp buffalo based on whole-genome sequence data (Luo et al. 2020). These differences may be explained by taking demographic effects into account. Inference drawn from whole-genome sequences showed that around 100 thousand years ago river buffalo ancestors experienced a four times larger population expansion compared to swamp buffalo ancestors (Luo et al. 2020). This expansion of the ancestral river buffalo likely led to increased diversity of which only a small amount was sampled when domestication occurred, resulting in a reduction in uniparental lineages while nuclear genomic diversity was maintained in the background. Another explanation may be that in swamp buffalo domestication captured a proportion of diversity from wild population with a high variability of uniparental markers but a generally lower nuclear genomic diversity. Following domestication swamp buffalo variation increased due to a further introgression from wild populations (Zhang et al. 2016).

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3 Advances in Buffalo Breeding: A Journey from Classical Breeding to Genomic Selection

G. R. Gowane and Vikas Vohra

Abstract

Buffalo is a domesticated species for milk, meat, and draft and it is naturally found in the Asian region. Importance of buffaloes varies within region, as in Indian subcontinent milk from buffaloes is a top commodity; however in South-east Asia, the meat followed by milk is important. Buffalo breeding in China has shown significant improvement in milk production due to crossbreeding of Riverine with Swamp buffaloes. The South East Asian region had the slow pace of progress. Buffalo breeding in India is very important owing to the contribution of buffaloes in total milk production (~50%). The traditional approaches of selecting the bulls of high genetic merit using progeny testing have resulted in improvement in significant per animal productivity for milk yield. However, owing to the sex-limited nature of trait, long generation interval, and low selection intensity, the progress was slow. Genomic selection (GS) has brought the paradigm change in the cattle breeding across the world. Similarly, the buffalo breeding may gain a significant leap in productivity enhancement, if the genomic selection is implemented in true spirit. GS can dramatically increase accuracy, reduce generation interval and can predict breeding values for traits which are recorded late in life, recorded after death, or recorded only on one sex. These properties make the GS a method of choice for milch buffalo breeding. However, as a breeder we must understand that in parallel, accurate recording of phenotypes, sufficient investment in traditional infrastructure and management is equally essential. Existing infrastructure for buffalo breeding schemes can be supplemented with genotyping of animals. Key issues in genomic selection for

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buffaloes will be creation of reference population which can be used for prediction of breeding values of selection candidates.

Keywords

Buffalo breeding · Genomic selection · Reference population · Single-step

3.1 Introduction

The population of buffaloes in the world is estimated to be nearly 207 million, which is distributed in 42 countries, out of which nearly 97% are homed in Asia and 3% are found in other part of the world (FAOSTAT 2019). Amongst the water buffaloes, the river buffaloes have high production performance as compared to swamp buffaloes. The differences in these two types are not only phenotypic but also genetic. The River buffalo has 50 chromosomes, out of which 5 pairs are sub-metacentric and 20 acrocentric, whereas for the Swamp buffalo, there are 48 chromosomes, out of which 19 pairs are metacentric (Borghese and Mazzi 2005). The riverine buffaloes constitute nearly 69% of total buffaloes in Asia and are mainly found in the Indian subcontinent (Sivarajasingam 1987). According to Livestock census (2019), the population of Buffalo in India is 109.9 million, out of which 55 million are adult female buffaloes which along with 81.4 million cattle produce 187.7 million tonnes of milk in India. Buffaloes produce 49% of total milk yield, out of which, 35% is contributed by Indigenous buffaloes and 14% by non-descript buffaloes (BAHS 2019). The share of milk production by buffaloes has always been nearly 50%, in spite of their low population size. Figure 3.1 depicts the population status of adult breeding female cattle and buffalo since independence and also total milk production. The swamp buffaloes are multipurpose and have nearly 30% share of population in buffalo species. Swamp buffaloes are mainly found in the China, Indonesia, Philippines, Thailand, and Vietnam. In this region, they are commonly engaged for draft purpose and meat and also for milk in some regions. The third category of buffaloes, the Mediterranean are predominant mostly in west Asia and constitutes nearly 1% of total buffalo population. The Indian subcontinent has world's best germplasm for buffaloes as far as milk production and adaptability to harsh climate is concerned. India has river and swamp type of Asian buffaloes. The wild Asian buffaloes are also part of the buffalo genetic diversity in India. Across the world, the genetic Improvement of Buffaloes is a recent trend and no systematic buffalo selection and breeding programs have been in place since long. Usually the buffaloes are selected for their size or growth rate, breeding and selection is practiced for milk, beef, and draft separately or in combination.

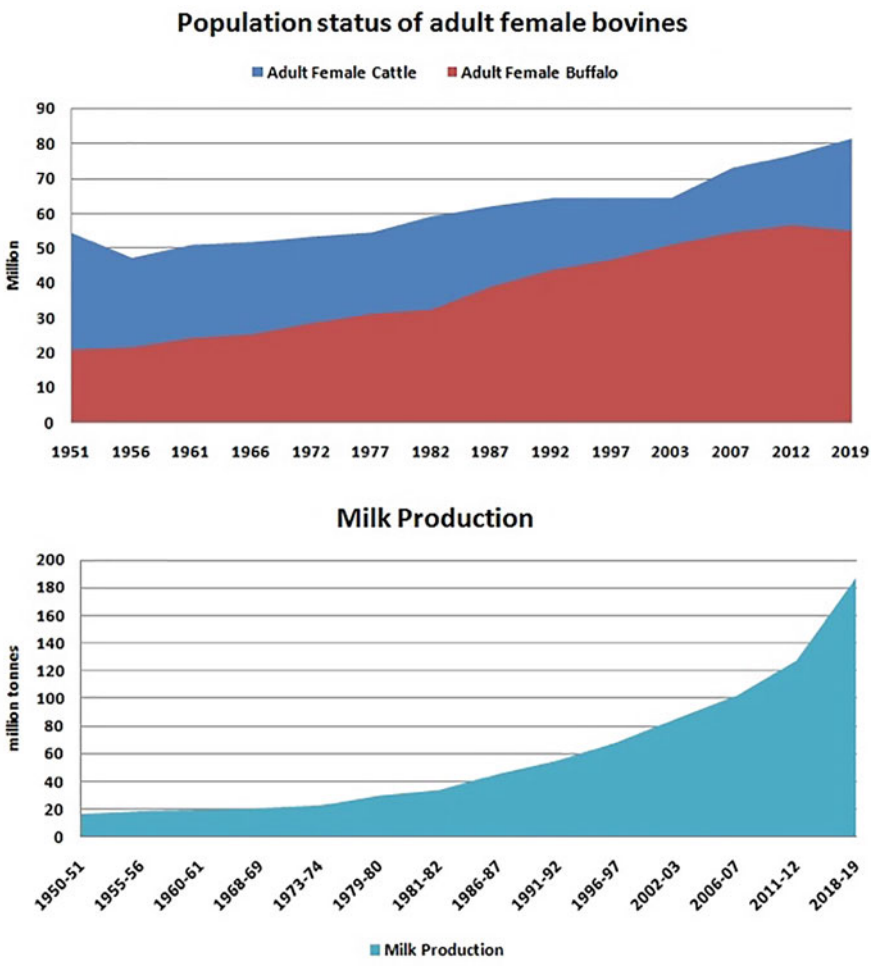

Fig. 3.1 Population status of adult breeding female cattle and buffalo

3.2 Buffaloes Play a Major Role for Milk Production in India

The importance of buffalo in India is mostly for the higher milk yield. As buffalo meat doesn't have any taboos associated with it, so the buffalo meat also forms a major source of income for many farmers. Importance of buffaloes was rightly identified in India during "white revolution" and we can see that the trend of increase in population of buffalo is more as compared to cattle. The increased production of milk has resulted in per capita availability of milk at 394 g per day (BAHS 2019). India has the best river-type milk breeds of buffalo in Asia such as Murrah, Nili-Ravi, Surti, and Jaffarabadi. They all have originated from the north-western part of

India and have a high potential for milk and fat production, as well as meat production (Sethi 2003). Currently India has 17 established and registered breeds (www.nbagr.res.in) which accounts for 35% descript buffaloes. There is still a great need to identify and record the non-descript germplasm. In different geographical regions of India, we can also observe the following types of buffaloes, viz. Tarai, Manda/Ganjam, Sambalpuri, South Kanara, Dharwari, Kuttanad, Jerangi, Godavari, Assamese/Mongoos, Sikamese, and Paralakhemundi (www.buffalopedia.cirb.res.in). The spread of buffaloes in India is ubiquitous; however, the majority of the population (72%) is concentrated in the north and western states, viz. Haryana, Punjab, Uttar Pradesh, Rajasthan, Gujarat, and Maharashtra (Kumar and Singh 2010). The important breeds and their details are as given in Table 3.1 below.

3.3 Improving Production Potential of Buffaloes in the World

Across the world, the genetic improvement for buffaloes mainly focused on either milk, carabeef, draft, or combination of everything, depending upon the region and utility of the product. Improvement programs in China mainly focused on swamp buffaloes which are numerous with population of 23.38 million (Yang et al. 2013) and mostly were used for draft purpose as the 99% population is swamp type with very low milk production. The genetic improvement involved crossbreeding of swamp buffaloes with exotic river-type dairy buffalo breeds such as Murrah and Nili-Ravi from India and Pakistan in 1957 and 1974, respectively (Yang et al. 2007). The improvement was significant and showed 1240.5 kg and 1423.3 kg average for first and second generation of Murrah crossbreds and 2041.2 kg and 2351.3 kg for Nili-Ravi crossbreds (Yang et al. 2013) as compared to the earlier average of 500–700 kg.

In Southeastern Asia, swamp buffaloes are primary animal; however, the focus from draft to beef, milk, and hide production has increased. The main reason is mechanization that requires almost no draft power for agriculture (Pineda et al. 2021). Several studies in southeast Asian region have shown the slow pace of genetic improvement programs in this region (Salas et al. 2000; Suryanto et al. 2002; Othman 2014; Ariff et al. 2015; Cruz 2015; Sanghuayphrai et al. 2013; Herrera et al. 2018; Komariah et al. 2020). People in this region especially in the rural areas are dependent on the local buffalo population for source of animal protein, both in the form of milk and meat. The genetic improvement for buffaloes in Thailand was initiated only in 1979 using BLUP. In Philippines, crossbreeding of riverine and swamp buffaloes was carried out to improve the performance as compared to local swamp buffaloes. The crossbreeding led to decline in reproductive performance. Backcrossing of these crossbreds with again the swamp- or river-type buffaloes was carried to produce 75% Philippine swamp-type for draft and 75% river-type for dairy purpose. Efforts for the genetic selection for superior germplasm with an aim to increase milk has also been done in the Philippine dairy buffaloes. Sporadic regional efforts in Malaysia, Indonesia, and Vietnam for increasing reproductive performance, meat yield, and milk yield have been done. In Indonesia, the

Table 3.1 Major buffalo breeds of India and their production status

Breed	Breeding tract	Lactation length (days)	Average lactation yield (kg)	Milk composition
Bhadawari	Agra, Etawah, Jaluan, Aurriya, Jhansi and Lalitpur districts of Uttar Pradesh and Bhind and Morena Districts of Madhya Pradesh	272	1294	Fat: 7.88% SNF:9.57%
Jaffarabadi	Saurashtra region of Gujarat especially areas in and around Gir forest, viz. Junagarh, Bhavnagar, Jamnagar, Porbandhar, Amreli, and Rajkot districts	271–305	2239	Fat: 7.68–8.25%
Marathwadi	Parbhani, Nanded, Beed, Jalna, and Latur districts of Maharashtra	302	1118	Fat: 8.8%
Mehsana	Mehsana, Sabarkantha, Banaskantha, Ahmedabad, and Gandhinagar districts of Gujarat	305	1988	Fat: 6.83%
Murrah	Rohtak, Jind, Hisar, Jhajhar, Fatehabad, Gurgaon, and the Union Territory of Delhi	305	1752	Fat: 7.3%
Nagpuri	The natural breeding tract of the breed is Ellichpur (Achalpur), Paratwada, Daryapur, Anjangaon-Surji tehsils of Amravati districts and Arvi tehsil of Wardha district, and also Nagpur and Yavatmal districts	286	1039	Fat: 8.25%
Nili-Ravi	Belt between the Sutluj and Ravi rivers of the undivided Punjab Province. Amritsar, Gurdaspur, and Ferozepur districts of Indian Punjab	294	1850	Fat: 6.8%
Pandharpuri	Solapur, Sangli, and Kolhapur districts of Maharashtra state	305	1790.6	Fat: 8.01% SNF 9.28%
Surti	Anand, Nadiad, Kaira, and Baroda district of Gujarat. The animals are also commonly found in South Rajasthan	305	1667	Fat: 7.02%
Toda	Nilgiri hills of Tamilnadu	200	500	Fat: 8.22% Protein 4.45%
Banni	Banni area of kachchh, which is a part of Kachchh district of Gujarat. Animals are found is Bhuj, Nakhatrana, Anjar, Bhaahau, Lakhpat, Rapar, and Khavda talukas	290–295	2857.2	Fat: 6.65–7.0%

(continued)

Table 3.1 (continued)

Breed	Breeding tract	Lactation length (days)	Average lactation yield (kg)	Milk composition
Chilika	Cuttack, Ganjam, Puri, and Khurda districts of Odisha	255–262	500	Fat: 8.7% Protein: 8.62–8.81%
Kalahandi	Kalahandi and Rayagada districts of Odisha	285	680–737	Fat: 7.86% SNF: 8.64%
Luit (Swamp)	Jorhat, Sibsagar, Lakimpur, Dibrugarh, Tinsukia, Dhemaji, Golaghat, Majuli, and Biswanath districts of Assam. The Luit (Swamp) buffaloes are mostly distributed in the upper Brahmaputra valley of Assam covering nine districts of upper Assam	NA	449.4	Fat: 8.68%
Bargur	Erode district of Tamilnadu	NA	1.5 to 2.0 L/day (~450 kg/lactation)	Fat: 8.59%
Chhattisgarhi	Common breeding tract is north hilly, central plains and Bastar plateau region of Chhattisgarh	240	1180	Fat: 9.49%
Gojri	Chamba and Kangra districts of Himachal Pradesh; Hoshiarpur, Gurdaspur, Ropar, Pathankot, and SAS Nagar (Mohali) districts of Punjab	192	700–1000	Fat: 6.5%

Sources: www.nbagr.res.in; https://www.buffalopedia.cirb.res.in/; https://www.dairyknowledge.in/; https://en.wikipedia.org/wiki/Banni_buffalo; http://www.fao.org/3/y4924t/y4924t0b.htm#bm11

buffalo breeding and management follows the traditional approach that leads to low production and accumulation of inbreeding.

Buffalo breeding in Pakistan is important owing to availability of superior germplasm and nearly 18% buffalo population of the world (Siddiky and Faruque 2017). The emphasis is on milk and meat both. Pakistan had 26.3 million buffaloes (Bilal et al. 2006), having a good production potential. Apart from meat and milk, for which Nili-Ravi and Kundi are famous in Pakistan, the draft power is also one of the major utility of buffaloes. However, there is a lack of systematic breeding and selection, owing to poor data recording and inefficient PT programs. Selection in right direction can pave a way for enhancing genetic profile of buffaloes in Pakistan. Bangladesh breeds buffaloes for meat, milk, and draft and genetic improvement of local swamp and riverine buffaloes by crossing with Murrah, Nili-Ravi, and Mediterranean buffaloes have been tried. The impact of improvement programs was not satisfactory due to poor planning and execution.

3.4 Genetic Improvement in Buffalo Has Seen Tremendous Leap in the Production Status in India

As per the estimates of FAOSTAT (2019), total milk production from buffaloes was 11.087 million tonnes in 1961 that increased to 91.82 million tonnes during 2018 (Fig. 3.2). This contributed to nearly 2.48% annual increment over these years. The contribution of the "white revolution" assisted by government efforts to increase per animal productivity was instrumental behind these achievements. According to BAHS (2019), the per animal productivity of buffaloes is continuously increasing as a result of genetic progress and since last 6 years (2013–2019), there has been 710 g enhancement in per day productivity for buffaloes. For the year 2018–2019, per buffalo productivity was 5.62 kg per day. The programs run by Government of India under the department of animal husbandry has executed several schemes for buffalo breeding, which involved mostly selective breeding in pure breeds and

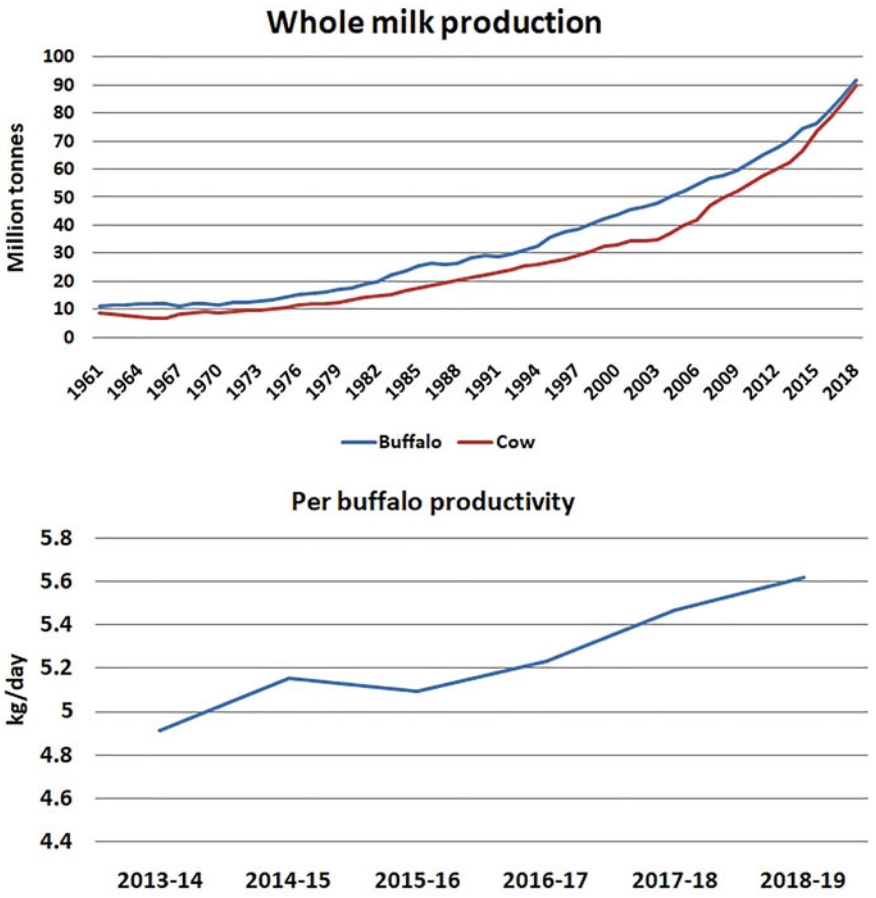

Fig. 3.2 Milk production and productivity of buffaloes

upgrading in non-descript buffaloes. However, unlike cattle, where huge technological and financial input is provided by developed nations and also top organizations such as International Committee for Animal Recording (I-CAR), InterBull, buffaloes remained behind cattle for investments in data recording and genetic improvement strategies across world. In the following 2 paragraphs, we will have a crisp look at progress made in the past for buffalo breeding in India.

Following programs and schemes for buffalo improvement have been implemented by GoI

- Establishment of Central/State Cattle (181) and Buffalo (29) Breeding Farms.
- Key Village Scheme (1950).
- CHRS—Identification of superior germplasm through performance recording under farmer herds.
- Establishment of sire evaluation units and Progeny testing schemes.
- National Project for cattle and buffalo breeding (2000).

After year 2010

- Dairy entrepreneurship development Scheme (2010).
- National Programme for Bovine Breeding and Dairy Development (2014).
- National Dairy Plan I (2011–2012 to 2018–2019) and NDP II (2020–2025).
- National Kamdhenu Breeding Centre for development and conservation of 39 indigenous cattle and 13 buffalo breeds (2014–2015).
- Rashtriya Gokul Mission (2014).
- National Livestock Mission (2014–2015).

In India, the importance of buffaloes has been well known to Government and hence central and state governments have been actively participating in the breed improvement programs with some importance for data recording and evaluation. In the third five-year plan, the progeny testing (PT) scheme in Murrah bulls was started to identify the best bulls based on the evaluation of their daughter's performance rather than relying on mother's milk production level (Thiruvenkadan et al. 2013). The PT selected and proven bulls were further used for insemination using artificial insemination technique for faster spread of better genetics. The collaboration from research institutes, state and central governments, NGOs and cooperatives used similar models for PT of Murrah and other indigenous breeds of buffaloes. The national Project on Cattle and Buffalo Breeding (NPCBB) since year 2000 was run by DAHD (GoI) for 10 years with an objective to deliver the improved genetics at farmer's door-step and improve the then available population for milk yield. 28 states and 1 union territory participated with establishment of 71,341 AI centers (DAHD 2020). The Central Herd Registration Scheme (CHRS) was implemented by GoI at some locations invested in recording of data and selection of elite animals available with farmers.

ICAR-Network Project on Buffalo Improvement (NPBI): The NWBI funded by Indian Council of Agricultural Research (ICAR-GoI) is a mega scheme, which was

initiated as "All India Coordinated Research Project on Buffaloes (AICRP-B)" during 1970–1971 and then transformed to NPBI since 1993. The scheme aimed at genetic improvement of important defined breeds through selective breeding. Murrah being the most important buffalo breed, the progeny testing (PT) in Murrah is carried out at 6 participating institutes, viz. CIRB Hissar, NDRI Karnal, IVRI Izatnagar, GADVASU Ludhiana, LUVAS Hissar, and ICAR research complex Patna (NPBI 2018). There are also three field units for Murrah at CIRB Hissar, NDRI Karnal, and GADVASU Ludhiana. The major aim is to evaluate the bulls across herds and in field for better selection accuracy. Apart from Murrah, other breeds such as Nili-Ravi, Jaffarabadi, Pandharpuri, Swamp, Surti, and Bhadawari are improved in institutional herds by selective breeding. Seven important buffalo breeds are currently covered through 18 principal and associated centers, which include associated herds/main units of Murrah (8), Pandharpuri, Surti and Jaffarabadi, field units for Murrah (3) and centers for genetic improvement and conservation for Swamp, Bhadawari, and Nili-Ravi breeds. A bull certification laboratory for disease diagnosis and testing of young bulls has been established as part of the project at CIRB, Hisar (Haryana). About 10 to 15 buffalo bulls are being tested in each cycle of 18 months on 1000 breeding females. Each bull under test is used to produce 60 to 70 pregnancies to obtain 20 to 25 performance recorded daughters. NDDB is organizing a similar program for the genetic improvement of Mehsana involving farmers' animals.

Studies at different centers and sub-centers of network project have shown improvement in both productive and reproductive performances. At present the target assigned for Murrah breed to various centers are average age at first service as 24 months with 300 kg body weight, average age at first calving as 40 months, average age for initiating training of bulls as 18 months with 350 kg body weight, average age at first collection of semen as 30 months with 400 kg body weight, average service period of 130 days, calf mortality (0–3 months) to be less than 5%, and wet and herd averages of greater than 8.5 and 5.5 kg, respectively. However, different targets are assigned to other buffalo breeds, keeping in view their genetics, production, and reproduction potential.

The National Dairy Development Board (NDDB) helped farmer's cooperatives by adopting national policies for buffalo breeding. This is producer owned organization and cooperative network operated in 285 districts. The concrete results in buffalo improvement by NDDB were observed in Gujarat, where under Dairy herd Improvement Program (DIPA), buffalo recording was started since 1987. The genetic merit of bulls was evaluated using nearly 30–50 daughter's performance (Moioli 2005). Under the National Dairy Plan (NDP)-I Murrah and Mehsana breeds were covered under progeny testing in Gujarat, Uttar Pradesh, and Haryana. Under PT 595 high genetic merit (HGM) Murrah bulls and 161 HGM Mehsana bulls were distributed apart from several thousand AI done. For the pedigree selection program, where PT was difficult, 30, 32, and 14 HGM bulls of Jaffrabadi, Pandharpuri, and Nili-Ravi, respectively, were made available (NDDB 2020). The NDP is now supported under Rashtriya Gokul Mission (RGM) of GoI for meeting out the supply of HGM bulls for semen stations.

3.5 Concept of Selection and Classical Problems of Animal Breeding

Selecting animals of best genetic worth was a great challenge for animal breeders until modern statistical genetic methods of selection were developed. Prior to this, usually animals with phenotypic information, or on immediate relative record (e.g., selecting bull based on its dam's milk yield) were followed. Inclusion of multiple sources of information and using multi-trait selection were great achievements in animal breeding for selection of best animals. Henderson (1975) showed that the Best Linear Unbiased Prediction (BLUP) provides unbiased estimates of breeding values in populations under selection; however, it is essentially required to include all the information used in the selection decisions. This was proved using simulation study by Sorensen and Kennedy (1984). The relationship that follows from the recorded pedigree can be structured in the numerator relationship matrix (NRM). BLUP is the most robust method of breeding value prediction as compared to any other method of prediction in the animal breeding programs as it accounts for the effect of selection and bias of culling, allows selection across age classes and also account for unequal usage of sires in different herds. Selecting best animals in each generation and propagating them should bring significant positive change in the average genetic merit of subsequent generations. Improvement in the overall genetics of next generation due to selection of parents in previous generation is called as the selection response. In the text below, we will try to understand the factors which may influence the selection response in a typical animal breeding experiment.

The ***"Breeder's equation"*** (Lush 1943) defines how the genetic improvement takes place in the breeding program. The hurdles in the traditional genetic improvement programs, especially in India are many. Given the principles of animal breeding, the response to selection in any selection experiment is bound to be influenced by factors in Eq. (3.1).

$$R = h^2 S \tag{3.1}$$

where R is response to selection, h^2 is heritability of a trait, and S is the selection differential, i.e., superiority of the parents to their contemporaries.

In a real life animal breeding program, obtaining response to selection is not so straightforward and there exists overlapping generations and also differential generation interval for males and females. The rate at which males and females are chosen to make them parents of next generation is also different. Thus, the response to selection is further expanded to include these factors (Falconer and Mackay 1996)

$$R = \frac{i_m + i_f}{L_m + L_f} \times \sigma_a \times r \tag{3.2}$$

where intensities (i) and generation intervals (L) can be attributed to the male and females in the population separately. σ_a is the estimate for additive genetic standard deviation and r is accuracy of selection.

3.5.1 Long Generation Interval

It is evident from Eq. (3.2) that the generation interval (L) always has an inverse relationship with the selection response, hence larger the L, lesser will be response. The generation interval can be defined as the average age of parents when the next generation (progeny) is born. Buffalo has long generation interval. In a breeding program, if older parents (males and females) are used for mating, there is increase in generation interval and the genetic gain is much slower. This is so, because with every generational progress we assume that the individuals born are better in genetic worth than the earlier generations. Use of younger parents (males and females) by replacing older generations, we can cut short on generation interval making rapid genetic gain. In the traditional breeding programs, we highly rely on the progeny testing as a gold standard for selecting the best bulls. The progeny testing of a bull takes somewhere between 10 and 15 years, by which time the bull's genetics is really outdated. However, as we do not have any means to identify the genetics of those bulls earlier, we relied heavily on the PT programs making the pace of the genetic progress slow.

There has not been any alternate way to reduce generation interval, as alternate approaches involve a great cost on the reproductive efficiency. In cattle breeding, the efforts were made for adoption of Multiple Ovulation Embryo Transfer (MOET); however, results were not encouraging due to high cost in MOET, so if system cannot afford, then it is better to invest on more basic things, like recording of phenotypes, pedigree, extension activities, and evaluation. Marker Assisted Selection (MAS) has proven to be the best bet for reducing generation interval in many plant breeding programs and a few animal breeding programs, where potential marker for the trait under selection was positively selected. However, as we know that most of the traits of economic importance are polygenic, mostly controlled by not one or two genes but several of them and each may contribute for less than significant proportion of variance. Hence, very few examples of MAS in animal breeding are available such as Booroola fecundity gene in sheep (Sharma et al. 2016) and MSTN (GDF8) mutation that underpins muscle hypertrophy (Clop et al. 2006).

3.5.2 The Intensity of Selection (*i*)

This is another factor which is very important and mostly ignored factor in any institutional animal breeding program. In simple analogy, students who crack national level entrance and get admission in top class business school are bound to be brilliant than those who somehow manage to get admission in local school. The difference is of selection intensity. In similar way, we need to select best parents from a huge number of individuals so that the best are selected with very high selection differential. Higher intensity of selection has been greatly practiced by livestock breeding in Europe, America, and Australia. The InterBull helps to link several herds together across regions so as to increase the intensity of selection and select the better genetics. In India, one of the major concerns since the

implementation of the projects have been low or very low intensity of selection. To meet the demand of the breeding program and mating design, it is essential to select a fixed number of bulls from every batch. What if we do not have the sufficient number of candidates for bull selection? Such a scenario will result in near zero intensity. For female path, most of the breeding programs consider zero intensity. Ironically, due to several reasons, the institutional herds face problem of low intensity of selection. A serious look at Eq. (3.2) will tell us that if the intensity is compromised, it will be next to impossible to obtain the response for the selection experiment. What we need here is to expand the population, from where we are selecting the parents.

3.5.3 The Genetic Variability of the Trait Affects Selection Response

The genetic variability for the trait of economic importance (e.g., milk yield in buffaloes) within the recorded population is measured by the standard deviation (σ_a). The σ_a measures dispersion of how many observations range around the mean. If the animals are close to the average, then the σ_a is low and if there is more variability and spread is wider then the σ_a is large. Now, we must understand here that the breeders do not have control over this component in a given population, as it is a property of the population for that trait. It is also essential to have wider spread of σ_a to obtain better genetic gain. One interesting thing is when we carry out selection continuously, there is decline in genetic variance resulting from the generation of linkage disequilibrium under selection (Bulmer 1976). However, is there a point of no return where variance vanishes completely due to selection? Simulation study carried over for >10 generations where intense selection was practiced, revealed decline in the additive genetic variance to a significant level (Gowane et al. 2019b). However, many animal breeding programs show contrasting results, as with several generations of continued selection pressure the variation still remains in the population. This is so, as mutations and crossover during meiosis help to create more and more variation every generation. Not only this, but also, purchase of new animals in the breeding program may also help to maintain the level of genetic variance to the desired level, thus helping to gain the results of selection experiments.

3.5.4 The Accuracy of Selection Also Affects the Response to Selection Directly

The accuracy depends on square root of h^2, if selection is on individual's own measurement (e.g., weaning weight). However, when selection is done using expected progeny difference (EPD) or differences in performance between 2 animals based on their future offspring when each is mated to animals of the same average genetic merit, the accuracy is larger.

Selection of buffaloes for milk improvement has a classical problem of sex-limited trait. We are actually selecting bulls for higher milk yield; however, bulls don't give milk. Thus, the progeny testing of bulls is the only proven method for selection. The selection of buffalo bulls has therefore been tedious, time and resource consuming exercise, which is however essentially required. Limiting the rate if inbreeding is very important in a breed improvement program. Especially in the closed herd, it is essential to use more males and keep sufficient sire lines so as to avoid the breeding within same sire line. Effective population size needs to be estimated and care needs to be taken to keep it above 50.

3.6 Buffalo Breeding Also Faced the Inherent Challenges of Classical Breeding

The buffalo breeding in India has also faced challenges of low intensity of selection, low selection differential, long generation interval, and selection for the sex-limited trait, where males need to be selected for milk yield potential. Record keeping in India is a huge challenge, due to which most of the field-related improvement activities could not achieve desired success. The nucleus of breeding animals which are mostly with government organizations are supposed to spread the better genetics in the field through two or three tier system. Replacement of the breeding stock from within the nucleus and also outside is also required. However, owing to limitations as mentioned above, the success achieved was limited. Figure 3.3 depicts the multi-tier breeding structure with selection in nucleus and also in other downline tiers and also upward tiers migration of superior genetics (Van der Werf 2000). Although the structure depicted here is highly desired, it is not followed in principle due to hurdles as mentioned above. Thus, the need to shift from the traditional breeding programs to the newer platforms was felt. Genomic selection seems to fulfill the gaps which were observed in the traditional program and support the selection schemes efficiently. Today we know that the genomic selection is a one-step solution for reduction of generation interval, selection for traits with low heritability, selection for sex-limited traits and later in life or post death measured traits. Milk yield in buffalo being the sex-limited trait had always have a problem of selecting best bulls as we need to rely on closer relatives or daughter's records. The GS has advantage of selection of the bull based on its own genetic merit. The details of the GS are as discussed below.

3.7 The Paradigm Change: Genomic Selection Has Prospects for Better Genetic Gains in Buffalo Breeding

During the year 2001, the methodology for the genomic selection (GS) was developed (Meuwissen et al. 2001). The GS is an expanded form of the MAS in which genetic markers covering the whole genome are used to make selection decisions, so that all quantitative trait loci (QTL) are in linkage disequilibrium (LD) with the panel

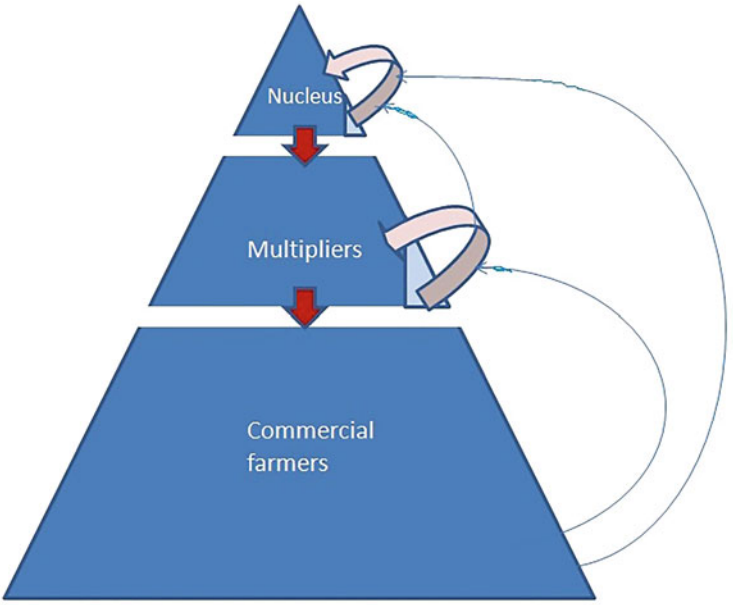

Fig. 3.3 Multi-tier desired breeding scheme. (Source: Van der Werf 2000)

of dense markers (Goddard and Hayes 2009). In the GS, benefit of LD of markers with QTL is harvested.

With advancements in genotyping technology and publication of human and bovine genome, the race for development of SNP-chips fast paced. The Axiom buffalo genotyping array were the first of its kind to provide services for identifying large-scale genome-wide detection of SNP in buffaloes. Thakor et al. (2018) studied 7 buffalo breeds and observed low inbreeding coefficient. The breeding program for Murrah buffalo in India under Network program has six institutional herds at various locations throughout the country along with the field progeny testing centers in three locations. The total number of breeding females ranges near 1170. Annually, approximate 15,000 inseminations are made in field. Marshall et al. (2017) gave a roadmap for creation of reference by genotyping at least 3000 animals which also have phenotypic data and are a part of breeding program, using the Axiom® Buffalo Genotyping Array with 90K SNPs developed from eight river buffalo breeds (Italian Mediterranean, Murrah, Nili-Ravi, Jaffarabadi, Kundhi, Aza-Kheli, Egyptiana, and Swamp type from Philippines) and subsequently verified on the Italian Mediterranean and Brazilian Murrah breeds. This reference if created can straightway help in switching from traditional to genomic selection platform.

Ananthasayanam et al. (2020) developed high quality haplotype phased buffalo genome assembly in Murrah. These developments lead NDDB along with Animal Breeding Research Organisation (ABRO) for development of genotyping microarray chip for buffaloes. Two hundred ninety six animals of 9 buffalo breeds

were sequenced for identifying SNPs to develop the genotyping chip in buffaloes "BUFFCHIP" which is suitable to genotype Indian buffalo breeds and their crosses (Nayee et al. 2020). The BUFFCHIP developed by NDDB along with AGIL, ARS-USDA is a custom buffalo genotyping chip developed on Illumina-Infinium HTS platform. It has 59,967 SNPs in final array with a median gap size of 41 kbps between SNPs. The usable SNPs on array are 54,776. Nayee et al. (2020) reported the use of chip increased the accuracy of genomic estimates of breeding value (GEBV) by 75% as compared to pedigree based EBV and 140% as compared to dam's yield as selection criteria.

3.7.1 Slow Pace of Buffalo Genomic Selection in the World

In other countries, the genome-wide association of SNPs with milk production has been studied. In Italy, the selection of riverine buffalo has improved the production potential for milk significantly. Liu et al. (2018) showed that the reliability of genomic estimated breeding values (GEBV) of 6 milk production traits ranged from 0.06 to 0.22, and the correlation between estimated breeding values and GEBV ranged from 0.23 to 0.35, which was a significant step towards implementation of GS. Pakistan brought significant improvement in Nili-Ravi buffaloes through traditional selection. The implementation of GS for Nili-Ravi has shown to reduce the generation interval in male-to-male pathway from 9.5 to 3.3 years and brought two-fold increase in response to selection as compared to progeny testing. It has also shown to reduce the cost of proving bulls by 88% (Moeen-ud-Din et al. 2014). Many reports are available where genome-wide association of SNPs have been tried for production traits of buffaloes (Liu et al. 2018; Mokhber et al. 2019); however, implementation of GS is yet not common as it is in cow across the world.

3.7.2 How Does the Genomic Selection Actually Work?

The procedure of genomic selection has two steps. They mainly deal with estimation of marker effects and prediction of genomic estimated breeding values (GEBVs). The details of these steps are as below:

Step 1: Estimating the Effect of Chromosome Segments in a Reference Population A sufficiently large reference population is essentially required for estimation of the effects of chromosome segments, or SNPs which are closely related to QTLs. Reference is a group of animals which have both genotypes (SNPs ~50 K) and phenotypes (of desired traits, such as milk yield, growth rate, etc.) meticulously recorded. The Genome-Wide Association Study (GWAS) is performed to estimate the effect of the markers. Least squares, ridge regression, Bayes A, and Bayes B are some of the methods for assigning marker effects.

The step 1 is difficult with respect to creation of reference as creating a reference of random samples will not be much accurate. Genotyping as many number of

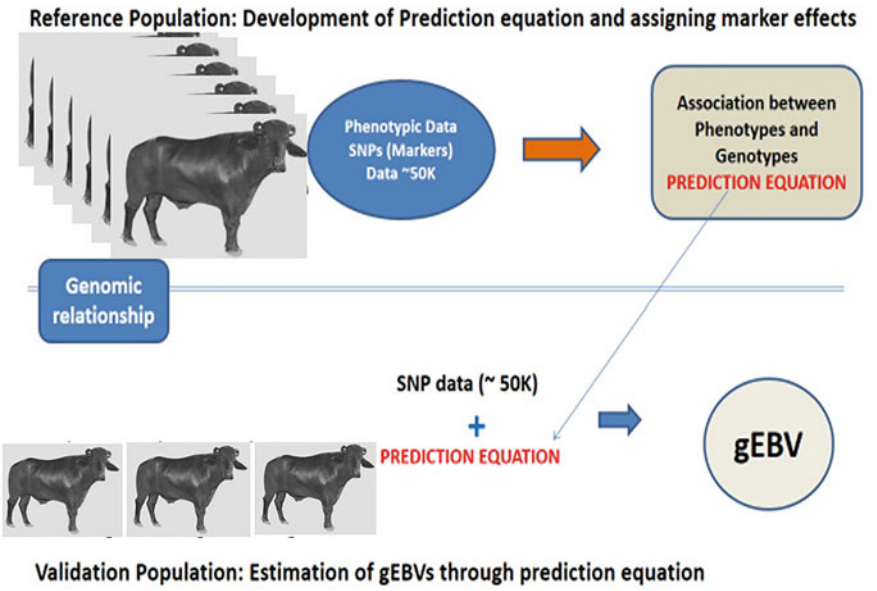

Fig. 3.4 A typical genomic evaluation program using genomic data of 50 K for prediction of GEBV in validation population

animals as possible is also not a solution as genotyping is a costly affair. Thus, creation of a reference is the first and most important step in GS methods.

Step 2: Prediction of Genomic EBVs (GEBVs) for Animals Not in the Reference Population This is relatively easy step, if genotypes are available for the selection candidates. The selection candidates are the individuals like progeny population, for which genomic breeding value is expected and phenotypes are not available (e.g., for young bulls, predicting their merit for milk yield potential). Figure 3.4 describes in pictorial format the method adopted for prediction of breeding values using GS.

Step 2 also uses the BLUP (Henderson 1975); where, the numerator relationship matrix (**NRM**) is replaced by genomic relationship matrix (**GRM**) based on marker relationships between the individuals. **GRM** is a realized relationship matrix and hence the explained relationship is more accurate than approximations based on pedigree relationships. **GRM** plot the relationships exactly based on the genomic information; however, **NRM** is based on expected relationships and many relationships take 0 values, which actually has a negative or near 0 values, when estimated through **GRM**.

The mixed model equations for Genomic BLUP (GBLUP) is

$$\mathbf{Y} = \mathbf{Xb} + \mathbf{Zg} + \mathbf{e} \tag{3.3}$$

where **Y** is the vector of phenotypic values, **X** is a vector of 1s, **g** is a vector of additive genetic effects due to the *i*th marker allele or haplotype, **Z** is an incidence matrix and has a 0, 1, and 2 for the number of alleles for the marker effect at locus *i* as present in the *j*th animal.

The equation can also be written as

$$\begin{bmatrix} \mathbf{X}'\mathbf{X} & \mathbf{X}'\mathbf{Z} \\ \mathbf{Z}\mathbf{X}' & \mathbf{Z}'\mathbf{Z} + \frac{n\sigma_e^2}{\sigma_{\mathbf{G}}^2}\mathbf{I} \end{bmatrix} \begin{bmatrix} \boldsymbol{\beta} \\ \mathbf{G} \end{bmatrix} = \begin{bmatrix} \mathbf{X}'\mathbf{Y} \\ \mathbf{Z}'\mathbf{Y} \end{bmatrix}$$

where **X** is a vector of 1s, **G** is a vector of genetic effects due to the *i*th marker allele or haplotype, **Z** is an incidence matrix and has a 0, 1, and 2 for the number of alleles of type Gi present in the *j*th animal.

The traditional selection using pedigree information was accurate and unbiased and replacing the pedigree based BLUP with genomic BLUP (GBLUP) has issues of not including the selection decisions, and pedigree information that leads to significant bias of prediction in selected populations such as institutional herds (Gowane et al. 2019b). However, there are also other issues like many a times we do not have sufficient funds to create a reference and also do not have complete pedigree, or pedigrees are truncated and available only for a few recent generations. In such a case merging the pedigree information with the genomic information can give much better results for compromising bias and increasing the accuracy of prediction.

3.7.3 Single Step Can be a Better Solution for Indian Genomic Selection Program

Single Step combines the pedigree relationships with genomic relationship matrices and extends the genomic information from genotyped animals to non-genotyped animals which are available in the pedigree. The relationship matrix that combines pedigree and markers is then called **H** matrix. Fitting $\mathbf{H^{-1}}$ in Henderson's MME gives the Single Step Genomic BLUP (SSGBLUP), which is a single estimator of breeding values that includes all available information. SSGBLUP (Legarra et al. 2009; Christensen and Lund 2010) combines genomic relationships from genotyped and pedigree relationships with non-genotyped individuals, hence this integration should allow information on unselected animals and trace back to a conceptual unselected base population.

$$\mathbf{H}^{-1} = \mathbf{A}^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & \mathbf{G}^{-1} - \mathbf{A}_{22}^{-1} \end{bmatrix} \tag{3.4}$$

where $\mathbf{A^{-1}}$ is inverse of **NRM**, $\mathbf{G^{-1}}$ is inverse of **GRM**, and $\mathbf{G}^{-1} - \mathbf{A}_{22}^{-1}$ is a correction to avoid double counting of genotyped animals.

In our recent study (Gowane et al. 2019b), we observed that even in the highly selected populations over 10 current generations, if the selective genotyping of small percentage of individuals is done and used in single-step approach, significant improvement in accuracy of prediction and relatively unbiased estimates are obtained as compared to only genomic or only pedigree selection method. Hence in country like India where still genotyping is a costly affair and authentic pedigree information with phenotypes is available with many government farms, SSGBLUP approach seems to be the better option for implementation of genomic selection.

3.7.4 Where Do We Stand and Can We Benefit from This Technology?

One major problem with the genetic evaluation program in Asia as a whole and India in particular is lack of pedigree records for field livestock. Recording daughter's performance for milk is tough task that need huge investment on part of government. This has restricted the inclusion of sufficient numbers of animals in genetic evaluation. With the possibility of genomic selection (GS), these animals without pedigree can also be included in the selection program for more intense selection, which may lead to increased accuracy and reduced generation interval (Gowane et al. 2019a). The cost of genotyping of the animals is still high (US$ 100 per sample) by Indian standards. However, with availability of low cost SNP chip for buffalo (Nayee et al. 2020) and several other efforts in ongoing programs in India and other countries, the application of GS for mainstream selection programs seems reality. Large ruminants have direct relation to industry, as milk is the commodity that has somehow succeeded in creating the link to industry (quantity of milk produced, fat content of milk) and thus commodity can directly be converted to money. However, a few things are essential for translating the gains of GS to farmers such as strong linkages between livestock producers and market and its related aspects.

The models which need to be applied for implementation of the genomic selection is a matter of concern as we have a loosely bound livestock structure, where main-stakeholder is farmer and he/she has a small herd of varying genetic makeup. Thus, we need to understand the impact of creation of reference using such population on the overall prediction accuracy as well as bias. Dedicated research on this aspect may come out with models for creation of reference population. Other approach may be to utilize the existing genetic improvement schemes and infrastructure available with the Govt and NGOs for buffalo breeding (e.g., Network project on buffalo in India), where existing pedigree and PT based programs can be easily supplemented for genomic data creation and implementation of single step genomic selection schemes. The high genetic merit bulls from this scheme can then be used for dissemination of superior genetics in the field as shown in Fig. 3.3. However, we must not forget the importance of parallel investment in the conventional data recording and management system while implementing GS.

Gaining insight from the cattle breeding programs, we must also understand that selecting buffaloes only for higher milk and ignoring every other aspect may bring

unknown challenges in future, especially in the developing world. Hence, breeding goals must be set up with futuristic vision where selection programs can be directed for desired gains in limited time and resources. Use of multi-trait selection with proper weightage to the trait of importance combined with genomic technology can lead to holistic selection of breeding buffaloes. Advent in the technology of GS can also be supplemented by modern aids such as machine learning. A strong technical knowhow at implementing agencies is also essential which needs investment on human resources. Genetic improvement is ongoing process which is benefitted by modern evaluation tools. BLUP with GS will increase the rate of genetic gain inside the breeding nucleus of buffaloes. The superior genetics can therefore be spread from the breeding nucleus to the commercial herds or multiplier herds for realizing the gains of selection under breeding program.

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Reproductive Management of Dairy Buffaloes

4

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Abstract

Buffalo has a fundamental role in the economic sustainability because of its considerable contribution in meat and dairy industry; however, reproductive performance of this species is compromised. Timing of artificial insemination on detected estrus (AIDE) has been optimized using AM-AM or PM-PM rule to maximize the pregnancy per AI (P/AI) in buffaloes. Initially, estrus synchronization was performed either injecting single shot of prostaglandin $F_{2\alpha}$ or applying standard ovsynch protocol to perform AIDE and fixed-time artificial insemination (FTAI), respectively; however, the P/AI varied substantially between these protocols. The advent of progesterone releasing devices such as PRID and CIDR along with the incorporation of either estradiol benzoate or gonadotropin releasing hormone has revitalized the synchronization programs with greater intensity of estrus and tighter synchrony of ovulation in buffaloes. Besides traditional regimens of synchronization programs, the standardization of doses of equine or human chorionic gonadotropins along with CIDR protocol ensure their safe application and usage to improve fertility in summer anestrus buffaloes.

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Although the solutions to reproductive problems have been encapsulated, their applications are still challenging in small or commercial buffalo herds.

Keywords

Synchronization · Fixed-time AI · Pregnancy per AI · Fertility · Dairy buffalo

4.1 Introduction

Buffaloes (*Bubalus bubalis*) are domesticated farm animals in tropics and sub-tropics having unique abilities to convert poor quality roughages into high-fat milk, greater adaptability to harsh climatic conditions, and somewhat resistant to various tropical diseases (Gordon 1996). The total buffalo population exceeds 200 M and found in 46 countries with over 20 distinct breeds, producing almost 1500–4500 L of milk per lactation that meet more than 13% of the world's milk requirements. Interestingly, nearly 97% of the world's population of buffaloes prevail in Asia primarily in India, Pakistan, and China, and often termed as "Black Gold of Asia." Initially, prior to domestication, buffaloes were raised mainly by nomads along the coast of rivers and marshy areas of the Indus valley, thereafter, buffalo rearing farms were established to meet the growing requirements of milk and dairy products.

Buffaloes contribute a major share of the agricultural economy and ensure food security to the farmers of the Southeastern part of Asia. Nili-Ravi buffaloes can be considered as an ideal dairy animal with a genetic potential to produce 30 L of milk per day (Bhatti et al. 2010) with 6.5% contents of fat in the milk (Hussain et al. 2006). Besides these remarkable productive traits, the reproductive parameters such as age of puberty, estrus expression, calving interval, conception rate, and pregnancy survival are still not up to the mark in dairy buffaloes (Singh et al. 2000; Ahmad and Arshad 2020; Campanile et al. 2016). To overcome these reproductive issues, many hormonal strategies have been attempted to enhance the reproductive outcomes of dairy buffaloes (De Rensis and Lopez-Gatius 2007; Carvalho et al. 2016). Reports have indicated that estrus and ovulation synchronization are the key strategies to enhance the fertility of buffaloes.

Initially, protocols primarily developed for estrus synchronization in cattle (Stevenson and Britt 2017) were used in buffaloes during the last six to seven decades. The basic principle of these protocols comprised of either shortening the luteal phase with the help of prostaglandins (PG) $F_{2\alpha}$ (Lauderdale 2009) or prolonging the luteal phase using progesterone (P_4) releasing devices (Martinez et al. 1997) to achieve estrus synchronization. Furthermore, the application of conventional single or double PG (Chohan 1998) or standardized ovsynch protocols (Warriach et al. 2008), and usage of new or used P_4 devices (Naseer et al. 2011) helped to achieve acceptable pregnancy rate in buffaloes. The administration of estradiol benzoate (EB) in conjunction with P_4 treatment such as controlled internal drug release (CIDR) device enhanced the intensity of estrus signs specifically to treat

summer anestrus in nulliparous and parous dairy buffaloes (Yousuf et al. 2015). Furthermore, administration of equine chorionic gonadotropin (eCG) hormone has also been reported to improve the ovarian dynamics in buffaloes (Carvalho et al. 2013). More recently, the use of gonadotropin-releasing hormone (GnRH) 36 h before AI seemed to further tighten the synchrony of ovulation with improved pregnancy per AI (P/AI) in dairy buffaloes (Haider et al. 2015).

Although synchronization of estrus proved to enhance the reproductive efficiency, however, the advent of resynchronization of estrus has emerged as an aggressive approach to enhance herd fertility in buffaloes. The concept of resynchronization was opted from dairy cows in order to enhance pregnancy rate, and also in an attempt to increase pregnancy survival and reduce number of days open in buffaloes (Arshad et al. 2017). Resynchronization protocol involves the earliest diagnosis of pregnancy and timely re-breeding of non-conceived females, subsequently improving cumulative pregnancy rate of commercial buffalo herds (Neglia et al. 2018). Despite several strategically advancements, the main objective of enhancing fertility has always been a continuous challenge because of greater incidence of embryonic mortality in buffaloes (Qayyum et al. 2018). Different factors reported to be responsible for embryonic mortality including photoperiodicity (Di Francesco et al. 2012), reduced circulatory concentrations of P_4 (Campanile et al. 2010), uterine milieu (Balestrieri et al. 2013), and decreased luteal blood flow because of lower size and quality of luteal tissue in buffaloes (Neglia et al. 2012). Previously, many hormonal therapies were utilized to curb the incidence of embryonic losses in buffaloes. For instance, one of the strategies is to inject either GnRH or human chorionic gonadotropin (hCG) or P_4 on day 5, 12, 23, or 25 (Arshad et al. 2017; Pandey et al. 2013, 2015; Campanile et al. 2007, 2008) post-AI. The principle for using these hormonal preparations is to enhance the circulatory concentrations of P_4 through formation of accessory corpus luteum (ACL) that facilitates the embryonic attachment and placentation around day 25 post-breeding, and subsequently increasing the survival rate of buffalo embryos (Campanile et al. 2016). In addition to hormonal manipulations, reproductive physiology is greatly influenced by different factors including body condition score, parity (Yousuf et al. 2015), day-light length (Di Francesco et al. 2012), and cyclic or acyclic status of the ovaries (Neglia et al. 2003) for the successful accomplishment of breeding programs in buffaloes. Previously, numerous reviews have excellently summarized the various aspects of buffalo reproduction (Singh et al. 2000; Ahmad and Arshad 2020; Campanile et al. 2016; De Rensis and Lopez-Gatius 2007; Carvalho et al. 2016; Perera 2008; Das and Khan 2010; Warriach et al. 2015); however, the main objectives of this book chapter are to encapsulate the solutions of routine reproductive problems using key principles of improved reproductive management in dairy buffaloes.

4.2 Breeding Programs in Buffaloes

4.2.1 Natural Breeding

Natural service is the conventional method of breeding and predominates and practiced in different sub-continent countries than artificial insemination (AI; Thomas 2008). The higher cost of feeding, labor, transmission of sexually transmitted diseases, and steady dissemination of genetics are the main issues of this breeding method. Qayyum et al. (2018) reported that P/AI after natural service was significantly greater ($P < 0.05$) during peak breeding season (PBS; 63%) than low breeding season (LBS; 48%), when determined at day 30 post-breeding, in dairy buffaloes. Although natural service had a higher conception rate, the absence of bulls with superior genetics and an approximately low ratio of male to females (1:140) pushed the farmers to adopt AI in buffaloes.

4.2.2 Artificial Breeding

Artificial breeding is the reproductive biotechnology used for rapid genetic improvement in the shortest possible time. Initially, AI was performed using liquid semen, later on, liquid semen was replaced with frozen semen, and recently AI using sex sorted semen has also been attempted in dairy buffaloes. Using liquid semen, a lower fertility rate was reported in buffaloes as compared to cows; however, buffaloes inseminated with frozen semen showed a lower pregnancy rate than that of the liquid semen. AI has played an imperative role in selecting superior genetics, controlling diseases, and reducing breeding costs in dairy cattle (López-Gatius 2012). Owing to the fact of similar anatomical and physiological aspects of reproductive organs between cattle and buffalo, the methods of AI which were initially developed for cattle also adopted in dairy buffaloes. Therefore, without having any preliminary knowledge about buffalo breeding, a common practice to perform AI in cattle using AM-PM rule (AI after 12 h of standing estrus) was also adopted in dairy buffaloes (Drost 2007). Whenever buffaloes were inseminated using AM-PM rule, the number of services per pregnancy increased, whereas the P/AI decreased, and subsequently the adaptability of AI was not conceived by the farmer community (Anzar et al. 2003). In this background, Riaz et al. (2018) conducted an experiment to optimize the timing of AI at detected estrus in dairy buffaloes. The authors detected standing estrus using penile deviated buffalo bull, and then randomly enrolled buffaloes to be inseminated artificially either at 0, 12, 24, or 36 h after the beginning of standing estrus. The authors observed that P/AI at 24 h (53%) was greater ($P < 0.05$) as compared to 12 h (37%) after the onset of standing estrus, primarily because of delayed timing of ovulation (34.5 ± 0.96 h (range: 30 to 42 h) in buffaloes as shown in Fig. 4.1a. This delayed ovulation in buffaloes suggests that they might have a distinct patterns of release of luteinizing hormone (LH) regulating hypothalamo-hypophyseal gonadal axis differently than dairy cows. In order to improve the reproductive performance, various hormonal regimens have been investigated to

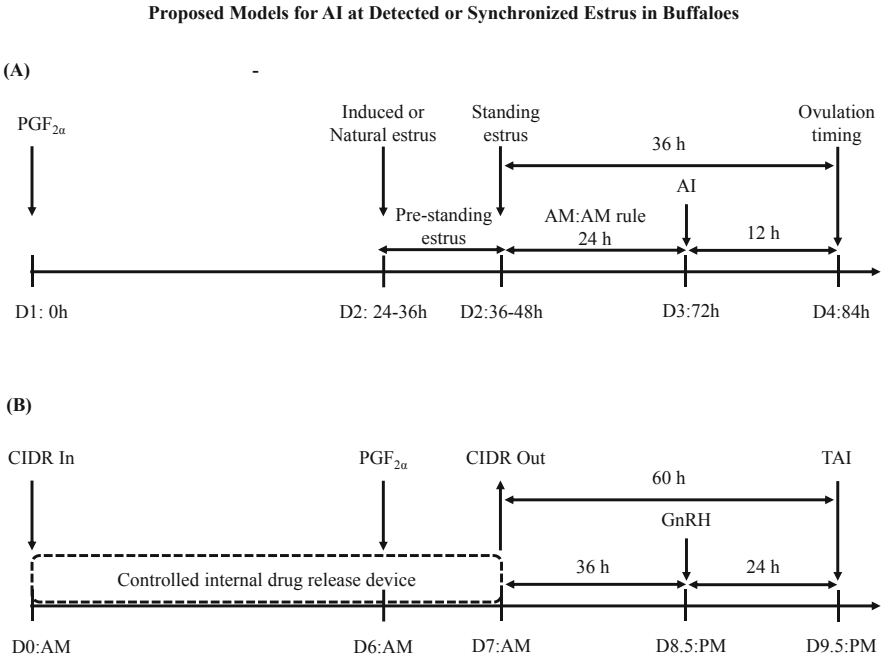

Fig. 4.1 Represents the unique models for timing of AI for detected or synchronized estrus in dairy buffaloes. (**a**) Schematic diagram presenting AI at detected estrus using AM-AM or PM-PM rule in which prostaglandin ($PGF_{2\alpha}$; 25 mg dinoprost tromethamine) is administered in cyclic buffaloes on Day (D) 1 which induces estrus almost 24 h later or buffaloes detected in natural or spontaneous estrus. The pre-standing estrus was observed for almost 9 to 12 h leading to standing estrus in which buffaloes stand to be mounted for 12 to 14 h. The AI was performed 24 h post-standing estrus (AM: AM) to attain maximum pregnancy per AI in buffaloes. Generally, the ovulation window is delayed and takes almost 36 h post-standing estrus in buffaloes. (Adapted from Riaz et al. 2018). (**b**) Presents schematic representation of CIDR-GnRH based modified protocol to adopt during summer anestrus in dairy buffaloes. (Adapted from Haider et al. 2015)

synchronize the estrus and ovulation in buffaloes (De Rensis and Lopez-Gatius 2007; Carvalho et al. 2016).

4.3 Synchronization Programs in Buffaloes

4.3.1 Use of Prostaglandins and Gonadotropin-Releasing Hormones

The detailed description of synchronization protocols that have been investigated is presented in Table 4.1. In modern-day animal agriculture, AI is regarded as the backbone of breeding programs; however, it is associated with different limiting factors including estrus detection and timely insemination. Generally, buffaloes are

Table 4.1 List of references including parity, breed, season, breeding method, pre- and post-insemination hormonal interventions, timing of AI with reference to onset of standing estrus or respective synchronized protocols, pregnancy per AI, and late embryonic losses in dairy buffaloes

Reference	Parity[a]	Breed	Season[b]	Breeding[c]	Spontaneous or synchronized or resynchronized estrus		Pregnancy/AI[f], %	Embryonic losses[g], %
					Pre- and post-insemination interventions[d]	Timing of AI[e]		
Paul and Prakash (2005)	P	Murrah	TBS	FTAI	GnRH–PG–GnRH	12 and 24 h after GnRH	33 (5/15)	–
Baruselli et al. (2007)	P	–	LBS	FTAI	GnRH–PG–GnRH	12 h after GnRH	28 (11/39)	–
	P		LBS	FTAI	CIDR in+EB–PG–CIDR out–eCG–hCG	14 h after hCG	54 (46/86)	–
Warriach et al. (2008)	P	Nili-Ravi	PBS	AIDE	PG	12 and 24 h after OSE	48 (25/52)	–
	P		PBS	FTAI	GnRH–PG–GnRH	12 and 24 h after GnRH	28 (8/29)	–
	P		LBS	AIDE	PG	12 and 24 h after OSE	38 (15/39)	–
	P		LBS	FTAI	GnRH–PG–GnRH	12 and 24 h after GnRH	23 (7/23)	–
Naseer et al. (2011)	P	Nili-Ravi	LBS	AIDE	CIDR in–PG–CIDR out	12 and 24 h after OSE	37 (11/30)	–
Husnain et al. (2013) [UB][h]	P	Nili-Ravi	Both	FTAI	CIDR in–PG–CIDR out–*hCG*	48 and 60 h after CIDR out	65 (44/68)	5 (2/44)
	N		Both	FTAI	CIDR in–PG–CIDR out–*hCG*	48 and 60 h after CIDR out	59 (20/34)	–
Rosi et al. (2014)	P	Italian	TBS	FTAI	GnRH–PG–GnRH+*Resynch with ovsynch*	18 and 24 h after GnRH	46 (115/250)	12 (16/131)[i]
	P		LBS	FTAI	GnRH–PG–GnRH+*Resynch with ovsynch*	18 and 24 h after GnRH	49 (203/414)	9 (19/223)

	P		TBS	FTAI	GnRH–PG–GnRH+*Resynch with ovsynch*	18 and 24 h after GnRH	52 (230/444)	7 (16/246)
	P		PBS	FTAI	GnRH–PG–GnRH+*Resynch with ovsynch*	18 and 24 h after GnRH	47 (112/240)	8 (10/122)
Hoque et al. (2014)	P		PBS	FTAI	GnRH–PG–GnRH	16 h after GnRH	28 (7/25)	–
	P		PBS	FTAI	PG–GnRH–PG–GnRH	16 h after GnRH	36 (8/22)	–
	P		PBS	FTAI	GnRH–PG–GnRH– GnRH–PG–GnRH	16 h after GnRH	44 (8/18)	–
Mirmahmoudi et al. (2014)	P	Murrah	PBS	FTAI	PG–GnRH–PG–EB	48 and 60 h after EB	56 (16/29)	–
Devipriya et al. (2015)	P	Murrah	PBS	AIDE	P_4 sponge in–PG–P_4 sponge out	–	67 (16/24)	–
Yousuf et al. (2015)	N	Nili-Ravi	PBS	FTAI	CIDR in–PG–CIDR out	48 and 60 h after CIDR out	41 (19/46)	–
	P		PBS	FTAI	CIDR in–PG–CIDR out	48 and 60 h after CIDR out	47 (50/106)	–
	N		PBS	FTAI	CIDR in–PG– CIDR out–EB	48 and 60 h after CIDR out	38 (17/45)	
	P		PBS	FTAI	CIDR in–PG– CIDR out–EB	48 and 60 h after CIDR out	55 (55/101)	
Haider et al. (2015)	N, P	Nili-Ravi	PBS	AIDE	CIDR in–PG–CIDR out	60 h after CIDR out	37 (19/51)	–
				FTAI	CIDR in–PG–CIDR out–GnRH	24 h after GnRH injection	59 (10/17)	–
Waqas et al. (2016)	P	Nili-Ravi	PBS	FTAI	PG_GnRH–GnRH–PG–GnRH	16 h after GnRH injection	56 (14/25)	–
Arshad et al. (2017)	N, P	Nili-Ravi	PBS	FTAI	CIDR-GnRH+*Resynch with GnRH*	–	63 (40/63)	13 (5/40)

(continued)

Table 4.1 (continued)

Reference	Parity[a]	Breed	Season[b]	Breeding[c]	Spontaneous or synchronized or resynchronized estrus		Pregnancy/AI[f], %	Embryonic losses[g], %
					Pre- and post-insemination interventions[d]	Timing of AI[e]		
Qayyum et al. (2018)	P	Nili-Ravi	PBS	NS	–	–	63 (25/40)	20 (5/25)
				AIDE	–	24 h after OSE	43 (17/40)	24 (4/17)
			LBS	NS	–	–	48 (12/25)	33 (4/12)
				AIDE	–	24 h after OSE	32 (8/25)	50 (4/8)
Riaz et al. (2018)	P	Nili-Ravi	PBS	AIDE	–	0 h after OSE	26 (8/30)	–
					–	12 h after OSE	37 (10/27)	–
					–	24 h after OSE	53 (15/28)	–
					–	36 h after OSE	13 (3/24)	–
Khan et al. (2018)	N, P	Nili-Ravi	PBS	FTAI	CIDR in+EB–eCG–CIDR out–EB	48 and 60 h after CIDR out	56 (25/44)	–

[a]*P* parous buffaloes, *N* nulliparous buffaloes, *N and P* nulliparous and parous buffaloes
[b]*PBS* peak breeding season (September–December), *TBS* transition breeding season (March–April), *LBS* low breeding season (June–August). Both = PBS and LBS throughout the year
[c]*AIDE* artificial insemination at detected estrus, *FTAI* fixed-time artificial insemination, Natural service = introduction of bull into herd for natural service (NS)
[d]$PGF_{2\alpha}$ prostaglandins $F_{2\alpha}$, *GnRH* gonadotropin-releasing hormone, *CIDR* controlled internal drug release device, *EB* estradiol benzoate, *eCG* equine chorionic gonadotropin releasing hormone. Only 2 experiments had post-insemination interventions (italicized) started on days 7 and 23 post breeding, respectively
[e]*OSE* onset of standing estrus
[f]Pregnancy per AI was determined between days 28 and 35 post insemination in all the experiments
[g]Late embryonic mortality was assessed using ultrasonography between days 30 and 45 after insemination
[h]The findings of this experiment are based on a master's dissertation
[i]They reported late embryonic mortality from synchronized and resynchronized groups during different periods of synchronization programs

considered as seasonal breeders as they calve between February and June in South-Asian, whereas they give birth to young ones between June and January in South-American countries (Ahmad et al. 1981). Generally, majority of buffaloes undergo calving during summer months; however, a considerable number of buffaloes also calve in winter months, therefore, during this particular period, the milk production becomes less available, concurrently with an increased demand for milk in the food markets. One option is to synchronize buffaloes during summer months, therefore, the calvings could be programmed during the lean winter period. In this connection, buffalo workers have an opportunity to inject single shot of $PGF_{2\alpha}$ to synchronize the estrus; however, the response to this single dose of luteolytic hormonal administration is greatly dependent on the presence of CL (Dhaliwal et al. 1987) which is not present during proestrus, therefore, double shots of $PGF_{2\alpha}$ might be another approach to cover all stages of estrous cycle and increase the proportion of estrus induction in dairy buffaloes.

Based on above-mentioned facts, Warriach et al. (2008) conducted an experiment to compare $PGF_{2\alpha}$ and standardized ovsynch protocols during PBS and LBS in dairy buffaloes. The authors observed that induced luteolysis using $PGF_{2\alpha}$ resulted in better intensity of estrus as compared to the ovsynch protocol. However, buffaloes in ovsynch group took less time to ovulate after the onset of standing estrus (15.0 ± 0.8 h vs. 30.6 ± 1.5 h; $P < 0.05$) as compared to $PGF_{2\alpha}$ synchronized group, indicating a tighter ovulation window with respect to FTAI. Surprisingly, P/AI of buffaloes synchronized either using $PGF_{2\alpha}$ protocol during PBS and LBS (48% and 38%) or standard ovsynch protocol in both seasons (28% and 23%) remained similar ($P > 0.05$). However, P/AI was lower in ovsynch as compared to $PGF_{2\alpha}$ synchronized buffaloes within both seasons. One of the limitation of this study was the lack of enrollment of cyclic buffaloes in ovsynch group and most likely, higher P/AI in $PGF_{2\alpha}$ group could be because of enrollment of cyclic buffaloes. Therefore, Waqas et al. (2016) enrolled cyclic buffaloes and conceptualized the idea of comparing the standard ovsynch and a unique G6G protocol (administration of $PGF_{2\alpha}$ at D0; GnRH at D2; GnRH at D8; PGF2α at D15; GnRH at D17 followed by FTAI between 16 and 24 h). In G6G protocol, initial treatment with $PGF_{2\alpha}$ leading to complete luteolysis, followed by GnRH administration to promote folliculogenesis leading to increased ovulatory response to the first GnRH of ovsynch, concurrently with greater concentrations of plasma P_4, that ultimately increase the risk of P/AI ($P < 0.05$) in G6G (56%) as compared to ovsynch (32%) protocol in buffaloes. This particular study suggested that cyclic buffaloes had higher fertility synchronized either using $PGF_{2\alpha}$ or G6G protocols, irrespective of the season in order to calve during the months of low availability of milk. However, this also leads to find the solutions to address the issues of summer anestrus in buffaloes.

4.3.2 Progestin-Based Protocols

4.3.2.1 New Vs. Used Controlled Internal Drug Release Devices

In traditional farming setup, the detection of estrus or reduced compliance to AI primarily influenced because of summer anestrus in dairy buffaloes (Das and Khan 2010). Therefore, pregnancy persists lower in majority of buffalo herds during hot and humid months leading to extended calving intervals. One approach to enhance the intensity of estrus signs is to prime the hypothalamus using P_4 releasing devices that facilitates the growth of follicles, concurrent with greater synthesis of estradiol, and leading to increase the expression of estrus in buffaloes (Barile 2005). Higher pregnancy was achieved by using the CIDR device among progestogens that was efficiently and consistently used in estrus synchronization in cattle (Colazo et al. 2004). In fact, CIDR is extensively used and becoming popular for reproductive management because of short period of treatment, i.e., approximately 5 to 7 days CIDR insertion period along with decreased occurrence of persistent follicles in beef cows (Ahmad et al. 1995). It is a common practice to reuse CIDR despite of the fact that reuse of CIDR is not recommended in dairy cattle (Colazo et al. 2004). In order to compare the efficacy between new and used CIDR devices, Naseer et al. (2011) conducted an experiment and determined the impact of both devices on estrus response and P/AI in dairy buffaloes. Interestingly, the authors observed that rates of estrus expression and P/AI remained similar between new and used CIDR devices, therefore, a single device can be used at least two times to synchronize the estrus in buffaloes. However, this approach is limited for the usage at commercial dairy farms because of laborious estrus detection based on visual observation, and unavailability of activity monitors or tools to detect estrus automatically, therefore, a more robust approach was required to optimize FTAI protocols using CIDR devices in buffaloes.

4.3.2.2 Progesterone and Estradiol Benzoate-Based protocol

At first, administration of EB along with CIDR-based protocol was evaluated in nulliparous and multiparous dairy buffaloes. In this context, Yusuf et al. (2015) demonstrated the efficacy of low dose of EB on estrus intensity, ovulation synchrony, and P/AI using FTAI in CIDR-treated buffaloes. The aim of administering low dose of EB along with CIDR removal is to enable pre-ovulatory surge of LH for the better ovulation synchrony as described in *Bos indicus* cows (Bó et al. 2003). Administration of a low concentration of EB after CIDR withdrawal seemed to be a useful tool to improve the estrus intensity; however, P/AI did not increase in dairy buffaloes. Initially, it was anticipated that addition of EB might enhance size of ovulatory follicle and rate of ovulation in buffaloes. On the contrary, an injection of low dose of EB had no influence on ovulatory follicle size, timing of ovulation, and ovulation rate in CIDR synchronized buffaloes. Therefore, these findings indicated an utmost need for the development of CIDR protocol for better ovulation synchronization with reference to FTAI in buffaloes.

4.3.2.3 Progesterone and Gonadotropin Releasing Hormone Modified Protocol

In order to tightly synchronize the ovulation window, a CIDR-GnRH based modified protocol was adopted in dairy buffaloes (Fig. 4.1b). At day 0, CIDR was inserted in the anterior vagina, and removed at day 7. At day 6, a single dose of $PGF_{2\alpha}$ was administered, whereas GnRH was injected at 36 h after CIDR removal, and FTAI was performed either at 12 or 24 or 36 h later to assess the accurate timing of insemination in dairy buffaloes (Haider et al. 2015). The P/AI was greater in buffaloes inseminated either at 12 h (50%) or 24 h (59%) when compared with 72 h (18%) in CIDR-GnRH synchronized buffaloes. This CIDR-GnRH based modified protocol might be interacting with the physiological actions of hormones as well as their effects on ovarian structures in two possible ways: (1) priming of hypothalamo-hypophyseal gonadal axis to encourage the growth of developing oocyte in a P_4 enriched environment and (2) priming the brain with P_4 to promote the regulation of GnRH bearing neurons for follicular wave emergence in the subsequent estrous cycle. The administration of $PGF_{2\alpha}$ was to reduce the circulating P_4 concentrations to minimum level before AI by partial or complete luteolysis of the functional CL. Thereafter, the removal of CIDR concurrent with an injection of ovulation inducing hormone, i.e., GnRH, not only regulates the pulsatile secretion of LH but also improves the LH surge to optimize the ovulation. Lastly, the deposition of semen using FTAI approach increases the possibility of fertilization and successful pregnancy. Undoubtedly, it seems that synchronization of estrus using CIDR-GnRH based modified protocol is greatly effective, as a treatment for summer anestrous buffaloes. Following establishment of FTAI in buffaloes, another question was to develop strategies to increase concentrations of P_4 after AI to improve fertility and reduce embryonic and fetal losses.

4.3.2.4 Progesterone and Equine Chorionic Gonadotropin-Based Protocol

In another study conducted by Khan et al. (2018), the authors determined the effect of eCG on estrus response, ovarian structures, P/AI, and concentrations of P_4 in CIDR-EB synchronized buffaloes. Interestingly, whenever buffaloes were injected with eCG, the authors not only observed greater expression of estrus signs, but also reported increased growth rate of dominant follicle, concurrent with greater ovulation rate and P/AI as compared to buffaloes which were not treated with eCG in that particular study. Moreover, the increased concentrations of P_4 were also observed in eCG-treated buffaloes. Although, administration of eCG improved overall reproductive performance probably because of increased binding of FSH to its receptors on granulosa cells (Butnev et al. 1996), reduced rate of apoptosis of granulosa cells (Tilly et al. 1995), enhanced production of insulin-like growth factor-1 (Sun et al. 2013), and increased E_2 production, causing an enhanced size of the Graafian follicle leading to better estrus response and fertility in anovular buffaloes during PBS; however, the effect of eCG on pregnancy survival has not been determined between peak and low breeding seasons in buffaloes (Monteiro et al. 2018). Therefore, there was a need to standardize the dose and evaluate the efficacy of different

tropic hormones such as hCG for the induction of ACL for greater concentrations of P_4 post AI to increase the pregnancy survival in buffaloes.

4.3.2.5 Progesterone and Human Chorionic Gonadotropin-Based Protocol

Fertility in buffalo is comparatively low compared to dairy cows in spite of the development and modification of synchronization programs (Drost 2007). The key factor for this reduced fertility might be because of reduced pregnancy survival between days 25 and 40 post AI in buffaloes (Campanile et al. 2005). Early and late embryonic losses were reduced by interventions of different hormone in dairy buffaloes (Campanile et al. 2016). However, injection of GnRH, hCG, or P_4 at day 5 after AI seemed non-beneficial to enhance the pregnancy survival in Italian Mediterranean buffaloes (Pandey et al. 2015). On the contrary, when gonadotropins were administered late such as at days 23 or 25 post AI it seemed beneficial to reduce embryonic mortality (Arshad et al. 2017; Campanile et al. 2008). In dairy heifers and cows, several studies have shown that hCG can enhance the pregnancy rate (Stevenson et al. 2007), primarily because of greater binding to LH receptors during metestrus (Niswender et al. 2000), or diestrus (Schmitt et al. 1996; Santos et al. 2001), which resulted in increased concentrations of plasma P_4 either because of luteotropic action on the prevailing CL or by helping the development of ACL (Rajamahendran and Sianangama 1992).

In this connection, recently, Ahmad et al. (2021) conducted a meta-analysis to determine the effects of administration of GnRH, hCG, or P_4 post AI on concentrations of P_4, P/AI, and pregnancy loss in dairy buffaloes. The literature was systematically reviewed and 10 manuscripts, comprising 12 experiments, having 42 treatment means and 1582 buffaloes, were included in the analyses. Buffaloes were randomly assigned to receive treatments at mean ± SD (range) 10.8 ± 8.5 days (1–25) post AI as: (1) CON (n = 491), (2) GnRH (n = 361), (3) hCG (n = 396), and P_4 (n = 334). Mixed models were fitted using the MIXED and GLIMMIX procedures of SAS. Models contained the fixed effect of treatments and random effect of experiment, and data were weighted by the inverse of the SEM squared to account for the precision of each experiment. Plasma concentrations of P_4 (ng/mL) post treatments increased (P = 0.02) in hCG (3.36 ± 0.72) and tended (P = 0.09) to be greater in GnRH (3.00 ± 0.79); however, it did not increase (P = 0.15) in P_4 (2.91 ± 0.97) treated buffaloes as compared to CON (1.81 ± 0.76). Administration of GnRH (58.2% ± 4.0) or hCG (63.4% ± 4.0) also increased ($P < 0.01$) P/AI at day 30 post AI as compared to P_4 or CON buffaloes (51.4% ± 4.0 and 42.6% ± 3.8), respectively. The relative risks of P/AI in GnRH, hCG, and P_4 treated buffaloes have been presented as a forest plot and explained in Fig. 4.2. The pregnancy loss was significantly lower (P = 0.01) in GnRH or hCG-treated (13.2% ± 3.1 and 14.2% ± 3.1) buffaloes, than in P_4 or CON (23.4% ± 4.0 and 30.2% ± 4.0); however, fetal losses did not differ due to treatments at day 45, 60, or 90 post AI. Collectively, administration of GnRH or hCG post AI enhances P/AI and pregnancy survival in dairy buffaloes.

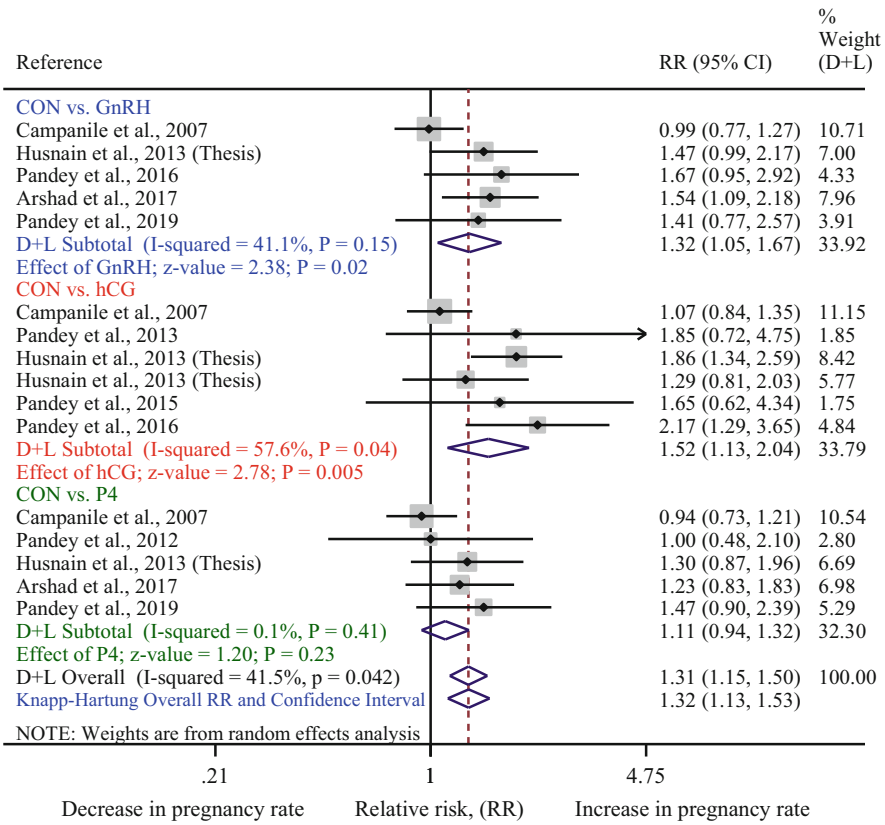

Fig. 4.2 Forest plot represents the relative risk (RR) of pregnancy rate at Day 30 in dairy buffaloes (n = 1124). Collectively, 15 comparisons from 9 experiments, and 8 manuscripts are depicted in the forest plot. Administration of saline was adjusted as reference control (CON; n = 380), whereas interventions included an injection of gonadotropin-releasing hormone (GnRH; n = 255), or human chorionic gonadotropin-releasing hormone (hCG; n = 250) or progesterone (P_4; n = 239) in the comparative analysis. Whenever the experiments reported data having factorial arrangements of treatments, the CON group was correctly matched to the respective treatment groups (GnRH, hCG, or P_4). Each black diamond having a black horizontal line represents point estimate and respective 95% confidence intervals (CI). The central black line indicates no effect of the interventions (GnRH, hCG, or P_4), and point estimates moving left to the central line indicate a decrease, whereas point estimates directing towards the right side represent an increase in the pregnancy rate. The relative weighting of the comparisons to account for the precision of each experiment is represented as grey box around the black diamonds. If the grey box is larger, it is contributing more weight to the analysis as compared to small box. The weight that each comparison contributed is mentioned in the right-hand column. The upper and lower limit of the line connected to the square represents the upper and lower 95% CI for the effect size. The D + L indicates a mixed effects model using the DerSimonian and Laird (1986) method to estimate the between study variance with respective I-squared (I^2), whereas the Knapp and Hartung (2003) random effects approach for the effect size is characterized by the red dashed line (super-imposed due to central black line) with the 95% CI depicted by the width of the diamond shape in the figure. The heterogeneity measure, I^2, is a measure of variation beyond chance among treatments included in the meta-analysis. Overall, the

In dairy cows, it was reported that administration of hCG using 1000 IU is sufficient for the formation of ACL (Buttrey et al. 2010); however, this information was not available in dairy buffaloes. In order to validate the dose of hCG in buffaloes, Husnain et al. [Unpublished data] executed an experiment and reported that 2500 IU of hCG was sufficient to induce ovulation and formation of ACL in dairy buffaloes. Besides this, the advantages to hCG treatment in terms to enhance concentrations of P_4 (5.68 ± 0.4 vs. 2.34 ± 0.4; $P < 0.05$), P/AI (65% vs. 35%; $P < 0.05$), and pregnancy survival (95% vs. 78%; $P < 0.05$) were greater than saline-treated buffaloes. The increasing concentrations of P_4 is associated with better development of growing conceptus that produces greater concentrations of interferon-tau (Demmers et al. 2001) which is directly linked to the length of embryo (Mann et al. 2006) in dairy cows. Based on findings of Husnain et al. [Unpublished data], administration of hCG at day 7 post-AI has been proposed to increase the pregnancy survival in buffaloes. Interestingly, the recent literature suggests that ACL formation, i.e., induced on the opposite side of the gravid uterus, usually regress (74%) between days 33 and 67 of gestation in dairy cows (Megan et al. 2019); however, this phenomenon has not been evaluated in dairy buffaloes.

4.4 Resynchronization Programs

An approach to resynchronize the dairy cows has been attempted for the last two decades (Stevenson et al. 2003); however, few studies have been documented to evaluate its merits in buffalo breeds including Nili-Ravi (Arshad et al. 2017) and Italian Mediterranean buffaloes (Rossi et al. 2014). Resynchronization has been regarded as an aggressive approach to manage reproduction that involves the insemination of all cows at the start of the breeding season, isolating the non-pregnant cows, and timely re-insemination of the non-pregnant cows (Stevenson et al. 2003). Recently, Arshad et al. (2017) conducted an experiment where authors synchronized estrus using CIDR-GnRH modified protocol as described previously (Haider et al. 2015). At day 23 post-AI, the authors randomly assigned buffaloes either to inject them with GnRH or saline. Interestingly, the P/AI in GnRH-treated (63%) was greater ($P < 0.05$) than control (41%) buffaloes. Besides this, the authors observed reduced embryonic losses in GnRH-treated (13%) as compared to control (35%) buffaloes. Similarly, Neglia et al. (2018) investigated the effects of two different regimens (ovsynch vs. progestogens) for consecutive six resynchronized FTAI approaches on overall herd fertility during LBS in nulliparous individuals of a commercial buffalo herd. Resynchronizing the buffaloes using P_4-based protocol resulted in 89.3% overall herd fertility. These results suggested that

Fig. 4.2 (continued) buffaloes who were administered with GnRH or hCG had 32% and 52% greater risk to achieve pregnancy as compared to CON, respectively, whereas buffaloes who were administered with P_4 did not differ from CON

resynchronization may be considered as an effective tool to attain higher herd fertility in dairy buffaloes.

4.5 Conclusion

The improved reproductive management programs increased the profitability of a dairy herd. The application of AM-AM rule to breed buffaloes on detected estrus seems an exciting finding to increase P/AI. Administration of $PGF_{2\alpha}$ and ovsynch based protocols increase P/AI across breeding and non-breeding seasons; however, responses to these protocols require an ultrasonographic monitoring of ovarian structures. The application of P_4 releasing devices alone or in combination with other hormonal regimens such as GnRH or eCG or EB can be used and reused to enhance the estrus intensity, concurrent with improved P/AI in buffaloes. An injection of hCG at day 23 post-AI can be used successfully to resynchronize buffaloes with reduced embryonic losses and improved herd fertility in commercial herds. Although, regimens to improve reproductive management are available, however, the application of these strategies are challenging especially in small-holder farming systems, and concurrent with less availability of resources.

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Behavior and Welfare of Dairy Buffaloes: Calving, Milking, and Weaning

5

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Abstract

This chapter addresses basic aspects of the behavior of female buffaloes during farrowing, milking, and weaning while also discussing the neurophysiological mechanism of the pain that normally accompanies parturition and describing the pain that female buffaloes may experience when suffering from mastitis. This is a common medical condition, but one that requires preventive measures because if it progresses it compromises not only the health and welfare of the buffaloes, but also the economic success of the productive unit. Finally, we outline novel strategies for weaning that can reduce stress in calves. The birthing process places huge physical demands on the parturient buffalo that are associated with pain.

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Birth is a dynamic process characterized by constant, painful uterine contractions that increase in frequency and intensity. This type of pain has both visceral and somatic components. Pain surges in the peripheral nervous system as pain signals form synapses with the nerve tracts that extend towards the central nervous system. Recent studies demonstrate that milking routines can affect the comfort level of female buffaloes if they have not been habituated to the specific actions involved in a daily routine in which milking is always performed at the same time of day through certain kinds of handling during human-animal interaction. This chapter focuses too on traditional and current weaning strategies to improve productivity and calf welfare for river buffaloes. Wean calves as old as possible. Older calves display less signs of stress than young animals. Deworming calves prior to weaning, may produce heavier and healthier calves at weaning. Avoid combining stressful procedures like castration or branding with weaning. Weaning itself is a stressful procedure and subjecting a calf to further stress, notably harms their welfare. The knowledge of specific weaning methods might contribute to enhance the welfare of the offspring and to improve the reproductive efficiency of the buffalo cow.

Keywords

Buffalo · Behavior · Welfare · Calving · Milking · Weaning

5.1 Introduction

Achieving success in reproductive efficiency and, as a result, in dairy production, depends on the birth of calves. Female river buffaloes (*Bubalus bubalis*) generally give birth to just one calf with twin births being exceptional (De Rosa et al. 2009b). Gestation entails adaptations to the gradual changes that these dams undergo as their fetuses grow for a period of approximately 300–310 days, a period during which it is important to focus on her care and feeding (Punia and Singh 2001). The changes generated at the moment of farrowing, in contrast, are sudden and severe, occur relatively quickly, and cause some degree of pain due to physiological and morphological changes that also produce stress and anxiety (da Pereira et al. 2011; Mota-Rojas et al. 2019a, b, 2020; Orihuela et al. 2021; González-Lozano et al. 2019).

Normal (eutocic) births involve a complex physiological process that triggers a cascade of physiological and hormonal responses accompanied by morphological and behavioral changes (Titler et al. 2015). For this reason, birth is normally considered a stressful event. In abnormal cases of expulsion (dystocic births), additional stimuli intensify the normal stress of parturition (Sathya et al. 2007). It is not the objective of this chapter to address dystocia, but it is necessary to mention that complications during birth in female buffaloes are rare (2%) compared to bovine cattle of the genus *Bos*, though the stress generated by such births brings on a condition that affects both animal welfare and dairy production (Purohit et al. 2011, 2012; Mota-Rojas et al. 2019a, b). Then, it is necessary to understand better the

dynamics of birth in buffaloes so as to distinguish normal birth processes from pathological cases.

Another fundamental element of the productive life of female dairy buffaloes is the efficiency and efficacy of milking, which plays a decisive role in their productivity and state of health. Diverse factors can negatively influence the productive performance of female buffaloes during milking: poor handling practices by workers who shout, strike, shove, or use instruments to move animals; nervous behaviors by the animals; the milking technique utilized (especially with the increased use of mechanical versus manual means due to the intensification of breeding); prior negative experiences with humans; and lesions. It is essential that all human-buffalo interaction during daily routines be positive (Polikarpus et al. 2014b). The intensification of buffalo milk production has meant incorporating automatic milking machines into handling routines, a drastic change that entails physical and psychological factors that can impede milk extraction; for example, incorrect connection to the machine and deficient maintenance, perhaps exacerbated by inadequate handling procedures (Saltalamacchia et al. 2007). Female buffaloes are more sensitive to stressful stimuli during milking than dairy cattle (Thomas et al. 2005). The noise of the machines, for example, can cause stress and discomfort (Polikarpus et al. 2014b) that can cause them to retain the milk (Borghese et al. 2007). In contrast, calm, gentle handling yields positive effects that reduce nervousness during milking (Ellingsen et al. 2014).

This chapter addresses basic aspects of the behavior of female buffaloes during farrowing, milking, and weaning, while also discussing the neurophysiological mechanism of the pain that normally accompanies parturition and describing the pain that female buffaloes may experience when suffering from mastitis. This is a common medical condition, but one that requires preventive measures because if it progresses it compromises not only the health and welfare of the buffaloes, but also the economic success of the productive unit. Finally, we outline novel strategies for weaning that can reduce stress in calves.

5.2 Calving

During the prepartum stage of calving, female river buffaloes display natural behavioral patterns (similar to those of dairy cows of the genus *Bos*) indicative of an impending birth. One of these patterns in dairy cows is isolation (Rørvang et al. 2018). Similarly, though the river buffalo is a gregarious species, just prior to parturition females separate from the group to give birth in isolated sites far from the herd where they are protected from predators (De Rosa et al. 2009b), just as occurs with cows raised for milk and meat (*Bos taurus*, *B.* indicus) in pasture systems. This isolation, free of interference by other females, is extremely important because it fosters the establishment of the dam-calf bond (De Rosa et al. 2009b) and the survival of the offspring. By facilitating mutual recognition, this bond allows the calf to quickly accede to the udder and ingest colostrum (von Keyserlingk and Weary 2007).

The onset of parturition in female buffaloes is characterized by extensions the tail and flexing of the hips and hind legs while standing. If they are in a recumbent posture, they will be stretching their neck and extremities (Mohammad and Abdel-Rahman 2013). Like other ungulates, female buffaloes expel their calves while standing (von Keyserlingk and Weary 2007). Exploration is another behavior that most parturient females exhibit during this first stage. This is characterized by extensive sniffing of the ground, while if provided with a substrate such as straw they will push it around with their head and hooves to form a rest area. This is because as the first stage progresses most females will lie down and get up several times, paw at the ground, and observe their abdomen. A study by Mohammad and Abdel-Rahman (2013) interpreted the changes in exploratory behavior during the first stage of parturition in female buffaloes as signs of increased pain and stress during dystocic births, so they may provide a way to identify births that could become complicated, thus giving handlers time to take necessary precautions in a timely manner.

Labor consists of three sequential phases. It begins with dilatation of the cervix that ends with the rupture of the chorioallantois in the vagina. In the second stage, the calf is visible in the vulva and is expelled. The third stage involves elimination of the fetal membranes (Mohammad and Abdel-Rahman 2013; Proudfoot et al. 2013). It is important to note that in normal conditions the first stage of labor is prolonged in primiparous female buffaloes compared to multiparous dams (Mohammad and Abdel-Rahman 2013). After the chorioallantois breaks and the amniotic fluid spills out, the dam will likely begin to lick it up. It is believed that this rupture relieves some of the pain associated with the first stage of the birthing process (Wehrend et al. 2006).

When the cervix begins to dilate during the first stage as the calf positions itself for expulsion, the mother shows other behavioral changes including more frequent episodes of lying down and then standing up, which reflects restlessness (Huzzey et al. 2005). Her restlessness is also characterized by an increase in the amount of time spent walking around (Wehrend et al. 2006). The occurrence of uterine contractions increases this restlessness and leads to frequent changes of position (Huzzey et al. 2005; Miedema et al. 2011; Jensen 2012). At this time, she begins to pay more attention to her abdomen (Jensen 2012) while standing and tends to raise her tail more frequently (Miedema et al. 2011). The onset of rhythmic abdominal contractions and the release of the amniotic sac are obvious indications of the onset of the second stage of labor (Noakes et al. 2001) (Fig. 5.1).

In stage two the intensity and frequency of uterine and abdominal contractions help expel the calf (Schuenemann et al. 2011). As the fetus passes through the birth canal, it produces cervical-vaginal stimulation that activates the mother's hypothalamus, releasing oxytocin. This hormone acts on the buffalo's olfactory bulb that, in turn, allows the secretion of dopamine. This initiates the delicate period during which the mother identifies her calf (Singh et al. 2017).

During the third and final stage of labor, the placenta is expelled so uterine involution can begin.

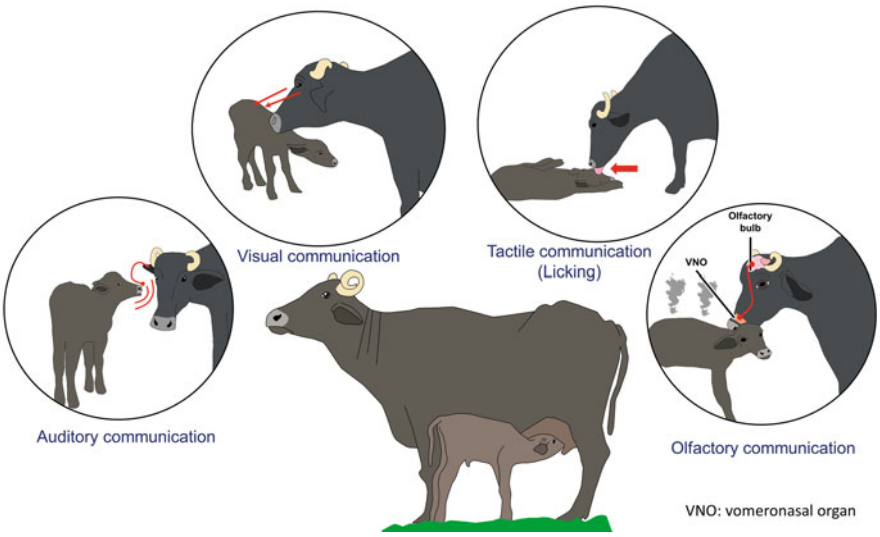

Fig. 5.1 Imprinting of the female buffalo with her calf

5.2.1 Origin and Transmission of Painful Stimuli During Labor

The birthing process places huge physical demands on the parturient buffalo that are associated with pain. Birth is a dynamic process characterized by constant, painful uterine contractions that increase in frequency and intensity. This type of pain has both visceral and somatic components (Hashemi et al. 2018; Alimoradi et al. 2019). Pain surges in the peripheral nervous system as pain signals form synapses with the nerve tracts that extend towards the central nervous system (CNS) (Fig. 5.2). The pain felt during the first stage of labor—dilatation—is predominantly visceral and caused by painful stimuli derived from the mechanical distension of the inferior uterine segment and cervical dilatation (Mainau and Manteca 2011). Lowe's (2002) study of women found that these nociceptive stimuli (dilatation phase) are transmitted to the ganglions of the posterior nerve root at T10 (spinal nerve of the thoracic segment) that originates in the spinal column beneath the tenth thoracic vertebra, and passes through L1 (spinal nerve of the lumbar segment), which begins in the spinal column beneath the first lumbar vertebra.

The (visceral) pain associated with labor can be referred progressively to the abdominal wall, the lumbosacral region, iliac crests, gluteus, and thighs. The onset of abdominal contractions, breaking of the allantochorion sac, and the expulsion of the fetus all characterize the second phase of birth, during which the final widening of the cervix occurs (Taverne 1992). This means that somatic pain predominates due to the distension and traction of the pelvic structures surrounding the vagina, and the distension of the pelvic floor and perineum. The uterine contractions persist into the third stage of labor, but are less frequent and may decrease in amplitude, while

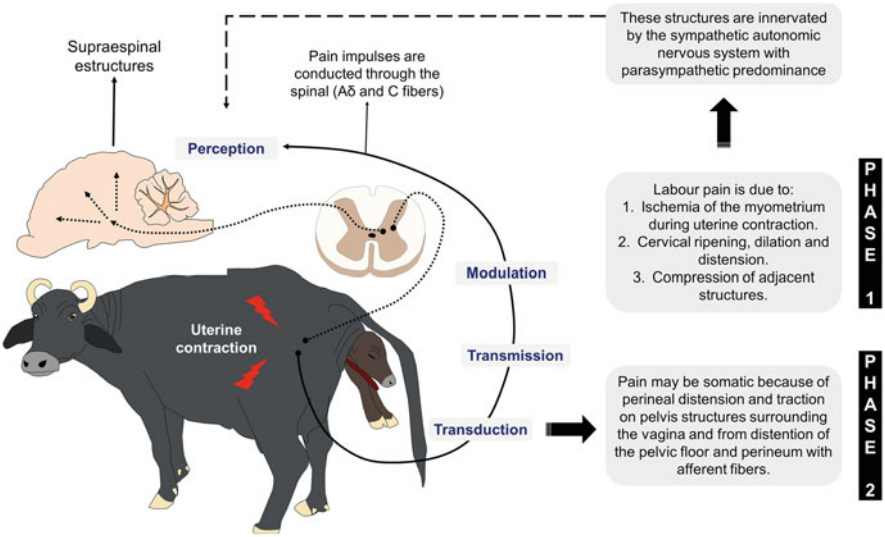

Fig. 5.2 Transmission of the painful impulse during labor in female buffaloes (Mota-Rojas et al. 2019c, d)

maintaining the same intensity (Noakes et al. 2001; Mota-Rojas et al. 2019c; Orihuela et al. 2021).

5.3 Behavior and Welfare of the Dairy Buffalo During Milking

Recent studies demonstrate that milking routines can affect the comfort level of female buffaloes if they have not been habituated to the specific actions involved in a daily routine in which milking is always performed at the same time of day through certain kinds of handling during human-animal interaction. The design of the stable and milking facilitations in general is another important aspect to be considered (Polikarpus et al. 2014a), as these measures will help reduce or eliminate sources of stress during milking (Munksgaard et al. 2001; Rushen et al. 2001). Thomas et al. (2005) affirm that female buffaloes are more sensitive to stressful factors during milking than dairy cows. Adrenaline production in stressed animals can cause a reduction in the release of the oxytocin necessary to maintain milk flow during milking (Borghese et al. 2007). Even a slight change in the milking routine can alarm female buffaloes and cause discomfort (Napolitano et al. 2013) due to fear provoked by neophobia (Polikarpus et al. 2014b). The result may be reduced milk production.

Another important feature of female river buffaloes is that their udders have only a small cistern, so most of their milk (approximately 95%) is stored in the alveolar compartment, which requires oxytocin to release the milk stored there (Thomas et al. 2005). At the onset of milking, the female buffalo's milk is thus located at two levels. The first fraction is in the cistern (cisternal milk) and can be extracted easily and

simply by applying pressure to the teat. The second fraction is stored in the alveolar region and lobular ducts (alveolar milk). Extracting this milk is more difficult because it requires previous stimulation to obtain the correct milk ejection response (Singh et al. 2017), which can only be achieved through the release of oxytocin (Cavallina et al. 2008).

In traditional, small-scale milk production systems, the calf is kept close to the dam to stimulate milk release (Singh et al. 2017). In pasture systems, in contrast, stimulation occurs during the cleaning of the udder through manual washing and massaging the teats with running water for 5 s (Cavallina et al. 2008). Once again, the timing and procedures prior to milking must follow a routine in order to foster milk release, regardless of the type of production system (Cavallina et al. 2008). The stress generated in milking rooms has an exacerbated effect on milk production in buffaloes because, as mentioned above, it reduces the supply of oxytocin required to release the milk from the alveolar fraction (Neglia et al. 2008). For this reason, establishing and maintaining the order in which female buffaloes are milked is essential for good production (Polikarpus et al. 2014b, 2015). Various authors coincide in noting that a particularly important aspect of the social system of dairy cattle is establishing a fixed order for entry into the milking room (Grasso et al. 2007; Berry and McCarthy 2012). Establishing such an order may require considering factors like social rank (Melin et al. 2006), health (Flower et al. 2006), and level of productivity (Górecki and Wójtowski 2004), among others.

Female buffaloes tend to prefer one side of the milking room (left or right). Indeed, few fail to show a preference for the site or section of the room where they will be milked, so any handling practice that interferes with their preference of order and site inside the milking room will generate stress that can compromise the welfare, health, and productivity of female buffaloes (Polikarpus et al. 2015). Prelle et al. (2004) pointed out that this lateralization of the behavior of female buffaloes depends on factors that include the social domain and structure of the herd as a whole, and such individual traits as anxiety, fear, stress, and sensitivity. Impeding the animals from performing specific behavior patterns can provoke a negative psychological state that affects their welfare (Jensen and Toates 1993).

Inadequate practices in milking routines can also result in reduced flow and production, and predispose the buffaloes to mastitis if the emptying of the udder is deficient (Cavallina et al. 2008; De Rosa et al. 2009b). In pasture production systems, milking can come to represent a chronic stressor by provoking fear or discomfort in the buffaloes if the milking machine is not set to operating constants appropriate for this species (vacuum operation at 50 kPa, 70 cycles of pulsations/min at a proportion of 65:35) (Cavallina et al. 2008), or if equipment maintenance is inadequate (Cavallina et al. 2008; De Rosa et al. 2009b, a). Other stressful stimuli that may appear during milking are separation from calves, the new, unfamiliar environment that the animals are forced to confront (Cavallina et al. 2008), and the behavior of handlers (Saltalamacchia et al. 2007). For example, if the herding into the milking room by the personnel is aggressive or brusque it may provoke a more severe loss of milk production (Saltalamacchia et al. 2007; Cavallina et al. 2008; De Rosa et al. 2009b). Some researchers propose administering exogenous oxytocin

(e.g., Cavallina et al. 2008) to counteract the negative effects of human-animal interaction and minimize behaviors related to acute anxiety and stress, such as kicking, shifting position, and urinating, in primiparous female buffaloes upon first contact with milking machinery. Other suggestions are for handlers to always use positive, calm, gentle handling procedures inside the milking room (Saltalamacchia et al. 2007), and the implementation of habituation programs for primiparous females on days 0, 3, and 6 before farrowing (Polikarpus et al. 2014b). Exogenous oxytocin must always be used with great care and exclusively with animals that experience difficulties in milk release. Finally, the doses applied must never exceed the recommended amount.

5.3.1 Factors That Predispose Female Buffaloes to Mastitis

Certain characteristics of buffalo milk, such as its high content of total solids, mean protein content of 3.91 ± 0.61%, and fat content of 6.87 ± 0.88% (Tonhati et al. 2000), ensure high yields in the processing of byproducts, so river buffalo milk production is a livestock resource of great economic importance in the dairy industry (Tanamati et al. 2019). However, the production and composition of buffalo milk can be affected significantly if aspects of animal health (like udder hygiene) are compromised in any way (Rainard and Riollet 2006). Poor health conditions will, of course, also exacerbate negative effects on animal welfare. One especially clear case of this is the incidence of mastitis, which can make buffalo milk less apt for consumption and processing, and cause significant economic losses due to the sub-clinical course and potentially chronic evolution of this disease (Rainard and Riollet 2006). As mentioned above, poor practices in milking routines can predispose female buffaloes to mastitis due to deficient emptying of the udder (Cavallina et al. 2008; De Rosa et al. 2009a; Mota-Rojas et al. 2019c).

Mastitis of bacterial origin in large ruminants is one of the most common diseases in the dairy industry, and one that represents a significant global challenge (Bradley 2002). But it also constitutes an important problem in the area of animal welfare (de Boyer des Roches et al. 2017; Mota-Rojas et al. 2019a, b, c). Mastitis is characterized by inflammation of the mammary gland caused by metabolic or physiological changes, trauma, or pathogenic infection (Oviedo-Boyso et al. 2007). In the female buffalo, the pathogens most often involved in mastitis are coliform bacteria (*Klebsiella pneumoniae* and Escherichia coli), followed by *Staphylococcus aureus*, *Streptococcus uberis*, and *S. agalactiae* (El-Khodery and Osman 2008). However, fungi and yeasts are also associated with mastitis in bovines (Petzl et al. 2008). Mastitis caused by *E. coli* is typically self-limiting and of short duration, but may be associated with severe clinical signs, reduced milk production, and serious tissue damage to the mammary gland (Burvenich et al. 2003).

Although dairy buffaloes are traditionally considered less susceptible to mastitis than bovine cattle (Guccione 2017), some researchers have found similar incidences in both species (Guccione et al. 2014). It may be that the high prevalence of sub-clinical intramammary infections (IMI) skews estimates of the real dimensions

of this problem (Pisanu et al. 2019). Such key management strategies as carefully monitoring milking efficiency and implementing adequate hygienic measures largely limit the propagation of gram positive bacteria and help significantly reduce the proportion of isolates of *S. aureus* and sub-clinical mastitis worldwide (Burvenich et al. 2003). Despite this, mastitis is still one of the most common diseases in the dairy industry and one with negative effects on the economy (de Boyer des Roches et al. 2017), as shown by a case study in southern Italy (Restucci et al. 2019). We must recognize that our understanding of the health of the river buffalo's udder is quite limited compared to what we know about the udder of dairy cows. In both species, however, the harmful effects of mastitis are evident. Unfortunately, the pain associated with clinical mastitis is rarely measured or treated (Leslie and Petersson-Wolfe 2012), an omission that has significant repercussions for animal welfare.

5.3.2 Perception of Pain During Milking in Female Buffaloes with Mastitis

Pain is a term generally associated with human experience and one considered relatively subjective. Identifying experiences of pain in animals depends on monitoring changes in their physiological and behavioral reactions. The International Association for the Study of Pain (IASP) defines this phenomenon in humans as "an unpleasant sensory and emotional experience with real or potential tissue damage" (Merskey and Bogduk 1994). In 2020, the IASP stipulated that non-human animals' inability to communicate the sensation of pain does not negate the possibility that they may suffer it. Hence, the definition of pain should be applicable equally to humans and non-human animals (Raja et al. 2020). Defining pain in animals can, however, be a complicated undertaking because of the differences in responses that exist among species, individual animals, different stages of disease, and between acute and chronic conditions (Leslie and Petersson-Wolfe 2012). Severe clinical cases of mastitis in large ruminants are painful and produce visible signs and discomfort accompanied by low milk production, weight loss, and abnormal body postures (Huxley and Hudson 2007). In contrast, in mild-to-moderate clinical cases of mastitis, or those in the sub-clinical stage, it may be difficult to determine if they cause a painful condition (Fitzpatrick et al. 1998).

Pain in the teat and udder produces chemical, mechanical, or thermal stimulation of the free nerve endings that contain nociceptors. The cells of injured tissues release inflammatory mediators such as prostaglandins, histamine, and bradykinin that stimulate nociceptors located in the proximal nerve endings. In this way, a stimulus that affects a relatively small number of nerve endings will stimulate others, thus amplifying the sensation of pain. The impulses that derive from this stimulus are channeled through the ventrolateral part of the spinal cord to the brainstem and thalamus in a process that generates even greater amplification. The activation of certain areas of the cerebral cortex through the thalamus then allows the conscious perception of pain (Fig. 5.3). In theory, pain is a central "experience" caused by

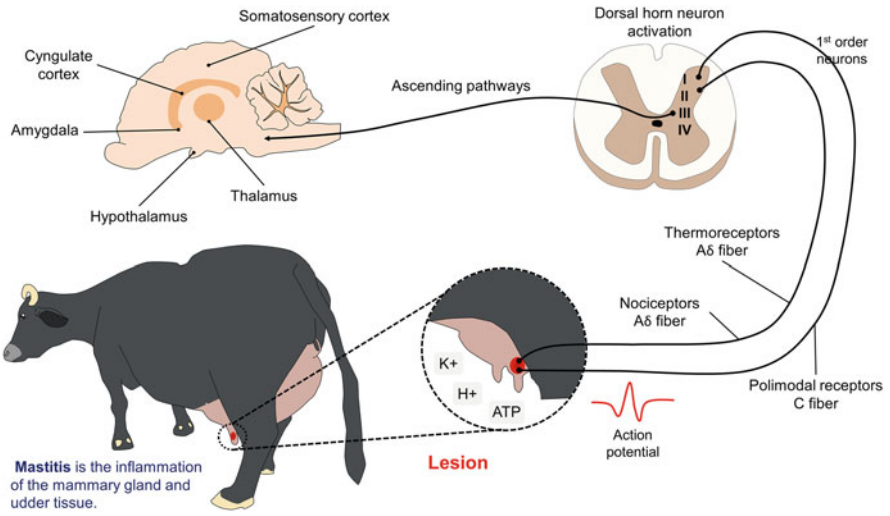

Fig. 5.3 Transmission mechanism of the pain impulse in a process of chronic mastitis in the dairy buffalo

nociception in the peripheral nerves. Acute pain is the result of tissue lesions with activation of the autonomous sympathetic nervous system. In cases of chronic pain, observations show the presence of high levels of inflammatory mediators around the site of the lesion with a persistent activation of the pain fibers in the spinal cord that triggers hyperalgesia (Hudson et al. 2008). The pain and discomfort that accompany mastitis can severely compromise animal welfare (Leslie and Petersson-Wolfe 2012), so in recent years special attention has been paid to pain as an important aspect of this disease in line with considerations of the basic principles of animal welfare (Giovannini et al. 2017; Mota-Rojas et al. 2016, 2019a, b, c). This is because mastitis has been shown to interfere with four of the five established freedoms (FAWC 2009) (Fig. 5.4).

Among the indicators that are useful for recognizing and quantifying pain caused by bovine mastitis, we propose:

1. Alterations of physiological parameters such as heartbeat, respiratory frequency, and temperature (Leslie and Petersson-Wolfe 2012). These measures are considered good indicators of disease severity in cases of mastitis that appear spontaneously (Kemp et al. 2008).
2. Modified postural and feeding behaviors (that is, the time devoted to eating, ruminating, and lying down) (Cyples et al. 2012; Yeiser et al. 2012).
3. Expression of active behaviors like walking, kicking, and raising the extremities (Siivonen et al. 2011; Medrano-Galarza et al. 2012).
4. Alterations of nociceptive thresholds (Rasmussen et al. 2011; Fitzpatrick et al. 2013).

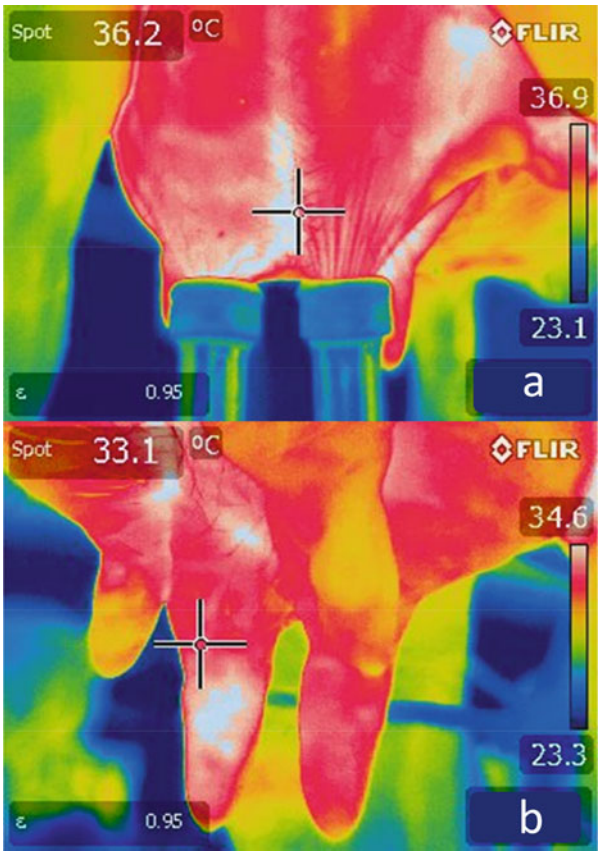

Fig. 5.4 Monitoring the udder by infrared thermography to detect inflammatory processes based on changes in dermal microcirculation. (**a**) Mechanical milking: the udder tissues can be seen in red and white, while the blue color shows the teat cups of the milking machine. (**b**) An udder post-milking: in the early stages, the buffalo experiences a long period of suction but no milk ejection (Caria et al. 2011). The use of heavy duty suction, combined with the absence of milk flow, can irritate the delicate mammary tissues (Bruckmaier and Blum 1996) and make the female susceptible to opportunistic pathogenic agents (Mota-Rojas et al. 2019c)

Giovannini et al. (2017) induced sub-clinical mastitis experimentally (using lipoteichoic acid, LTA, and the lipopolysaccharide derivatives of *S. aureus* and *E. coli*, respectively) in *B. taurus* cows (Holstein and Swiss Fleckvieh breeds), and then developed a scoring system for the characteristically multidimensional pain associated with this disease. Their system includes two principle categories based on general vs. local signs. The general signs are classified into six sub-categories: (1) general subjective evaluation (without signs of pain or severe pain); (2) postural behaviors, the first three related to facial expression (drooped, asymmetric ears, corrugated upper eyelids, flared nostrils, restlessness, apathy, hind hooves separated); (3) interactive behaviors (interest or lack of interest); (4) response to food (with appetite or total lack of appetite); (5) position of the sacral area (normal, downwards with arched back, no reaction); and (6) reaction to palpation of the back (no reaction or severe reaction). The local signs are classified in two sub-categories: (1) edema of the udder (with or without severe swelling) and (2) palpation of the udder with no reaction, only a mild reaction (tail movement, raising of extremities), or a severe reaction (kicking, moving away).

This system made it possible to obtain an index of total pain with a maximum possible score of 42, obtained by adding up the scores assigned to each sub-category. It is important to note that this study also utilized temperature measurements taken by infrared thermography; however, no changes related to treatment were observed, perhaps because factors like ambient temperature, circadian oscillations, or physical activity were not taken into consideration. Studies of this kind in which a disease is induced experimentally have enhanced our ability to recognize and quantify the pain caused by bovine mastitis (by evaluating pain thresholds), and to characterize animals' physiological and behavioral responses to pathogenic invasions of the mammary gland.

5.4 Weaning in Buffaloes

5.4.1 Widely Used, Routine Approaches

Compared to cattle calves, the offspring of buffaloes are less mature, as shown by their comparatively low milk intake and delayed eruption of teeth. This leaves them more susceptible to harmful elements in the environment (Mota-Rojas et al. 2020), whether in the form of pathogens or extremely high or low temperatures (Zicarelli 2006; Orihuela et al. 2020). Because this is a tropical species, the low temperatures that may occur in other breeding regions can affect their welfare. But neonate buffaloes are also more vulnerable than other ruminant species to certain illnesses, especially if females give birth in wintertime (Mingala and Gundran 2008). A factor that impacts weaning is a phase difference between the buffaloes normal breeding season and the periods of greatest demand for one of the principle products made from their milk: mozzarella cheese. This has led many breeders in Italy to program reproduction by allowing bulls to mix with herds from April to September (Di Francesco et al. 2011). Whether buffalo-raising operations employ this technique or not, farms sometimes have to deal with periods of intense calving when farrowing pens may become overcrowded (Orihuela et al. 2020). This is an undesirable condition because it can allow contagions to spread more easily, leading to increased morbidity and death.

Studies of intensive dairy buffalo-breeding farms around the Mediterranean show that standard weaning practices are similar to those commonly employed with dairy cattle, including the separation of calves from their dams shortly after birth followed by administration of colostrum that they would normally ingest from their mother's udder (Zicarelli et al. 2007). The health condition of these calves and, perhaps, even their survival, depend to a significant degree on how colostrum is provided, since this nutritional element impacts both their weight gain and future development. Because buffalo calves are born agammaglobulinemic, they require the IG in their dams' colostrum for protection from pathogenic organisms (Godden et al. 2019). Indeed, it is imperative that these neonates ingest sufficient amounts of colostrum that is both hygienic and of high-quality in the early hours immediately postpartum. In Holstein calves, 3–4 L of colostrum, or feed containing 10–12% of this substance

by body weight ensures adequate protection, but means that each calf must receive at least 150–200 g of IgG soon after expulsion. Under ideal conditions, providing 300 g of IgG will help ensure a high level of passive transfer (Godden et al. 2019). After this initial period, the amount of milk substitute administered to neonates should increase (24% protein, 21% fat) to around 6–7 liters/day, and should be offered in two feedings until the age of 4–5 weeks (De Rosa et al. 2017). At this age, it is advisable to gradually lessen the amount of substitute offered and replace it with hay and concentrated feed ad libitum. If this process is begun at the age of 7–10 days, then it is feasible to plan on weaning the calves when they are about 90 days old and have achieved a body weight in the range of 80–90 kg, as this indicates that they have the capacity to gain 800–900 g of weight—or more—per day (De Rosa et al. 2017). Another recommendation is to house calves individually in separate pens for a period of 8 weeks. In fact, this is stipulated as a protective measure in European legislation. After that, the calves can be grouped indoors in larger corrals that hold various individuals. A recent study of 70 farms located in southern regions of Italy (De Rosa et al. 2017) found that handlers there separated neonates from their dams after 3 days, sufficient for them to ingest natural colostrum from their udders. The quantity of milk substitute provided there ranged from 2 to 8 L/day, with an average of 4.8 L. Concentrated feed and hay were made available to the calves on around day 18 postpartum, and they were weaned, on average, at 88 days of age, though some were weaned as early as day 60, while others were not weaned until around day 120. Mean body weight at weaning was 77 kg (min. 50, max. 115) (De Rosa et al. 2017). The findings from this study suggest that some calves did not have their nutritional requirement satisfied before weaning, a fact that negatively impacted their later performance as was manifested, for example, by body weights below expected levels.

A second factor that can affect calf welfare in this period is the amount of space set aside for the weaning area. Restricting this part of the farm may allow breeders to lessen their financial investment but it is a controversial measure when viewed from the angle of requirements for the calves' welfare (Grasso et al. 1999). In their study, Grasso et al. (1999) found that while a space allowance in the area of 1.0–2.6 m^2 per unweaned calf did not affect their growth, numerous variations in endocrine and immune parameters (such as antibody responses and levels of cortisol) were verified and interpreted as responses to restrictions on the space provided. Significantly, those researchers observed that moving the calves to an outdoor paddock favored the responses of their humoral and cellular immune systems, so this measure has the potential to impact mortality rates favorably. A study of Murrah buffalo calves by Bharti et al. (2015) compared two groups: one with neonates removed from their mothers at birth and given colostrum drawn from the dams; the second with dam-reared calves. While the two groups were found to have similar immunoglobulin levels, daily weight gain in the first was low. The conclusion to which all these findings leads is clear: it is necessary to ensure that buffalo calves, whether nourished by their mothers or separated soon after birth, ingest sufficient colostrum in the first days of extrauterine life to obtain protection from the pathogens and other potentially harmful conditions to which they may normally be exposed on the farm. Having said

this, it is also important to understand that the stress caused, for example, by early removal, can affect immune responses (humoral and cellular) to novel threats in the environment. Therefore, it is essential to ensure excellent hygienic conditions and provide ample space: two measures that will reduce deaths in this vulnerable animal species (Grasso et al. 1999).

An earlier study was carried out in Egypt on an experimental farm under controlled conditions. Those authors reported that buffalo calves in a control group weaned at 120 days had weight gains comparable to those registered in the study group of calves weaned as early as day 45 postpartum (Khatri et al. 1986). In contrast, a more recent analysis of river buffaloes, this one carried out on a commercial farm in Egypt, determined that early weaning can result in lower indices of growth and deteriorate the performance of calves in ways similar to animals that suffer pathological afflictions (diarrhea, for example), or those housed under inadequate conditions (Ali et al. 2015). A study by Abbas et al. (2017) of Pakistani Nili-Ravi calves that were weaned at 56 days also measured growth rates inferior to those of a group of calves weaned at 84 days. Moreover, regardless of group, all those calves were found to have decreased daily weight gains when milk substitute was administered at 10% vs. 15% of body weight. The situation of dairy buffalo calves in Asia contrasts sharply because (1) they are usually raised with their mothers from birth-to-weaning, and (2) in traditional operations they suck milk from one or two teats after milking (Kantharaja et al. 2018).

Artificial rearing is a topic that has been widely studied, but most reports document low growth rates in buffalo calves raised under these conditions, where they are given milk substitute at 10% of body weight (see Bharti et al. 2015; Kantharaja et al. 2018). Observations from work in Australia, where swamp buffaloes are raised in extensive conditions, have also verified reduced performance in calves reared by artificial means; indeed orphaned calves reared artificially there had death indices of up to 25% (Standing Committee on Agriculture 1995). The authors recommended a space allowance of 1.5–2 m^2/calf and emphasized the importance of increasing the frequency of handling as a way to reduce the stress caused by obligatory interaction with beasts that are not used to contact with humans. On a final note, Lemcke (2015) recommends weaning dam-raised calves when they attain a body weight of 120–150 kg, though the availability of feed and the mother's physical condition are two additional factors that must be evaluated.

5.4.2 Novel Weaning Strategies

The issue of separating calves from their dams at an early age is now a point of contention in dairy circles due, in part, to growing awareness among consumers and the wider society (Sirovnik et al. 2020). In the case of dairy cattle, breeders are currently working to devise novel methods that will prolong the duration of the dam-calf bond (Sirovnik et al. 2020). To the best of our knowledge, no studies of this kind have appeared for dairy buffaloes, but similar ethical questions are beginning to

deeply influence orientations towards the demand for mozzarella cheese, which is today the primary product derived from buffalo milk (De Rosa et al. 2015).

Buffalo calves are similar to other ruminant offspring in the sense that if they do not receive enough high-quality colostrum in the immediate postpartum period and, as a result, do not absorb the required quantity of immunoglobulins, the risk of disease and death during the neonatal period will rise, even though the colostrum produced by female river buffaloes is of greater nutritional quality than that of dairy cows, because it provides essential nutrients (IGF-1, ash, total solids, fat, phosphorus, lactose, vitamin E) at higher concentrations, however it has less Mg, vitamin A, Na, K, Zn, and lactoferrin (Abd El-Fattah et al. 2012). Buffalo calves have relatively high mortality rates, and this may be due, at least in part, to inadequate immune protection. For this reason, the use of esophageal feeders—now a common practice with dairy calves—has been recommended (Adams et al. 1985), though this method produces somewhat lower levels of serum immunoglobulins than bottle-feeding, especially if the amount of colostrum ingested is low (Godden et al. 2009). This effect may result from the fact that feeders introduce colostrum into the calf's rumen, not its abomasum, so it takes 2–4 h longer to reach the intestine (Lateur Rowet and Breukink 1983), when the intestinal walls may be less permeable. A second drawback of esophageal feeding may be the additional labor required, though this could be offset by reducing feedings from two to just one per day (Hopkins and Quigley 1997). Finally, great care must be taken when introducing the feeding tube so as not to injure the animal's oral-esophageal tract (epiglottis, larynx, pharynx, etc.), or insert it into the trachea, as that will introduce milk into its lungs.

Another important issue involves the workforce on buffalo farms, which may be inadequate if, for example, farrowing is concentrated at certain times of the year. In this case, the number of calves produced could make it difficult to provide the necessary care to each one. A second consideration here is the high economic outlay required to purchase milk substitutes. With respect to dairy cattle, this point has led Gleeson et al. (2007), Vecchio et al. (2013), and De Martino et al. (2018) to recommend administering this complement only once a day, but at double the concentration (36% DM) to produce growth rates comparable to those of calves that ingest the substitute twice a day (at 18% DM) up to weaning (90 days). This approach will also maintain the required levels of bactericide activity provided by haptoglobin and lysozyme.

In 2019, Serrapicad et al. designed an experiment to explore the possibility of developing "pre-stomachs" as a way to reduce weaning age and, in this way, enhance the profitability of breeding operations. They gave one group of calves, ad libitum, long hay (20 cm) combined with commercial pelleted starter; a second group received chopped hay (3–4 cm) with the same starter; while a third consumed only the pelleted starter, all beginning at day 15 of extrauterine life. Option 2 increased the size of the calves' stomachs compared to conditions 1 and 3 and lessened non-nutritive oral behaviors, but those calves had reduced growth, perhaps due to the fact that they devoted less time to eating the starter. In that experiment, group 3 (starter only) had the highest daily weight gains. Serrapica et al.'s findings suggest that while the latter dietary approach can raise indices of growth and reduce

Fig. 5.5 Common weaning practices in intensive Mediterranean dairy buffalo operations do not differ from those used with dairy cattle. These include early separation of the calves soon after parturition, and administering colostrum. Numerous studies of artificial rearing have been conducted, and most report lower growth rates in artificially reared buffalo calves (i.e., receiving milk substitute at 10% of body weight) compared to dam-reared ones

age at weaning, in the long run ingesting greater quantities of fiber likely stimulates pre-stomach development (as in the calves in group 2) and improves the weaned animals' subsequent performance. An earlier work found inferior weight gain in free-ranging buffalo heifers that were left to pasture and, as a result, consumed a diet higher in fiber than a control group of corralled animals that received concentrated feed. Those authors found no between-group differences in age at puberty (Sabia et al. 2014) and concluded that the animals which received more fibrous types of feed made more efficient utilization of nutrients (Fig. 5.5).

5.5 Final Considerations

Based on the information available on buffaloes, the stress generated during parturition is a condition that affects both animal welfare and dairy production. Thus, it is necessary to analyze and understand the dynamics of birth in order to distinguish the point at which normal conditions end and pathology begins. Another fundamental element of the productive life of female dairy buffaloes relates to milking efficiency

and efficacy, as these decisively influence their productivity and state of health. During milking, diverse factors can negatively impact the productive performance of female buffaloes, especially poor handling practices. Studies affirm that female buffaloes are more sensitive to stress factors than dairy cows during milking. Some researchers propose counteracting the harmful effects that may arise from human-animal interaction, and reducing behaviors associated with acute anxiety and stress in primiparous female buffaloes when they first come into contact with a milking machine by administering exogenous oxytocin and ensuring positive interactions with handlers in the productive unit and milking room. It is suggested that workers remain calm and treat the animals gently. Another recommendation is to implement habituation programs for buffaloes.

Though it has long been thought that female dairy buffaloes are less susceptible to mastitis than dairy cows, more recent evidence on disease incidence suggests that it is similar in both species. Our knowledge of the health of the buffalo udder is limited compared to what we know about dairy cows, but in both species the severely harmful effects of mastitis are evident. Despite this, the pain associated with cases of clinical mastitis are rarely measured or treated, and this has significant repercussions for levels of animal welfare. Fortunately, the tendency in recent years is to devote special attention to pain as a key aspect of mastitis. Additional studies are required to improve our ability to recognize and quantify the pain caused by mastitis (i.e., evaluating pain thresholds), and to characterize buffaloes' typical physiological and behavioral responses.

It is extremely important to gain a better understanding of fundamental aspects of the female buffaloes' behavior during parturition and milking, the neurophysiological mechanism of the normal pain that accompanies parturition, and the pain that a buffalo affected by mastitis may experience. This latter kind of pain must be prevented because if allowed to progress it will compromise the state of health and welfare of these animals, and affect the economic yield of breeders.

Giving calves access to a silvo-pastoral system at the time of weaning has beneficial effects on the live weight change of both the calves and their dams, and supplementation of the calves during the weaning period provides further benefits. The knowledge of specific weaning methods might enhance the welfare of the offspring and improve the reproductive efficiency of the buffalo cow. However, there is still little information available about such strategies. By now, even though some results are available, there are many questions without answers, leaving the field open for the development of future research.

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Buffalo Milk and Its Products: Composition, Nutrition and Benefits

6

Yogesh Khetra, G. S. Meena, and Sumit Arora

Abstract

Buffalo milk represents the second largest type of milk globally and contributes approximately 13% to the world's total milk production. Significant differences exist between buffalo and bovine milk, particularly with respect to higher fat and mineral contents. Differences also exist in the type and amount of micronutrients present in buffalo milk. These differences bring upon changes in physico-chemical and techno-functional properties and hence the conventional processing conditions used for bovine milk need to be altered sometimes for processing of buffalo milk. Nutritional value of buffalo milk is also significantly different from bovine milk. Owing to the inherent advantages it offers, buffalo milk is preferred for manufacturing several dairy products including Mozzarella cheese, paneer and khoa among others. Recent popularity in buffalo milk has attracted research attention owing to its micronutrient content and health claims. Several interesting components such as gangliosides and ceramides have also been identified. This chapter describes in detail the composition, nutritive value, important physico-chemical and technological properties and also highlights the major differences with bovine milk.

Keywords

Buffalo · Cow · Milk · Physico-chemical properties · Mozzarella cheese · Paneer

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6.1 Introduction

Global milk production is mainly dominated by five major animal species, namely cow, buffalo, goat, sheep and camel with their respective contributions of 83%, 13%, 2%, 1% and 0.4% (Food and Agriculture Organization 2020) and thus buffalo milk can be said to contribute significantly. Contrary to the developed countries, where cattle produce most of the milk, buffaloes, sheep, camel and goats collectively produce 33% of total milk production in developing countries (Food and Agriculture Organization 2020). India and Pakistan have the highest population of water buffaloes and also are the top most buffalo milk producing countries (Food and Agriculture Organization 2020). Buffalo milk occupies prime importance in Asian subcontinent and some of the world's famous buffalo breeds are available in India (Pandya and Khan 2006). However, population of buffaloes as well as interest in buffalo milk and milk products is now spreading to other countries as well.

Production and diversification of buffalo milk for manufacture of various dairy products is gaining importance in different buffalo milk producing countries. Scientifically, domesticated buffaloes or water buffaloes are known as *Bubalus bubalis* and are the main source of milk production in South Asia. Asian water buffalo is known as Bhains, Al-Jamoos, Karabue or Kwai, Carabao and Karabo in India, Arab countries, Thailand, Philippines and Malaysia, respectively. Buffaloes are now playing a prominent role in global milk production since they are the major milk producing animal in several countries. The river buffalo and swamp buffalo are two subspecies of domesticated buffaloes. Buffaloes are majorly populated in Asia, representing 97% of the total global populations while these are lesser populated in regions such as South America (0.7%), Australia and Europe (0.2%) and Africa (2%, particularly in Egypt). Further, river buffalo and swamp buffalo account for ca. 70% and 30% of world's buffalo population, respectively. The milk yield of river buffaloes (1500–4500 L milk per lactation) is significantly higher than swamp buffaloes which are smaller and mainly raised in eastern Asia for draught power (Food and Agriculture Organization 2020). Mehsana, Bhadawari, Jaffarabadi, Surti, Kunndi, Nili-Ravi and Murrah are some of the well-established breeds of dairy buffaloes. Indeed, as per Acharya (2009), Indian buffaloes are considered as the Black Gold, while Pandya and Khan (2006) reported buffaloes as "living tractor of Asia" and "a walking fertilizer factory".

6.2 Chemical Composition of Buffalo Milk *Vis-a- Vis* Bovine Milk

The chemical composition of buffalo milk and bovine milk is shown in Table 6.1. Milk fat, lactose and proteins are the first, second and third major constituents present in buffalo milk. Thus, in comparison to bovine milk, the concentration of all major milk constituents is naturally higher in buffalo milk. The concentration of milk fat in buffalo milk is almost double as compared to that present in bovine milk (Pandya and Khan 2006). According to Ahmad (2013), the ratio of total solids (TS),

Table 6.1 Chemical composition of buffalo milk (gram per 100 g)

Milk constituents → Species↓	Total solids (TS)	Fat	Solid-not-fat (SNF)	Protein	Lactose	Ash
	Range (g/100 g)					
Buffalo milk[a]	16.3–18.4	6.6–8.8	8.3–10.4	2.7–5.2	4.5–5.2	0.71–0.85
Bovine milk[bcdef]	12.8–13.7	3.7–4.3	9.1–9.6	2.9–3.7	4.6–5.0	0.70–0.72

Modified from [a]El-Salam and El-Shibiny (2011), [b]Kukovics and Németh (2013), [c]Pandya and Khan (2006), [d]Ahmad (2013), [e]Arora and Khetra (2017), [f]Rafiq et al. (2016)

fat and protein present in buffalo milk to that present in bovine milk are 1.28, 1.71 and 1.30, while the ratio for solid-not-fat (SNF), lactose and ash contents is 1.09. Thus, buffalo milk is richer in energy value and nutrition over bovine milk. Further, Pandya (2019) reported that due to higher TS, 100 g buffalo milk can provide higher (~100 calories) energy as compared to that provided by similar amount of bovine milk (~70 calories). Although, many scientific reports indicate that the average fat content of buffalo milk is nearly 7.5%, however, as per Ahmad (2013) this can also reach up to 15% under normal conditions. Thus, in nutshell, all macro components present in milk are higher in buffalo milk than in bovine milk. This results in better remuneration to the producers by fetching a higher price than bovine milk in Asian countries. Other than the differences in content of macronutrients, significant differences also exist in configuration of these macronutrients which are discussed in subsequent sections.

6.2.1 Milk Fat

Buffalo milk contains higher fat than bovine milk with larger size of the fat globules. Average fat globules size of buffalo milk is reported to be ~5 μm as compared to ~3.5 μm of bovine milk (Ahmad et al. 2008; Ménard et al. 2010). Ganguli and Bhavadasan (1980) reported the compactness, sphericity, surface roughness, surface area (μm^2), length (μm), width (μm) and orientation of fat globules present in buffalo milk and bovine milk as 0.71, 0.59, 0.91, 58.34, 9.85, 4.15, 107.46 and 0.81, 0.91, 0.99, 12.26, 4.67, 2.02, 90.79, respectively. Further, Ménard et al. (2010) opined that compositional differences in milk fat globule membrane (MFGM) led to variation in zeta-potential values of buffalo fat globules (-11.0 ± 0.7 mV) and bovine fat globules (-9.4 ± 0.6 mV). The fatty acid composition of buffalo and bovine milks has been presented in Table 6.2.

Myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1c9) are the main fatty acids present in buffalo and bovine milks, with the former containing higher amount of saturated fatty acids (buffalo 70.8 ± 0.8 and bovine 69.6 ± 1.7, % weight of total methyl ester) and the latter contain higher amount of unsaturated fatty acids (buffalo 29.2 ± 0.8 and bovine 30.4 ± 1.7, % weight of total methyl ester) (Ménard et al. 2010). Buffalo milk contains markedly

Table 6.2 Fatty acid composition of buffalo and bovine milk (up to nearest decimal, % of total methyl esters)[a]

Fatty acid	Buffalo	Cow	Fatty acid	Buffalo	Cow
C4:0	2.8–5.2	2.5–4.2	C6:0	1.4–3.0	1.4–2.9
C8:0	0.9–3.9	1.4–2.9	C10:0	1.5–3.8	2.3–4.6
C12:0	2.1–4.1	1.0–2.9	C14:0	9.1–11.8	9.1–13
C14:1 *cis*-9	0.5–1.2	0.7–1.4	C15:0	0.9–1.74	1.2
C16:0	25.7–36	24.7–33.8	C16:1*cis*- 9	1.0–2.8	1.0–2.8
C17:0	0.4–0.7	0.4–0.7	C18:0	8.6–14.5	8.9–13.9
C18:1 *cis*-9	17.4–26.2	22.13–26.4	C16:1 *trans*- 9	0.2–0.6	0.2–0.6
C18:1 *trans*-11	0.1–2.7	0.2–3.1	C18:2 *trans-9 trans-12*	0.2–0.7	0.2–0.6
C18:2 *cis-9 trans-11*	0.3–0.9	0.4–1.0	C18:2 *trans-10 cis-12*	0.02–0.03	0.03–0.05
C18:2 *cis-9 cis-12*	1.0–2.9	1.0–2.1	C18:3 n-3	0.2–1.4	0.5–1.1
C20:4 n-6	0.1–0.2	0.1–0.5	C20:5 n-3	0.1–0.3	0.1–0.4
C22:6 n-6	0.1–0.2	0.1	C20:0	0.2	0.1
Short-chain fatty acids (SCFA)	8.0–14.9	8.6–14.5	Saturated fatty acids (SFA)	62.1–70.8	55.7–69.6
Monounsaturated fatty acids (MUFA)	24.0–29.4	25.0–30.3	Polyunsaturated fatty acids (PUFA)	2.3–3.9	2.7–3.0
Conjugated linoleic acid (CLA)	0.4–0.9	0.5–1.1			

[a]Modified from El-Salam and El-Shibiny (2011)

higher amounts of long-chain fatty acids such as myristic acid (C14:0) and palmitic acid (C16:0) while the contents of short-chain fatty acid in both milks were similar. Among monounsaturated fatty acids, bovine milk contains markedly higher contents of oleic acid (C18:1 c9) while buffalo milk has distinctly higher concentrations of C18:1 trans-fatty acid, particularly vaccenic acid (C18:1 tr11). Rumenic acid (C18:2 c9 tr11), also known as the key conjugated linoleic acid (CLA) is present in prominently higher amounts in buffalo milk in comparison to bovine milk, hence, consumption of buffalo milk also advocates the health benefits of CLA. Ménard et al. (2010) also reported that the contents of total polyunsaturated fatty acids were lower in buffalo milk. However, considering the individual polyunsaturated fatty acids, it contained significantly higher amounts of linolenic acid (C18:3, ω-3). Triglycerides present in buffalo and bovine milk were separated according to their crystallization behaviour into low-melting triglycerides (LMT), medium-melting

triglycerides (MMT) and high-melting triglycerides (HMT). According to Sindhu and Arora (2011), the percent proportion of HMT in buffalo and bovine milks ranges as 5–6 and 9–12%, respectively. Further, these triglycerides were also separated as per their molecular weight into low-molecular weight triglycerides (LMDT), medium-molecular weight triglycerides (MMWT) and high-molecular weight triglycerides (HMWT) which clearly indicated that their proportion in bovine and buffalo milk were 28.2, 18.9, 52.9 and 40.5, 17.0, 42.5%, respectively.

6.2.2 Milk Proteins

The major and minor protein fractions present in buffalo and bovine milks are shown in Table 6.3. Casein and whey proteins are naturally present in a ratio of 4:1 in both buffalo and bovine milk. Quantitatively the casein content in buffalo milk is ~4% (80% of total protein) and whey protein content is 0.9% which in combination with non-protein nitrogen represents 20% of the total proteins (Sindhu and Arora 2011). In total casein and whey proteins present in buffalo milk, the percent of their individual fractions such as α_{s1}-casein, α_{s2}-casein, β-casein, γ-casein, κ-casein, α-lactalbumin, β-lactoglobulin, serum albumin, proteose peptone and lactoferrin are 1.4–1.8, 0.2–0.28, 1.25–1.6, 0.15–0.16, 0.4–0.5, 0.15, 0.40, 0.03, 0.32 and 0.03, respectively. Compared to bovine milk, the average proportion of α_{s1}-casein and β-casein fractions are higher (40 and 35%) in buffalo milk. Contrary to that, α_{s2}-casein and κ-casein fractions (6.3 and 12%) are lower in buffalo milk than bovine milk. El-Salam and El-Shibiny (2011) reported that α_{s2}-casein and κ-casein contents of buffalo milk were higher than that of bovine milk. Thus, presence of higher

Table 6.3 Major casein and whey protein fractions in buffalo milk and bovine milk

Protein fractions		Buffalo milk[a]	Bovine milk[a]	Buffalo milk[b]	Bovine milk[c]
		Concentration in milk (g/kg)		Percent of total casein/whey proteins	
Casein	α_{s1}-casein	14.4–18	12–15	30.2–31[d]	26.9–35.6[d]
	α_{s2}-casein	2.2–2.8	3–4	13–17.6[d]	7–12.1[d]
	β-Casein	12.6–15.8	9–11	28–33.9[d]	27.8–37.1[d]
	γ-Casein	1.6	1–2	–	4.70[d]
	κ-Casein	4.3–5.4	3–4	7–15.4[d]	8.5–19.6[d]
Whey proteins	α-Lactalbumin	1.4	2–4	16.2[e]	3.6–3.9[f]
	β-Lactoglobulin	3.9	1–5	50.3[e]	9.6–10.5[f]
Minor proteins	Proteose peptone	3.3	6–18	10.0[e]	–
	Serum albumin	0.3	1–4	8.7[e]	–
	Lactoferrin	0.3	0.05	–	–

Modified/compiled from [a]Ahmad (2013), [b]El-Salam and El-Shibiny (2011), [c]Kukovics and Németh (2013); [d]Percent of total casein, [e]Percent of total whey proteins, [f]Percent of total protein

κ-casein in buffalo milk resulted in faster rennet induced coagulation. Apart from major protein fractions, the minor protein fractions such as immunoglobulins were also present in buffalo milk. The content of IgG, IgM and IgA in buffalo milk are 8.71, 1.91 and 0.04 mg per mL. Campanella et al. (2009) determined IgG by new immunosensors and reported that the average concentration of IgG in bovine milk (0.77 mg per mL) was higher than that obtained in buffalo milk (0.67 mg per mL).

Wide variations have been reported in major and minor protein fractions of buffalo and bovine milks as presented in Table 6.3. However, as indicated earlier in Table 6.1, the total protein content of buffalo milk is higher than bovine milk which signifies better yield of different high-protein dairy products from buffalo milk.

6.2.3 Amino Acids

Table 6.4 indicates the quantity of essential and non-essential amino acids present in buffalo and bovine milks. All essential amino acids (valine, methionine, isoleucine, leucine, threonine, phenylalanine, lysine and histidine) are present in higher concentrations in buffalo milk as compared to bovine milk except arginine. Among the non-essential amino acids, bovine milk contains tyrosine in higher concentration while the concentration of aspartic acid, serine, glutamic acid, proline, glycine, alanine and half cystine are higher in buffalo milk. These quantitative differences in amino acids are attributed to the variation in protein content of bovine and buffalo milks (Ahmad 2013).

6.2.4 Minerals

Buffalo milk contains both major and minor minerals and the total mineral content is higher as compared to bovine milk (Table 6.1). Calcium and magnesium contents are ~1.5× higher in buffalo milk than bovine milk, while the latter contains higher concentrations of chloride, sodium and potassium (Khedkar et al. 2016). The breed, stage of lactation, health and nutritional status of animal, method of estimation as well as genetic and environmental factors collectively attribute to large variations in calcium content of buffalo and bovine milk as shown in Table 6.5. The ratio (buffalo milk: bovine milk) of total, diffusible and colloidal forms of calcium, magnesium, phosphate and chloride (Ahmad 2013) are as follows:

- calcium—1.54, 0.95, 1.78,
- magnesium—1.54, 0.95, 1.78,
- phosphate—1.59, 1.17, 2.38,
- chloride—0.94, 0.87, 2.0.

Table 6.4 Essential and non-essential amino acids in buffalo and bovine milk

Amino acids	Buffalo milk				Bovine milk			
	(g/kg[a])	(g/100 g of whole casein[b])	(g/100 g of protein[b])	(mg/100 g of milk[c])	(g/kg[a])	(g/100 g of whole casein[b])	(g/100 g of protein[b])	(mg/100 g of milk[c])
Essential amino acids, average								
Valine	2.52	7.1	5.85	234	2.41	7.2	5.88	188
Methionine	0.62	2.7	2.33	93	0.61	2.8	2.28	73
Isoleucine	2.48	6.0	4.85	194	1.98	6.1	5.03	161
Leucine	4.24	10.1	9.20	368	3.68	9.2	8.13	260
Threonine	1.22	4.4	4.35	174	1.05	4.9	4.38	140
Phenylalanine	2.31	5.4	4.58	183	1.89	5.0	4.50	144
Lysine	3.51	7.2	7.48	299	3.14	8.2	4.28	137
Histidine	1.66	2.9	2.73	109	1.49	3.1	2.28	73
Arginine	1.17	3.0	2.55	102	1.43	4.1	2.28	73
Tryptophan	–	1.4	–	–	–	1.2	2.28	73
Non-essential amino acids, average								
Aspartic acid	2.94	6.9	7.13	285	2.64	7.1	7.25	232
Tyrosine	0.48	5.8	4.53	181	0.53	6.3	4.63	148
Serine	0.72	6.1	4.65	186	0.58	6.3	3.25	104
Glutamic acid	9.96	22.7	21.4	857	8.51	22.4	19.8	634
Proline	4.44	11.9	12.0	481	3.42	11.3	10.4	334
Glycine	0.81	1.8	1.93	77	0.67	2.7	2.28	73
Alanine	1.57	3.0	3.03	121	1.34	3.0	3.16	101
Half cystine[1]/ Cysteine[2]	–	0.3[1]	0.83[2]	33[2]	–	0.3[1]	0.50[2]	16[2]

Compiled from [a]Ahmad (2013), [b]Pandya and Khan (2006), [c]Medhammar et al. (2012)

Table 6.5 Composition of major minerals present in buffalo milk and bovine milk

	Buffalo milk[a] (mg/100 mL)			Bovine milk[a] (mg/100 mL)			Buffalo milk[b] (mmol/L)			Bovine milk[b] (mmol/L)			Buffalo milk[c] (mg/100 g)	
Mineral	Total	Dissolved	% of dissolved phase	Total	Dissolved	% of dissolved phase	Total	Diffusible	Colloidal	Total	Diffusible	Colloidal	Total	Soluble
Calcium	183 [163–224]	40	22	114	39	34	47.1	8.2	38.9	30.5	8.6	21.9	112 ± 40–195	32.8 ± 8–55.2 ± 10.9
Magnesium	18 [16–30]	8	46	11	8	70	7.3	3.5	3.8	4.6	3.0	1.6	8 ± 2–35.5 ± 9.1	8.4 ± 0.2–17.7 ± 6.3
Sodium	44 [45–57]	42	95	50	47	94	20.3	–	–	17.5	–	–	35.0 ± 11–95.5	–
Potassium	107 [102–148]	101	95	148	143	98	28.7	–	–	42	–	–	92.0 ± 25.0–181.5 ± 21.2	
Phosphorus	82 [89–137]	26	31	85	38	45	–	–	–	–	–	–	99.0 ± 32.0–145.3 ± 26.9	28.5 ± 1.3–56.9 ± 12.9
Citric acid	159 [158–218]	115	84	166	152	96	–	–	–	–	–	–	–	–
Chloride	58 [57–106]	57	99	106	106	100	16.6	–	–	21.8	–	–	58.1 ± 2.8–74.9 ± 6.0	–
Phosphate	–	–	–	–	–	–	27.7	9.2	18.5	19.2	9.9	9.3		
Citrate	–	–	–	–	–	–	8.3	7.1	1.2	8.8	8.2	0.6	143.9 ± 28.3–321.60	114.8 ± 24.1–136.4 ± 7.7
Ca/P	1.71	1.11	–	1.04	0.84	–	–	–	–	–	–	–	–	–
(Ca + Mg)/(P + Citrate)	1.52	0.82		0.94	0.66	–	–	–	–	–	–	–	–	–

Compiled from [a]Pandya and Khan (2006), [a]Ahmad (2013), [c]El-Salam and El-Shibiny (2011)

Pandya and Khan (2006) reported that concentration of calcium plus magnesium (total divalent cations) in buffalo milk (200 mg per 100 mL) was ~1.5× higher as compared to that present in bovine milk (132 mg/100 mL). Further, it could be safely concluded that these cations concentrate in the colloidal phase as they are present in ~2× higher (150 mg per 100 mL) concentration in buffalo milk with respect to bovine milk. The range of copper, iron, boron, zinc, sulphur, iodine, cobalt, fluoride (in ppm) and magnesium (μg per100 mL) contents in buffalo milk are 0.07–2.6, 0.4–13, 0.5–1.4, 3.2–7.3, 157–314, 8.6–19.4, 0.7–1.6, 0.4–18.5 and 38.2–65.8, respectively (Pandya and Khan 2006). According to Khedkar et al. (2016), the calcium to phosphorous (Ca/P) ratio of buffalo milk (1.8) is higher than bovine milk (1.2).

6.2.5 Vitamins and Enzymes in Buffalo Milk

Buffalo milk contains both water and fat soluble vitamins. Khan et al. (2019) reported that niacin, vitamin B6, biotin and vitamin C contents (mg/100 g) of buffalo milk were higher as compared to bovine milk; however, these milks contained similar thiamine and vitamin D contents. Further, vitamin A, riboflavin, vitamin E, pantothenic acid and vitamin B6 was higher in bovine milk over buffalo milk as indicated in Table 6.6.

Table 6.7 indicates different enzymes present in bovine and buffalo milks. Sindhu and Arora (2011) reported that buffalo milk contained higher lipase, alkaline phosphatase, lactoperoxidase, ribonuclease and protease as compared to bovine milk. However, the concentrations of xanthine oxidase and lysozyme were higher in

Table 6.6 Vitamin concentration in buffalo milk and bovine milk

Vitamins	Buffalo milk[a]	Bovine milk[a]	Buffalo milk[b] (mg/100 g)	Bovine milk[b] (mg/100 g)
Vitamin A (IU/mL)	340	230	69	46
Thiamine (mg/L)	0.2–0.5	0.2	0.05	0.05
Riboflavin (mg/L)	1.59	2.33	0.11	0.17
Pyridoxine (mg/L)	3.25	2.6–3.0	–	–
Ascorbic acid (mg/L)	2.2	1.94	–	–
Tocopherol (μg/g)	334.2	312.2	–	–
Vitamin E	–	–	0.19	0.21
Niacin	–	–	0.17	0.09
Pantothenic acid	–	–	0.15	0.37
Vitamin B6	–	–	0.33	0.04
Vitamin B12	–	–	0.40	0.45
Biotin	–	–	13	2.0
Vitamin C	–	–	2.5	0.09
Vitamin D	–	–	2	2

Compiled from [a]Sindhu and Arora (2011), [b]Khan et al. (2019)

Table 6.7 Enzyme concentration in buffalo milk and bovine milk

Enzymes	Buffalo milk[a]	Buffalo milk[b]	Bovine milk[a]
Lipase (U/mL)	0.16–1.13	0.2–1.1	0.1–0.6
Alkaline phosphatase (U/mL)	0.12–0.18	0.1–0.2	0.08–0.12
Xanthine oxidase (U/mL)	0.075	0.1	0.092
Lysozyme (μg/mL)	0.152	0.2	0.18
Lactoperoxidase (U/mL)	5.2–9.8	5.2–9.8	4.36–7.16
Ribonuclease (μg/mL)	9.78	9.8	8.23
Protease (U/mL)	0.78	0.8	0.68

[a]Sindhu and Arora (2011), [b]Sahai (1996)

bovine milk over buffalo milk as shown in Table 6.8. Pandya and Khan (2006) reported that the activity of alkaline phosphatase and lipase is lower in buffalo milk as compared to bovine milk. Further, both bovine and buffalo milks have similar xanthine oxidase activity, but protease activity is slightly lower in bovine milk than buffalo milk.

6.3 Important Physico-Chemical and Technological Properties of Buffalo and Bovine Milk

Physico-chemical constants of buffalo and bovine milk are presented in Table 6.8. The pH of buffalo milk is higher than that of bovine milk. Prajapati et al. (2017) reported that specific gravity, viscosity, freezing point and electrical conductivity of buffalo milk were higher than that of bovine milk. However, acidity, surface tension and refractive index values were higher in bovine milk. As evident from Table 6.8, the upper value of the reported range of different physico-chemical constants advocates that melting point of milk fat, acid value, RM value and buffer value (at pH 5.1), BR reading, curd tension, grain size, size and number of fat globules (per mm^3), L^* value and density values of buffalo milk were higher than that of bovine milk. However, saponification, iodine and Polenske values, softening point of milk fat, urea content, oxidation reduction potential and phosphatase activity values were higher in bovine milk than buffalo milk. Ahmad (2013) reported that the heat coagulation time (HCT, the resistance shown by milk sample towards its heat induced coagulation usually at 140 °C) of buffalo milk (8 min 48 s) was higher than that of bovine milk (6 min 30 s). Contrary to that, Prajapati et al. (2017) observed markedly higher HCT values in bovine milk (53 ± 4.86 min) as compared to buffalo milk (41 ± 3.51 min). El-Salam and El-Shibiny (2011) opined that buffalo milk exhibit lower heat stability than bovine milk owing to different urea, fat and calcium content. HCT of buffalo milk changes as a function of change in pH, exhibiting behaviour similar to type A milk, showing maxima at pH ~6.7 and minima at pH ~6.9 in HCT-pH curve. Buffalo milk also contains higher values of thermal expansion and thermal conductivity over bovine milk, while the latter milk exhibits higher heat capacity. These parameters are of vital significance for dairy

Table 6.8 Physico-chemical constants of buffalo and bovine milk

Parameters[abc]		Buffalo milk	Bovine milk
Softening point, (°C)		34.3–36.3	33.5–35.9
Melting point, (°C)		33.4–46.4	31.5–35.2
Acid value, (μm)		0.17–0.352	0.25–0.27
Refractive index		1.3448–1.4533	1.3338–1.4530
BR Reading		41.00–43.50	41.05–42.40
Saponification value		218.23–236.10	221.0–238.0
Iodine value		27.00–33.90	27.70–37.32
Reichert-Meissl value		27.83–35.50	24.6–29.7
Polenske value		0.7–1.6	1.3–1.8
Grain size, mm		0.20–0.41	0.098–0.190
Buffer value at pH 5.1		0.0417	0.0359
Curd tension, (g)		32–85	28–54
Density at 20, (°C)		1.0310	1.0287
Fat globules size, (nm)		0.5–7.5	3.85
Fat globules, millions per mm^3		3.2	2.96
Fluorescence under UV light		Greenish yellow	Pale bluish
Colour	White (L) (a.u.)	74	73
	Green (−a) (a.u.)	−1.6	−2.0
	Yellow (b) (a.u.)	5.6	7.4
Energy (kcal/kg)		1035	701
pH		6.74–6.81	6.60–6.76
Acidity	% lactic acid	0.13	0.15
	(D°, 1°D = 0.1 g/L)	16.2	15.3
Urea, (mg/L)		175–237	304–400
Freezing point, (°C)		−0.518 to −0.590	−0.530 to −0.521
Heat coagulation time (HCT)		8 min 48 s	6 min 30 s
Heat capacity, (Cal/g/°C)at 20, (°C)		0.852 ± 0.017	0.933–0.954
Oxidation reduction potential, Eh (+V)		0.31–0.5391	0.258–0.5367
Phosphatase activity (units/100)		28	82
Surface tension, (dynes/in)		42.75	42.57
Thermal conductivity, (kcal/h m °C)		0.5689 ± 0.00734	0.460
Electrical conductivity, (mmhos)		6.69–9.17	6.615–11.12
Thermal expansion		4.106×10^{-4}	–
Viscosity, (cp)		2.04	1.86

Modified/compiled from [a]Ahmad (2013), [b]El-Salam and El-Shibiny (2011), [c]Pandya and Khan (2006)

product formulation involving heating and cooling of these milks. A similar trend was also reported by Sindhu and Arora (2011) for these parameters. Buffalo milk is richer in energy than bovine milk, which is evident by virtue of its chemical composition (Table 6.1). However, digestibility of bovine milk is better than buffalo milk as advocated by lower curd tension (Table 6.8). Higher buffer index values as indicated by slow decrease in pH during acidification were observed in buffalo milk

as compared to bovine milk due to the presence of higher content of proteins and minerals particularly caseins and inorganic phosphate in buffalo milk (Ahmad et al. 2008). Buffalo milk and bovine milks were acidified with glucono-delta-lactone (GDL) and pH at gelation ranged between 5.1 and 5.2 for bovine and 5.5 and 5.9 for buffalo milk (Abd El-Salam et al. 1996; Kim and Kinsella 1989). Further, owing to the differences particularly in casein and calcium content in colloidal phase, buffalo milk exhibits shorter rennet coagulation time (RCT). Even after similar heat treatment, the resultant increase in RCT is lower in buffalo milk. The appearance of buffalo milk is bright white while bovine milk is yellowish-white in colour.

6.4 Significance of Buffalo Milk in Product Formulation

Unlike Italy, buffalo milk industry is mainly unorganized in Africa and Asia (Ahmad 2013). Indians are big fan of buffalo milk because boiled whole milk forms a thick layer of cream (also called *malai*) during cooling to room temperature and its thickness further enhances during subsequent storage. Further, consumers are more attracted towards buffalo milk owing to its higher viscosity and better mouthfeel. Higher concentration of whey proteins present in buffalo milk liberates higher concentration of sulfhydryl compounds upon boiling that imparts a cooked and nutty flavour in boiled, concentrated milks and other heat desiccated products (such as *khoa*, r*abri*, b*asundi,* etc.), which improve their acceptability among consumers (Ahmad 2013). Buffalo whole milk fetches higher market price than bovine milk owing to its chemical make-up, sensory attributes and ability to provide products with higher yield in shorter time.

In India, buffalo milk is either admixed with bovine milk or used separately since it is better suited for the manufacture of number of dairy products (as shown in Fig. 6.1). This is primarily attributed to the higher TS, fat, protein and calcium content of buffalo milk. The presence of higher TS advocates higher yield of dairy products (such as fat-rich dairy products, condensed and dried milks, high-protein powders, traditional Indian dairy products, etc.) in shorter processing time. Buffalo milk contains higher solid fat content (SFC) along with larger fat globules as compared to bovine milk which helps in separation of cream followed by more efficient churning of cream to butter accompanied with better fat recovery. Hammad (1993) also reported that in comparison to bovine milk, fat globules present in buffalo milk are less stable, due to low Laplace pressure (0.8–1.6 kPa) as compared to that of bovine milk (1.1–2.2 kPa); hence, they can be churned in shorter time (Ménard et al. 2010). The yield (calculated on milk basis) of fat-rich dairy products such as cream, butter, butter oil and ghee (clarified milk fat) is always higher when produced from the buffalo milk.

Production of concentrated milk, sweetened condensed milk (SCM) and evaporated milks involves a major step of water removal via evaporation in multiple effect evaporators (vacuum pan in case of SCM), reverse osmosis or their combination. Additional water removal is required for the conversion of such concentrated milks into dried milks (such as skim milk powder and whole milk powders) via roller

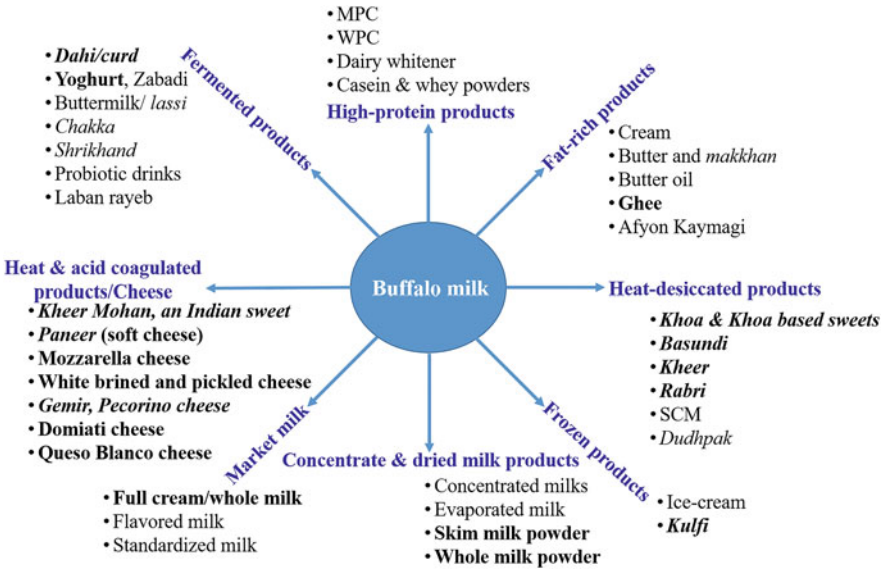

Fig. 6.1 Various dairy products manufactured from buffalo milk (preparation of products marked bold are preferred from buffalo milk)

or spray drying. Thus, these processes consume markedly higher energy. Cooling and chilling also demand high energy during milk processing. Hence, by virtue of natural composition of buffalo milk (particularly higher total solids content), lower heat capacity, higher thermal expansion as well as higher thermal conductivity value underlines lower energy requirement during preparation of different dairy products from similar weight or volume of buffalo vis-a-vis bovine milk (Pandya 2019). Thus, utilization of buffalo milk results in saving of processing time, energy and manpower along with better throughput. Presence of higher casein and whey proteins in buffalo milk are also responsible for imparting a distinct whitening effect to tea and coffee. Among the major milk components, presence of higher β-casein (favours humanization of buffalo milk), lactose, phosphorous, calcium, calcium/phosphorous ratio and lower potassium and sodium and in minor components higher concentration of lactoferrin and taurine while lower urea concentration in buffalo milk as compared to bovine milk collectively enables buffalo milk as a better ingredient for infant formula (Pandya 2019). The presence of higher peroxidase activity in buffalo milk advocates its better keeping quality over bovine. Moreover, its higher TS and fat content enables toning of larger volume of milk to enable liquid milk supply at far distant places (Pandya 2019).

High-protein powders such as milk protein concentrate (MPC), milk protein isolate (MPI), native casein powder or micellar casein isolate (MCI) are currently gaining popularity and penetration in food and pharma industries due to their unique nutritional and functional properties. These powders are produced from skim milk employing microfiltration (MF), ultrafiltration (UF), diafiltration (DF, if required),

optional evaporation and spray drying. Buffalo milk is rich in total milk proteins; hence, manufacturing of these value added, high-proteins powders from buffalo milk definitely curtails their overall production time and markedly improves plant economy via decreasing the extent of milk proteins (casein or casein plus whey proteins) concentration and purification as compared to bovine milk. Apart from this, permeate obtained from microfiltration (MF) is rich in whey proteins while that produced by ultrafiltration (UF) is rich in lactose content. Hence, such permeates will act as ideal raw material for production of different powders such as ordinary whey powders, demineralized whey powders, whey protein concentrates (WPC), whey protein isolate (WPI) and lactose powders with comparatively higher yield as compared to bovine milk MF/UF permeates. Patil et al. (2018, 2019) and Mahadev and Meena (2020) manufactured and characterized MPC60, MPC68 and MPC80 powders from buffalo milk. Buffalo milk based MPC60 expressed higher water binding, oil binding capacity and heat stability than similar products prepared from bovine milk. The dispersibility and solubility of buffalo MPC60, MPC68 and MPC80 powders were reported as 63, 48, 7% and 67.13, 82, 24.40%, respectively, and these values were markedly lower than MPC60-MPC80 powders from bovine milk (Patil et al. 2018, 2019; Mahadev and Meena 2020). Presence of higher casein and calcium in buffalo milk favoured casein-casein and calcium-casein interactions during spray drying but decreased dispersibility and solubility of MPC60; dispersibility, porosity, heat stability, solubility and flowability values of MPC80 powder which was also advocated by a tendency of clustering in particles of these powders. Production of such powders from buffalo milk poses numerous challenges since the existing technological processes for bovine milk may not deliver the desired techno-functional properties. Therefore, concerted scientific interventions are required for the manufacture of buffalo milk based dairy products including high-protein powders (MPC, MPI, MCI, etc.) and several cheese varieties.

Buffalo milk is suited better for the preparation of *paneer* (soft cheese), a heat and acid-coagulated product as compared to bovine milk. Khan and Pal (2011) reported that the key sensory attributes of a good quality *paneer* include marble white colour, nutty flavour, and sponge like body, close knit and smooth texture along with sweetish, mildly acidic taste. They summarized that the presence of higher casein and mineral (calcium, phosphorus) in buffalo milk imparted desired firm and rubbery body, while higher amount of total fat with bigger size of fat globules, larger casein micelles exhibiting relatively lower solvation and voluminosity attributed towards imparting desired spongy character to buffalo milk *paneer*. These desired attributes are lacking in bovine milk *paneer* due to differences in characteristics of fat globules and casein micelles.

The popularity of buffalo milk based cheeses is now rising globally. Production of number of soft, semi-hard and hard varieties of cheese as well as cheese spreads exhibiting unique sensory attributes (such as mild flavour and typical body and textural characteristics) from buffalo milk is now a reality. Mozzarella and Stracciatella (Southern Italy), Blu di Bufala, an Italian blue-veined (Bergamo, Italy), Domiati (Egypt), Paneer (India), Queso Blanco (South and Central America), White Brined and Pickled cheese (Balkan countries) are some of the examples of

well-established buffalo milk cheeses. Table 6.9 shows the chemical composition of buffalo milk-based selected milk products.

6.5 Nutritive Value of Buffalo Milk

Buffalo milk contains higher amounts of all macronutrients viz. fat, protein, lactose and minerals as compared to bovine milk which makes it superior in terms of nutritional value and energy. Buffalo milk consumption has been reported to be beneficial for conditions including atherosclerosis, milk allergy, anaemia and dental problems (Ahmad 2013). This section of the chapter summarizes nutritional significance of all macro- and micronutrients present in buffalo milk.

6.5.1 Milk Fat

Buffalo milk contains significantly higher amount of fat which contributes to higher energy and nutritive value (Pandya and Khan 2006). It has been reported that buffalo milk is rich in PUFA with significantly higher percentage of phosphatidylcholine (Ménard et al. 2010). Differences also exist in the amounts of medium chain triglycerides (MCT) in buffalo and cow milk with the former containing significantly higher amounts (Table 6.2). This difference enables buffalo milk fat to act as weight loss agent since these MCTs are absorbed more efficiently than long-chain fatty acids in the gastrointestinal tract (Papamandjaris et al. 1998). Buffalo milk is also rich in conjugated linoleic acid (CLA) which has been shown to inhibit the growth of some cancers, including prostate, stomach and breast cancer (Kim et al. 2016; Koba and Yanagita 2014). Moreover, buffalo milk contains significantly lower amount of cholesterol (0.5 g/L) than cow milk (3.14 g/L) and thus it can be a good source of nutritious fat for the population suffering from cardiovascular diseases.

6.5.1.1 Gangliosides

Buffalo milk is unique in having gangliosides (GS) that are absent in bovine milk. A ganglioside is a molecule composed of a glycosphingolipid (ceramide and oligosaccharide) with one or several sialic acids (e.g. *n*-acetylneuraminic acid, NANA) attached to the sugar chain. The oligosaccharide and sialic acid moieties of GS are hydrophilic. A sphingoid long-chain base and its amide-bound fatty acid form the lipophilic ceramide moiety (Colarow et al. 2003). Individual GS classes have high structural diversity as compared to other lipids owing to the differences in their sphingoid and fatty acid moieties.

Milk fat globule membranes (MFGM) contain about 90% of total dairy GS. The molar concentration of lipid bound sialic acid (LBSA) in mature human milk is 6.8–14.0 μmol/L after 16–35 days of lactation. Mature bovine milk (30–180 days) contains 4.1–4.4 μmol/L LBSA (Colarow et al. 2003). Mature Italian buffalo milk has 40–100% more LBSA as compared to Swiss cow milk (Colarow et al. 2003). Gangliosides are important bioactive compounds that exhibit beneficial

Table 6.9 Chemical composition of selected milk products exclusively manufactured from buffalo milk

Product name		Total solids (moisture)	Fat	Protein	Lactose/(total carbohydrates)	Ash	pH	References
Kheer Mohan		66.18	6.22	8.34	1.28/(50.6)	1.02		Baghubhai et al. (2015)
Yoghurt		13.76	3.25	3.95	5.72	0.84	4.32	Yadav et al. (2018)
MPC 60		98.12	3.93	58.47	27.49	8.24	7.17	Patil et al. (2018)
MPC 80		96.47	2.24	77.59	6.68	9.99	7.28	Mahadev and Meena (2020)
Khoa		76.06	35.13	17.56	20.59	2.74	6.48	Choudhary et al. (2019)
Paneer[a]		43.01–49.08	18.10–27.97	14.89–18.43	2.29–2.69	1.80–2.20	–	Kumar et al. (2014)
WPC powder[b]		95.10	1.46	76.84	10.50	6.30	–	Sawhney et al. (2014)
WMP		96.37	33.67	21.35	–	4.84	–	Borges et al. (2017)
Buffalo milk powder		–	4.34	46.68	41.22	7.84	–	Hammes et al. (2015)
MFLDW[d]	Liquid	32.89	3.32	16.39	–/(10.82)	2.36	–	Khatkar et al. (2014)
	DM basis[c]	100	10.09	49.82	32.89	7.17	–	
Cheeses	Mozzarella	49.72	23.11	20.13	–	3.17	5.60	Fasale et al. (2017)
	Cheddar	61.35	32.45	20.50	–	4.40	4.76	Pagthinathan and Nafees (2016)
	Soft pickled	46.70	21.60	14.40	–	2.70	–	Shahein et al. (2014)
Sandesh		73	21.9	12.4	–/(37.1)	1.6	–	Sanyal et al. (2011)
Chakka		31.61	13.93	14.04	3.17	0.89	4.39	Panda (2010)
Shrikhand		61.06	6.31	6.96	–/ (47.24)	0.52	4.38	

[a]Manufactured from buffalo milk containing 3.5–6% fat
[b]Per 100 g on dry matter (DM) basis
[c]Calculated
[d]*MFLDW* medium fat liquid dairy whitener

anti-infective, anti-inflammatory and neuronal development effects (Perea-Sanz et al. 2018).

6.5.1.2 Carnitines and Valerobetaine

Buffalo milk has been reported to have higher levels of carnitine precursors, carnitine and short-chain acylcarnitines. L-Carnitine (Cnt) is a water soluble quaternary amine which is present ubiquitously in plants and animals. Servillo et al. (2018) indicated significantly higher levels of carnitine precursors including valerobetaine (δ-VB) and GABA betaine (ɣ-BB). The contents of carnitine (Cnt), acylcarnitine (C2Cnt), propionylcarnitine (C3Cnt), butylcarnitine (C4Cnt) and 3-methyl butylcarnitine (3MeC4Cnt) were also reported to be significantly higher as compared to cow milk (Table 6.10). These carnitine precursors, carnitine and short-chain acylcarnitines have been demonstrated to possess antioxidant and anti-inflammatory functions. The neuroprotective potential of these compounds has also gained much research attention recently. The role of these compounds in Alzheimer's disease, hypoxia-ischemia and brain injury has been investigated. Further, owing to higher concentrations of these carnitines, buffalo milk can be a very good source for infant formula preparations. D'Onofrio et al. (2019) studied the beneficial effects of buffalo milk δ-VB and reported that buffalo milk enriched with 0.5 mM δ-VB had significantly higher antioxidant and anti-inflammatory activities. The efficacy of buffalo milk δ-VB to counteract oxidative stress and cytokine release induced by hyperglycaemia was also reported and a decrease in reactive oxygen species (ROS), malondialdehyde (MDA), cytokine levels was observed. Thus, the involvement of δ-VB in antioxidant and anti-inflammatory potential of buffalo milk was suggested.

6.5.2 Minerals

Buffalo milk contains significantly higher amount of calcium and hence carries great potential for controlling osteoporosis. Recommended Daily Allowance (RDA) for

Table 6.10 Concentration (in μmol/L) of carnitine precursors, carnitine and short-chain acylcarnitines in buffalo, cow and human milk

		Buffalo milk	Cow milk
		μmol/L	
Carnitine precursors	δ-VB	106.8	58
	ɣ-BB	32[a]	21[a]
Carnitine and short-chain acylcarnitines	Cnt	125[a]	250[a]
	C2Cnt	100[a]	150[a]
	C3Cnt	40[a]	115[a]
	C4Cnt	8[a]	56[a]
	3MeC4Cnt	2[a]	15[a]

[a]Approximate values estimated from bar chart given by Servillo et al. (2018)

calcium is 600–800 mg/day (Kamala et al. 2011) and accordingly a 200 mL serving of cow milk provides approximately 28–38% of RDA for calcium while the same serving of buffalo milk provides 45–60% of RDA for calcium. Thus, buffalo milk consumption enhances dietary calcium consumption required for combating osteoporosis.

Caseinophosphopeptides (CPP) and glycomacropeptide (GMP) have been proven beneficial against dental carries. CPP and GMP act against the cariogenic organism *Streptococcus mutans*. Moreover, CPP also forms nanoclusters with amorphous calcium phosphate at the surface of the tooth and acts as a reservoir of calcium along with phosphorous ions. This facilitates maintaining a state of supersaturation with respect to tooth enamel and thus reduces pH drop and also provides ions for tooth enamel remineralization.

6.5.3 Proteins

Buffalo milk proteins are a rich source of essential amino acids as well as biologically active peptides derived from them mostly through in vitro enzymatic hydrolysis. Such peptides have been reported to confer several health benefits on the host. Both substrate (variation in native protein across species) and enzyme specificity have a major influence on the release of such bioactive peptides and their activities as well.

Total protein content of buffalo milk is higher than bovine milk. However, the proteins in milk of both are majorly homologous (~92%). This underlines the prominent use of buffalo milk casein in obtaining bioactive peptides expressing active ACE-inhibitory, antimicrobial activity, and antioxidant property as well as osteoblast proliferation activity via replacing bovine milk caseins. Proteolysis of buffalo milk casein fractions (α_{s1}, α_{s2} and β) result in formation of phosphopeptides similar to that obtained from casein fractions of bovine milk, since casein fractions present in both species have similar phosphoserine clusters. Caseinophosphopeptides (CPP) from buffalo milk showed almost equal calcium solubilization and binding abilities as compared with bovine casein. Saini et al. (2014) identified 8 phosphopeptides exhibiting calcium binding property and the sequences were reported to be different than that of bovine milk.

Bovine and buffalo milks have similar amino acid sequences for β-casein f (165–209). Further, liberation of peptides from this bovine milk casein fraction showed ACE-inhibitory and immunomodulatory activities (Minervini et al. 2003; Pandya and Haenlein 2009; El-Salam and El-Shibiny 2013; Abdel-Hamid et al. 2017; Li et al. 2020). Hence, to achieve similar activities in liberated bioactive peptides, β-casein fraction of buffalo milk was exploited as a prominent substrate.

ACE-inhibitory peptide sequence LVYPFPGPI has been identified from buffalo milk β-casein. The hydrolysis was carried out by using *Lactobacillus helveticus PR4* proteinase. Bovine sodium caseinate hydrolysate and buffalo milk β-casein hydrolysate were compared for their IC50 values. Buffalo milk β-casein f(58–66) showed an IC50 value of 112.6 μg/mL while that of bovine sodium caseinate ranged between

16.2 and 57.2 μg/mL suggesting significantly higher ACE-inhibitory potential of buffalo milk peptides (Minervini et al. 2003). Abdel-Hamid et al. (2017) prepared ACE-inhibitory bioactive peptides from buffalo skim milk retentate using papain, trypsin and pepsin. Peptides prepared using papain exhibited maximum ACE-inhibitory activity. Size exclusion chromatography of the hydrolysates suggested that the molecular weight of the fraction with significant ACE-inhibitory activity was in the range from 1.8 to 0.5 kDa.

Peptides with significant antioxidant potential have also been prepared from buffalo milk (Shanmugam et al. 2015; Shazly et al. 2017a, b, 2019). Hydrolysates were prepared from all the fractions of casein using pepsin, trypsin and chymotrypsin and several peptides with potential antioxidant activity were reported (Shanmugam et al. 2015). EDVPSER (f84–90), NAVPITPTL (f115–123), VLPVPQK (f170–176) and HPHPHLSF (f98–105) derived from buffalo's α_{s1}-casein, α_{s2}-casein, β-casein and κ-casein**,** respectively, were identified as the active fractions with VLPVPQK (f170–176) possessing the maximum activity. Similarly antioxidant peptides viz. RELEE (f18–20 β-CN), MEDNKQ (f69–74 α_{s1}-CN) and TVA (f163–165 κ-cn) were obtained from buffalo casein with activities similar to the cow milk peptides (Shazly et al. 2017b). Antioxidant peptides prepared using different enzymes have been reported to have different activities. Shazly et al. (2019) hydrolysed cow and buffalo casein by alkalase, trypsin, pepsin or papain and reported that buffalo casein hydrolysates contained more hydrophobic amino acids when pepsin was used followed by trypsin, alkalase and papain. Buffalo casein hydrolysates prepared using alkalase exhibited greater antioxidant capacity due to high level of hydrophobic amino acids as compared to its cow casein counterpart. Further, the polymorphism of αs1-casein has been reported to have significant effect on the digestive properties and bioactivities of buffalo milk protein in simulated gastrointestinal digestion (Li et al. 2020) (Table 6.11).

In addition to ACE-inhibition and antioxidant properties, buffalo milk protein derived peptides have also been investigated for potential osteoblast proliferation and antimicrobial activity. Reddi et al. (2016) reported buffalo milk protein derived peptides with osteoblast activity and suggested the use of buffalo milk for improving bone health. Bajaj et al. (2005) prepared peptides from buffalo α_{s1}-CN and α_{s2}-CN and reported that the peptides possess antimicrobial activities against *Micrococcus luteus*, *Escherichia coli* and *Bacillus cereus*. Chanu et al. (2018) reported the presence of several buffalo milk derived antimicrobial peptides including defensins, cathelicidins and hepcidin with broad spectrum antimicrobial activity against gram positive and gram negative bacteria.

6.6 Conclusion

The basic composition of buffalo milk and its properties have been well documented. Buffalo milk has an edge over cow milk in many aspects including higher amounts of all macronutrients. Buffalo milk is preferred globally for manufacturing several cheeses with Mozzarella being the prominent variety. In

Table 6.11 Protein fractions from buffalo milk with active peptide sequence and their biofunctional properties

Protein fraction	Peptide sequence	Biofunctional property	Reference
α_{s1}-casein	EDVPSER (f84–90) MEDNKQ (f69–74) FFVAPFPEVFGK (f38–49)	Antioxidant and osteoblast proliferation Antioxidant ACE-inhibition	Shanmugam et al. (2015), Reddi et al. (2016), Shazly et al. (2017a) and Li et al. (2020)
α_{s2}-casein	NAVPITPTL (f115–123)	Antioxidant and osteoblast proliferation	Shanmugam et al. (2015) and Reddi et al. (2016)
β-Casein	LVYPFPGPI	ACE-inhibition	Minervini et al. (2003)
	VLPVPQK (f170–176)	Antioxidant and osteoblast proliferation	Shanmugam et al. (2015) and Reddi et al. (2016)
	RELEE (f18–20)	Antioxidant	Shazly et al. (2017a)
κ-Casein	HPHPHLSF (f98–105) TVA (f163–165)	Antioxidant and osteoblast proliferation Antioxidant	Shanmugam et al. (2015), Reddi et al. (2016) and Shazly et al. (2017b)

countries like India, buffalo milk is successfully being utilized in the manufacturing of selected traditional dairy products and even preferred over cow milk for *paneer*, *khoa*, butter, *ghee*, milk powders, etc. Production of high-protein powders from buffalo milk such as milk protein concentrate, dairy whiteners, etc. is possible after suitable technological interventions. Recently, the micro-constituents of buffalo milk have been studied largely in relation to their health benefits. Several interesting components have been identified in buffalo milk particularly from MFGM. Still, significant scope exists in the area of exploring the properties of micronutrients such as gangliosides, ceramides, etc. and also in validating their health claims. The differences between the breeds may also be of interest and needs further investigation.

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Welfare of Buffaloes at Slaughter: Signs of the Return of Sensibility

7

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Abstract

Indicators of consciousness and insensibility are tools that make it possible to evaluate, though indirectly, cerebral functioning and so determine if stunning has been performed adequately. When stunning is effective all signs of consciousness disappear and those of unconsciousness or insensibility become manifest. It is extremely important to emphasise that slaughtering cannot be allowed under

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circumstances that produce pain, so correct stunning methods are essential for ensuring the welfare of animals throughout the production process. Key indicators that aid in evaluating stunning quality are the absence of the righting, pain withdrawal, palpebral, and corneal reflexes, the animal's respiratory rhythm, and the absence of vocalisations. The objective of this chapter is to evaluate recent scientific findings regarding how to accurately identify states of unconsciousness or insensibilisation, the neurophysiology of pain impulses, and the importance of recognising the signs of the return to sensibility or consciousness before death in large ruminants (*Bubalus bubalis, Bos indicus, and Bos taurus*), especially the river buffalo. A second aim is to raise operators' awareness of the importance of using adequate stunning techniques and recognising the signs of the return to sensibility as essential tools for assessing stunning quality.

Keywords

Buffalo · Slaughter · Welfare · Stunning

7.1 Introduction

River buffaloes (*Bubalus bubalis*) are often raised in the same infrastructure and installations on ranches designed for bovine cattle (genus *Bos*), and are subjected to identical processes in abattoirs. But this compromises their welfare. The most important ante-mortem stages in the meat supply chain involve movement to the abattoir—loading, transport, and unloading—followed by stunning to render the animal unconscious (Mota-Rojas et al. 2010; Guerrero-Legarreta et al. 2019, 2020; Mota-Rojas et al. 2021). The stunning of these species is commonly performed by applying a conventional captive bolt device to the animal's forehead, but this method has a serious drawback because, according to Schwenk et al. (2016), it produces severe brain lesions regardless of the animal's sex or age. Those authors suggest that these conventional devices are more effective in buffaloes when applied through occipital contact in the ventral depression of the intercornual protuberance and the dorsal region of the insertion points of the nuchal ligament (HSA 2014). Their proposal is based on the anatomy of the buffalo's skull, which differs markedly from that of domestic bovines.

One consequence of the slaughter of buffaloes by religious methods that prohibit all methods of stunning or insensibilisation is that it increases the interval during which the animals lose their cerebral function and manifest the signs of cephalic death (Mota-Rojas et al. 2019, Mota-Rojas et al. 2021). This has been demonstrated experimentally by monitoring animals' cerebral somatosensory responses before collapse (Blackmore 1984). During slaughter, pain and fear are the main sources of stress in animals. To prevent this, two techniques are employed in most cases: (1) stunning to induce complete loss of consciousness or an adequate level of unconsciousness; and (2) bleeding or exsanguination to induce death by hypovolemic shock. Stunning renders buffaloes unconscious and insensible to

environmental stimuli because their brains are no longer able to process sensory information (Farouk 2013; Terlouw et al. 2016a; Mota-Rojas et al. 2019, Mota-Rojas et al. 2021). However, there is another important consideration here: that of animal welfare, which is considered a key factor in ensuring food quality and safety (Fike and Spire 2006). This view holds that the objective of stunning is to prevent pain and stress in animals during death (OIE 2012); but what exactly is the nature of stunning? Basically, it is a technique that was developed to perturb the animal's senses by inducing an immediate loss of consciousness through the application of a blow, electrical discharge, or anaesthesia with CO_2 (i.e., mechanical, electrical, or chemical means). All three procedures render animals insensible during slaughter (Tardio et al. 1999) and are an elemental requirement prior to the death of animals destined for human consumption (Zivotofsky and Strous 2012).

Regulations on the use of different stunning methods vary from country to country. The European Union, for example, prohibits slaughtering cattle by discharging the captive bolt gun in the nape, stipulating that it must be applied to the frontal area. This is because discharging through the nape does not achieve the same depth of penetration, so the stunning achieved is sub-optimal (Gregory 2008). The stunning methods authorised by the European Union up to 1999 included: (a) the penetrating captive bolt gun; (b) percussion; (c) electronarcosis; and (d) exposure to carbon dioxide (Tardio et al. 1999).

These normative procedures specify that non-humane slaughtering causes pain, anxiety, fear, and other forms of suffering in animals, even under the best technical conditions. This is why it is important to monitor the loss of consciousness in buffaloes during slaughter. With some exceptions—like electro-immobilisation or other forms of induced paralysis—an animal can be considered unconscious when it collapses from its natural standing position, does not wake up, and ceases to present signs of positive or negative emotions, such as fear or excitation. Methods that do not cause instantaneous death (called simple stunning) must be followed, as quickly as possible, by a procedure that irremediably causes death, such as exsanguination, electrocution, or prolonged exposure to anoxia (Mota-Rojas et al. 2019, Mota-Rojas et al. 2021). In the case of the river buffalo, stunning requires certain adjustments because these beasts should not be stunned using the techniques applied to domestic bovines (genus *Bos*) due to certain features of their skull that impede the 90-mm captive bolt gun from penetrating sufficiently deeply into the cranial cavity. What are these features?: a broader frontal sinus, harder bony plates, and thicker skin. In fact, even longer bolts (up to 180 mm) may not be effective in inducing loss of consciousness in buffaloes (Mota-Rojas et al. 2019, Mota-Rojas et al. 2021). These findings led Meichtry et al. (2018) to evaluate a bullet gun developed specifically for stunning river buffaloes. Their device consists of a double 9-mm shotgun that uses 357-calibre ammunition and is equipped with a safety device that allows a second discharge to be applied when necessary. This gun effectively induces a deep loss of conscious, except in older buffaloes. It is important, finally, that operators at all levels understand that they cannot assume that the feeding, handling, installations, and stunning methods used with bovines will be suitable for river buffaloes.

In countries like India, religious beliefs prohibit slaughtering cows (*Bos indicus*) but permit sacrificing buffaloes (Bruckert 2018). Against all recommendations concerning animal welfare, however, Islam disdains stunning methods and stipulates that animals slaughtered for human consumption must be beheaded without stunning (halal method) (Gregory et al. 2008).

A recent development that is attracting wide attention is the steady increase in buffalo consumption meat due to certain advantages over beef, including lower levels of cholesterol and saturated fats (Irurueta et al. 2008), a more attractive, intense red colour, and its tender texture (Spanghero et al. 2004). A significant advantage for producers, meanwhile, is that buffaloes tend to yield higher meat production with lower economic investment than bovines (Kandeepan et al. 2013). Unfortunately, concern for the welfare of these animals has not developed at the same rhythm as the popularity of buffalo meat, as is reflected in the fact that no specific norms exist to regulate the breeding, transport, and/or slaughter of this species (Bornett-Gauci et al. 2006).

Against this background, the goal of this chapter is to evaluate recent scientific findings on (i) distinguishing between unconsciousness and insensibility, (ii) the neurophysiology of pain impulses, and (iii) the importance of recognising the signs of the return of sensibility or consciousness during death in large ruminants (*Bos indicus, Bos taurus*, *Bubalus bubalis*), especially the river buffalo. It is important to raise the awareness of all personnel involved in raising buffaloes regarding the use of suitable techniques for inducing insensibility, to continue insisting that under no circumstances can animals be allowed to perceive pain during death, and to emphasise that operators' must learn to recognise the signs of return of sensibility as an essential tool for evaluating stunning quality. Though the mechanisms used to induce insensibility are highly-effective, they are subject to the usual problems that reduce the efficacy of stunning gear; that is, infrequent or null maintenance, inadequate infrastructure for restraining and turning the animals, and handling by untrained operators. To remedy this situation, all personnel involved must have a thorough understanding of the tools and procedures they use, always utilise them in strict adherence to the techniques stipulated in regulations, ensure that adequate equipment is available for each animal species, and verify that all gear is correctly maintained to prevent unnecessary suffering.

7.2 Loss of Consciousness and Insensibility

Perceiving the environment requires the good functioning of the primary cerebral cortex and associated abilities to recognise, understand, and make sense of perceptions (Laureys 2005; Mota-Rojas et al. 2021). This state of consciousness is lost when cortical and subcortical structures are rendered incapable of producing and/or integrating images of oneself and the environment (Damasio 2010). States of unconsciousness are caused by disfunctions in the cerebral hemispheres, reticular formation, or middle bilateral thalamus (Brown et al. 2012), so consciousness and insensibility are opposite phenomena. The former is associated with waking states

and the capacity to perceive and experience sensations, including negative ones like pain (Gibson et al. 2015); that is, the ability to perceive and interact and communicate with the environment and other beings (Zeman 2001). Unconsciousness (perhaps more appropriately the less anthropomorphic term insensibility), in contrast, refers to the temporary or permanent alteration of cerebral functioning that occurs when an interruption leaves them unable to respond to stimuli, even those of a painful kind (European Food and Safety Authority [EFSA] 2006). A state of unconsciousness or insensibility can be induced by cerebral concussion (e.g. discharging a captive bolt gun), anaesthesia, a process of anoxia, or electroconvulsive shock (Verhoeven et al. 2015). Insensibility, finally, refers to the complete inability to perceive any kind of stimulus or sensation, whether pleasant or painful (Hemsworth et al. 2009; Mota-Rojas et al. 2020).

The goal of stunning animals prior to slaughter is to induce a state of unconsciousness or insensibility that lasts up to the moment of death (Gregory et al. 2010; Mota-Rojas et al. 2021), thus preventing by all possible means pain and suffering (Verhoeven et al. 2015). At the moment insensibility is induced by any stunning method (e.g. discharging an explosive cartridge or compressed air through a penetrating or non-penetrating captive bolt) the impact triggers a series of changes that depolarise the cellular membrane of the nervous system (Terlouw et al. 2008). This technique affects primarily the reticular system causing the animal to collapse because the cerebral cortex can no longer maintain it upright (Verhoeven et al. 2015). These factors make the thalamus and midbrain suitable targets for effective stunning (Zeman 2001). When the cerebral cortex is damaged by, for example, a captive bolt gun, the neuronal integration of the stimuli transmitted by the central nervous system (CNS) that is necessary for consciousness and the perception of experiences, ceases to be performed and the animal is rendered unconscious (Adams and Sheridan 2008). Figure 7.1 is a schematic representation of the stunning of a river buffalo (*Bubalus bubalis*). The procedure described follows Glardon et al.

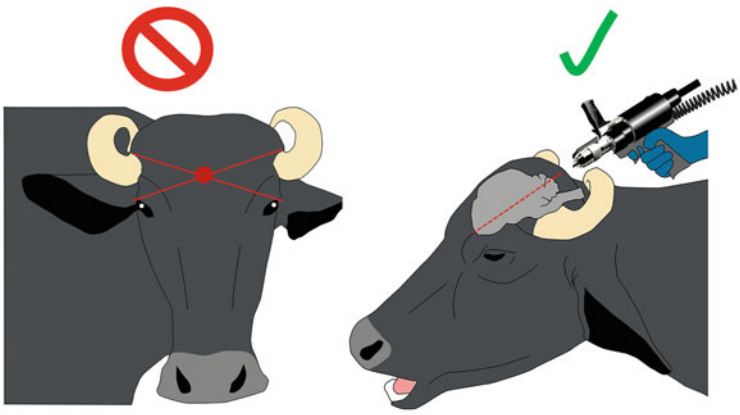

Fig. 7.1 Schematic representation of the correct stunning of river buffaloes

(2018) who used a penetrating captive bolt gun held at a distance of 5–10 cm from the animal's frontal region. The non-penetrating captive bolt gun, in contrast, should be held 20 mm above the position used for the penetrating type (HSA 2006) Insensibility is produced immediately by a combination of concussion and alterations of intracranial pressure (Barros and Castro 2004). River buffaloes can be stunned effectively by a high-power captive bolt gun when it is applied carefully, at the correct site, and at the correct angle. For relatively light buffaloes (up to 380 kg) non-perforating pneumatic hammers function efficaciously, but the stunning of larger animals is usually done by electronarcosis at 2–2.5 A, or with a perforating pneumatic hammer. The conventional cartridge-activated pistol is not effective for stunning buffaloes.

As illustrated on Fig. 7.1, the bovine brain is situated in the upper, or dorsal, part of the head. A. - The ideal site for stunning animals of the genus *Bo*s is in the centre of the forehead at the point of intersection of two imaginary lines traced between the eye and the centre of the base of the opposite horn. But this site is not adequate for buffaloes because of the thickness and resistance of the bones in this area of the skull. B. - The recommendation for buffaloes is to apply the discharge in the nuchal region, in the depression below the intercornual protuberance but above the attachment points of the nuchal ligament. C.- Other authors suggest that the optimal site for trepanation of the frontal bone is on a line that joins the median points of the temporal sides to a point halfway between the midline and the lateral margin of the head. The mouth of the stunning gun should be held at a right angle to the skull to direct the bolt against the dorsal or high area of the brain in the direction of the medulla oblongata. When the discharge is applied directly and strongly (here, with a captive bolt gun) it produces an immediate concussion that displaces the brain causing it to strike against the cranium. This interrupts normal electrical activity due to a sudden massive increase in intracranial pressure, followed by a sudden reduction of that pressure. The damage to the nerves and blood vessels causes disfunction and/or destruction of the brain, impedes blood flow, and causes the animal collapse.

The immediate effect of stunning in the animal is unconsciousness accompanied by what is known as tonic-clonic activity (Gregory et al. 2007). Stunning by the captive bolt gun method causes abrupt trauma in the skull in the form of a cerebral concussion and/or contusion that affects the blood vessels, triggering certain physiological signs depending on the exact site where the bullet or plunger strikes the animal's skull, the velocity or force of impact, and the depth of penetration into the brainstem (Appelt and Sperry 2007). Mechanical stunning by a penetrating captive bolt gun causes cerebral concussion and insensibility in 1 ms by damaging the brain and interrupting cerebral activity. It produces a state of unconsciousness that may exceed 60 s. By comparison, the non-penetrating gun causes cerebral concussion and insensibility in 2 milliseconds, but produces tissue deformation in the CNS and increases intracranial pressure. In this case, loss of consciousness can last for 25–35 s (Ghezzi 2017). When rendered unconscious, the animal collapses, ceases to breathe, and becomes rigid with its head stretched out and its hind limbs flexed towards the abdomen. This period of rigidity normally lasts 10–20 s after stunning (HSA 2014).

The animal's head, ears, tail, and back will likely hang down, and normal reflexes are absent. Other indicators that should be monitored are the absence of nose movements (rabbit nose), hanging tongue, visible flaccidness of the mouth, and the absence of vocalisations. The combination of these signs indicates that stunning was performed correctly. Another sign may be the loss of muscle tone (Meichtry et al. 2018), though the animal's forelimbs may flex initially before gradually stretching out. If, however, an animal shows movement of its fore- or hindlimbs immediately after collapsing, it is almost certain that it was not completely stunned. This movement is often accompanied by the absence of arching of the spine and by signs of the righting reflex (slight lateral flexion is permitted). Other signs that should not be observed are the palpebral and corneal reflexes, nystagmus, and eyeball rotation (Grandin 2002). In conclusion, effective stunning occurs when the animal is rendered fully and immediately unconscious and insensitive to pain. After this process, exsanguination must be performed without delay.

The duration of unconsciousness and insensibility depends on the stunning method used, so the time required for beheading must be adjusted accordingly. The duration of stunning plus exsanguination must be less than half a minute when the non-penetrating gun is used, less than a minute when the penetrating gun is used, and less than 15 s for electrical stunning, so as not to compromise animal welfare and meat quality (HSA 2006).

While from the perspective of animal welfare mechanical stunning systems do not offer any operational advantages over other methods, their use does have advantages for meat quality. In the case of electrical stunning, for example, excessive application times or excessively high voltage can cause fractures and haemorrhages that will appear in the canal (Figueroa et al. 2011).

The definitive physical signs of effective stunning are: a) collapse; b) absence of rhythmic breathing; c) a fixed, glassy expression in the eyes; d) absence of the corneal reflex; e) slack jaw; and f) hanging tongue (Gregory et al. 2007; HSA 2014). In order to opportunely detect an animal's return to sensibility, the personnel must learn to recognise the opposite of these signs in the stunned beast. Since no specific sign is conclusive on its own, the HSA (2006) stipulates that any animal which shows at least one sign should be considered conscious and must be re-stunned (Figueroa et al. 2011).

7.3 Neurophysiology of the Ascendent Transmission of the Painful Impulse from the Reticular Formation to the Cerebral Cortex

Animals' brains are irrigated by the basi-occipital plexus and carotid arteries, which supply blood to the caudal tissues (mainly the occipital cerebral cortex), and by the basilar arteries that carry blood rostrally (Gregory 2008; Johnson et al. 2015). Exsanguination during slaughter is performed by cutting the carotid arteries and jugular veins or brainstem to interrupt the supply of nutrients and oxygen to the brain to induce death by hypovolemic shock (Robins et al. 2014; Mota-Rojas et al. 2021).

However, when the cutting of the large blood vessels for exsanguination or the stunning method is performed incorrectly, the result will be acute pain because the animal's skin, muscles, arteries, veins, and connective tissue all contain physiological sensors called nociceptors which generate the electrical impulses that are sent to the CNS where they are recognised as pain (Johnson et al. 2015).

The brainstem contains the information that controls the cardiovascular system, respiratory apparatus, sensibility to pain, and states of awareness. The reticular formation is necessary, but not sufficient, for the development of consciousness. Sensory information is transmitted from the reticular formation to the thalamus and from there to the cerebral cortex, where the pain sensation is perceived. Therefore, the aim of all stunning methods is to interrupt the ascending transmission of information from the reticular formation to the cerebral cortex (Terlouw et al. 2016a; Glardon et al. 2018). Understanding how painful stimuli are transmitted requires discussing the role of reflexes, which are defined as automatic movements mediated by the CNS in response to certain stimuli (Carlson 2007). The so-called central reflexes are regulated by the brainstem and spinal cord, while the brainstem reflexes are regulated by the 12 pairs of cranial nerves that enter through afferent or sensitive branches and exit through the efferent or motor branches of the brain, and are not under cortical control. The efferent or sensitive branches of cranial nerve pairs I and II enter the forebrain. Cranial pairs III-XII are called "mixed" since they have both sensitive branches that enter the brainstem and motor branches that exit it (Rubin and Safdieh 2007).

Brainstem reflexes are commonly used to evaluate an animal's degree of consciousness after stunning. They include the palpebral (a), corneal (b), pupilar (c), and threat reflexes (d) (Dugdale 2010). The first two require the correct functioning of cranial pairs V (trigeminal) and VII (facial), and the eye muscles (Adams and Sheridan 2008), while the pupilar reflex is regulated by cranial pairs II (optic) and III (oculomotor), and can be evaluated by shining a light beam towards the eye to observe the reaction of the pupil (Fig. 7.2) (Blackman et al. 1986). The threat reflex is regulated by cranial pair VII (facial) and the action of the motor cortex (Grillner et al. 2008).

Figure 7.2 Neurophysiology of the ascending transmission of pain impulses from the reticular formation to the cerebral cortex.

7.4 Signs of the Return to Sensibility to Assess Stunning Quality

Stunning can be reversible or irreversible. In the first case, animals can recover sensibility before death occurs. As mentioned above in the description of the intervals necessary for humane slaughter, the time between stunning and exsanguination is a determining factor for the efficacy of stunning (Tardio et al. 1999). For this reason, it is important to understand that stunning techniques which function adequately in some animals are not recommended for others. As we have seen, due to differences in the crania of cows (genus *Bos*) versus female river buffaloes,

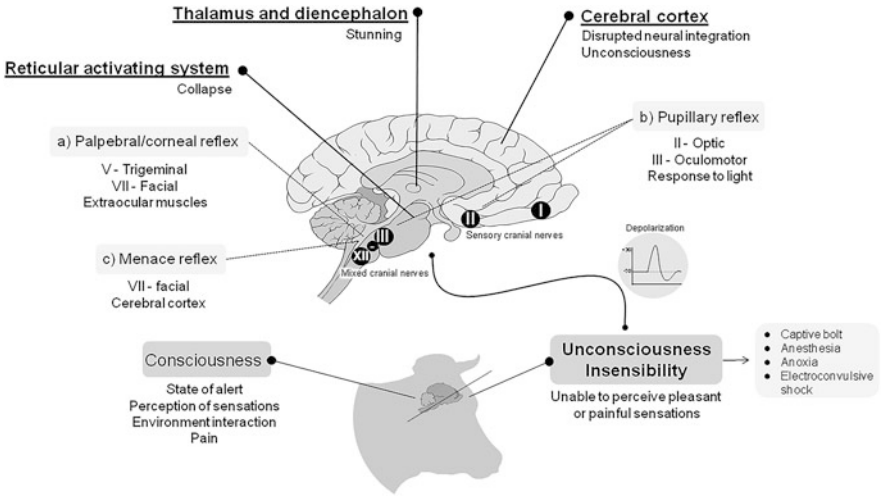

Fig. 7.2 Schematic representation of the neurophysiology and factors that participate in transmitting pain impulses from the reticular formation to the cerebral cortex

stunning with lower-calibre bolt guns is effective in the former but not in the latter because those animals have a broader skull with harder bones and thicker skin (Schwenk et al. 2016).

Ensuring humane slaughter requires that no signs of the return of sensibility are manifested. This means that animals must be carefully monitored after stunning and during exsanguination (Gregory et al. 2007). Atkinson et al. (2013) and Terlouw et al. (2016b) identified several signs that help veterinarians determine whether stunning was performed adequately or not. They are specified in Table 7.1, which shows that inadequate stunning leaves the animal susceptible to feeling pain, a condition that can potentially affect meat quality by subjecting the animal to unnecessary suffering and a poor quality of death. The main indicators of unconsciousness are the absence of rhythmic breathing, ocular reflexes, muscle tone, ocular rotation, and nystagmus, accompanied by a null response to painful stimuli and spontaneous movements of the neck and hooves. The most important indicators of consciousness, in contrast, are upright posture, vocalisations, spontaneous blinking, ocular movements, and the threat reflex (Terlouw et al. 2016b).

Several behavioural, physiological, physical, and pathological parameters have been proposed to evaluate animal welfare and the elements of the handling of cattle in abattoirs. Behavioural aspects include vocalisations, falls, slipping, ear movements, struggling, bristling, trembling, and standing without advancing. Physiological parameters commonly examined are cortisol levels, fasting, dehydration, and indexes of fear and excitation, while physical events identified are hernias, falls, fractures, lesions, and contusions, and pathological manifestations are the index of corporal condition, prevalence of disease, immunological indicators, and signs of mortality (Romero et al. 2013). Table 7.2 identifies and describes the various

Table 7.1 Protocol for determining stunning quality and animal welfare

Stun Quality Rating (SQR)	Action	Symptom	Definition
SQR3[a]	Re-stun immediately	Failed to collapse	Animal does not immediately fall to the ground after shot with all legs collapsed
		Attempt to regain Posture	Animal attempts to stand up or lift the head before hoisting
		Vocalisation	Repeat vocalisations or groans can be heard not associated with a one-time exhalation animal reacts to a painful stimulus such as a prick to the inner skin of the nostril with a sharp instrument while on the stun crate or shackle
		Pain response Blinking Corneal reflex	Animal opens/closes eyelid on own (fast or slow) without stimulation Animal blinks (fast or slow) in response to stimulus of the cornea
		Rythmic breathing	Continuous rhythmic inhalation and exhalation in the form of expansion/contraction of the trunk area can be seen or exhalations can be felt with the hand
SQR2[a]	Re-stun immediately	Full eyeball rotation Nystagmus	The eyeball rolls so mostly pink sclera can be seen and little or no iris Rapid side-to-side (twitching) movements of the eyeballs
SQR1[a]	Monitor closely and re-stun if $\geq$2 symptoms are observed	Absence of tonic/clonic phase	Absence of tonus in whole body and muscle spasms for over 20 s after stunning
		Partial eye rotation	The eyeball rolls so that only half of the iris is still visible
		Groaning	A groaning sound can be heard upon exhalation and not repeated
		Head raising	The head is flexed upwards while animal hangs on the shackle line
		Gasping	Repetitive contraction and retraction of the lips and slight opening/closing of the mouth
		Reactions to sticking	Severe kicking and body or head movements during skinning or sticking procedures
		Ears not pointing downwards	When the ears face backwards towards body at sticking and do not hang downwards
		Tongue up	When tongue is retained in mouth (not hanging down and relaxed out of mouth) at sticking

aIf an animal shows any one symptom of SRQ2 or 3, it is considered inadequately stunned
Description of the signs that assess the risk of the return of sensibility from high (3) to low (1)
If the animal shows any of the CA3 or CA2 signs, stunning is deemed inadequate (Atkinson et al. 2013)

Table 7.2 Reflexes used to assess unconsciousness in livestock after stunning (Modified from Verhoeven et al. 2015)

		Present in animals that are			
Reflex	Definition	Conscious	Unconscious	Based on	Remarks
Brainstem reflexes	Reflexes that originate from the brainstem			Functional cranial nerves originating from the brainstem	Reflexes may be present in animals that are unconscious, depending on the stunning method used. The absence of these reflexes is considered a valuable indicator for assessing unconsciousness. Cannot be tested when seizures occur.
Corneal reflex	Involuntary blinking in response to stimulation of the cornea	+ (−)	- (+)	Functional cranial nerves V and VII and eye muscles	One of the most commonly-used reflexes after stunning. Normally the last reflex lost in anaesthetised animals. May be present after electrical stunning, but never after effective captive bolt stunning.
Palpebral reflex	Involuntary blinking in response to touching the medial canthus of the eye	+	–	Functional cranial nerves II and III and eye muscles Cranial nerves V and VII and eye muscles	Disappears earlier than the corneal reflex in anaesthetised animals. The ophthalmic branch of the trigeminal nerve (pair V) is active, as is the auricular-palpebral branch of the facial nerve (pair VII).
Pupillary light reflex	Narrowing of the pupil in response to	+	–	Functional cranial nerves V	Considered of little value during

(continued)

Table 7.2 (continued)

		Present in animals that are			
Reflex	Definition	Conscious	Unconscious	Based on	Remarks
	light that falls on the retina			and VII and eye muscles Cranial nerves II and III and eye muscles	exsanguination, as blood supply to the retina is restricted during this period Pupillary dilatation is considered a sign of total brain dysfunction. May be absent in animals that are paralysed but conscious. Pupilar activity is mediated by an AFFERENT (visual) optic nerve (pair II) and an EFFERENT (motor; miosis-mydriasis) oculomotor nerve (pair III). Miosis refers to the function of the sphincter of the pupil innervated by parasympathetic fibres. Mydriasis refers to the function of the dilator of the pupil. It is controlled by sympathetic fibres from cranial pair III of the oculomotor nerve which, after exiting the large sphenoid cleft, divides into two branches, dorsal and ventral. The latter includes

(continued)

Table 7.2 (continued)

		Present in animals that are			
Reflex	Definition	Conscious	Unconscious	Based on	Remarks
					the pupilar fibres that make synapses in the ciliary ganglion. From there, it goes through the short ciliary branches towards its destination in the ciliary muscle that acts on the iris and pupil.
Threat reflex	Involuntary blinking or withdrawal of the head in response to moving a finger or hand quickly towards an animal's eye	+	–	Functional cranial nerve VII, eye muscles and integration with motor cortex	Cannot be tested if eyes are closed.
Spinal reflexes	Reflexes that originate from the spinal cord			Require a functioning spinal cord, but not necessarily cerebral coordination	May occur more vigorously when there is a lack of inhibition from the brain (e.g. after captive bolt stunning).
Pain withdrawal reflex	Withdrawal of the body part that has received a painful stimulus	+ (−)	(+)		In a survey on expert opinion, the pain withdrawal reflex was ranked high, and thus valued highly as an indicator for assessing unconsciousness after all types of stunning.
Pedal reflex	Withdrawal of the foot in response to pinching the skin between	+ (−)	(+)		Difficult to assess when convulsions occur. Not easy to perform in all

(continued)

Table 7.2 (continued)

		Present in animals that are			
Reflex	Definition	Conscious	Unconscious	Based on	Remarks
	an animal's toes				species. Used mainly in poultry.
Righting reflex	Returning the body to its normal position after being taken out of its normal upright position	+ (−)	(+)		Difficult to assess when convulsions occur.

[a]The presence or absence of reflexes is indicated as follows: + present; − absent; (+) may be present; (−) may be absent (Verhoeven et al. 2015)

brainstem and spinal reflexes used to assess the level of unconsciousness after stunning.

Unconsciousness caused by the permanent or temporary interruption of cerebral communication is usually evaluated by monitoring behavioural indicators that may reflect internal responses to external stimuli (Levitis et al. 2009). These responses can include reflexes that originate in the brain (ocular and pupillary reflexes) or those that occur as withdrawal from painful stimuli (Fig. 7.3), and the ones that originate in the spinal cord, such as pedal reflexes. But this should include behavioural indicators like loss of posture, vocalisations, and rhythmic respiration as well (Table 7.2) (Verhoeven et al. 2015). In some cases, these behavioural indicators may not be specific, for a study by Miranda-de la Lama et al. (2012) found that 10% of animals vocalised and only 51% of them were correctly insensibilised and continued to show signs of sensibility, even though 95% collapsed at the first discharge. For this reason, the authors recommend that head restraint systems are included to improve slaughtering at abattoirs as this measure increases the effectiveness of the stunning method employed (Miranda-de la Lama et al. 2012).

Electroencephalography (EEG) has been shown to be potentially useful in evaluating insensibility or unconsciousness because marked changes occur in EEG patterns immediately after the discharge that stuns the animals (especially in the delta and theta waves, which tend to become isoelectric). Animals are assumed to be unconscious by analogy to similarities to EEG findings in humans (European Food and Safety Authority [EFSA] 2004). EEG recordings in animals show relatively small waves that increase in amplitude during the tonic phase and reduce their frequency in the clonic phase, causing a period of reduced electrical activity, as has been demonstrated in pigs, sheep, and cows (Lambooy 1982; Anil and McKinstry 1992). The question is whether EEG recordings can determine if an animal is truly unconscious. The electrical activity recorded in EEGs is classified in

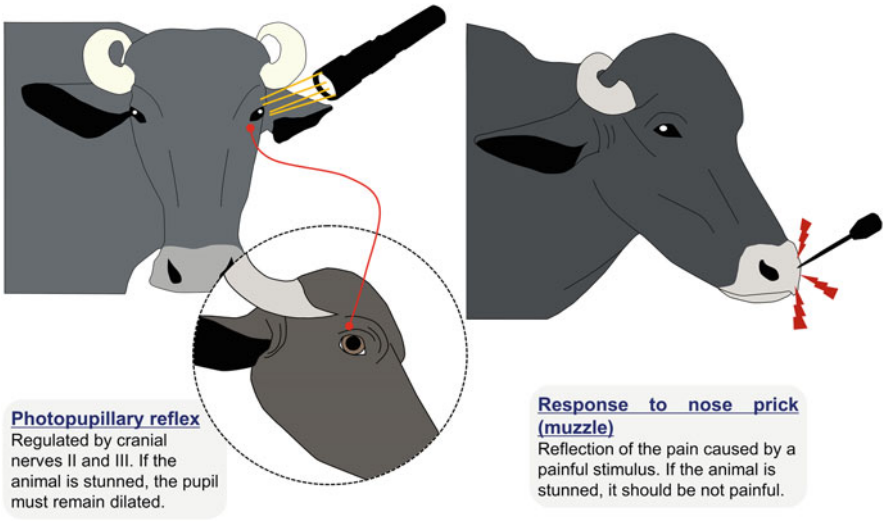

Fig. 7.3 Shows the ocular reflex when a light is shone on the retina. This reflex involves cranial pairs II (optic nerve, afferent pathway) and III (oculomotor nerve, efferent pathway) and the eye muscles. It is of only minor value during beheading and exsanguination because little blood reaches the retina at those times. Pupilar dilatation is considered a sign of total cerebral disfunction that may occur in animals that are paralysed but conscious. The reflex response to painful stimuli (nose prick) is an indicator that helps determine consciousness or unconsciousness after any type of stunning

delta (0–4 Hz), theta (4–7 Hz), alpha (8–13 Hz), and beta (above 13 Hz) waves. In this approach, an animal is considered conscious if it presents alpha and beta rhythms (Kooi et al. 1978), so EEG is a tool that could aid in evaluating stunning quality.

7.5 Why Must the River Buffalo be Stunned Differently than Cattle?

As discussed earlier, this is mainly because of the anatomical differences between the skulls of these two species (Mota-Rojas et al. 2021). In cattle (genus *Bos*, *Bos taurus, Bos indicus*), the ideal site for applying the discharge from the captive bolt gun to produce effective stunning is the intersection of two imaginary lines traced from the external corner of the eye to the centre of the base of the opposite horn (AVMA 2020) on the animal's forehead, 3–5 cm above the intersection of the lines (depending on breed) (Grandin 2013). The head of the river buffalo (*Bubalus bubalis*), however, has significant differences that make standard captive bolt gun models inefficacious when applied to buffaloes because they may fail to produce the required loss of consciousness or stunning quality. These differences are in the depth of their frontal sinuses, skin thickness, and the hardness of their bony plates (Schwenk et al. 2016). Studies show that the buffalo's frontal bones are 4–8 cm

thicker than those of bovines (Gregory 2008). Free bullets are potentially adequate for stunning buffaloes, but this method entails risks for the safety of operators (Schwenk et al. 2016). Taking all these factors into account suggest that buffaloes should be stunned with captive bolt guns of a calibre above 120 mm, though in some cases even 125-mm guns are not effective and, therefore, are judged inadequate for stunning animals of this species (Glardon et al. 2018; Meichtry et al. 2018).

Another important anatomical difference between the skulls of female buffaloes and cows was highlighted in a study by Alsafy et al. (2013), who used computerised tomography to compare Egyptian buffaloes (*Bubalus bubalis*) to bovines (*Bos taurus*). They found that the nasal septum of the buffalo reaches the floor of the nasal cavity with a vomeronasal organ on each side of the septum, while in bovines the septum does not touch the floor of the nasal cavity but forms a central channel that continues to the nasopharynx (Alsafy et al. 2013). According to Gregory et al. (2009), the discharge from a captive bolt gun positioned in the frontal region can be effective in stunning river buffaloes, but it produces only a superficial orifice that causes less brain concussion than the frontal discharge applied to cattle. These authors suggest directing the discharge towards the base of the tongue to avoid damaging the spinal cord, pointing out that otherwise the results may be only acute vertigo, ataxia, and loss of equilibrium, without necessarily achieving the required stunning quality.

7.6 Final Considerations

Indicators of consciousness and insensibility are tools that allow us to evaluate, indirectly, cerebral functioning and determine if stunning has been performed adequately. Quality stunning is confirmed when signs of consciousness are absent and those of unconsciousness or insensibility appear. The most important indicators that help assess SQ are body posture or the righting reflex, respiratory rhythm, and the absence of vocalisations and the palpebral and corneal reflexes. Only if all of these signs are absent can we assume that an animal has been correctly stunned and is totally unconscious. This means that any sign of the return to sensibility in large ruminants must be recognised and verified to evaluate SQ. However, none of these signs on its own can be used to determine SQ, so any animal that shows at least one sign of the return of sensibility should be considered conscious and be stunned a second time. Clearly, it is of the utmost importance to develop stunning and slaughtering methods that ensure insensibility throughout the process so as to prevent unnecessary pain during death in large ruminants. Another key point is that the insensibilisation technique chosen for, and applied to, buffaloes must be suitable for this species' particular corporeal conformation, anatomical characteristics, and the category of each animal. Two additional recommendations are (i) to ensure that the stunning equipment receives regular maintenance according to the manufacturers' instructions, and (ii) that the technique chosen is applied by qualified, trained personnel.

In closing, we would reiterate the importance of stunning as a critical step in the slaughter of cattle and buffaloes destined for human consumption that can affect animal welfare if three fundamental conditions are not met: applying the technique at the optimal anatomical site; utilising adequate equipment; and ensuring that it is performed only by qualified operators.

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Part II

Omics Approaches to Understand Buffalo's Genome, Physiology, and Reproduction

8

Molecular Evolution and Genome Architecture of Water Buffalo (*Bubalus Bubalis*), the "Living Bank" for Marginal Farmers in Developing Countries

Ayan Mukherjee and Sachinandan De

Abstract

A useful livestock animal, the water buffalo has made significant contributions to the socioeconomic upliftment of agricultural populations in developing countries. Water buffaloes are found on all five continents and have an estimated worldwide population of 200 million. Asian water buffalo and African buffalo are two taxonomic species of buffalo that developed from a common ancestor, *Bubalus arnee* (wild buffalo). There are two varieties of domesticated Asian water buffalo (riverine and swamp), each with its own set of characteristics. The river buffalo travelled west as far as Egypt, the Balkans, Anatolia, and Italy after becoming domesticated on the western Indian subcontinent (about 6300 years ago). Following domestication in the China/Indochina border area around 3000–7000 years ago, swamp buffaloes expanded over Southeast Asia and China as far as the Yangtze River basin. High-quality reference genome and a medium-density marker panel for buffalo genotyping have become accessible thanks to the progress of high-throughput genome sequencing techniques and bioinformatics algorithms. This has given a tremendous boost to genome-wide studies aimed at learning more about molecular genetic diversity, structural genomic variants, and their links to diverse phenotypes. As a result, sensible breeding techniques and conservation initiatives in buffaloes across the world gained a new dimension. The present chapter will concentrate on the water buffalo's evolutionary history, chromosomal structure, and autosomal and sex chromosomal variation. The structural differences in the buffalo genome have

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also been characterized, as well as their influence on productivity, reproduction characteristics, and innate immunity.

Keywords

Buffalo · Molecular evolution · Genome · Autosomes · Sex chromosomes

8.1 Introduction

From the time immemorial livestock rearing has become an integral component of agrarian human civilization globally. Water buffalo, an important livestock species, contributed significantly in socioeconomic development in different countries where agriculture is the mainstay of their economy. This exceptionally hardy animal can subsist on poor quality roughages, adapt to difficult climatic conditions, and show resistance to numerous bovine tropical diseases, in addition to contributing milk, meat, leather, dung, hide, horns, traction power, and so on. Due to this resilience that developed over the centuries by natural or man-made selection, the species has been considered as "convertible currency" and "living bank" to serve economically backward rural communities of the world.

Of the global population of approximately 200 million water buffalo, the majority are found in South Asia, where they provide more milk than cattle. However, it shows comparatively poor reproductive fitness across different parts of the world including delayed puberty, anestrus, lengthier postpartum ovarian quiescence, and poor conception rates following artificial insemination (Gordon 1999). Required research efforts have not been directed earlier to circumvent this problem as buffaloes are being reared by resource-poor developing countries of the world (Warriach et al. 2015). The Food and Agriculture Organization has aptly termed buffalo as "an asset undervalued". But with the hyperbolic nature of human population growth pattern and to secure "food for all," strategies have been adopted to boost production from economically important livestock species like buffalo by means of scientific breeding and management practices. The meteoric rise of application-based high-throughput genome sequencing technologies in the last decade of this century have generated heaps of buffalo genomic, transcriptomic, and other omics information. Simultaneously, this has opened the avenues for adopting accurate selection and breeding strategies including genomic prediction, genome-wide association studies, and identification of candidate genes. This chapter elaborately discusses the architecture of water buffalo genome in the light of recent advanced genomic studies in water buffalo. This knowledge will be useful to increase production potential, address the problem of reproductive failure, improve product quality, and highlight immunity aspect.

8.2 Different Types of Buffaloes and Their Evolution

Buffalo is a member of family Bovidae, sub-family Bovinae. There are two main species of buffalo: Asian water buffalo and African buffalo (Iannuzzi and Di Meo 1995). Wild buffalo (*Bubalus arnee*) is considered as their common ancestor. Asian water buffalo originated in Asia and subsequently dispersed from Asia to Europe during the Pleistocene era. Domesticated species of buffalo are of two types: riverine buffalo and swamp buffalo (Macgregor 1941). Controversy prevails in their taxonomic status as sometimes they are considered as individual species (*B. bubalis* for river buffalo and *B. carabanensis* for swamp buffalo) or subspecies (*Bubalus bubalis bubalis* and *Bubalus bubalis carabanensis*). The main differentiating features of two types of buffaloes are presented in Table 8.1:

Different important breeds of river type and swamp types with their diverse distribution pattern have been presented in Fig. 8.1.

8.3 Migration History of Buffaloes Across the World

Figure 8.2 shows the migratory pathways of river and swamp buffaloes from their origin. The two distinct water buffalo subspecies (river and swamp) are descendent of different populations of the wild Asian water buffalo. They diverged approximately 900,000 years before present (BP). Since then two independent domestication events have taken place for river and swamp buffalos. The river buffaloes were domesticated probably at ~6300 yr. BP in north-western part of India. From there domesticated river buffaloes migrated west, across south-western Asia, Egypt, and Anatolia, and reached the Balkans and the Italian peninsula (S. Kumar et al. 2007). River buffalo populations exhibit a weaker phylogeographic structure but higher phenotypic diversity, thus resulting in more breeds (Zhang et al. 2020). With Arab invasion around the eighth century the water buffalo was subsequently introduced in

Table 8.1 Main differences between riverine and swamp buffalo

	Riverine buffalo	Swamp buffalo
• Chromosome number	2n = 50	2n = 48
• Morphology	Massive in size, curled horns	Like *Bubalus arnee*
• Behavior	prefers to wallow in clean water and rivers	Natural habitat is wet grassland, swamp or marshland
• Distribution	Mostly in India, Pakistan, Bulgaria, Hungary, Turkey, Italy, Egypt, Brazil, and Caucasia	Mostly in Thailand, Philippines, Vietnam, and China
• Purpose	Milk purpose	Low milk yielder. Mainly used as draft purpose

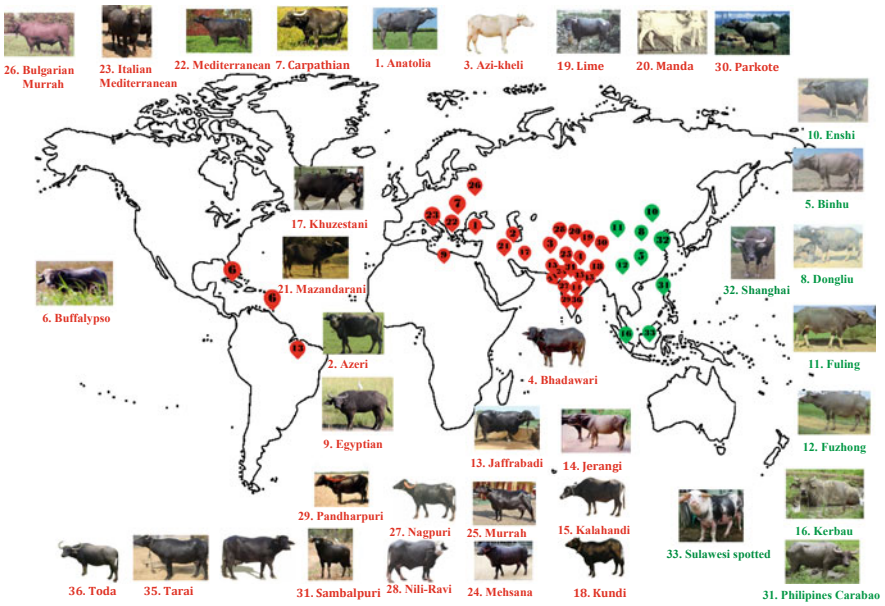

Fig. 8.1 Diversity of buffalo breeds across the world. Breeds typed in red color font is river type and those in green color font is swamp type

Egypt and Italy. Buffaloes migrated in the Balkans and Turkey during the expansion of the Ottoman Empire in the fifteenth century by crusaders and by the Selgiukid invasion (Borghese 2013). The border between China and Indochina is assumed to the place of domestication of swamp buffaloes that took place at ~3000–7000 years BP. According to the phylogenetic analysis, Chinese swamp buffalo population may be divided into two distinct lineages, A and B. For predominant lineage A, South-western China is presumed to be the most likely location for the domestication. However, exact location of domestication cannot be pinpointed for lineage B due to complex pattern of diversity for lineage B. The presence of a long-term, strong gene flow among swamp buffalo populations as a consequence of extensive migrations of buffaloes and frequent human movements along the Yangtze River is considered as the reason behind this (Dutta et al. 2020).

8.4 Chromosomal Organization of Asian and African Buffaloes

The karyotype is the organization of the genome expressed as an arrangement of chromosomes (usually smallest to largest). The success or failure of de novo genome sequencing effort is dependent upon whether this can assign all or most of the sequences to the appropriate chromosomes in the karyotype, thus creating a genetic map. As far as marker assisted selection (MAS)-based livestock breeding strategy is concerned, karyotype plays pivotal role to determine genotype-phenotype

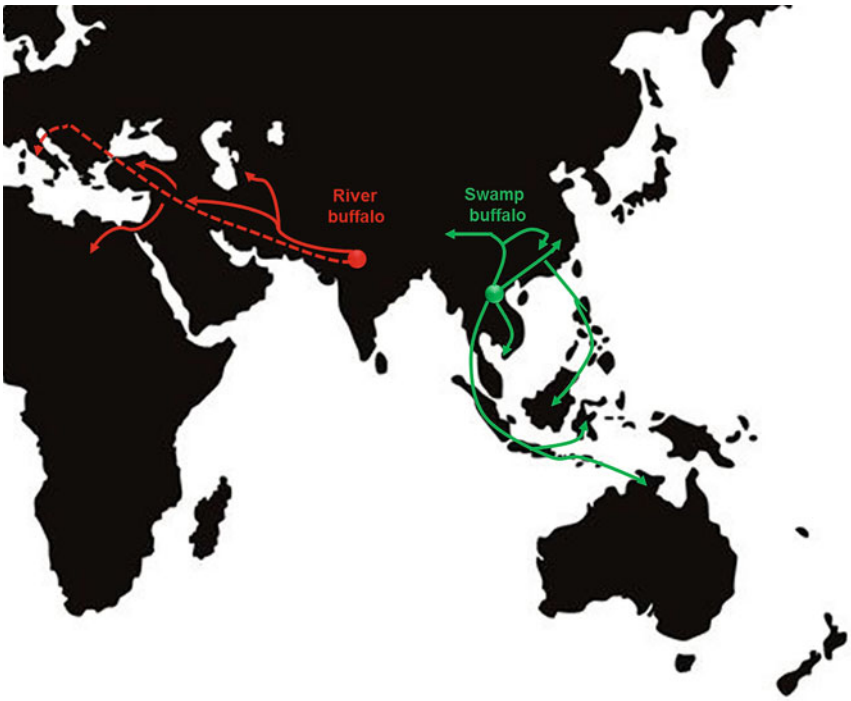

Fig. 8.2 Pathway of migration of river and swamp buffaloes. The dashed line indicates independent migration of river buffaloes to Europe

correlations. As already stated, river and swamp buffaloes have 25 and 24 of chromosomes pairs, respectively. Swamp buffalo has one less number of chromosome than river buffalo because of a tandem fusion translocation between river buffalo (BBU) chromosomes 4 and 9 (telomere of BBU4p and centromere of BBU9) occurred to form chromosome 1 (the largest one) of swamp buffalo (Berardino and Iannuzzi 1981). River buffalo has 5 biarmed chromosomes and the remaining chromosome 20 pairs are acrocentric including the sex chromosomes.

In African buffalo subspecies, there are 26 chromosome pairs in cape buffalo and 27 chromosome pairs in forest buffalo. There are four biarmed chromosomes in *Syncerus caffer* and three biarmed chromosomes in *S. nanus*. All other chromosomes, including sex chromosomes, are acrocentric in this species.

8.5 Similarity in Chromosome Organization Among Ruminants: A Comparative Cytogenetics Approach

8.5.1 Autosome

River buffalo shows close cytogenetic relationship with domestic cattle (another member of Bovidae family) as evident from resemblance in chromosome banding pattern and gene order homology between two different species. The river buffalo has five biarmed and 19 acrocentric chromosome pairs along with sex chromosomes while cattle genome has 29 acrocentric chromosome pairs with sex chromosome pair (Amaral et al. 2008). Centric fusion translocations between each two cattle acrocentric chromosomes, vis-à-vis BTA1 and BTA27, BTA2 and BTA23, BTA8 and BTA19, BTA5 and BTA28 gave rise to BBU1, BBU2, BBU3, BBU4, and BBU5 biarmed chromosome pairs of river buffalo, respectively, during evolutionary course of time. Therefore, all the acrocentric chromosomes of river buffalo are cytogenetically similar to one of the cattle chromosomes (Amaral et al. 2008).

Syncerus caffer and *Syncerus nanus* have four and three biarmed chromosomes, respectively. Rest of the chromosomes, including sex chromosomes, is acrocentric in this species. Like river buffalo similar type of fusion of cattle chromosomes vis-à-vis BTA1 and BTA13; BTA2 and BTA3; BTA5 and BTA20; and BTA11 and BTA29 have been evidenced in *Syncerus caffer* biarmed pairs of chromosomes (Gallagher Jr and Womack 1992).

8.5.2 Sex Chromosome

Unlike high degree of resemblance in autosomes among the members of Bovidae family, sex chromosomes in these species have very complex rearrangements of loci order (Cockett and Kole 2008). This might have occurred during the karyotype evolution of river buffalo and cattle. Probably, this topsy-turvy of loci order arose among the species during centromeric translocation event during evolution. Consequently, different centromeric locations in X chromosome have been noticed in bovids, viz. the submetacentric cattle type, the acrocentric eland (or river buffalo) type, and the acrocentric sheep (and goat) type with small and visible p-arms. However, comparative mapping of sex chromosomes by fluorescence in situ hybridization (FISH) shows similar gene order (Cockett and Kole 2008). Comparative cytogenetics of Y chromosomes an inversion including the centromere and breakage points in both arms (pericentric inversion) have occurred between cattle and buffalo. Also, buffalo Y chromosome is slightly larger in comparison to cattle Y chromosome due to gain of heterochromatin regions (Cockett and Kole 2008).

8.5.3 Nuclear Organizer Region (NOR)

Nucleolus organizer regions (NORs) are chromosomal milestones consisting of tandem repeats of ribosomal genes (rRNA). Combinedly, they determine the chromosomal dynamics during cell division. These NORs along with conserved regions of bovine chromosomes constitute nucleolus organizer centers (NOCs). The number of NOR-bearing chromosomes differs between domestic buffaloes also. NORs are present in six chromosomal pairs (3p, 4p, 6, 21, 23, and 24) in the river buffalo (*B. bubalis bubalis*), but only five (4p, 6, 20, 22, and 24) in the swamp buffalo (*B. bubalis Kerebau*). (Iannuzzi et al. 1990, 1996; Tanaka et al. 2000). Number and location of NOR vary among the closely related bovids. Sheep, goat, cattle, and river buffalo have one common NOC. River buffalo and cattle share two NOCs signifying a strong evolutionary relationship between two bovids (Cockett and Kole 2008).

8.6 Genome Diversity in Buffalo

Analysis of highly variable nuclear DNA loci such as microsatellites (Barker et al. 1997) or uniparental markers such as the maternally inherited mitochondrial DNA (Lau et al. 1998) and the paternally inherited non-recombining part of the Y chromosome (Yindee et al. 2010) are frequently used methods for investigating domestication dynamics, population relationships, and history (Lenstra et al. 2012).

8.6.1 Microsatellite Markers

Microsatellite markers or short tandem repeats (STRs) are continuous repeats of one, two, three, or four nitrogenous bases. These microsatellite markers are found in the genome, mostly in the non-coding region.

There are several advantages of microsatellite markers for which these are frequently used to detect parentage, breed characterization, and genetic diversity.

1. Microsatellite markers are highly polymorphic.
2. Due to small product size, a microsatellite marker can be amplified by single set of primer only.
3. The amplified products of these microsatellite markers can be studied using genotyping alone.

Due to high level of polymorphism microsatellite markers are frequently used to characterize genetic diversity in buffalo. Microsatellite loci analysis of Chinese swamp buffaloes identified four major geographical clusters and one highly distinct Dehong population (Zhang et al. 2007). Another microsatellite-based study including 26 different Asian swamp buffalo populations by Zhang et al. (2011) showed that the genetic differentiation among the Chinese populations was much less than other populations from south-east Asia. In the same study, highest genetic variability

was detected in Thai buffaloes. Several microsatellite marker-based studies have been carried out to detect genetic diversity in river buffalo populations (El-Kholy et al. 2007; Gargani et al. 2010; Mishra et al. 2015; Moioli et al. 2001; Özkan Ünal et al. 2014; Saif et al. 2012a; Zhang et al. 2011). Results of all these studies show that three Indian breeds of buffalo, viz. Murrah, Nili-Ravi, and Kundi populations show maximum microsatellite diversity (Satish Kumar et al. 2006; Vijh et al. 2008) as evident from highest expected heterozygosity values ($H_E = 0.71–0.78$). Subsequent decrease in heterozygosity is observed in populations to the west towards the Mediterranean (Elbeltagy et al. 2008; Gargani et al. 2010; Moioli et al. 2001; Özkan Ünal et al. 2014; Saif et al. 2012a). In a recent study, Uffo et al. (2017) validated a panel of 30 microsatellite markers suggested by FAO/ISAG for studies of biodiversity in cattle to improve the characterization of Cuban buffalo populations. Twenty-eight of the thirty regions analyzed were amplified, with one (ETH10) found to be monomorphic. In the Cuban water buffalo population, a total of 143 alleles were found. The number of alleles per polymorphic locus varied from two (INRA 63 and MM12) to nine (ETH185). The polymorphic information content (PIC) ranged from 0.097 (MM12) to 0.806 (INRA32), and the overall value for these markers was 0.482. Within the population, inbreeding estimates (FIS) was positive in 14 of the 30 loci analyzed (Uffo et al. 2017).

In a nutshell, all these studies prove the usefulness of highly polymorphic microsatellite markers to investigate intraracial diversity, levels of inbreeding, genetic differentiation between breeds, genetic kinship, mixed breeds introgression within diverse buffalo population, which subsequently helps to rational breeding and conservation practices in buffaloes around the world.

8.6.1.1 Microsatellite Markers in Indian Buffalo Breeds

India is abode to large and diverse population of domestic buffaloes with 17 number of registered breeds. Buffalo contributes to ~49% to the milk pale of the country. Due to continuous grading up program with high yielding breeds of buffalo different level of breed admixture has taken place in different parts of the country. Some of the local breeds like Toda which is maintained by a tribe of the same name in the Nilgiri hills of South India is endangered due to scarcity of pasture lands and upward social mobility of this tribe. To devise effective conservation and breeding strategies in buffalo, therefore, it is crucial to accumulate information about the genetic structure of different breeds of buffaloes of the country. Few systematic studies have been carried out to map the microsatellite diversity of Indian cattle breeds in the last two decades (Table 8.2) with International Society of Animal Genetics (ISAG)/FAO recognized microsatellite markers. They show high degree of polymorphism as evident from observed heterozygosity (F_o), expected heterozygosity (F_e) and polymorphism information content (PIC). Table 8.2 represents microsatellites loci analyzed by different research groups of the country.

Table 8.2 Microsatellite diversity of Indian buffalo breeds

Breeds	Microsatellite loci	References
 Murrah	HEL5, BM1818, ILSTS030, ILSTS011	Sukla et al. (2006)
	BMS462, BMS4012, ILSTS089, BMS1724, MSBQ, BL1134, BMS462, CA004, BMS1747, BL1036, BL1029, BMS518, BM757	Kumar et al. (2006)
	BM1818, CSSM29, ILSTS11, ILSTS52, ILSTS59, CSSM43, CSSM45, ILSTS05, ILSTS49, ILSTS58, ILSTS72, ILSTS30, ETH152, CSSM47, CSSM33, CSSM08, CSRM60, CSSM06, CSSM19, CSSM57, ILSTS38, ILSTS29	Vijh et al. (2008)
	CSRM 60, ILSTS 026, HEL 13, ILSTS 030, ILSTS 033, ILSTS 017, ILSTS 019, ILSTS 045, ILSTS 034, ILSTS 058, ILSTS 056, ILSTS 089, CSSM 66, ILSTS 036, ILSTS 095, ILSTS 029, ILSTS 028, ILSTS 025, ILSTS 052, ILSTS 031, ILSTS 073, ILSTS 060, BM 1818, ILSTS 061, ILSTS 068	Mishra et al. (2009)
 Bhadawari	HEL5, BM1818, ILSTS030, ILSTS011	Sukla et al. (2006)
	BMS462, BMS4012, ILSTS089, BMS1724, MSBQ, BL1134, BMS462, CA004, BMS1747, BL1036, BL1029, BMS518, BM757	Kumar et al. (2006)
	BM1818, CSSM29, ILSTS11, ILSTS52, ILSTS59, CSSM43, CSSM45, ILSTS05, ILSTS49, ILSTS58, ILSTS72, ILSTS30, ETH152, CSSM47, CSSM33, CSSM08, CSRM60, CSSM06, CSSM19, CSSM57, ILSTS38, ILSTS29	Vijh et al. (2008)
 Surti	BMS462, BMS4012, ILSTS089, BMS1724, MSBQ, BL1134, BMS462, CA004, BMS1747, BL1036, BL1029, BMS518, BM757	Kumar et al. (2006)
	BM1818, CSSM29, ILSTS11, ILSTS52, ILSTS59, CSSM43, CSSM45, ILSTS05, ILSTS49, ILSTS58, ILSTS72, ILSTS30, ETH152, CSSM47, CSSM33, CSSM08, CSRM60, CSSM06, CSSM19, CSSM57, ILSTS38, ILSTS29	Vijh et al. (2008)
 Mehsana	HEL5, BM1818, ILSTS030, ILSTS011	Sukla et al. (2006)
	BMS462, BMS4012, ILSTS089, BMS1724, MSBQ, BL1134, BMS462, CA004, BMS1747, BL1036, BL1029, BMS518, BM757	Kumar et al. (2006)
	BM1818, CSSM29, ILSTS11, ILSTS52, ILSTS59, CSSM43, CSSM45, ILSTS05, ILSTS49, ILSTS58, ILSTS72, ILSTS30, ETH152, CSSM47, CSSM33, CSSM08, CSRM60, CSSM06, CSSM19, CSSM57, ILSTS38, ILSTS29	Vijh et al. (2008)

(continued)

Table 8.2 (continued)

Breeds	Microsatellite loci	References
 Pandharpuri	BM2113, BM1818, CSSM66, HEL13, INRA037, ILSTS05, HAUT27, INRA023, INRA035, HEL5, ETH3, NRA063, MM12, ETH10	Khade et al. (2020)
	BMS462, BMS4012, ILSTS089, BMS1724, MSBQ, BL1134, BMS462, CA004, BMS1747, BL1036, BL1029, BMS518, BM757	Kumar et al. (2006)
	BM1818, CSSM29, ILSTS11, ILSTS52, ILSTS59, CSSM43, CSSM45, ILSTS05, ILSTS49, ILSTS58, ILSTS72, ILSTS30, ETH152, CSSM47, CSSM33, CSSM08, CSRM60, CSSM06, CSSM19, CSSM57, ILSTS38, ILSTS29	Vijh et al. (2008)
 Nagpuri	HEL5, BM1818, ILSTS030, ILSTS011	Sukla et al. (2006)
	CSRM 60, ILSTS 026, HEL 13, ILSTS 030, ILSTS 033, ILSTS 017, ILSTS 019, ILSTS 045, ILSTS 034, ILSTS 058, ILSTS 056, ILSTS 089, CSSM 66, ILSTS 036, ILSTS 095, ILSTS 029, ILSTS 028, ILSTS 025, ILSTS 052, ILSTS 031, ILSTS 073, ILSTS 060, BM 1818, ILSTS 061, ILSTS 068	Kataria et al. (2009)
	BMS462, BMS4012, ILSTS089, BMS1724, MSBQ, BL1134, BMS462, CA004, BMS1747, BL1036, BL1029, BMS518, BM757	Kumar et al. (2006)
	BM1818, CSSM29, ILSTS11, ILSTS52, ILSTS59, CSSM43, CSSM45, ILSTS05, ILSTS49, ILSTS58, ILSTS72, ILSTS30, ETH152, CSSM47, CSSM33, CSSM08, CSRM60, CSSM06, CSSM19, CSSM57, ILSTS38, ILSTS29	Vijh et al. (2008)
 Toda	BMS462, BMS4012, ILSTS089, BMS1724, MSBQ, BL1134, BMS462, CA004, BMS1747, BL1036, BL1029, BMS518, BM757	Kumar et al. (2006)
	BM1818, CSSM29, ILSTS11, ILSTS52, ILSTS59, CSSM43, CSSM45, ILSTS05, ILSTS49, ILSTS58, ILSTS72, ILSTS30, ETH152, CSSM47, CSSM33, CSSM08, CSRM60, CSSM06, CSSM19, CSSM57, ILSTS38, ILSTS29	Vijh et al. (2008)
 Banni	CSRM 60, ILSTS 026, HEL 13, ILSTS 030, ILSTS 033, ILSTS 017, ILSTS 019, ILSTS 045, ILSTS 034, ILSTS 058, ILSTS 056, ILSTS 089, CSSM 66, ILSTS 036, ILSTS 095, ILSTS 029, ILSTS 028, ILSTS 025, ILSTS 052, ILSTS 031, ILSTS 073, ILSTS 060, BM 1818, ILSTS 061, ILSTS 068	Mishra et al. (2009, 2010)
 Jaffarabadi	HEL5, BM1818, ILSTS030, ILSTS011	Sukla et al. (2006)
	BMS462, BMS4012, ILSTS089, BMS1724, MSBQ, BL1134, BMS462, CA004, BMS1747, BL1036, BL1029, BMS518, BM757	Kumar et al. (2006)
	BM1818, CSSM29, ILSTS11, ILSTS52, ILSTS59, CSSM43, CSSM45, ILSTS05, ILSTS49, ILSTS58, ILSTS72, ILSTS30, ETH152, CSSM47, CSSM33, CSSM08, CSRM60, CSSM06, CSSM19, CSSM57, ILSTS38, ILSTS29	Vijh et al. (2008)

8.6.2 Mitochondrial DNA Variation

Initial studies based on sequencing data and haplotype analysis of *Cytochrome b* (*Cytb*), a mitochondrial gene, supported the clear divergence between swamp and river buffalo (Kikkawa et al. 1997; Tanaka et al. 1996). Subsequently, analyses of the complete mitochondrial D-loop sequence of Chinese swamp buffaloes (Lei et al. 2007) and Indian river buffaloes (Kumar et al. 2007) revealed evolutionary evidence of domesticated swamp and river buffalo from ancestral swamp-like animals, and identified two distinct mitochondrial lineages (major A and minor B) in the Chinese swamp buffalo. Mitochondrial DNA has been extensively used to identify haplotype diversity of buffalo populations of the world. This has been summarized in Table 8.1. An extensive investigation studying entire mitochondrial D-loop and *Cytb* sequences from 913 swamp buffaloes confirmed five major (SA1, SA2, SB1, SB2, and SB3) and three rare and highly divergent (SC, SD, and SE) swamp buffalo haplogroups (Zhang et al. 2016). Further research employing analysis of 16 kb mitogenome sequences fine-tuned swamp buffalo mtDNA subhaplogroups as SA1 (SA1a, SA1a1, SA1a2, SA1a3), SB1 (SB1a, SB1a1, SB1a2, SB1b), SB2 (SB2a, SB2b), SB3 (SB3a, SB3a1), and SD (SD1, SD2). Simultaneously, the demographic history of swamp buffalo matrilineal lineages were restructured.

In river buffalo, various investigations have confirmed the presence of three haplogroups, viz. R1 (major and most frequent), R2 (less frequent), and R3 (rare) (Kumar et al. 2007; Zhang et al. 2016). Repeated introgression of wild buffalo mtDNA variants has been evidenced into the Indian domestic stocks after domestication (Nagarajan et al. 2015). Therefore, swamp buffalo has more diverse haplotypes and stronger phylogeographic structure compared to river buffalo. From all these mtDNA-based evidences it was hypothesized that Indian domestic buffalo stock migrated gradually after domestication from the Indian subcontinent towards western Asia and Europe without considerable population bottlenecks (Zhang et al. 2016) (Table 8.3).

8.6.3 Y-chromosome Variation

Compared to mtDNA, less number of studies have been carried out so far involving buffalo Y chromosome variations for phylogeographic analysis. In a study by (Yindee et al. 2010), sequencing and analysis of *DBY*, *ZFY,* and *SRY* gene fragments of water buffalo Y chromosome revealed distinct divergence pattern of river and swamp buffalo from different wild populations. Like mtDNA-based evidences, buffalo Y chromosome analysis also reveals that swamp buffalo population possesses lower diversity and weaker phylogeographic structure compared to swamp buffaloes (Yindee et al. 2010; Yi Zhang et al. 2016). Amplification and sequence analysis of 2310-bp Y-chromosome segment (GenBank Accession nos. GQ259327-GQ259332, KT186376-KT186427) revealed four major and seven rare haplotypes defined by nine SNPs in 450 swamp buffalo bulls (Fig. 8.4a) and one SNP (Fig. 8.4b) in 45 river buffalo bulls of Chinese, Indochinese, and Bangladeshi

Table 8.3 Mitochondrial DNA analysis in diverse buffalo breeds across the world

Breeds of buffalo	Country	Sample size	Mitochondrial target region	Number of haplotypes	Reference
Murrah, Bhadawari, Mehsana, Suri, Jaffarabadi, Nagpuri, Pandharpuri, Toda	India	217	D-loop	135	Kumar et al. (2007)
Azi-Kheli, Kundi, Nili Nili Ravi, Ravi	Pakistan	123	D-loop	52	Babar et al. (2012)
Toda, South Kanara, Murrah, Marathwada	India	176	D-loop	28	Kathiravan et al. (2011)
Nili Ravi, Kundi	Pakistan	50	*Cytochrome b*	10	Saif et al. (2012a)
Nili Ravi, Kundi	Pakistan	50	*Cytochrome b*	7	Saif et al. (2012b)
Haizi, Shanqu, Dongliu, Wenzhou, Xinglong, Fu'an, Binhu, Jianghan, Enshi, Xiajiang, Xinfeng, Xinyang, Hanzhong, Guizhou, Dechang, Fuling, Guangxi, Fuzhong, Xilin, Diandongnan, Yanjin, Dehong	China	455	D-loop	157	Yue et al. (2013)
Upper Assamese, Manipuri, lower Assamese, Chilika, South Kanara, Marathwada	India	86	D-loop	173	Mishra et al. (2015)
Swamp and river buffalo breeds	China, Laos, Myanmar, Thailand, Vietnam, India	107	Complete mitogenome sequencing	87	Wang et al. (2017)
Swamp, crossbred and Murrah	Malaysia	105	D-loop	10	Shaari et al. (2019)
Egyptian river buffaloes	Egypt	29	Complete mitogenome sequencing		Youssef et al. (2021)

origins (Zhang et al. 2016). River buffalo Y chromosome diversity has not been explored to much extent till now. Analysis of the 2310 bp-long Y-chromosome sequences in a small population of river buffaloes ($n = 45$) showed only one polymorphic site defining two haplotypes (Zhang et al. 2016).

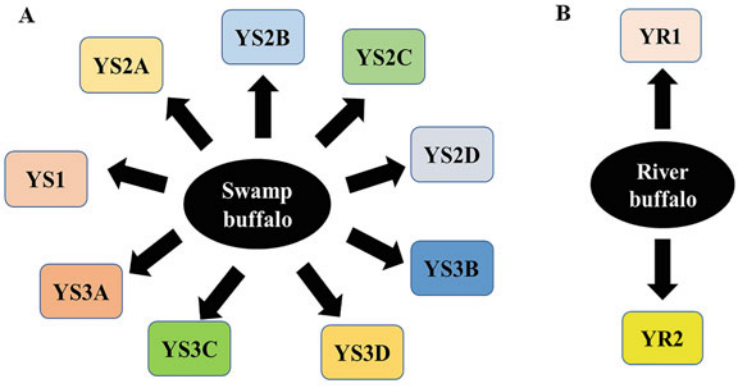

Fig. 8.4 Y chromosomal haplotype diversity in swamp buffalo (**a**) and river buffalo (**b**)

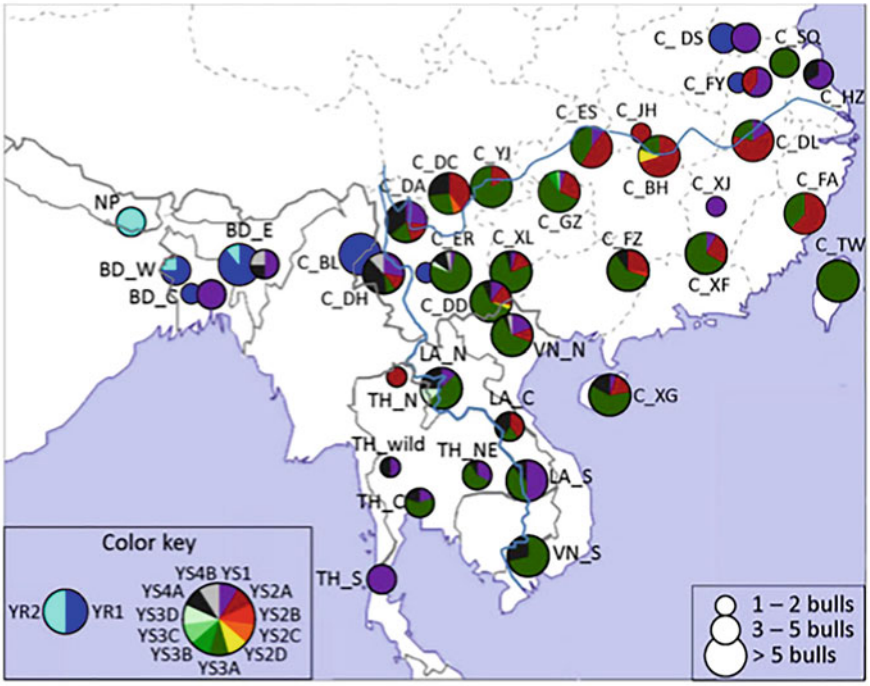

Fig. 8.5 Y-chromosomal haplogroup distributions of river and swamp buffalo populations. The area of the circles is proportional to the number of bulls sampled from each population. Photo taken from Zhang et al. (2016)

This is evident from Fig. 8.5 that only the southern Chinese populations harbor all major haplotypes. This is in accord with the earlier established fact that wild females were integrated into the domestic buffalo population, which began in south China–

north Indochina and proceeded west of the Mekong. Bulls of river buffalo breeds such as Murrah, Nili Ravi, and Mediterranean from India, Pakistan, and mediterranean regions have been introduced from time to time for crossbreeding program in China in the last century. The presence of river buffalo Y haplotypes as evident in some of the lower Yangtze River populations are nothing but their traces of these events in the population.

This noteworthy haplotype diversity in swamp buffalo was further confirmed by Y-chromosome specific microsatellite markers-based study that identified nine haplotypes (Wang et al. 2018). A total of 40 bovine Y -chromosome-specific microsatellite (Y-STR) markers were examined in this study. Out of 40 markers, seven Y-STR markers (UMN2405, UMN0504, UMN0103, UMN1307, BC1.2, UMN0304, and INRA008) were specific and polymorphic in male swamp buffaloes. Based on these Y-STRs, nine Y-haplotypes corresponding to four Y-haplogroups (Y1, Y2, Y3, and Y4) were defined. Y1 haplogroup was predominant (83.4%) in all swamp buffalo populations. This indicated that major domestication event of swamp buffalo occurred in Y1 haplogroup around the Yangtze river (Wang et al. 2018). The most ancient lineage, Y4 haplogroup was found in Hainan Island which was separated by Qiongzhou Strait from the mainland China. Y chromosomal marker based paternal origin has been determined for swamp buffaloes of China and Southeast Asia. Future research efforts directed to determine paternal lineages of diverse buffalo populations of the world with Y chromosome based markers will unravel important information about the history of buffalo domestication and migration events across the globe.

8.6.4 Single Nucleotide Polymorphisms (SNPs) Marker in Buffalo Genome

Initially, cattle specific arrays were used to score SNPs in buffalo genome. Affymetrix developed the Axiom® Buffalo Genotyping Array (Affymetrix 90 K chip) for genotyping of water buffalo in collaboration with the International Buffalo Genome Consortium (Iamartino et al. 2017). The array was developed based upon the bovine UMD 3.1 genome and contains 89,988 SNPs, 5799 probes for quality control and 1784 probes for gender determination (Iamartino et al. 2017). The SNP discovery was carried out using important river buffalo breeds like Mediterranean, Murrah, Jaffarabadi, and Nili-Ravi. About 25% of the SNP markers were found to be polymorphic in swamp buffalo populations (Iamartino et al. 2017). With the release of latest version of the buffalo reference genome candidate SNP profile was further updated (Low et al. 2019). Using this SNP panel, 31 water buffalo populations across the world, including pure river, pure swamp, and river × swamp crossbred buffaloes have been characterized (Colli et al. 2018). Molecular analysis revealed three distinct gene pools in pure river as well as in pure swamp buffalo populations. Genomic admixture was seen in the Philippines and in Brazil, confirming importations of animals for breed improvement (Colli et al. 2018).

8.6.5 Copy Number Variation (CNV) in Buffalo Genome

The copy number variation (CNV) consists of duplications and deletions of DNA sequence ranging from 50 bp to several megabases. CNVs are important source of gene evolution and structural variation within mammalian genomes. CNVs in buffalo genome have not been delved in much extent although this may have substantial contribution in determining several biological functions and inherent qualities of buffaloes such as disease resistance, milk quality, etc. A single study involving genome-wide analysis of segmental duplications (SDs) and associated copy number variations (CNVs) in the water buffalo has been carried out so far (Liu et al. 2019). A total of 1344 CNV regions spanning over 59.8 Mb of variable sequence has been detected in 14 additional water buffaloes. The CNV regions harbor 1245 genes that impart many biological functions including immune response, oxygen transport, sensory system, and signal transduction (Liu et al. 2019). These CNV regions are of invaluable importance for phenotypic and evolutionary studies.

8.7 Genotype Meets Phenotype: Buffalo Structural Genome Variations in Determining Economic Traits

Understanding the correlation between genotype and complex trait phenotype is the crux of genetic studies, especially in economically important livestock species. Plethora of studies have been carried out so far to understand the genomic variations and important economic traits like growth, milk production and quality, reproductive efficiency, and disease resistance.

8.7.1 Candidate Gene Approach

8.7.1.1 Milk Production and Growth

Polymorphisms of several candidate genes have been confirmed to be associated with milk quality and quantity of milk production and growth in buffaloes. A total of 47 mutations in 19 candidate genes have been reported to be associated with milk production traits in different buffalo breeds so far (Du et al. 2019). *BTN1A1*, *INSIG2*, *LALBA*, *LEP*, *MC4R*, *OXT*, *PRL*, *SCD*, *SREBF1*, *STAT1,* and *STAT5A* are associated with milk yield; *A2M* and *GHRL* have influence on milk fat yield; *A2M*, *DGAT1*, *GHRL*, *LEP*, *MC4R*, *PRL*, *SCD*, *SREBF1*, STAT1, and TG are related to milk fat percentage; and *ADRA1A*, *A2M*, *CSN1S1*, *DGAT1*, *GHRL*, *INSIG2*, *MC4R*, *MTNR1A*, *PRL,* and *SPP1* have an effect on milk protein percentage. A non-synonymous mutation in exon 10 of the insulin-like growth factor 2 (*IGF2*) gene is probably associated with higher average daily gain from birth to 9 months of age in Egyptian river buffaloes (Abo-Al-Ela et al. 2014).

8.7.1.2 Reproductive Traits

A considerable number of studies have been performed to identify association between gene mutations with reproductive traits in both male and female. Mutations in luteinising hormone beta (*LHβ*) polypeptide have been recently shown to be linked with semen quality traits, including ejaculate volume and sperm qualities, in Chinese swamp buffaloes (Cheng et al. 2017) and in Indian Murrah buffaloes (Reen et al. 2018). Analysis of eight SNPs in Gonadotropin-releasing hormone receptor (*GnRHR*) gene revealed significant association between ejaculate volume, sperm concentration, post-thaw sperm motility, and sperm abnormality (Wang et al. 2017, 2020). In Egyptian river buffalo female, four SNPs, one in the 5'UTR and three in exon 10 of the cytochrome P450 aromatase (*CYP19A1*) gene have been observed to influence the susceptibility to anoestrus (El-Bayomi et al. 2018; Pandey et al. 2019) reported an association between various genotypes of melatonin receptor 1A (*MTNR1A*) gene and pregnancy rate in Murrah river buffaloes.

8.7.1.3 Innate Immunity and Disease Resistance

River buffaloes are resistant to many tropical diseases compared to cattle breeds of the world (Borriello et al. 2006). The genetic polymorphisms of several innate immune genes contribute significantly to confer resistance in buffaloes. Diversity in C-terminal antimicrobial domain of cathelicidins (CATH) gene family, an antimicrobial peptide gene, plays crucial role in innate immunity of buffaloes (Brahma et al. 2015). Polymorphisms in ten candidate genes related to innate immunity (*CHGA*, *CHGB*, *CHGC*, *Slc11a1*, *Slc11a2*, *DEFB1*, *BNBD4*, *BNBD5*, *LAP,* and *TAP*) have been shown to be associated with disease resistance in economically important Indian buffalo breeds, viz., Jaffrabadi, Mehsani, and Murrah (Patel et al. 2015).

Resistance to several diseases of buffaloes such as brucellosis, bovine tuberculosis, and mastitis is affected by specific genetic variants. Microsatellite polymorphism identified in the 3′UTR region of the water buffalo *Nramp1* gene contributes to susceptibility or resistance to *B. abortus* by determining a low or high quantity of the NRAMP1 protein (Borriello et al. 2006). A significant association between bovine tuberculosis susceptibility and G > A mutation in an SNP at position 4467 in the 3′-untranslated region (3'-UTR) of buffalo interferon gamma (*IFNG*) gene have been observed (Iannaccone et al. 2019). This may be due to the disruption of the target sequence for a microRNA (miR-125b). Milk somatic cell score which is an important indicator of clinical mastitis has been reported to be influenced by a C > A substitution in exon 27 of the complement component 3 (*C3*) and component 5 (*C5*) in Egyptian buffaloes (El-Halawany et al. 2017, 2018). Recently, mucin 1 (*MUC1*) gene polymorphism has been observed to be associated with mastitis resistance in buffalo (da Rosa et al. 2020).

8.8 Future of Buffalo Production

Buffaloes are present in 77 countries in five continents with a population of more than 208 million head (Minervino et al. 2020). The buffalo is better converter of poor quality feed to high quality animal products and adapt in a wide range of topographical, climatic conditions (Mengwei et al. 2020). Water buffaloes play an important role in the economy of many tropical and subtropical countries, as they are used for meat and milk production and as draft animals. Buffalo milk has higher fat content and total solids content compared to cow (Costa et al. 2020). In terms of energy, 1 kg of buffalo milk equals 5.10 Mj, which is much higher than the 2.90 Mj/kg of 1 kg of cow's milk (Varricchio et al. 2007). The water buffalo has a longer productive life when compared to cattle. Buffalo milk contains lower level of cholesterol, sodium, potassium, and it is a rich source of calcium, phosphorous and vitamins, particularly vitamins E and A (Borghese and Moioli 2016; Wahid and Rosnina 2011). With all these exceptional qualities of buffalo and buffalo-derived products, this animal is a natural choice for husbandry practices in developing world and its demand is expanding worldwide. However, factors which may limit the development of the dairy buffalo industry are lower milk production than that of high-yielding cattle breeds such as the Holstein (Deng et al. 2019).

The recent advancement in high-throughput sequencing technologies has opened up new opportunities to understand the molecular basis behind unique phenotypes of water buffalo. Genome wide identification of candidate gene regions, quantitative trait loci (QTL), and selection signatures are extremely useful for selecting animals for production, adaptation, and disease resistance. With accumulating molecular information about the genetic make-up of this species following future measures may be taken to augment the production and increase the adaptability in this animal:

- Adoption of genomic selection by incorporating superior buffalo bulls on the basis of genomic estimated breeding values.
- Adoption of DNA-based methods for paternity assessment through the estimation of parentage matrices.
- Use of advanced genome-editing techniques to propagate important genes associated with desirable traits within buffalo genome.

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Advances in Buffalo Bull Fertility Prediction

9

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Abstract

Although it is not wise to compare buffalos with cattle, often, when talking about reproduction, buffaloes are compared with cattle and are said to have comparatively higher sub-fertility problems. Both male and female contribute to infertility; however, the role of males is amplified because one bull is used to artificially breed several thousands of females. Buffalo spermatozoa is unique in terms of structural, compositional, and functional attributes, and thus the fertility prediction methods/tools used in cattle may not hold good for this species. During the last one or two decades, there have been a quantum of information generated on buffalo spermatozoa in relation to their fertilizing potential. In this chapter, the molecular composition and phenotypic and functional attributes of buffalo spermatozoa in relation to fertility are discussed. In the absence of specific information in buffaloes, wherever needed, the information from other species, especially from cattle bulls are intentionally incorporated so as to facilitate the reader's understanding on the recent developments on male fertility and to open up new avenues for research on buffalo bull fertility.

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9.1 Introduction

With the increasing importance of buffalo as a milk producer, the practice of artificial insemination (AI) with frozen semen has become a prerequisite to improve their production by using better germplasm. However, poor conception rate with AI using cryopreserved semen in an obstacle hindering the faster genetic improvement in this species. Although females contribute equally to the success/failure of conception through AI, the male assumes much significance in AI because not all the males yield similar conception rates and using semen from a low-fertile bull will lead to colossal loss to the farmers. For artificial breeding, like cattle bulls, buffalo bulls are also selected based on pedigree and breeding soundness evaluation (BSE). However, a considerable proportion of bulls that passed these evaluations produced ejaculates with poor quality of semen that are not suitable for cryopreservation. Moreover, the bulls that qualified the BSE and inducted into semen stations for artificial breeding, differed among themselves by 20–25% in field fertility indicating that the currently used BSE method do not precisely assess the fertility of the bulls. These low performing bulls can be identified at a very later stage only, upon large scale use of semen samples through AI and subsequent feedback from the field, hampering the genetic progress and economic loss (Kumaresan et al. 2017b).

Generally, post thaw sperm motility of cryopreserved semen is used to determine its suitability for artificial breeding. Although good post thaw motility is prerequisite for the spermatozoa to reach the site of fertilization upon insemination, it has little value in predicting the fertility of bulls (Rodríguez-Martínez and Peña Vega 2013). Buffalo spermatozoa differ from cattle spermatozoa in terms of membrane profile and their sensitivity to altered environments encountered during cryopreservation. For instance, there is a clear difference between lipid profile of buffalo and cattle spermatozoa, the polyunsaturated fatty acid content is high in buffalo semen. The differences in biochemical composition and other less known mechanisms all collectively lead to altered membrane fluidity, high oxidative damage, premature capacitation, and high apoptosis like changes during cryopreservation (Martin et al. 2004), which ultimately results in poor freezability and fertility of buffalo spermatozoa (Andrabi 2009). With the developments in sperm analytical tools/methods, now it is possible to assess several specific functions of spermatozoa in less time, which led to development of sperm function-based fertility prediction tools. During the last decade, a lot of information have been generated on sperm composition in terms of transcripts, proteins, and metabolites and their relationship with fertilizing potential (Feugang et al. 2010; Aslam et al. 2019; Saraf et al. 2020). Using high throughput techniques, sperm molecular signatures for fertility have been identified at the level of transcripts (Prakash et al. 2020; Paul et al. 2020), proteome (Aslam et al. 2019), and metabolites (Saraf et al. 2020) in cattle. Although such studies on buffalo spermatozoa are limited, few recent studies open up such possibilities of identification of fertility associated sperm transcripts (Paul et al. 2020) and proteins (Aslam et al. 2019; Fu et al. 2019).

Here, we made an attempt to compile the available information of buffalo sperm functional assays, sperm transcriptome, and proteome and delineate their utility in

buffalo bull fertility prediction. Information on cattle spermatozoa that have the potential to be tested in buffaloes are also discussed.

9.2 Sperm Phenotypic Characteristics and Bull Fertility: What to Assess?

To be able to fertilize the oocyte, spermatozoa should possess certain phenotypic and functional characteristics. Spermatozoa must be motile, retain their ability to produce energy via metabolism in mitochondria, maintain normal plasma membrane configuration and integrity, intact acrosome membrane, intact receptors that permit the spermatozoa to bind to the zona pellucida, maintain enzymes within the acrosome to allow penetration of ova and a nucleus capable of decondesation (Graham and Mocé 2005). Disruption of any of these functions or abilities will significantly affect the sperm's ability to achieve fertilization. The basic characteristics of viable spermatozoa required for fertilizing an oocyte are given in Box 9.1.

Box 9.1: Sperm Characteristic Required for Fertilization of an Oocyte

1. Motility - Motility is paramount important for the sperm to traverse the barriers of female reproductive tract and to reach the site of fertilization.
2. Morphology – Normal morphology is important. Abnormalities like misshapen head, suggestive of chromatin damage, can adversely affect early embryonic development.
3. Intact membrane - To maintain normal functionality during passage in male as well as female reproductive tract and to participate in fertilization, intact plasma membrane is a prime requirement.
4. Intact acrosome – Important for retaining the acrosomal enzymes responsible for sperm penetration into oocyte. Poorly formed acrosome or premature loss of acrosome can compromise the fertility.
5. DNA integrity - Indispensable for the fertilizing sperm to initiate and sustain fertilization process, to substantiate embryonic development and important for offspring fitness.
6. Mitochondrial stability - To produce ATP by oxidative phosphorylation for the metabolism, membrane function and motility.
7. Ability to undergo capacitation in female reproductive tract – Essential to attain fertilizing capacity.

On the other hand, if we consider cryopreserved spermatozoa, its structural and functional competencies are altered by the process of freezing and thawing. Freezing and thawing of buffalo spermatozoa cause considerable damages to DNA (Kumar et al. 2011), motility apparatus (Rasul et al. 2001), plasma membrane, acrosomal cap (Rastegarnia et al. 2013), leakage of intracellular enzymes (Dorostkar et al. 2012), lipid peroxidation (Kumaresan et al. 2006; Kadirvel et al. 2009), apoptosis (Khan

et al. 2009), and thus reduce fertility. Traditionally semen quality control involves subjective assessment of motility, sperm morphology assessment, and an estimate of the concentration of spermatozoa (Gillan et al. 2008). These tests determine minimum standard for the frozen semen to be used. However, they have limited value for predicting the fertility (Rodriguez-Martinez 2001). As a result, attention has been directed towards the assessment of other aspects of semen quality as predictors of fertility, such as viability, acrosomal integrity, membrane status, capacitation status, lipid scrambling, mitochondrial potential, Ca^{2+} uptake, apoptosis, DNA integrity, membrane proteins, and the ability of spermatozoa to swim-up. Few in vitro sperm parameters show a reliable and repeatable correlation with field fertility (Rodriguez-Martinez 2001; Rodríguez-Martínez 2018; Kumaresan et al. 2017a). With developments in sperm cryobiology, a combination of in vitro tests has been utilized to produce fertility prediction model. Several such studies, conducted in this aspect, have estimated one or two sperm functions and related with bull fertility. Detailed studies involving assessment of several sperm functions in bulls with different fertility indices and estimation of their relationship with fertility would help us to arrive at a battery of tests that could be used to fairly assess the bull fertility.

In this direction, Kumaresan et al. (2017b) developed a fertility prediction model using specific sperm function tests in cattle bulls. These researchers found that the proportion of live ($r = 0.53$) and live acrosome-reacted spermatozoa ($r = 0.50$) were significantly positively related, whereas the proportion of dead spermatozoa ($r = -0.53$) and %DFI (DNA Fragmentation Index; $r = 0.61$) were significantly negatively related to field fertility in cattle bulls. The developed fertility prediction model could effectively differentiate below-average and above-average fertility bulls. The accuracy of the developed model for fertility prediction in bulls was high ($R^2 = 0.83$).

$$\text{NRR} = 55.71 + (0.39 \times \text{live}) + (0.37 \times \text{live } H_2O_2\text{negative}) - (0.39 \times \%\text{ DFI})$$

(Kumaresan et al. 2017b)

In case of buffaloes, information on the relationship of sperm functional and phenotypic attributes are very limited. Using field fertility, Singh et al. (2016) classified Murrah buffalo bulls into high, medium, and low fertile and studied several sperm phenotypic characteristics and functional competencies. They identified the subtle differences among the spermatozoa from bulls with different fertility ratings and their relationship with field fertility. They reported that the proportion of moribund spermatozoa, protamine deficient spermatozoa, spermatozoa with unstable membrane, apoptotic and necrotic spermatozoa in a given sample was negatively and significantly related with bull fertility. On the other hand, the proportion of live acrosome intact spermatozoa and fertility was positive and significant. Based on these tests, these researchers developed a fertility prediction model for buffaloes with accuracy of 73%.

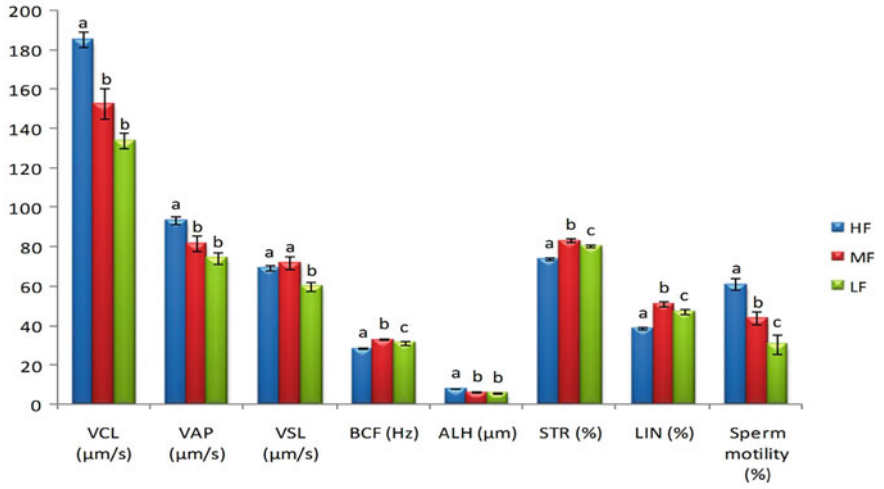

Fig. 9.1 Kinematics of frozen-thawed spermatozoa of Murrah buffalo bulls belonging to high-, medium-, and low-fertile groups (HF- high fertile; MF- medium fertile; LF – low fertile). Adopted from Singh et al. (2016)

Few other studies identified sperm motion characteristics related to fertility. Kumar et al. (2012) concluded that average path velocity, curvilinear velocity, straight linear velocity, and total motility, acrosomal integrity, and percentage of apoptotic sperm were useful for evaluating the semen quality of buffalo bull in relation to fertility. Kumar et al. (2016) identified that buffalo bull fertility could be predicted on the basis of sperm motion traits (VCL, VAP, VSL, ALH, and LIN), motility, viability, and membrane integrity. Ahmed et al. (2016) reported that assessment of CASA parameters and some sperm structural and functional parameters like integrity of plasma membrane and acrosome, and transmembrane potential of mitochondria were able to predict the in vivo fertility of water buffalo bull during low-breeding season. Similarly, Singh et al. (2016) found that spermatozoa from high-fertile buffalo bulls had significantly higher ($P < 0.05$) BCF, STR, ALH, and LIN compared to either medium- or low-fertile bulls. They concluded that buffalo bull fertility was significantly and positively correlated with sperm motility, VAP, VSL, VCL, and ALH. Collectively, these findings offer scope for bull fertility prediction using sperm phenotypic and functional differences between high- and low-fertile bulls. The differences in sperm kinematic parameters between high-, medium-, and low-fertile buffalo bulls are shown in Fig. 9.1. However, more and more information need to be generated on sperm phenotypic and functional attributes using large number of bulls with different fertility ratings for widespread application of such tools for fertility prediction in buffalo bulls.

9.3 Sperm-Oviduct Explants Binding Assay for Fertility Prediction

It is well understood that a stringent selection process occurs at the oviduct for allowing the eligible spermatozoa to establish sperm-oviduct reservoir. There is consensus among researchers that the dam's oviduct operates a mechanism (cryptic female choice) to select a superior spermatozoon to father her offspring (reviewed by Holt and Fazeli 2010). It has been reported that the sperm selection process in the oviduct can result from a female-controlled process that ensures the availability of good quality spermatozoa for fertilizing the oocyte. It is reported that if a bull is unable to populate the sperm reservoir with a sufficient number of spermatozoa or for a sufficient period of time, fertility could be adversely affected (De Pauw et al. 2002). In this direction, Kumaresan et al. (2017a) proved that spermatozoa from high-fertile bulls responded in a different way from that of spermatozoa from low-fertile bulls when they were exposed to oviductal fluid. They reported that spermatozoa from bulls with higher fertility showed a lower degree of tyrosine phosphorylation and a higher degree of capacitation and acrosome reaction upon exposure to oviductal fluid.

To carry out such studies in buffaloes, development of a quantitative and reproducible in vitro sperm-oviduct explant model is required; however, such model was not available earlier for the buffalo. For the first time, such sperm-oviduct explant model (Fig. 9.2) was developed for buffaloes by Saraf et al. (2017a). Using the developed model, it was been shown that spermatozoa with high mitochondrial membrane potential and low tyrosine phosphorylation preferentially bound to oviduct explants in the buffalo (Saraf et al. 2017b). Furthermore, it was also shown that the ability of buffalo spermatozoa to bind to oviductal explants differed between bulls with different fertility ratings (Saraf et al. 2019). They concluded that the number of spermatozoa bound to the unit area of oviduct explants was significantly higher in high-fertile bulls compared with low-fertile buffalo bulls, indicating the possibility of using sperm-oviduct explant binding model as a fertility prediction tool in this species.

9.4 Sperm Molecular Signatures for Fertility and beyond

9.4.1 Sperm Transcripts

For a long period of time, it was believed that spermatozoa are transcriptionally and translationally inert and only act as a vehicle for delivering paternal DNA. Of late, scientists discovered traces of RNA in sperm head which arises several questions regarding their origin and potential roles (Pessot et al. 1989; Miller et al. 2005). Since then, several researches were conducted to identify the origin of RNA's in spermatozoa and it was believed that the cargo of RNA present in spermatozoa were originated from residual cytoplasm during spermiogenesis. Spermatozoa head possesses a diverse RNA population including messenger RNAs (mRNAs),

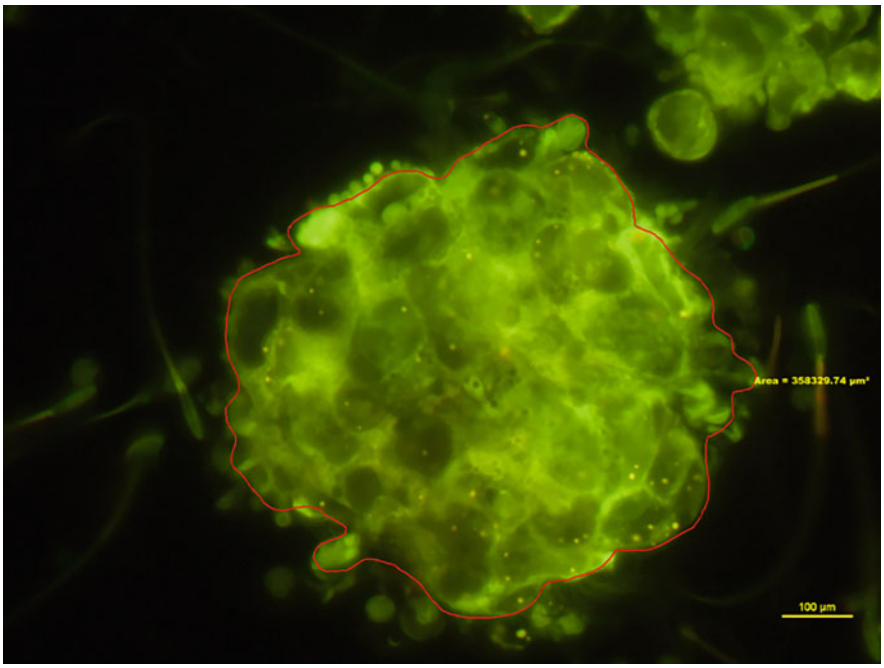

Fig. 9.2 Sperm-oviduct explant binding model for buffaloes

Table 9.1 Localization of sperm RNAs (Zhang et al. 2019)

Localization	Types of RNA
Sperm outer membrane	mRNAs, fragmented rRNAs
Sperm nucleus	mRNAs, tsRNAs, and miRNAs, may form RNA-DNA hybrid structure
Nuclear matrix	Types of RNAs present is still under study
Sperm tail	Cytoplasmic droplet containing RNAs present remnant of spermatogenesis, mitochondria may still possess active RNA transcription

ribosomal RNAs, mitochondrial RNA (mitoRNAs), annotated coding transcripts as well as small non-coding RNAs (Jodar et al. 2013; Prakash et al. 2020). Off late, the presence of diverse types of RNAs in different ultrastructural components of spermatozoa had been confirmed (Table 9.1). Recently, it has been found that mature spermatozoa contain on an average of 10–20 fg of total RNA, whereas somatic cell contains approximately 10–20 pg of total RNA which is nearly 1000 times more than that of the former (Table 9.2) (Krawetz 2005; Selvaraju et al. 2017). There is a significant evolution in technologies to study sperm RNA composition and in this line scopes for microarray and next generation sequencing (RNA sequencing) are promising. These high throughput technologies allow global detection of transcripts present in spermatozoa and helps identifying transcripts which are differentially expressed between samples (Boerke et al. 2007; Miller et al. 2005).

Table 9.2 Sperm-borne RNA's in different species

Species	Sperm-borne RNA quantity (fg)
	10–50
	100
	20
	20–30
	1–8

9.4.2 Types of RNA and their Role in Spermatozoa

Detailed investigation on bovine sperm composition revealed the presence of intact mature as well as immature mRNA and transcripts with only exonic reads or only intronic reads (Selvaraju et al. 2017). The coding transcripts provide key signal for oocyte activation, early embryonic development, and in epigenetic modification (Nomikos et al. 2013; Guo et al. 2017; Gannon et al. 2014). Recently, it has been found that nonprotein-coding RNAs accounts for a larger proportion of transcriptome as compared to protein-coding transcripts. These non-coding transcripts (ncRNAs) are composed of short as well as long ncRNAs based on their nucleotide content. The importance of non-coding RNAs have been realized recently owing to their diverse role in regulating genes at transcriptional, posttranscriptional, and epigenetic levels by interacting with nucleic acids and proteins (Wang et al. 2019). More importantly, it has been found that some of the non-coding RNAs are highly expressed in germline suggesting their roles in germline development (Saxe and Lin 2011). One of the interesting findings about short non-coding RNAs (less than 200 nucleotides) such as miRNAs and piRNAs is that they play crucial roles in regulating spermatogenic functions among mammals (Chuma and Nakano 2013; Yadav and Kotaja 2014; Robles et al. 2019). Recently, it has been found that spermatozoa carrying coding and non-coding RNAs influence

the spermatogenic functions which can be used as biomarkers to assess fertility also. For example, expression of BMP2 transcript is significantly upregulated in spermatozoa from high-fertile bulls as compared to low-fertile bulls which can be considered as a potential biomarker to assess spermatogenesis (Parthipan et al. 2017).

9.4.3 Role of Sperm Transcripts in Oocyte Activation

The first and most critical event for initiating mammalian embryo development after fertilization is oocyte activation. The sperm that takes part in fertilization delivers the paternal genome in oocyte. After the entry of the paternal genome, there is a time interval before the activation of the embryonic genome. Therefore, the maternal genome maintains activity in the early zygote. In Bos taurus, this transition (maternal gene expression to zygotic gene expression) occurs approximately 62 h after fertilization (Memili and First 2000). Because of this lag time in the embryonic genome activation, the hypothetical role of spermatozoal mRNAs before activation of the embryonic genome has been proposed (Ostermeier et al. 2002). Specific spermatozoal mRNAs such as clusterin (CLU), protamine 2 (PRM2), protamine 1 (PRM1), and DDX3Y are transferred to the oocyte at fertilization (Ostermeier et al. 2002; Swann et al. 2006). New discoveries found that the fertilizing sperm not only delivers mRNA into oocyte but also this mRNA remained intact for at least 3 h post-fertilization although specific functions of these spermatozoal transcripts are yet to be established. One of the spermatozoal transcript PLCZ1 has shown definitive function in oocyte activation at fertilization by triggering calcium oscillations in the oocyte (Swann et al. 2006). It has been found that microinjecting PLCZ RNA into oocytes causes Ca^{2+} oscillations very similar to fertilization in mouse and humans (Saunders et al. 2002; Rogers et al. 2004). It is evident that periodic Ca^{2+} oscillations during fertilization is not only necessary for oocyte or egg activation but also equally required for post-zygotic events (Yu et al. 2008).

9.4.4 Sperm Transcripts and Epigenetic Modification

Apart from the potential roles in early embryogenesis and oocyte activation, the role of spermatozoal RNA in deciding the phenotype of offspring has been proposed. In spermatozoa, three well characterized epigenetic markers have been identified, namely posttranslational modifications (PTMs) of histone, cytosine methylation of DNA, and small non-coding RNAs. Available studies show a trend that paternal preconception environmental exposures including lifestyle, diet, etc. can influence the performance of future progeny (Ramasamy et al. 2015; Schagdarsurengin and Steger 2016). The epigenetic transgenerational inheritance studies of sperm RNA were first done in mice which laid upon the basis of inheritance of paternal phenotype and behavior in offspring (Gapp et al. 2014). When total sperm RNAs from a mentally stressed male was injected into normal zygotes which produced offspring

with similar behavioral and metabolic effects to that of the father. Surprisingly, subsequent generations recapitulated this stress phenotype suggesting stable transgenerational epigenetic inheritance (Gapp et al. 2014). Similar results were observed when total sperm RNAs from high fat diet mouse model was introduced in normal zygotes and the offspring developed metabolic disorders suggesting the role of sperm RNA as epigenetic modifier (Chen et al. 2016). Paternal diet also alters the levels of small non-coding RNAs particularly tRNA fragments in male reproductive tract and in mature spermatozoa (Sharma et al. 2016). Zygote microinjection of sperm miRNAs from a chronically stressed parent where the offspring also yielded similar stress response mimicking to the father (Rodgers et al. 2015). Another way of paternal epigenetic inheritance is mediated by extra-testicular originated sperm sncRNAs which are absorbed and incorporated into sperm membrane by means of membrane bound vesicles (epididymosomes) during the process of sperm maturation (Sharma et al. 2018). Although the amount of sperm RNAs released into the oocyte is minimal, small changes in particular ncRNAs could impact on the expression of several crucial genes at the same time in a cascade-like fashion, at transcriptional and/or posttranscriptional level (Chen et al. 2016).

9.4.5 Small Non-coding RNAs and Early Embryogenesis

The functional significance of sperm-borne non-coding RNAs is still a matter of debate and remained to be unwrapped completely. However, newer studies are coming up showing influence of sncRNAs in fertilization and embryonic development. For example, it has been found that sperm-borne RNAs are being transferred to oocyte during the process of fertilization and provides essential cargo for early embryonic development. Liu et al. (2012) demonstrated that sperm-borne microRNA-34c is required for the first cleavage division in mouse by modulating Bcl-2 gene expression. Interestingly, paternal miRNAs and endo-siRNAs were found to initiate the normal developmental program during early preimplantation stage (fertilization to the 2-cell transition) (Yuan et al. 2016). The role of sncRNAs in embryogenesis was further confirmed by Guo et al. (2017) where they have performed ICSI on oocyte using RNA deficient mice spermatozoa. The resultant blastocyst formation rate and the live birth rate of embryos from partially RNA deficient sperm were significantly decreased as compared to control suggesting crucial roles of sperm mediated RNAs in embryonic development.

9.4.6 Transcriptomic Studies of Bull Spermatozoa

Several studies are available in the field of bull transcriptomics using microarray or next generation sequencing to identify differentially expressed as well as unique transcripts related to sperm functional attributes, oocyte activation, fertilization, and early embryonic development (Bissonnette et al. 2009; Nomikos et al. 2012; Das et al. 2013). In this line, many candidate genes have been identified influencing

Table 9.3 Candidate genes related to bull fertility

Gene name	Function	Reference
PRM1	Sperm DNA protection, indicates level of sperm chromatin compaction	Feugang et al. 2010
CRISP2	Role in spermatogenesis and associated to sire conception rate deviation (SCRD) score in Holstein bulls.	Arangasamy et al. 2011
RACK1	Protein phosphorylation	Prakash et al. 2020
SPATA7	Spermatogenesis	Elango et al. 2020
RPL31	Early embryonic development	Li and Gang 2010
HSP70	Sperm motility	Zhang et al. 2015
TSSK6	Sperm motility	Bissonnette et al. 2009
ADAM5P	Sperm motility	Bissonnette et al. 2009
BSP3, BSP5	Sperm capacitation and fertilization	Hung and Suarez 2012

sperm function and fertilization events (Table 9.3). One such study by Feugang et al. (2010) reported global transcriptomic profiling of spermatozoa collected from Holstein bulls with opposite fertility (high fertile and low fertile). Microarray result showed that a total of 415 transcripts were differentially expressed, out of approximately 24,000 detected in spermatozoa of both bulls (fold change ≥ 2.0; $P < 0.01$). Spermatozoa of the low-fertile bull were deficient in transcripts associated with extracellular space (CC), transporter (MF), transcription factor (MF), and translation machinery (BP), whereas spermatozoa of the high-fertile bull was lacking the transcripts (BP) essential for cell cycle. Validation of some of the genes from microarray study, namely PRM1, CSN2, CD36 molecule, and centromere protein A (CENPA), was done by quantitative PCR, which revealed that CSN2 was the highest expressed gene in both high and low bulls. High level of expression of such mRNA thus indicates the important role of protein and ion transport for a successful fertilization event. The high abundance of PRM1 transcript could be attributed to the presence of immature spermatozoa with residual cytoplasm as previously mentioned by Lambard et al. (2004). Upregulation of PRM1 transcript might also explain a short transitory translational activity in spermatozoa to aid in replacing protamine binding defects to sperm DNA during travel into female reproductive tract. Therefore, this study was all about the evaluation of spermatozoa specific transcripts and their differential expression in both high- and low-fertile bulls to help designing fertility prediction model in bovines. Similarly, several global transcriptomic analysis studies have been conducted in bull spermatozoa to deduce the functional significance of bovine transcriptome (Card et al. 2013, 2017; Chen et al. 2015; Selvaraju et al. 2017; Elango et al. 2020; Prakash et al. 2020). All the aforesaid

studies provide a basis of selecting bulls based on transcript abundance once validated on a large number of samples.

However, in buffaloes, data on spermatozoa global transcriptomic profiling is scanty. The available first report on comparative global transcriptomic profile of spermatozoa from high- and low-fertile buffalo bulls was carried out by Paul et al. (2020) where dysregulation of functionally relevant transcripts were identified. This study revealed 709 dysregulated transcripts, majority of which were involved in binding activity, catalytic activity, cellular and in important metabolic processes (Fig. 9.3). Pathway analysis identified that MAPK signalling, ribosome pathway, and oxidative phosphorylation pathway were significantly dysregulated in low-fertile buffalo bulls. Validation of some dysregulated transcripts related to embryonic development was shown to follow the same trend in their expression level as compared to transcriptomic profiling. Although not many studies are available in the area of buffalo sperm transcriptomics, future studies are needed to identify fertility biomarkers and to establish the molecular basis of infertility in buffalo bulls.

9.5 Sperm Proteins

Semen comprises of spermatozoa and seminal plasma. In addition to the various intrinsic proteins, the spermatozoa interact with several proteins during different phases of their lifecycle, starting from spermatogenesis, sperm maturation, capacitation, etc., to achieve fertilization ability. Any defects or altered expression of proteins at any of these stages may lead to fertilization failure. Seminal plasma is a complex mixture of secretions from various male sex organs, where proteins constitute the major part of macromolecules present in it. These proteins play important roles in many sperm functions and correlated with fertilizing potential of the spermatozoa (Manjunath and Thérien 2002). The composition and quantity of total proteins in seminal plasma are reported to be correlated with bull fertility. The protein concentration in the semen of buffaloes is less compared to cattle making the spermatozoa inferior in terms of motility, viability, and fertility (Dixit et al. 2016; Codognoto et al. 2018). As proteins hold key responsibility in carrying out various cellular functions, it is crucial to have detailed knowledge about the quantitative and qualitative expression of proteins in cells and tissues comprehensively, to understand these processes.

9.5.1 Techniques for Semen Proteomic Studies

Commonly, the gel-based approach and the shotgun/bottom-up approach are being used for the proteomic studies of semen samples. Initially, two-dimensional (2D) gel electrophoresis coupled with mass spectrometry technique was widely used to identify the proteomic biomarkers of fertility in the seminal plasma and spermatozoa of bulls with varying fertility index. Later, a modified version of 2D-gel

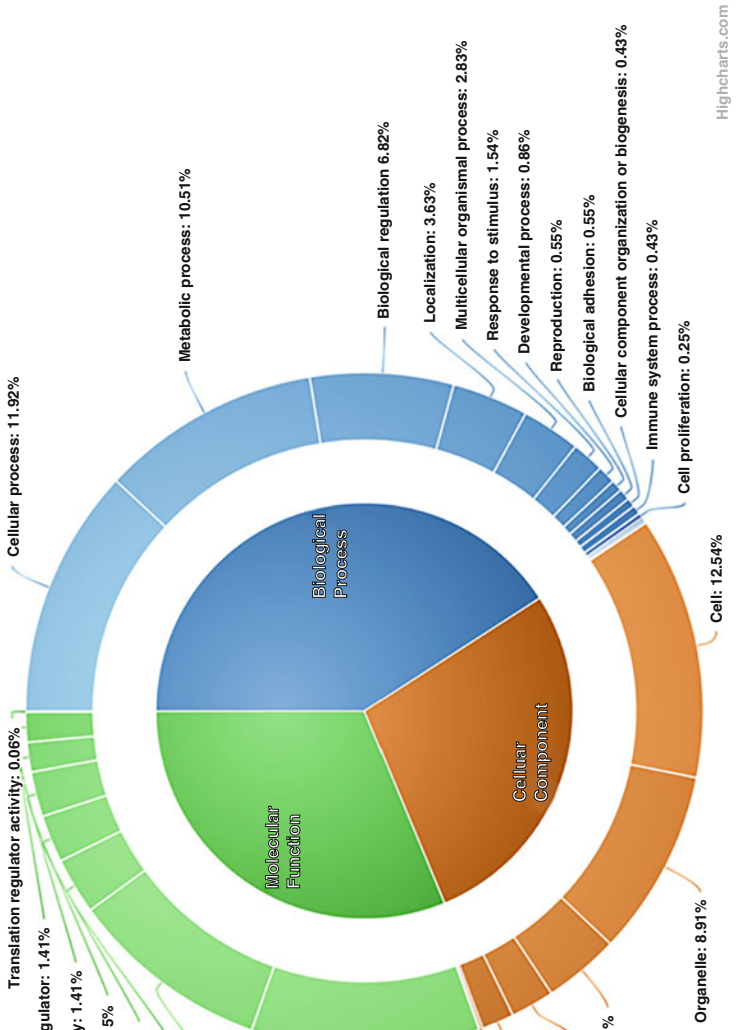

Fig. 9.3 Gene ontology analysis of dysregulated transcripts in low-fertile buffalo bull spermatozoa (Paul et al. 2020)

electrophoresis, known as Difference Gel Electrophoresis (DIGE), has been developed where two different sets of samples can be analyzed in a single experiment, with the minimum requirements of time and resources (Baker et al. 2005; Oliva et al. 2008). The gel-based techniques have certain intrinsic disadvantages in terms of sensitivity and the power to discover a greater number of proteins and scarce proteins in the samples. For the last few years, most of the researchers trusted the throughput shotgun approach, using LC-MS/MS technique for comparative proteomics and identifying proteomic biomarkers of fertility in the spermatozoa and seminal plasma of animals (Somashekar et al. 2017; Viana et al. 2018).

9.5.2 Semen Proteomics with Special Reference to Buffaloes

The earlier approaches in proteomic studies of buffalo seminal plasma were mostly concentrated on heparin-binding proteins, such as BSP A1/A2, A3, etc. (Arangasamy et al. 2005; Harshan et al. 2006, 2009; Kumar et al. 2008; Singh et al. 2007, 2013, 2014). Although these proteins are the major seminal plasma proteins in cattle, its presence and role are not well elucidated in buffaloes, making further applications limited. Codognoto et al. (2020) also reported that although BSPs are the main proteins of the seminal plasma in cattle, there seems to be no similar relevance in buffaloes. They also observed that several inhibitors of protein activity were overexpressed in buffaloes compared to cattle, which might contribute to reduced fertility in this species. Using a bottom-up LC-MS/MS approach, Fu et al. (2019) performed the global proteomic analysis of buffalo semen, and a total of 2147 and 864 proteins were identified from mature spermatozoa and seminal plasma, respectively. They reported that the most abundant proteins in the buffalo spermatozoa are ODF2, AKAP4, and TUBB, and those in the seminal plasma are ALB, CLU, and AZGP1. In another study, Brito et al. (2018) identified a total of 859 non-redundant proteins in the seminal plasma of buffaloes, among which the most abundant were WAP four-disulphide core domain protein 2, secretoglobin family 1D member, serum albumin, ribonuclease pancreatic-like, seminal plasma protein 30 kDa (BSP5), melanotransferrin, leucine-rich alpha-2-glycoprotein, epididymal-specific lipocalin-5, peptide YY, and osteopontin.

9.5.3 Proteins Associated with Sperm Functions in Buffaloes

In the recent past, few scientists identified some proteins associated with different sperm functions that could be considered as biomarkers of fertility in buffaloes. Using two-dimensional gel electrophoresis (2DE) coupled to matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOFMS), Huang et al. (2015) compared the proteomic profile of high- and low-motile spermatozoa. They reported that ODF2, ATP5A1 were upregulated in high motile spermatozoa, whereas SUCLG2 was upregulated in low-motile spermatozoa. Similarly, Divyashree (2019) reported that the proteins BSP-5,

AKAP4, and GRP-78 were associated with sperm motility and can be considered potential markers of buffalo sperm motility. In another study using the high throughput proteomics approach, it was observed that spermadhesin protein, ribonuclease, 14-3-3 protein zeta/delta, and acrosin inhibitor were in greater amounts in seminal plasma from the group with greater sperm motility, whereas prosaposin and peptide YY were significant in the group with reduced sperm motility (Codognoto et al. 2018).

For the first time, we compared the proteomic profile of spermatozoa from high-fertile bulls against low-fertile bulls and observed that ten proteins were overexpressed, and 15 proteins were under-expressed in the spermatozoa of high-fertile bulls at the level of two-fold or more ($P \leq 0.05$) compared to low-fertile bulls (Aslam et al. 2019). The proteins overexpressed in high-fertile spermatozoa were PDZD8, GTF2F2, ZNF397, KIZ, LOH12CR1, ACRBP, PRSS37, CYP11B2, F13A1, and SPO11, whereas those overexpressed in low-fertile spermatozoa were MT1A, ATP5F1, CS, TCRB, PRODH2, HARS, IDH3A, SRPK3, Uncharacterized protein C9orf9 homolog isoform X4, TUBB2B, GPR4, PMP2, CTSL1, TPPP2, and EGFL6. The differential expression ranged from 2.0 to 6.1-fold between the two groups, where CYP11B2 was high abundant in high-fertile spermatozoa, and MT1A was significant in low-fertile spermatozoa. Most of the proteins overexpressed in low-fertile spermatozoa were related to energy metabolism and capacitation factors, pointing out the possible role of premature capacitation and cryo-damages in reducing the fertility of cryopreserved buffalo spermatozoa.

Recently, Binsila et al. (2020)reported that serine protease inhibitor Kazal-type 2-like (SPINK2; 2.17-fold) and neddylin (NEDD8; 1.13-fold) were upregulated, and YBX2 was downregulated (0.41-fold) in good quality semen as compared with poor quality semen (one-fold). They reported that the identified proteins play a crucial role in oocyte maturation, fertilization process, and early embryonic development. The variations in the proteomic composition can be used as potential markers for the selection of breeding bulls.

9.6 Epilogue

Considering the vast loss incurred for rearing unproductive bulls, directly and indirectly, it is high time to evaluate the expression of identified biomarker candidates along with routine semen evaluation techniques. Despite increasing research on sperm biomolecules, the identification of fertility biomarkers in semen remains elusive in buffalo bulls. Though there are few reports of fertility related proteins in sperm and seminal plasma of buffalo bulls, there were no further attempts to validate the results in a large set of bulls. Moreover, there is lack of consistency among the identified biomarkers between various studies. Since the fertilizing ability of spermatozoa depends upon several factors and molecules, rather than going for a single molecule, a panel of biomarkers may be identified and used for the screening or evaluation. Besides, through innovative approaches, if the fertility biomarkers could be identified at an early stage, especially in testicular cells, it will aid to

identify and remove the inferior quality bulls at an early stage, thereby saving valuable resources.

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Being Sweet Is Being Smart: Lessons Learnt from Buffalo Spermatozoa

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Abstract

Artificial insemination (AI) with incorrectly selected spermatozoa accounts for a significant loss to the farmers and the dairy industry. Therefore, accurate prediction of male fertility is of major economic value, especially in a farm set-up. To maximize the efficiency of modern livestock systems, novel ways to ascertain the fertilizing ability of the spermatozoa are required. Numerous factors are known to govern the male fertility; however, the sugars (glycans) on the sperm surface are emerging as a novel and key factor regulating various aspects of the sperm fertilizing ability. A highly specialized epithelial cell, the mammalian spermatozoon is embellished with a rich array of functionally diverse and structurally complex glycans that manifest as a thick glycocalyx. These glycans which are often conjugated either to lipids or proteins exist as glycoconjugates (GCs) apart from their independent existence on the sperm surface. The glycans in the glycocalyx of mammalian spermatozoa are multifunctional molecules which perform reproduction-specific tasks such as cervical mucus penetration, communication with various cells in the female reproductive tract (FRT), and recognition of the oocyte, etc. The testicular spermatozoa only have a rudimentary glycocalyx which, however, is substantially transformed in thickness, composition, and structure during the epididymal sperm-surface remodeling (SSR) events. The sugars in the sperm glycocalyx have been proposed to exist in a specific three-dimensional conformation termed as Sperm Associated Glyco-Topography (SpAGT). This topography of glycans is recognized and presumably interpreted by the patterns recognition receptors (PRRs) on the female immune cells, e.g. neutrophils. The glycans in the sperm glycocalyx are known to interact

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with various inhibitory receptors on these cells which facilitate sperm survival in the FRT. Nevertheless, to decode the information about survival in the sperm glycome, the identity, valence, linkage, attachment sites, and structures of the individual sugar units in the sperm glycocalyx need to be determined.

Keywords

Buffalo · Artificial insemination · Spermatozoa · Glycocalyx · Fertility

10.1 The Epididymal Sperm-Surface Remodeling (SSR) Events and Acquisition of a Substantial Glycocalyx

The glycans on the sperm surface play a key role in their production in the testis, acquisition of motility, and fertilizing ability in the epididymis, their protection, selection, and secondary maturation (capacitation) in the FRT (Pang et al. 2011; Tollner et al. 2011, 2012). Many of these glycans are present on the testis-originated proteins, most of which are transmembrane and possess glycosylated extracellular domains. On the other hand, many proteins, e.g. the epididymal secreted glycoproteins and the glycosylphosphatidylinositol or GPI-anchored proteins are added post-spermatogenesis either in the epididymal lumen or in the ejaculatory or pre-ejaculatory ducts (Schröter et al. 1999).

The process of establishment of a spatiotemporally regulated glycocalyx starts with the de novo synthesis of the glycoconjugates (GCs) in the ER/Golgi of the nascent spermatozoa in the testis (Tecle and Gagneux 2015; Ma et al. 2016a). The testicular spermatozoa are incompetent to traverse the FRT and fertilize the egg. Therefore, to gain the functional competence for finishing the arduous journey in the FRT and to attain the maturity to eventually fertilize the oocyte, the spermatozoa undergo two maturation events: (a) Epididymal sperm maturation in the male reproductive tract MRT and (b) Capacitation, in the FRT (Gervasi and Visconti 2017). By the time a spermatozoon enters the epididymis, it loses its ability to synthesize glycans as it now lacks the endoplasmic reticulum and the Golgi apparatus. Hence, it requires a "passive maturation" in the epididymis (Caballero et al. 2010; Belleannée et al. 2012; De-Souza et al. 2017). Furthermore, the testicular spermatozoa are also quiescent in information pathways, nevertheless, the epiphenomena, like modifications or redistribution of existing glycans and changes in the lipid profile occur continuously during their epididymal transit. The synthetic activity of the epididymal epithelium and the type of secreted biomolecules vary along the various epididymal regions. This leads to changes in the composition of the luminal fluid in these regions creating distinct microenvironments for sperm maturation (Domeniconi et al. 2016; Robaire and Hinton 2015). The accompanying changes in the sperm glycocalyx occur majorly due to addition, removal or post-translational processing of glycoproteins, and the activity of glycan modifying proteins in the epididymal lumen (Tulsiani 2006). Besides, the microvesicles called epididymosomes carrying GPI-linked and other glycoproteins embed in sperm

surface which further diversify the layers of GCs in the sperm glycocalyx (Pini et al. 2017; Tecle and Gagneux 2015; Sullivan et al. 2007; Caballero et al. 2010). These biochemical modifications in the sperm surface occur in a progressive but controlled manner yielding functional changes in the spermatozoa. Consequently, the composition, arrangement of glycans, and structure of the sperm glycocalyx are extensively transformed during the epididymal transit (Tobón et al. 2020). These changes in the sperm surface not only assist the spermatozoa to overcome the various physio-chemical and immunological barriers in the FRT but also are required for a successful fertilization event (Fernandez-Fuertes et al. 2016; Tollner et al. 2012). The post-testicular sperm-surface remodeling events continue till ejaculation as a result of which the spermatozoa acquire a substantial glycocalyx in the MRT. The glycosylated proteins acquired by the spermatozoa during their transit in the epididymis and pre-ejaculatory ducts include the maturation antigens of the peripheral coats, e.g. Glycodelin S and CD52, the complement blocking proteins, the immunomodulatory molecules, and loosely bound anti-microbial peptides (e.g. beta-defensins, DEFB- 126 and 129) which are known to play crucial roles in successful fertilization. (Flickinger et al. 1990; Rooney et al. 1993; Yeung et al. 2006; Tollner et al. 2012; Batra et al. 2019). The majority of the sperm-surface molecules appear to follow the "last-in-first-out" (LIFO) or "first-in last-out" (FILO) functional and adherence strategy in the FRT. For example, the proteins of the testicular origin are retained on the sperm surface till fertilization whereas the proteins acquired post-spermatogenesis are shed before fertilization (Tecle and Gagneux 2015; Fernandez-Fuertes et al. 2018). The epididymis, therefore, acts as a site of high extracellular glycosyl metabolism involving somatic modifications of the glycocalyx which prepare the sperm for its ultimate goal, fertilization. Overall, the result of the epididymal SSR events is a 20–60 nm thick glycocalyx which is much diverse and structurally complex vis-à-vis the glycocalyx of typical somatic cells.

10.2 Diversity and Complexity of Sperm Glycans

The dynamicity of the sperm glycocalyx indicates the characteristics of both the sperm as well as its immediate surroundings (Diekman 2003; Gómez-Torres et al. 2012). Since the carbohydrates are the most dominant biomolecules on the extracellular surface, the sperm-surface glycocalyx is considered as the molecular frontier which acts as an interface between a cell and its surroundings. The huge variety of monosaccharide units, different types of linkages and branching add to the complexity of the glycocalyx. Moreover, evolutionary forces and sexual selection are also implicated in shaping the species-specific cellular glycome (Gagneux and Varki 1999; Ghaderi et al. 2011). For example, the self-associated molecular patterns (SAMPs) and their cognate receptors are under strong positive selection to hide and thus avoid being mimicked by pathogenic microbes (Varki and Gagneux 2009; Carlin et al. 2009; Khatua et al. 2010).

The spatial arrangement of the sperm-associated glycans in a glycocalyx possesses multiple hierarchical levels of complexity in its structure and an analogy

to the forest canopy has been drawn to describe the structural diversity and complexity of its constituent glycans (Cohen and Varki 2010; Tecle and Gagneux 2015). The oligosaccharides of the sperm membrane glycolipids have been compared to the "floor" of the glycan forest while the membrane-associated glycoproteins have been likened to short "shrubs." The long polysaccharide chains of glycosaminoglycans have been equated to the "vines" whereas the terminal monosaccharide sugars have been described as "leaves" that embellish the polypeptide trunk. The massive diversity in the spatial arrangement due to monomer units (individuality of sugars), their isomers, linkages, branching, and the terminal modifications is the hallmark of sperm glycocalyx (Maverakis et al. 2015; Diekman 2003; Gadella 2008; Transforming Glycoscience A Roadmap for the Future 2012). Each linkage and branching of glycans can result in a unique spatial presentation, referred to as "sperm-associated glycan topography" (SpAGT), which is recognized by the glycan-binding proteins, e.g. lectins and PRRs (Cohen and Varki 2010; Batra et al. 2020). Besides, the transport phenomenon, enzyme kinetics, thermodynamics, and the concentration of sugar-nucleotide donors also contribute to the diversity in SpAGT since these factors are known to affect the diversity and complexity of glycosylation patterns on proteins (Liu et al. 2008; Bousfield et al. 2014; Gil et al. 2009; Stavenhagen et al. 2013).

As mentioned earlier, the process of acquisition of the complex array of glycans begins in the testis and is continued during epididymal transit and in the seminal plasma. During spermatogenesis, many lipid-associated glycans are modified radically with the help of testicular glycan modifying enzymes such as GALGT1 (N-acetylneuraminyl-galactosylglucosylceramide N-acetylgalactosaminyltransferase), Siat9 (lactosylceramide α2, 3-sialyltransferase), and Siat8 (N-acetyl neuraminide α2, 8-sialyltransferase. The modifications by these enzymes are crucial to the completion of spermatogenesis in the testis (Tecle and Gagneux 2015). Many N-linked glycans associated with testicular glycoproteins are required for their proper folding in the ER/Golgi of the spermatozoa (Stanley et al. 2009). Besides, various mucins, O-linked, GPI-linked, and membrane interacting glycoproteins are also synthesized in testis (Hao et al. 2014; Fujihara et al. 2014). However, as the differentiated spermatozoa enter the epididymis, various enzymes such as glycosyltransferases and glycosidases (contributing to >80% of the total enzyme activity in the epididymal lumen) further modify the existing glycans (Ma et al. 2012; Pini et al. 2017; Schröter et al. 1999). Furthermore, the transmembrane proteins which arrive in epididymosomes and the proteins interacting with the plasma membrane via hydrophobic/electrostatic interactions continue to modify the glyco-topography of the sperm glycocalyx (Zhou et al. 2019; Martin-DeLeon 2015). The process of membrane tailoring continues in the seminal plasma where many glycosylated proteins, some of which act as de-capacitation factors, are added on sperm surface (Yeung et al. 2007; Tollner et al. 2012). Interestingly, the glycan moieties of the seminal plasma glycoproteins contain certain atypical glycans which are seldom found under normal, physiological conditions, in the tissues and other body fluids (Kratz et al. 2015). At the time of ejaculation, the secretions from the accessory sex glands add extracellular microvesicles such as prostasomes carrying

GCs and immune components to the spermatozoa membrane (Jodar et al. 2017). Further, post-ejaculatory modifications in the sperm glycocalyx occur in the FRT.

10.3 Localization and Functional Specialization of Sperm-Associated Sugars

A growing body of evidence suggests that the plasma membrane is laterally compartmentalized because of the heterogeneous distribution of (glyco) proteins and (glyco) lipids which leads to the formation of membrane micro-domains with the nanoscale organization (Garcia-Parajo et al. 2014; Sieber et al. 2007; Schröter et al. 1999; Tecle and Gagneux 2015). These functional micro-domains indicate a distinctive and spatially regulated distribution of the GCs associated with proteins and lipids (Schröter et al. 1999; Robles-Gómez et al. 2020). The spatial restriction in localization of various glycans on mammalian spermatozoa indicates that these glycan micro-domains exhibit region-specific physiological or functional specialization (Ma et al. 2016; Umezu et al. 2017; Torreno-Pina et al. 2014).

The sugar moieties of the GCs tend to confine themselves in a particular region on the mammalian spermatozoa. For example, the asialylated galactosyl (β-1,3) N-acetylgalactosamine is known to be restricted to the head region in human and bovine spermatozoa (Ravid et al. 1990; Medeiros and Parrish 1996; Umezu et al. 2017). The mannose residues are found to be clustered in the acrosomal region in feline spermatozoa (Toyonaga et al. 2011). It is now well accepted that the various glycans moieties on the plasma membranes are capable of forming clustered saccharide patches such as the sialylated clusters and glycosynapses (containing O-linked glycans) on the membrane surface (Hakomori 2004; Cohen et al. 2009). It has recently been established by single-molecule-localization-based super-resolution imaging that the carbohydrate clusters exist as functional domains where various interacting glycoproteins are localized (Chen et al. 2016). It was reported that in the Vero apical membranes, the sialic acids and the N-acetylglucosamine (GlcNAc) moieties aggregate into large and dense clusters, whereas the fucose was found to exist in relatively small, sparse clusters while the mannose, galactose and N-acetylgalactosamine (GalNAc) moieties organized into clusters without clear boundaries (Chen et al. 2016). Lectins are highly specific sugar-binding proteins which are often used to determine the localization patterns of various glycans. The lectins ConA, PNA SBA, and PSA have been observed to bind to the acrosomal region of the bovine spermatozoa indicating the abundance of their cognate glycans in this region (Kuroda et al. 2007; Yeung et al. 2006; Batra et al. 2020). Interestingly, the distribution pattern of various glycans is dynamic and it changes with either milieu, time or in response to intra and intercellular signals. Therefore, the glycocalyx has been described as a four-dimensional entity that changes its composition and structure with time (Schröter et al. 1999). Earlier work from our lab demonstrated a region-specific heterogeneity in the abundance of N-acetylglucosamine$_{2-4}$, galactosyl (β-1,3) N-acetylgalactosamine, mono or di-sialylated T-antigen, α-2,3 linked sialic acid, asialylated galactosyl (β-1,3)

N-acetylgalactosamine, and mannose/glucose moieties on the surface of spermatozoa obtained from high fertile (HF) and low fertile (LF) buffalo bulls. The spermatozoa from the HF and LF bulls appeared to possess unique glycan signatures specified by the region-specific abundance of glycan moieties which changed drastically on the induction of in vitro capacitation (Batra et al. 2020). Besides, the abundance and localization patterns of various glycans on the sperm surface are known to be species-specific. For example, the GlcNAc is restricted in localization to the acrosomal region in the caput spermatozoa whereas it is uniformly distributed along the whole sperm surface in the mature spermatozoa of rats, mice, and goats (Kumar et al. 1990). This is in contrast to C. mexicanus where the GlcNAc moieties are restricted to sub-acrosomal regions in caput sperm and after epididymal maturation, these moieties localize near sperm annulus (Tobón et al. 2020). The localization pattern of GlcNAc in mature buffalo spermatozoa is altogether different where it is uniformly distributed over the complete head region (Batra et al. 2020). Moreover, it has also been demonstrated that the different sugars such as fucose and GlcNAc tend to co-localize with each other and with other proteins such as Epidermal Growth Factor Receptor (EGFR) and band 3 proteins. This indicates that the glycan clusters are not independent, rather they can interact with each other forming mesoscale functional platforms (Chen et al. 2016).

The preferential localization of glycans in a particular region can narrate the history of testicular expressed genes, milieus encountered by the spermatozoa and apparently their quality (Maverakis et al. 2015; Tecle and Gagneux 2015). Moreover, the ensemble of sperm-surface glycans can mediate specific spatial interactions occurring during recognition, cell-communication, and fertilization. For example, the GCs associated with epididymosomes, prostasomes, glycolipids, and glycoproteins are capable of modulating local membrane properties and thus can affect the motility parameters of the spermatozoa (Purohit et al. 2008; Fernandez-Fuertes et al. 2018). In humans, a reduction in sperm sialic acid content has been found to affect their ability to penetrate the negatively charged cervical mucus enriched in anionic glycosaminoglycans (Tollner et al. 2011). Alterations in the O-glycan core structure of various glycoproteins are known to affect the structural integrity of the glycocalyx thereby disrupting biological functions (Gupta et al. 2010). The micro-patterning of sperm-surface glycans is therefore implicated in multiple biological functions on the plasma membrane, e.g. directing the glycoconjugate transport, regulation of cellular adhesion, protein–protein interactions, and signal transduction (Tecle et al. 2019; Froman and Engel 1989; Hakomori and Todeschini 2008; Tecle and Gagneux 2015; Dalziel et al. 2014).

Most of the studies that intended to define and characterize the GCs associated with the sperm glycocalyx have used lectins because of their complex specificities, ability to differentiate between glycoforms, branching, linkage, spacing, and multivalency, etc. (Accogli et al. 2016; Sharon and Lis 2004; Smith and Cummings 2013; Xin et al. 2014, 2016). The lectins are ubiquitous recognition molecules involved in a myriad of cellular and molecular interactions in many biological systems. They have been used to (a) visualize cell surface structures, (b) detect and analyze the expression and spatio-temporal distribution patterns of glycans, and

(c) monitor the perturbations in the sperm glycocalyx associated with the capacitation and acrosome reaction (Koehler 1981; Jiménez et al. 2002; Umezu et al. 2017; Gadella 2008; Robles-Gómez et al. 2020). Lectins facilitate understanding the organization and arrangement of glycans in SpAGT, which is crucial because the integrity of the glycocalyx can be used to evaluate sperm quality. Delineating the exact glycan distribution patterns involved in particular processes remains a challenge. Nevertheless, understanding the regional localization of glycans on the sperm cell surface would lead to the elucidation of the structure-function relationship between glycan distribution and their role in reproduction-specific physiology.

10.4 Changes in Glycan Localization after the Introduction of Perturbations in Sperm Glycocalyx

The changes in the molecular arrangement of the glycocalyx (SpAGT) after introducing perturbations such as capacitation (Tecle et al. 2019; Umezu et al. 2017), cryopreservation (Wu et al. 2017), flow cytometry (Bucci et al. 2012; Balao da Silva et al. 2013), or enzymatic deglycosylation (Fernandez-Fuertes et al. 2018; Tollner et al. 2011) have successfully been monitored using lectins as reporters. Following the LIFO/FILO functional and adherence strategy, the sperm proteins acquired in the testis which are required for successful fertilization become exposed in the FRT redistributing the sperm-surface GCs. Numerous reports are available on the changes in lectin binding after in vitro capacitation spermatozoa in various species like cow (Umezu et al. 2017; Medeiros and Parrish 1996), boar (Jiménez et al. 2003), and mice (Baker et al. 2004). Many glycoproteins like the sperm hyaluronidase PH-20 and AEG have been demonstrated to redistribute after capacitation and acrosome reaction (Myles and Primakoff 1997; Rochwerger et al. 1992). Likewise, the highly O-glycosylated anti-microbial proteins, β-defensins, and many GPI-anchored proteins effuse from the sperm surface during capacitation (Yudin et al. 2003; Tollner et al. 2004). These changes in the spatial distribution of proteins contribute to the redistribution of glycans occurring during capacitation (Robles-Gómez et al. 2020). It has been observed, in vivo that the sialidases act on the terminal sialic acid moieties of sperm-surface glycans cleaving them during capacitation thereby decreasing their abundance (Ma et al. 2012). An overall decrease in sialic acid content has been observed in human spermatozoa after in vitro capacitation (Ma et al. 2012). Previous work in our lab has also demonstrated that the abundance of galactosyl (β-1,3) N-acetylgalactosamine, galactose of mono or di-sialylated T-antigen [GlcNAc]1-3, N-acetylglucosamine, and α-2,3 linked sialic acid declines on the sperm surface after induction of in vitro capacitation in buffalo (Batra et al. 2020). This decrease indicated a major loss of the O-linked glycans from the buffalo sperm surface, after in vitro capacitation. This might imply a decline in the number of sites available for lectin/s-binding on the buffalo sperm surface after capacitation. It is now well established that during sperm capacitation most O-linked and a few N-linked glycans are removed from the sperm surface (Tollner et al. 2008; Tecle and Gagneux 2015). The loss of O-linked

oligosaccharides from the sperm surface of buffalo bulls in the capacitated spermatozoa could also indicate loss of non-covalent interactions between the sperm and the molecules acquired during the epididymal transit (Batra et al. 2020; Purohit et al. 2008). It, however, remains undetermined whether the observed decrease in abundance after in vitro capacitation was the result of either the re-arrangement or displacement or loss or a conformational change in O-linked glycans associated with the GCs on the sperm surface. The changes in the sperm-surface membrane during capacitation thus alter the SpAGT as a result of which the changes in lectin-binding pattern are observed. These changes associated with lectin binding are considered to be one of the earliest events of sperm capacitation. Not only the glycans but also the distribution pattern of cognate glycan receptors (e.g. mannose), on the sperm surface, has been found to alter during sperm capacitation (Tesarik et al. 1991). The appearance of affinity for mannose residues on the acrosomal region, for example, was interpreted to be the consequence of rearrangements in the GCs of sperm glycocalyx. These rearrangements presumably depend on membrane cholesterol efflux and are correlated with the acrosomal status of spermatozoa (Youssef et al. 1996).

Apart from capacitation, flow cytometry is also known to destabilize the sperm plasma membrane similar to capacitation (Bucci et al. 2012; Balao da Silva et al. 2013). For example, the pattern of PHA-E lectin binding has been reported to vary in sex-sorted and normal, mixed spermatozoa (Umezu et al. 2017). Moreover, the flow cytometry has been reported to induce precocious capacitation in bovine spermatozoa (Umezu et al. 2017). Besides, cryopreservation is also known to alter the SpAGT of the spermatozoa thereby changing its lectin-binding properties. Accordingly, the binding sites for 24 lectins reportedly increased while for 9 lectins the binding sites decreased significantly after cryopreservation of human spermatozoa (Wu et al. 2017). Deglycosylation is another perturbation that can affect the SpAGT structure and thus disrupt the associated molecular functions. The desialylation of spermatozoa has been reported to affect the sperm motility and its ability to penetrate cervical mucus and the oocyte eventually affecting the fertilization capability of the sperm (Fernandez-Fuertes et al. 2016, 2018). The removal of O-linked glycans from HF bull spermatozoa leads to enhanced spermiophagy vis-à-vis glycosylated HF control samples (Batra et al. 2020). It is now established that perturbations irreversibly damage the sperm glycocalyx and these might be one of the reasons associated with low CR observed with LF (Batra et al. 2020) or cryopreserved (Wu et al. 2017) or sex-sorted spermatozoa (Umezu et al. 2017).

10.5 Glycans Affect Sperm Survival: Determination of Self Vs. Non-self

Considering the massive diversity in the identity of individual glycan moieties, attachment, and their valence, the glycocalyx appears to be able to store a vast amount of information. For example, the identity of a cell to be distinguished as

"self" is known to be displayed by the complex glycocalyx of major cell types. It is now accepted that the spermatozoa also carry glycan specific signatures indicating their identity, quality, and origin. The components of the glycocalyx can function as self-associated molecular patterns (SAMPs) by interacting with glycan-pattern recognizing receptors on immune and epithelial cells in the FRT inhibiting elicitation of the immune response (Varki 2011; Springer and Gagneux 2013; Chen et al. 2009). It is well-known that both the innate and the adaptive arms of the female immune system respond to insemination by bringing a drastic reduction in sperm number in the FRT (Katilla 2012; Tecle and Gagneux 2015; Yudin et al. 2005a). The adaptive immune system responds to sperm glycocalyx since a majority of the antibodies raised against sperm-surface proteins tend to be directed preferentially against their carbohydrate moieties (Yudin et al. 2005b; Archana et al. 2019). Alternatively, the sperm glycocalyx interacts with the inhibitory receptors on the innate immune cells facilitating sperm survival in the FRT. It has recently been demonstrated that the spermatozoa engage the sialic acid binding Ig-like lectins, e.g. Siglec-9 on neutrophils and Siglec-10 on endometrial cells to reduce the post-insemination inflammatory responses (Tecle et al. 2019). The terminal sialic acid moieties of glycocalyx are considered as the most common determinant of SAMPs. These moieties and their cognate receptors, the female-expressed Siglecs on various cells in the FRT regulate the "leukocyte reaction" thereby inducing a state of immune-tolerance, at least for a fraction of spermatozoa (Ma et al., 2016a,b; Ali et al. 2014; Fernandez-Fuertes et al. 2018). Sialylation on sperm glycans appears to assist in the induction of a tolerant state by interacting with secreted as well as cell-bound PRRs of female immune cells (Varki and Gagneux 2012; Ram et al. 1998; Ma et al. 2016a). The Siglecs on the leukocytes reportedly act as SAMP receptors which can inhibit immune responses (Pillai et al. 2012; Geijtenbeek et al. 2004; Steinman 2003). Other carbohydrate-binding molecules in the FRT, e.g. Toll-like receptors (TLRs), α-defensins, and lectins also function as PRRs. They identify the arrangement pattern of the GCs such as pathogen-associated molecular patterns, PAMPs, danger-associated molecular patterns, DAMPs, and probably the SAMPs too (Zandieh et al. 2015; Medzhitov et al. 1997; Medzhitov and Janeway Jr 1997; Banda et al. 2011; Lehrer et al. 2009). Work in our lab suggests that a low abundance of O-linked glycans on the sperm surface of low fertile (LF) bulls renders them prone to recognition, phagocytosis, and NETosis by the female neutrophil cells. The non-covalent interactions between the buffalo sperm glycocalyx and its cognate receptors appear to be decisively influenced by the abundance and arrangement of the glycan moieties in the glycocalyx. This is because the lower abundance of glycans in LF-SpAGT appears to transduce a distinct recognition signal to the bound PRRs of the interacting female neutrophils which led to increased recognition and spermiophagy (Batra et al. 2020). The glycans are thus well capable of dictating the behavior of various immune cells during intercellular interactions (Maverakis et al. 2015). The arrangement and composition of glycans in the SpAGT appears to carry potential information about its identity and quality (Transforming Glycoscience A Roadmap for the Future 2012; Batra et al. 2020; Maverakis et al. 2015). Accordingly, the HF-SpAGT could effectively be perceived as SAMP that

helps to evade the recognition, adhesion, and spermiophagy by the female neutrophils. A deficiency in SAMP-mediated signaling might predispose the spermatozoa from LF bulls to be recognized as "non-self," followed by their phagocytosis. Moreover, other flexible glycans, e.g. the bisecting type fucosylated N-glycans have also been implicated in immune modulation in the FRT by the spermatozoa (Pang et al. 2007). The mobility and flexibility of individual glycan moieties are expected to increase the probability of glycocalyx entering a spatial conformation that is recognized by its cognate receptors like PRRs (Schröter et al. 1999).

It is now recognized that the spermatozoa interact with many female immune receptors in the FRT, nevertheless, many spermatozoa can evade the immune responses, partly because of the integrity of their SpAGT. The effect of alterations in the glycomic endowment of spermatozoa on the signaling cascades of various female immune and epithelial cells is not yet clear.

10.6 Role of Glycans in Inter-and Intra-Cellular Communication

The role of cooperation is imperative to the survival of a cell especially if it has to continually interact with other cells and respond to the adverse extracellular milieu. The glycans on the cell surface hold massive information capable of directing numerous intercellular interactions, as mentioned earlier (Dube and Bertozzi 2005; Schnaar 2015; Tra and Dube 2014; Rudd et al. 2001). The sperm glycocalyx plays crucial roles in many cooperative biological processes in vivo such as cell movement, immune response elicitation, immune-evasion, recognition, and formation of oviductal sperm reservoir (Gupta et al. 2010; Maverakis et al. 2015; Tollner et al. 2012). The insemination can be considered as the initiation of communication by the spermatozoa with the female which eventually allows successful pregnancy establishment. The various cells in the FRT are known to interact with the spermatozoa through surface glycans to facilitate their migration, storage, selection, and preservation of fertilizing ability (Suarez 2016). Carbohydrate–protein interactions are often used as a direct way to communicate with the extracellular milieu by PMN as well as spermatozoa (Töpfer-petersen et al. 2002; Wagner et al. 2002). For example, the glycan moieties on the oviductal epithelial cells (OECs) play a crucial role in interacting with the spermatozoa (Machado et al. 2014). Contrarily, the O-linked glycans associated with the epididymal secreted protein, DEFB-126 have been implicated in the binding of macaque spermatozoa to the OECs and zona pellucida (Tollner et al. 2004, 2008).

The sialylated clusters on the sperm surface can mediate intercellular signaling and are also known to be involved in cell-adhesion (Hakomori and Todeschini 2008). Interestingly, not only the identity, linkage, and branching of sugars but also the individuality of the underlying sugar in a linkage affects the specificity of glycan-binding proteins such as PRRs thereby mediating intercellular communication (Cohen and Varki 2010). Therefore, any alterations in the topography of cell surface glycans could potentially lead to defects in cell–cell recognition and

downstream information flow (Dalziel et al. 2014; Glavey et al. 2015). We have previously reported that the perturbations in the SpAGT occurring after the in vitro capacitation or in situ deglycosylation (O-linked glycans) lead to increased spermiophagy/NETosis by female PMN cells (Batra et al. 2020). This indicated either an increased availability of the PMN binding sites or a decrease in the anti-recognition molecules involved in the opsonin-independent phagocytic pathway after in vitro capacitation/deglycosylation of bull sperm. Interestingly, the terminally located of sialic acid moieties have also been demonstrated to interact with the neighboring cells and bring forth varied intercellular responses. As mentioned earlier, the sialic acids are known to facilitate immune-tolerance and survival of the sperm by interacting with the secreted or cell-bound innate female PRRs (Ma et al. 2012; Tecle et al. 2019; Varki and Gagneux 2012; Simon et al. 2013). They have also been implicated in cell–cell recognition and are regarded as anti-recognition molecules (Fernandez-Fuertes et al. 2018; Varki and Schauer 2009). Expectedly, a rise in recognition and phagocytosis has been reported for de-sialylated mice spermatozoa (Toshimori et al. 1991) and a decrease in phagocytosis was observed when the immature epididymal sperm were coated with sialic acid (Ma et al. 2016). The oligo-mannosidic chains have also been proposed to act as a recognition signal for eliminating the incompetent sperm during their transit in the FRT (Nardone et al. 1985; Accogli et al. 2017). Besides immune and epithelial cells, the spermatozoa interact with the oocyte in the oviduct, which involves the interaction of complementary molecules on sperm and the zona pellucida. The glycosylation associated with sperm maturation antigens, e.g. CD52 has been demonstrated to minimize the non-specific pre-emptive interactions before fertilization in the FRT (Schröter et al. 1999). The GCs on capacitated spermatozoa appear to be involved in the recognition and binding of the homologous zona pellucida during fertilization (Töpfer-petersen et al. 2002; Pang et al. 2011; Clark 2013). For example, the β-galactosyl residues at the non-reducing ends of the N- or O-linked oligosaccharides are known to mediate sperm–zona binding during porcine gamete interactions (Yonezawa et al. 2005). The O-linked carbohydrates are also known to be important in the sperm–zona binding in humans (Kratz et al. 2015). The dynamics of sperm glycocalyx structure and its composition is thus crucial not only to traverse the immunologic milieu in the FRT but also for gamete recognition and fertilization (Fernandez-Fuertes et al. 2018; Tecle et al. 2019).

Either any inherent incongruity in the SpAGT or introduction of perturbations in glycocalyx render the spermatozoa susceptible to recognition by innate immune cells in the female genital tract (Holt 1980; Batra et al. 2020; Kirchhoff and Schröter 2001; Xin et al. 2014, 2016). It is not yet clear if a reduction in sperm-surface glycans leads to defective intercellular communication of spermatozoa with female PMN cells.

10.7 Implications in Immune Recognition and Evasion with Special Reference to AI

The male gamete must be resilient enough to counter the immune responses mounted against it and concomitantly offer chances of selecting the competent sperm as the spermatozoa advance through the FRT. Since the spermatozoa are allogenic to females, it seems logical that many of the immune responses aim to eliminate them. These responses which include immune cells, complement proteins, and antibodies not only influence sperm cell selection but also induce immunological tolerance towards paternally derived antigens. The spermatozoa are potent enough to induce an influx of PMN cells in the FRT that can be as strong as a bacteria-induced influx (Miró et al. 2020; Matthijs et al. 2003; Gorgens et al. 2005). The insemination, rather the intromission of any liquid in the uterus has been found to trigger multiple immune mechanisms (Schuberth et al. 2008; Fichtner et al. 2020). Majorly, it initiates en-masse recruitment of the leukocytes, mainly PMNs in the uterine lumen through the sub-epithelial stroma, known as the leukocyte reaction. This recruitment is regarded as a normal physiological response to prepare the FRT for conception. It is worth mentioning that the neutrophils are known to recognize the allogenic spermatozoa and hinder their transport by rapidly ingesting/entrapping them (Eisenbach 2003; Matthijs et al. 2000; Alghamdi et al. 2015). This recruitment may be beneficial for the removal of microbial contaminants and unfit (e.g. post-capacitated) spermatozoa, nevertheless, may be counter-productive if seminal plasma components are diluted (Eisenbach 2003; Alghamdi and Foster 2005; Katilla 2012). This is especially relevant in the case of artificial insemination (AI) because much of the seminal plasma components (SPCs) that modulate the PMN activity in FRT are lost during semen processing (Pini et al. 2017; Marey et al. 2014). Nevertheless, AI with frozen semen has become a necessity for optimum utilization of semen, especially in farm animals (Srivastava and Kumaresan 2014; Singh et al. 2016). One such SPC is a seminal plasma DNase-like protein that is known to increase the fertility rate by digesting the DNA extruded by neutrophil cells (NETs).

The seminal plasma DNase frees the entrapped spermatozoa in these NETs thereby allowing more spermatozoa to reach the oviduct (Alghamdi and Foster 2005). The neutrophils are also known to interact with spermatozoa by direct attachment indicating the involvement of adhesion molecules. The possibility of molecules such as selectins, however, has already been ruled out because anti-P-selectin antibodies were not able to abrogate the sperm-PMN binding (Alghamdi et al. 2009, 2015; Marey et al. 2014). Based on our observation in buffalo, we proposed that the lectin-glycan interactions are implicated in the opsonin-independent phagocytosis of bull spermatozoa by the female PMN cells. The GCs of the sperm surface appeared to be the potential candidates because perturbations in the SpAGT elevated the rate of recognition, spermiophagy and NETosis by the female neutrophil cells (Batra et al. 2020).

The spermiophagy by neutrophils has been suggested to be a selective rather than a random process (Eisenbach 2003; Taylor et al. 2008). Data in our lab corroborated the suggestion that the neutrophils actively take part in sperm selection removing

non-competent, post-capacitated spermatozoa. This is because, despite the absence of any opsonins such as the serum, the spermatozoa were being recognized and actively phagocytized by the PMN cells, the incidences of which increased after capacitation (Batra et al. 2020). There may be other factors at play, in vivo, like antibodies and complement proteins which contribute to the reduction in sperm number along the FRT. Interestingly, lowering the inseminate volume has been found to reduce the interactions between boar spermatozoa and neutrophil cells (Matthijs et al. 2003). The exact nature of the molecules and interactions involved in sperm-neutrophil binding, however, remains to be fully elucidated.

Interestingly, the epididymal acquired GCs and the biomolecules in the semen endow the spermatozoa with immune-tolerance while traversing the immunologic milieu of FRT. The sperm glycocalyx is well-known to protect the spermatozoa from cellular and humoral immunity, and it masks the testicular sperm proteins involved in fertilization (Fernandez-Fuertes et al. 2018; Steinman 2003; Yudin et al. 2005a, b). An adequate level of temporally regulated sialylation, for example, is required for protection, survival, and function of the murine spermatozoa in the FRT (Ma et al. 2016). The N-acetyllactosamine repeats of the epididymis secreted, heavily glycosylated, GPI-anchored protein CD-52 have also been implicated in the modulation of the classical complement pathway thus evading recognition (Hardiyanto et al. 2012). The terminal sialic acid moieties of DEFB-126 have been compared to a Klingon cloak which purportedly shields the male-specific proteins from anti-sperm antibodies (Tollner et al. 2012; Archana et al. 2019). This pleiotropic molecule is required for the penetration of cervical mucus, the formation of oviductal sperm reservoir, capacitation, and zona recognition. Interestingly, most of the reproduction-specific functions of DEFB-126 have been ascribed to the abundant sialylation associated with O-linked glycosylation of this epididymal protein (Tollner et al. 2011, 2012; Yudin et al. 2008). The recently discovered polysialic acids on the sperm surface have been speculated to prevent the formation of neutrophil extracellular traps (NETs) thus regulating sperm survival (Simon et al. 2013). The seminal plasma prostasomes carry the CD59 surface glycoprotein which inhibits the formation of membrane attack complex (MAC) thus evading the complement attack in the FRT (Dorus et al. 2012).

The integrity of the SpAGT is important for sperm survival and the success of fertilization and therefore any changes on the structure of SpAGT by perturbations such as capacitation, flow cytometry cryopreservation, and deglycosylation may have undesirable or even detrimental effects (Wu et al. 2017; Umezu et al. 2017; Tobón et al. 2020). Based on observations in our lab, we proposed that these perturbations which lead to loss of glycans affect the inherent SpAGT of spermatozoa. This results in the increased proximity of female neutrophil cells to the existing antigenic sites on the buffalo spermatozoa surface (Batra et al. 2020). Conversely, Tecle et al. recently reported that induction of capacitation did not affect the in vitro activation of human PMN cells (Tecle et al. 2019), while Matthijs et al. reported a decrease in spermiophagy after in vitro capacitation of porcine spermatozoa (Matthijs et al. 2000, 2003). These species-specific differences in PMN activation could be ascribed to the high variability in the glycan abundance

and distribution amongst various species (Hahn et al. 2012; Tecle and Gagneux 2015; Ghaderi et al. 2011). Moreover, the post-capacitated and incompetent spermatozoa have been hypothesized to be preferentially phagocytized thus assisting in sperm selection (Eisenbach 2003). The differential SpAGT of the glycosylated, non-capacitated spermatozoa appeared to confer a different response modality to neutrophil activity, thus evading recognition by them (Batra et al. 2020). It has long been suggested that specific GCs, especially those associated with glycoproteins act as immune-suppressant in, the molecular mechanisms of which, however, remain largely unclear.

10.8 Regulation of Male Fertility

The maturity and integrity of the sperm glycocalyx appear to be crucial to male fertility (Tollner et al. 2011, 2012; Xin et al. 2016; Ma et al. 2016a; Gatti et al. 2004; Schröter et al. 1999). The female immune cells appear to filter the spermatozoa based on the abundance and 3-D spatial arrangement of glycans in the sperm glycocalyx because it is the interface directly in contact with the immunologic milieu in the FRT. Therefore, any aberrations in sperm glycocalyx structure are likely to negatively affect male fertility. A well-known example in humans is the mutations in DEFB-126 sperm-surface protein which cause anomalies in the structure of the sperm glycocalyx because of the paucity of O-linked glycans, as determined by lectin cytochemistry (Tollner et al. 2011; Xin et al. 2014, 2016). The lectins ABL and MPL could effectively distinguish the mutant DEFB-126 genotypes and have been proposed as biomarkers for identifying male subfertile genotype (Xin et al. 2016). Likewise, the sub-populations of spermatozoa could reportedly be separated based on their fertility potential using PNA lectin. Incubation of human spermatozoa with PNA yields two sub-populations: the major sub-population is agglutinated by the PNA (PNA^+) while the minor population that did not agglutinate (PNA^-) was found to be enriched in spermatozoa affected by acrosomal pathologies (Ravid et al. 1990). It was speculated that the removal of PNA^- sperm sub-population can improve the fertilizing ability of the spermatozoa from oligozoospermic specimens. This means that the level of asialylated galactosyl (β-1,3) N-acetylgalactosamine could be used to assess the fertility score of the spermatozoa. Similarly, the amount of GlcNAc in the spermatozoa from subfertile boars was found to be lower than the fertile boars and this reduction has also been found to be associated with reproductive problems in men suffering from oligospermia (Jiménez et al. 2002; Purohit et al. 2008). Although the relationship between the lectin binding and the condition of individual sperm is still unclear, it could provide the information regarding the quality of individual sperm mediating sperm selection in the FRT (Eisenbach 2003; Tecle and Gagneux 2015; Taylor et al. 2008; Batra et al. 2020). Interestingly, the removal of individual glycan moieties like the sialic acid by neuraminidase treatment of bovine sperm has been demonstrated to negatively affect the fertilization and embryo development (Fernandez-Fuertes et al. 2018). Although the glycan distribution pattern is species-specific, within a species the glycan abundance may

exhibit seasonal variations thereby varying glycan distribution pattern. For example, the O-linked glycans terminating with GalNAc and asialo N-linked glycans terminating with Gal-β1,4-GlcNAc and GlcNAc were abundant in buffalo spermatozoa during mating period vis-à-vis non-mating period (Accogli et al. 2017).

Work in our lab had revealed a differential abundance of O-and N-linked glycans on buffalo spermatozoa from bulls of contrasting fertility (HF and LF). The lack of O-linked glycans on LF spermatozoa or the enzymatic removal of these glycans from HF bull sperm led to preferential interactions of such spermatozoa with the peripheral PMN cells resulting in motility reduction and death (Batra et al. 2020; Miró et al. 2020). The cervical or vaginal leukocytosis and uterine neutrophilia during insemination is known to reduce fertility rate and is implicated in idiopathic infertility in many species (Alghamdi et al. 2004; Alghamdi and Foster 2005; Rozeboom et al. 2000; Ou and Su 2000). Apart from phagocytosis, the spermatozoa have been demonstrated to induce NET formation in a dose and time-dependent manner in multiple species (Miró et al. 2020; Fichtner et al. 2020; Batra et al. 2020). The neutrophil NETs play a vital role in mammalian reproduction as NETosis has been implicated in male infertility and low conception rates, e.g. because of spontaneous fetal loss (Hahn et al. 2012; Batra et al. 2020). Moreover, there has been observed a decrease in overall progressive motility of spermatozoa upon interacting with the NETs which is known to hamper successful fertilization (Zambrano et al. 2016). Many PTMs including glycosylation have been reported to differentially modulate the NET formation (Neeli et al. 2009; Papayannopoulos et al. 2010; Hahn et al. 2012; Keshari et al. 2012). Our work, however, specifically implicated the O-linked glycans in modulating NETosis and the subsequent spermiophagy by the female neutrophil cells. These glycans can be the regulatory factors of PMN-spermatozoon interaction because a rise in recognition and NETosis was observed after enzymatic deglycosylation of O-linked glycans but not N-linked glycans (Batra et al. 2020). Interestingly the NET formation only affects sperm transport, nevertheless, the bactericidal activities of PMNs remain unaffected (Alghamdi and Foster 2005). Further studies are warranted to better elucidate the relationship between the integrity of sperm glycocalyx and the regulation of male fertility which could be an epiphenomenon affected by molecular alterations in the sperm glycocalyx. Overall, the sweetness of the sperm supports its survival in the FRT which could be one of the factors involved in cryptic female choice.

10.9 Future Perspectives

The sperm-surface glycans could be a key factor regulating male fertility. The in situ profiling of GCs associated with sperm surface appears to be a promising approach for accurate characterization of the glycocalyx in a multiplexed lectin experiment. In a commercial farm set-up or semen production centers, the identification subfertile ejaculate is a necessity. The predictive capacity of "strict morphology" criteria to evaluate the fertilizing ability of the spermatozoa is limited. Therefore, novel means to assess the fertilizing ability such as assessment of glycocalyx integrity should be

incorporated in the existing SOPs at semen stations so that freezing of subfertile spermatozoa can be avoided. A comprehensive assessment of various aspects of sperm-sweetness may help to identify the glyco-biomarkers of male fertility in farm animals too. Further investigations focusing on the sugar-coated cross-talk between the spermatozoa and female epithelial and immune cells are required to provide new insights into the regulation of male fertility by sperm-surface glycans.

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Protein Signatures of Lactation and Early Pregnancy Diagnosis in Buffalo (*Bubalus bubalis*)

11

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Abstract

Proteins play crucial role as regulatory molecules, enzymes, hormones, signaling molecules in both lactation and pregnancy which include many complex physiological processes. Many studies have been performed to unravel the protein signatures of lactation and different pregnancy stages in various mammalian species. Out of all livestock animals buffalo (*Bubalus bubalis*) has been given more attention by the researchers due to its major contribution towards the milk production in India. In-depth understanding of the pregnancy and lactation process of this species in terms of involvement of proteins will help the researchers to promote successful pregnancy and enhancement of milk yield. Both reproductive and productive performances of the buffalo can be improved through various approaches with prior knowledge on proteins playing various roles in pregnancy and lactation. This chapter discusses various proteins and genes that are directly or indirectly connected to the pregnancy and lactation in buffaloes, and execute their function in different forms.

Keywords

Lactation · Pregnancy · Proteins · Buffalo · Mammary gland

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11.1 Introduction

Lactation (the process of secretion of milk by the mammary gland) is a very complex physiological process in mammals with involvement of various proteins as regulatory molecules, signaling molecules, enzymes, hormones, etc. Many aspects of the lactation process are understood in various species, beginning from milk synthesis, storage, and secretion by the mammary gland. Still many things to be explored in this context, to understand the complex process and plethora of components present in milk. The lactation physiology and the anatomical structure of the mammary gland varies across the species (Borghese et al. 2007).

According to the 20th Livestock census (India), 2019, the total buffalo population in India is 109.85 million out of the total livestock population of 535.78 million in our country. As per the data of 2019, buffaloes produced 91.82 million tonnes of milk which is around 50% of the total milk production (187 million tonnes) by various livestock species (Published by Statista Research Department, 2021). The lactation period of buffalo has been divided into three stages such as early lactation (14–100 days), mid-lactation (100–200 days), and late lactation (>200 days). The milk yield and the milk composition largely vary due to the genetic makeup of the animal, nutritional status, breed of the animal, age group, season, and the stage of lactation (Smith et al. 2000; Akingbade et al. 2003; Vijayakumar et al. 2017). Many studies have been performed to understand the involvement of various biomolecules in development of buffalo mammary gland during heifer stage, pregnancy, lactation, and involution, and more importantly the proteins play crucial role in these physiological processes. Proteins also play important role during early pregnancy beginning from embryo formation, implantation in the uterus, and further development to term. Many protein-derived hormones, regulatory molecules, cytokines, and enzymes execute their function and maintain a delicate balance for successful pregnancy and live birth of buffalo calves.

This chapter discusses the involvement of various proteins in these processes, with more focus on identification of the protein signatures in the lactation process. Besides, it also covers the gene expression studies related to lactation process, which will able the readers to plan future research strategies to augment the milk yield in buffaloes and fight against the deadly disease of the mammary gland i.e. mastitis. Moreover, the protein signatures of early pregnancy have also been discussed in the buffalo along with the gene expression studies so that a clear understanding of the pregnancy and lactation in buffaloes can be built in the context of involvement of proteins.

11.2 Protein Signatures Related to Lactation in Buffaloes

Various studies have been performed to identify the protein signatures during lactation process in buffaloes and many genes and proteins (Table 11.1) are found to have significant role during lactation. Study by Jena et al. (2015) on the comparative proteome analysis of mammary gland tissue from heifer and lactating buffaloes,

Table 11.1 Genes and Proteins significantly upregulated during lactation in buffalo

Sl. No.	Name of Protein	Stage of lactation	References
1.	78 kDa glucose-regulated protein (HSPA5)	Peak lactation	Jena et al. (2015)
2.	Serum albumin (ALB)	Peak lactation	Jena et al. (2015)
3.	Elongation factor 1-delta (EEF1D)	Peak lactation	Jena et al. (2015)
4.	Kappa-casein (CSN3)	Peak and late lactation	Jena et al. (2015)
5.	Annexin A4 (ANXA4)	Peak lactation	Jena et al. (2015)
6.	NADH dehydrogenase (NDUFV2)	Peak and late lactation	Jena et al. (2015)
7.	Heat shock protein beta-1 [Bos taurus] (HSPB1)	Peak lactation	Jena et al. (2015)
8.	Casein beta [Bubalus bubalis] (CSN2)	Peak and late lactation	Jena et al. (2015)
9.	AS2-casein (O62825)	Peak and late lactation	Jena et al. (2015)
10.	14–3-3 protein gamma (YWHAG)	Peak and late lactation	Jena et al. (2015)
11.	Rho GDP-dissociation inhibitor 1 (ARHGDIA)	Peak lactation	Jena et al. (2015)
12.	Programmed cell death protein 6 (PDCD6)	Peak and late lactation	Jena et al. (2015)
13.	Beta-lactoglobulin (LGB)	Peak and late lactation	Jena et al. (2015)
14.	Alpha-lactalbumin (LALBA)	Peak and late lactation	Jena et al. (2015)
15.	Fatty acid-binding protein (FABP3)	Peak and late lactation	Jena et al. (2015)
16.	Secreted Phosphoprotein1 (SPP1)	Early and late lactation	Arora et al. (2019)
17.	Translationally-controlled tumor protein (TPT1)	Early and mid-lactation	Arora et al. (2019)
18.	Fatty acid binding protein (FABP4)	Mid-lactation	Arora et al. (2019)
19.	Perilipin 2 (PLIN2)	Early and late lactation	Arora et al. (2019)
20.	Xanthine dehydrogenase (XDH)	Early and late lactation	Arora et al. (2019)
21.	Peroxisome proliferator-activated receptor alpha (PPARα)	Throughout lactation	Yadav et al. (2015)

revealed many proteins to be involved in the lactation process. This study identified 43 differentially expressed proteins (DEPs) between these two stages, through Difference Gel Electrophoresis (DIGE) technique, and found that there is 27-fold higher expression of the HSP27 protein in lactation stage. Moreover, some proteins such as EEF1D, HSPA5, HSPD1, and PRDX6 were identified for the first time in lactation stage of the buffaloes. Anand et al. (2012) have established a buffalo mammary epithelial cell line (BuMEC) from mammary tissue samples isolated from slaughter house. The characterization of BuMEC cells has been done using the epithelial cell marker cytokeratin18. Additionally, the functional differentiation of the cells was confirmed through observation of expression of β-casein, κ-casein, butyrophilin, and lactoferrin genes. This cell line is currently being used as an ideal in vitro model to study the physiological processes in buffalo mammary epithelial cells (Anand et al. 2012).

In an in vitro study (Jena et al. 2021), the proteins involved in lactogenic differentiation of BuMEC cultured cells were identified with controlled cells taken are the proliferative BuMEC cells. In this experiment, the proliferative cells were first differentiated (Anand et al. 2012) using lactogenic hormones such as insulin, prolactin, and cortisol. The cytosolic and mitochondrial fractions were prepared from both the proliferative and differentiated BuMEC cells, and further the proteins were analyzed by Difference Gel Electrophoresis (DIGE) and mass spectrometry technique to find out the differentially expressed proteins. The findings from this study revealed the involvement of annexins such as annexin-I, II, and V and the S100 proteins such as S100A4, S100A2, and S100A11 might play vital role in the differentiation initiation and induction of lactogenesis by the BuMEC cells (Jena et al. 2021).

To understand the physiological function of the protein MGP-40 (Mammary Gland Protein-40), BuMEC cell line was used (Anand et al. 2016). In this study the functional characterization of the MGP-40 protein was performed after transfecting the BuMEC cells with Pci neo expression vector. This study revealed the involvement of MGP-40 protein in maintaining the proliferative stage of cells. The overexpression resulted in reduced dome formation of the BuMEC cells, along with reduction in casein synthesis and acinar polarization. Moreover, this protein induced STAT3 phosphorylation and showed features of epithelial to mesenchymal transition (EMT). Besides above findings, MGP-40 is also observed to express in higher amount in serum-starved condition in BuMEC cells, supporting its protective role against stress condition. This protein is also associated with various molecular pathways to execute various functions in the cells. In another study on the proteome analysis of BuMEC cells (Jaswal et al. 2020), a total of 12,609 non-redundant proteins were identified from five different fractions such as cytosolic, membranous organelles, nuclear, cytoskeletal, and secretome components. Around 325 molecular pathways are associated with these proteins, as revealed from KEGG analysis. Some very highly enriched pathways were MAPK pathway, PI3 – AKT pathway, insulin signaling pathway, estrogen signaling pathway, and cGMP-PKG pathway. The newly identified proteins were found to be involved in notch signaling, secretory functions, and transport processes. A recent in-depth study on the proteome analysis

(by TMT-based mass spectrometry analysis) of differentiated BuMEC cells grown in vitro at different time points (3, 6, 12, 15 days) of differentiation in presence of lactogenic hormones, revealed the role of many proteins during the lactogenic differentiation and novel protein signatures were identified (Jaswal et al. 2021). A total of 4934 proteins were identified in this study and out of them 681 proteins were differentially expressed across various time points of differentiation. In this comparative proteome study it was observed that 307 number of proteins achieved highest expression at day 12 of differentiation. The new proteins that were identified in this study are ABCA13, IVL, VPS37, CZIB, RFX7, Rab5, TTLL12, SMEK1, GDI2, and TMEM131 in the BuMEC cells. This study concluded that proteins with higher abundance could serve as potential biomarkers of differentiation and significantly associated with the lactation process.

Negative energy balance during lactation in buffaloes occurs frequently due to increased energy demand for milk yield, fetus growth during pregnancy, and animal requirement for body physiological function. Diagnosing the negative energy balance by the body condition score only is not an efficient approach. The study by Golla et al. (2019) in Murrah buffaloes during the early lactation period, showed higher serum level of free fatty acids and lower serum level of leptin could be potential marker of negative energy balance in lactating buffaloes. The serum level of β-hydroxy butyrate, free fatty acids, growth hormone, IGF-1, insulin, and leptin were analyzed in this study. The level of free fatty acids was significantly higher in high milk yielders during the third and fourth week of parturition, as compared to that of low milk yielders and heifers. There was no significant difference in the level of β-hydroxy butyrate, growth hormone, IGF-1, and insulin between the lactating buffaloes and heifers under this study. This study concluded that negative energy balance could be assessed by observing both the higher free fatty acids level and lower leptin level together and this condition may be restricted during the first month of lactation in buffaloes (Golla et al. 2019). The comparative expression study of the protein insulin-like growth factor binding protein-5 (IGFBP-5) of milk sample from indigenous cows (*Bos indicus*) and buffaloes (*Bubalus bubalis*), revealed many interesting findings (Mohapatra et al. 2015). IGFBP-5 shows various functions such as apoptosis, proliferation, and differentiation. The present study showed that higher expression level of IGFBP-5 occurs during involution stage as compared to that of period during colostrum production, late lactation, and early lactation, in both the cattle and buffaloes. ELISA study of milk from cow and buffalo showed that lower level of IGFBP-5 is expressed in buffaloes as compared to that of cows. This study concludes that IGFBP-5 (proapoptotic) production is inversely proportional to the length of lactation and milk yield (Mohapatra et al. 2015).

11.3 Gene Expression Studies Related to Lactation in Buffaloes

The gene expression analysis of Murrah buffalo milk from early, mid, and late lactation stages revealed the involvement of many genes in the lactation process (Arora et al. 2019). RNA sequencing method was applied in this study and milk

transcriptome was analyzed. Highly expressed genes across all the lactation stages were CSN2, CSN1S1, CSN3, LALBA, SPP1, and TPT1 genes. In this study, a total of 12,833 transcripts were identified, and out of these 271 transcripts were unique to early lactation, 205 transcripts unique to mid-lactation, and 418 transcripts were unique to late lactation. Most of the genes were associated with protein metabolism, transport process, and immunological functions. There was an increase in immune response function towards the late lactation and many pathways associated with different functions throughout the lactation, were identified along with the gene profiling. This study revealed 14 differentially expressed and highly connected genes in the three lactation stages, which can be further used in future research strategies for further exploration in lactation physiology. In a study on the expression pattern of lipogenic genes at different stages in the lactation period of buffaloes revealed many interesting findings (Yadav et al. 2015). The gene expression studies were performed on the MECs isolated from milk and it was observed that the PPARα gene is abundantly expressed during lactation, whereas ABCG2 and ACSS2 genes were moderately expressed, SREBF and PPARγ genes were expressed at low level. Interestingly, the expression of genes such as BDH1, ACSS2, and LIPIN1 genes were positively correlated with the milk yield but negatively correlated with the fat yield. However, the ACACA gene expression showed negative correlation with milk yield but positive correlation with fat yield (Yadav et al. 2015). Comparative gene expression profiling of the fatty acid synthesis genes of MECs isolated from milk at 60 days of lactation in the Surti and Jafarabadi buffaloes revealed that there is no significant difference in the expression pattern of the important lipogenic genes such as butyrophilin subfamily 1 member A1 (BTN1A1), stearoyl-CoA desaturase (SCD), lipoprotein lipase (LPL), glycerol-3-phosphate acyltransferase mitochondrial (GPAM), acetyl-coenzyme A carboxylase alpha (ACACA), and lipin (LPIN) genes (Janmeda et al. 2017). In another comparative gene expression study of milk somatic cells of Murrah buffaloes and Sahiwal cattle revealed 377 upregulated genes and 847 down-regulated genes in buffaloes as compared to that of cattle (Ahlawat et al. 2021). Higher level of expression was observed with the genes related to host defence system (lysozyme, defensin β, and granzymes) in buffaloes, whereas higher expression of genes related to ECM-receptor interaction, cytokine ligand–receptor interaction, complement cascades, coagulation cascades, and keratinization pathway were upregulated in cattle. These findings concluded that robust immune mechanisms in buffaloes might implicate their lower susceptibility to udder diseases as compared to that of cattle (Ahlawat et al. 2021). Study on the expression pattern of glucose transporter 1 (GLUT-1) and apoptotic genes in MECs enriched from milk of riverine buffaloes revealed many interesting findings (Yadav et al. 2014). The expression pattern of GLUT-1 was positively correlated with the milk yield, as highest expression was found in the peak stage of lactation, i.e., 90 days after calving; whereas lower GLUT-1 expression was found during the early lactation stage. Higher rate of apoptosis was observed in the early lactation stage as evidenced from the higher BAX/BCL2 ratio; whereas lowered apoptosis was observed during mid-stage of lactation, evidenced by lower BAX/BCL2 ratio. Besides, highest level of apoptosis was observed in the late lactation stage (240 days of lactation),

coinciding with the decreased milk yield and entering to the involution stage towards the end of the lactation (Yadav et al. 2014). Study on the expression pattern of collagen gene family in water buffalo during lactation and their comparative study with that of cattle, revealed many interesting findings (Lu et al. 2020). Collagens provide structural integrity to MECs, along with other functions. A total of 128 collagen protein sequences were identified in this study which correspond to 45 collagen genes in buffalo that were classified to 6 groups depending on the evolutionary linkages. A duplicated gene pair of COL4A1 and COL4A2 expressed in higher concentration during lactation in buffalo than that of cattle. However, higher expression was observed in the gene pair COL6A1 and COL6A2 during lactation in cattle. Out of the collagen genes studied, 11 genes were upregulated during lactation in buffalo as compared to that of cattle, out of which 3 genes (COL12A1, COL17A1, and COL5A2) were upregulated in late lactation, whereas COL16A1 and COL4A4 genes were upregulated during early and mid-lactation. This study indicates the role of collagen genes in lactation process (Lu et al. 2020). Alpha-lactalbumin is a milk protein and its expression pattern explains the mechanism of milk yield during lactation. Study on the polymorphism analysis and gene expression pattern of LALBA genes in the Nili Ravi buffaloes revealed highest expression in transition phase (day 15), and gradual decrease in the mid (day 90) and late (day 250) lactation phase. The collinearity analysis of collagen genes showed 23 orthologous genes in the milk sample of buffaloes and cattle at different stages of lactation (Lu et al. 2020). Similar study in the stanniocalcin-1 (STC1) gene has been done with the lactating riverine and swamp buffaloes (Mishra et al. 2019). The STC1 gene plays crucial role in regulating the calcium ion level during involution of the mammary gland. Polymorphism was found in this gene in the 3'-UTR region by PCR-RFLP genotyping method in riverine and swamp buffaloes. Simultaneously expression profiling was done from peripheral blood mononuclear cells (PBMCs) and in the lactating mammary gland tissues during different lactational stages. The expression profiling revealed highest expression of the STC1 gene in the involution stage. The specific genetic variation has been found in STC1 gene in ruminants and it has importance during involution of buffalo mammary gland along with the coregulation of expression by miRNA binding in the 3′- UTR region, as observed in this study (Mishra et al. 2019).

Transcriptomic analysis of mammary epithelial cells isolated from milk of Sahiwal cows and Murrah buffaloes during early (day 5–20), peak (day 30–50), mid (day 90–140), and late lactation (>215 days) period, showed transcription kinetics of milk protein, fat synthesis, and their regulatory genes (Sharma et al. 2019). Significantly higher mRNA level of milk proteins and fat synthesizing genes were found in Murrah buffaloes as compared to that of cows. As compared with the different lactation stages, the casein genes (CSN1S1, CSN1S2, CSN2, and CSN3), whey protein genes (α-lactalbumin and lactoferrin), and milk fat synthesizing genes were abundant during early lactation. Moreover, some regulatory genes such as JAK2, STAT5, SREBF1, and EIF4BP41 genes were also upregulated during early lactation stages. This study shows that milk-derived MECs could be a better

alternative non-invasive source to understand the lactation physiology in large animals in terms of the gene expression studies.

In silico analysis of the candidate genes for milk production traits in water buffaloes (*Bubalus bubalis*) revealed the association of 516 candidate genes in milk production process (Du et al. 2020). Functional genomic analysis of these genes showed their association with cell proliferation and mitotic nuclear division. KEGG analysis of the physiological pathways showed the candidate genes enriched in various pathways such as ErBb pathway, AMPK pathway, JAK-STAT pathway, etc.

Expression and polymorphism studies of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) gene in various tissue samples of buffalo revealed that this gene is highly expressed in the muscle, heart, liver, brain, and kidney of both nonlactating and lactating animals; however, the expression pattern was different in muscle, heart, liver, small intestine, mammary gland, rumen, spleen, and lung between these two categories of animals (Qiu et al. 2020). This study suggests that buffalo PPARGC1A is a transcriptional coactivator which is induced and regulates the carbohydrate and fat metabolism, more specifically it may play role in milk fat synthesis and mammary gland development. The study on sterol regulatory element-binding protein cleavage-activating protein (SCAP) gene and the SNP identification in buffaloes revealed that this gene might be involved in milk production and affect the milk production traits (Deng et al. 2018).

The comparative stage-specific expression of ATP binding cassette (ABC), solute carriers (SLC), and glucose transporter (SLC) genes revealed interesting findings across different stages of lactation in buffaloes (Sharma et al. 2014). The ABCA1 and ABCG1 gene level was higher in heifer mammary gland followed by involuting and lactating gland. Similarly, ABCA7 gene expression was highest in heifer mammary gland and lowest in involuting mammary gland. Moreover, the ABCG2 gene had higher expression during lactation stage, and ABCG5 gene had higher expression in involuting mammary gland. The genes such as LXRα, SREBF1, and PPARA genes which regulate the expression of ABC transporters also showed interesting pattern of expression. The LXRα gene was expressed in higher amount in involuting gland, whereas the SREBF1 and PPARA gene expression was higher in lactating gland. The SLC transporters such as SLC2A1, SLC2A4, and SLC2A8 genes were highly expressed during lactating stage suggesting their active involvement for glucose uptake during milk synthesis in lactation stage. The SLC2A12 gene expression was higher in heifer mammary gland. Besides, the expression of lactoferrin gene was found to be higher in the involuting mammary gland (Sharma et al. 2014). Comparative genetic study of AKT3 gene between cow and buffalo showed some significant findings (Ullah et al. 2018). The AKT3 gene has vital role in milk fat and cholesterol synthesis by regulating the sterol regulatory element-binding protein (SREBP). The maximum expression of this protein occurs in intestine, followed by mammary gland and immune cells. The genetic analysis of this gene between cattle and buffalo showed that quite variations were there in some exons and other findings were identification of consensus sequence motifs, variation in 3' UTR region, and involvement of miRNA in regulation of expression of AKT3

gene in buffalo. The other studies of this gene like phylogenetic tree analysis, motif, and functional domain positions revealed its correlation with other species. This comparative study indicated the potential of buffaloes towards disease resistance, environmental fluctuations and less prone to mastitis, along with superior productive and reproductive traits. Similar genetic studies of prolactin gene in reverine buffalo showed its involvement with milk production traits (Nadeem and Maryam 2016). The polymorphism study of leptin and pituitary specific transcription factor genes revealed the potential of leptin as a genetic marker that can be used to increase the genetic potential of buffalo for milk production (Nasr et al. 2016). In a similar manner the polymorphism studies of melatonin receptor (MTRN1A) genes showed its involvement in the production as well as reproduction processes in buffalo (Zetouni et al. 2014). Study on the influence of CSN1S1-CSN3 (α(S1)-κ-casein) composite genotypes in the milk yield traits and milk coagulation property (MCP) in the Mediterranean water buffalo, revealed that casein genes play role in the variation of MCP in buffalo milk (Bonfatti et al. 2012). In another polymorphism study it was found that polymorphic A2M gene has certain influence on the production of good quality milk by Murrah buffalo (Freitas et al. 2016). In a comparative study on the gene expression of fat metabolism genes in *Bubalus bubalis*, using lactating and nonlactating mammary tissues, showed that the expression of ACACA, BDH, LIPIN, PPARG, LPL, and SREBF genes were higher in lactating mammary gland than that of nonlactating mammary gland (Yadav et al. 2012).

11.4 Protein Signatures Related to Pregnancy in Buffaloes

Gene and protein expression studies related to pregnancy in buffaloes have revealed the significant role of many proteins (Table 11.2) during pregnancy. A detailed analysis of the gene expressions of fetal cotyledons that are derived from 45-day old pregnant buffaloes were performed and the expression pattern was compared with that of non-pregnant buffaloes (Lotfan et al. 2018). A total of 497 upregulated genes and 578 down-regulated genes were identified. In-depth bioinformatic analysis revealed the involvement of gene clusters for different functions such as attachment of fetus, nutrient transport, and immunological tolerance towards the semi-allogenic fetus. The signaling pathways that were enriched in pregnant buffaloes were Hedgehog signaling, Calcium signaling, Wnt signaling pathways, cell cycle regulation pathways, and immune responses regulatory functions. Significant upregulation of IL-2 specific gene was observed, indicating its role in attachment of the embryo. The expression profile in 45-day old cotyledons of pregnant buffaloes revealed the existence of immunosuppressive environment, which is a requirement for existence of the semi-allogenic fetus inside the uterus (Lotfan et al. 2018). The study on the serum proteins of pregnant buffaloes showed five upregulated proteins during early pregnancy such as anti-testosterone antibody light chain, apolipoprotein A-II precursor, serum amyloid A, cytokeratin type II (component IV), and immunoglobulin lambda light chain protein (Buragohain et al. 2017). The transition from pregnancy to lactation involves many physiological changes and there is onset of lactogenesis

Table 11.2 Genes and proteins significantly upregulated during early pregnancy in buffalo

Sl. No.	Name of Protein	Type of sample	References
1.	Dickkopf WNT signaling pathway inhibitor 4 (DKK4)	Cotyledon of 45-day pregnant buffalo	Lotfan et al. (2018)
2.	WNT family member 10B (WNT10B)	Cotyledon of 45-day pregnant buffalo	Lotfan et al. (2018)
3.	Dickkopf WNT signaling pathway inhibitor 1 (DKK1)	Cotyledon of 45-day pregnant buffalo	Lotfan et al. (2018)
4.	Reelin (RELN)	Cotyledon of 45-day pregnant buffalo	Lotfan et al. (2018)
5.	Hedgehog interacting protein (HHIP)	Cotyledon of 45-day pregnant buffalo	Lotfan et al. (2018)
6.	Calmodulin like 5 (CALML5)	Cotyledon of 45-day pregnant buffalo	Lotfan et al. (2018)
7.	Cholecystokinin B receptor (CCKBR)	Cotyledon of 45-day pregnant buffalo	Lotfan et al. (2018)
8.	Anti-testosterone antibody light chain protein	Pregnant buffalo serum	Buragohain et al. (2017)
9.	Apolipoprotein A-II precursor	Pregnant buffalo serum	Buragohain et al. (2017)
10.	Serum amyloid A protein	Pregnant buffalo serum	Buragohain et al. (2017)
11.	Cytokeratin type II (component IV)	Pregnant buffalo serum	Buragohain et al. (2017)
12.	Immunoglobulin lambda light chain protein	Pregnant buffalo serum	Buragohain et al. (2017)
13.	MX2 protein	Pregnant buffalo serum	Buragohain et al. (2016)
14.	PAG-7 protein	Placental extract	Kumar et al. (2014)
15.	PAG-11 protein	Placental extract	Kumar et al. (2014)

in the mammary epithelial cells. A crucial study on the protein profiling during this transition period in water buffaloes revealed the predominance of acute phase proteins and markers of oxidative stress in the serum (Sauerwein et al. 2020). The various acute phase proteins under this study were haptoglobin, serum amyloid A, and acidic glycoprotein. The indicators of metabolic stress observed were non-esterified fatty acids (NEFA), ß-hydroxybutyrate (BHB) and adiponectin. The increased concentration of acute phase proteins reveal the inflammatory condition during calving period. Analysis of blood and milk samples from the Mediterranean buffaloes during the peripartum period revealed the modulation of total protein content, serum haptoglobin, albumins, milk proteins α2 and β2 caseins, and γ-globulins (Gianesella et al. 2019). In this study the blood and milk samples were collected from 30 buffaloes on day 7 before parturition, and on day 7, 30, and 50 after parturition. The pregnancy-associated glycoproteins (PAGs) are found to

play vital role during early pregnancy and could be used as markers of pregnancy diagnosis as early as day 28 of pregnancy in buffaloes (El-Battawy et al. 2009). In this study, the increased pattern of PAG concentration from day 28 to day 41 in pregnant buffaloes was observed through radio immune assay technique. Similar study on buffalo (*Bubalus bubalis*) PAGs using vicia villosa affinity chromatography revealed novel PAG, as studied in placenta derived from mid and late pregnancy placenta (Barbato et al. 2008). Amino terminal microsequencing was performed after the affinity chromatography and three distinct PAG sequences were identified with two novel sequences found. The western blotting with the anti-PAG sera revealed the molecular mass range of immune reactive bands from mid-pregnant placenta to be 59.5–75.8 kDa, and that from late pregnant placenta to be of 57.8–73.3 kDa range (Barbato et al. 2008). Another study on buffalo PAG protein identification using lectin-based affinity chromatography and peptide mass finger printing of the placental extract, revealed PAG-7 and PAG-11 could be potential biomarkers for early pregnancy diagnosis in buffaloes (Kumar et al. 2014).

11.5 Gene Expression Studies Related to Pregnancy in Buffaloes

Gene expression studies during pregnancy in buffaloes have revealed many interesting findings which facilitate understanding the molecular dynamics that occur during different stages of pregnancy. The study by Buragohain et al. (2016) has found the myxovirus resistance protein (MX2) can be used as a potential biomarker for early diagnosis of pregnancy in buffaloes. There is a sequential change in expression pattern of the MX2 transcript has been observed in the whole blood and the protein found in the serum of pregnant buffaloes at day 0, 7, 14, 21, and 35 of pregnancy. Interestingly, the transcript level was 28.16 ± 1.91 times higher on the 28th day of pregnancy as compared to day 0, which signifies its role as an early pregnancy biomarker in buffaloes. The increased MX2 expression both at the transcript and protein level between day 14 and 28 of pregnancy in buffaloes supports its potential as an early pregnancy biomarker (Buragohain et al. 2016). In another study in buffalo, it was found that OAS1 and MX2 genes can act as potential biomarker for early pregnancy diagnosis (day 18 post AI) (Thakur et al. 2017). This study involved the cloning and expression of interferon tau (IFNT) stimulated genes such as OAS1, MX1, MX2, and ISG15 genes, as IFNT is found to be the first pregnancy recognition signal. The IFNT signaling is observed during the maternal recognition of pregnancy. Out of the different interferon-stimulated genes, OAS1 and MX2 gene expression was higher on 18th day after AI, as confirmed by real-time PCR (Thakur et al. 2017). The study by Nag et al. (2018) revealed that the ISG15 and MX2 genes are expressed in higher quantity during the early pregnancy in buffaloes. In silico study and characterization of buffalo PAG-1 gene was performed and it was found that PAG-1 is having an open reading frame of 1140 bp which encodes for 380 amino acids. This 380 amino acid length PAG-1 carries a 15 amino acid signal peptide and a 365 amino acid long matured peptide. The phylogenetic analysis of buffalo PAG-1 gene revealed its relation with cattle, goat, and sheep PAG-1 genes

with more than 80% similarity (Jerome et al. 2011). In a study on polymorphism detection in CYP-19 (cytochrome P450 aromatase) gene in Murrah buffalo heifers by SSCP technique indicated that polymorphism may cause the variation in fertility performance in buffaloes (Suneel Kumar et al. 2009).

11.6 Conclusion

Various proteomic and transcriptomic studies related to pregnancy and lactation in buffaloes have revealed huge number of proteins playing crucial role in these two physiological processes. The transition of non-pregnant uterus to pregnant uterus has been marked with shift of protein signatures, and similarly the transition of nonlactating MECs to lactating MECs in mammary gland have identified group of proteins as biomarkers of these processes. Many researches are going on further to unravel these processes in molecular details. Understanding the molecular dynamics in the context of involvement of proteins in the early pregnancy and lactation of buffalo will help in enhancing the productive and reproductive performances in future.

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Pluripotent Stem Cells from Buffalo: Basic and Translational Applications

12

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Abstract

Buffalo, a multi-purpose and most valued animal among livestock species called as "Black Gold" due to its substantial contribution in the agricultural economy, providing milk, meat, and hides along with draught power in many developing countries, especially in Southeast Asia. There are several attempts have been made to generate pluripotent stem cells by means of derivation of embryonic stem cells or induced pluripotent stem cells in the species and limited success has been achieved so far. The generated pluripotent stem cells could be useful for the production of transgenic animals for improved traits, nucleus donors for improving cloning efficiency and for biomedical applications. Additionally, the availability of authentic pluripotent stem cells from buffalo would have suitable models studying human skin depigmentation disorders due to similar etiopathogenesis. Therefore, the present chapter summarizes the current updates on pluripotent stem cells generated from buffalo and their translational applications.

Keywords

Applications · Buffalo · Cellular reprogramming · Characterization · Culture condition · Culture media · Differentiation · Embryoid bodies · Embryonic stem cells · Foetal fibroblasts · iPS cells · Livestock · Pluripotency · Transcription factor · Teratoma

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12.1 Introduction

Pluripotent stem cells are those master cells that are able to self-renew, a constituent of three basic germ layers and potentially produce any cell or tissue of the body. The pluripotent stem cells could be obtained from embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. The ES cells can be derived from embryos produced through in vitro fertilization (IVF), parthenogenetic activation (PA), or nuclear transfer (NT) process, whereas iPS cells can be produced by reprogramming of somatic cells through the ectopic introduction of transcription factors (Fig. 12.1). ES cells derived from an inner cell mass (ICM) of a blastocyst, are pluripotent in nature i.e. they are able to produce any kind of cells or tissues other than extra-embryonic cells. The first successful ES cell line derived from the ICM of blastocysts of a mouse, which displayed unlimited proliferative capacity and retained an undifferentiated state of potency with genomic stability, and able to contribute to the germline (Evans and Kaufman 1981; Martin 1981). Soon after the derivation of mouse ES cells, many research groups worldwide started their effort to derive pluripotent ES cells from diverse species including livestock and human beings.

A decade after the derivation of mouse ES cells, few groups reported the derivation of livestock ES cells from the embryos of pig, sheep, and bovine which maintain only for few passages (Evans et al. 1990; Piedrahita et al. 1990). After 17 years of the successful establishment of mouse ES cells, the first human ES cells have been derived from the ICM of the blastocyst which grow indefinitely in vitro, maintaining pluripotent capacity with stable karyotype (Thomson et al. 1998). These cells showed differentiation potential to all cell types of the body which confirmed by several in vitro and in vivo assays. After that, ES cells lines have been derived from many farm animals such as pig (Chen et al. 1999; Li et al. 2003), cattle (Cibelli et al. 1998; Mitalipova et al. 2001; Yadav et al. 2005), sheep (Zhu et al. 2007), goat (Behboodi et al. 2011; Kumar et al. 2011a), and horse (Saito et al. 2002). Almost after 25 years of mouse ES cells derivation, the first report of buffalo ES cell-like

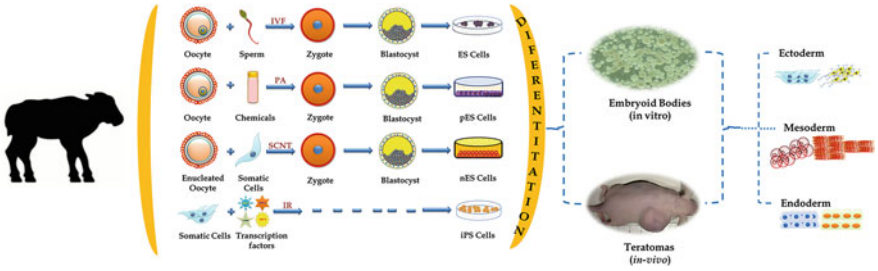

Fig. 12.1 Diagrammatic representation of derivation of pluripotent stem cells from buffalo (Adopted from Kumar et al. 2020b). *IVF* In vitro fertilization, *ES Cells* Embryonic stem cells, *PA* Parthenogenetic activation, *pES Cells* Parthenogenetically derived embryonic stem cells, *SCNT* Somatic cell nuclear transfer, *nES Cells* Nuclear transfer derived embryonic stem cells, *IR* Induced reprogramming, *iPS cells* Induced pluripotent stem cells

cells was generated from blastocysts of cloned Swamp buffalo (Kitiyanant et al. 2004). Subsequently, Riverine buffalo ES cell-like cells were established from in vitro fertilized embryos which maintained their pluripotency for prolonged periods of time in culture (Chauhan et al. 2005). Later, many workers produced buffalo stem cells from different sources such as embryonic, foetal, and adult using different protocols has been described (Kumar et al. 2015a).

ES cell production from embryos in animals is technically complicated and ethically complex for humans. Therefore, in order to prevent the use of actual embryos, many efforts have been made to produce pluripotent stem cells from other cells. The idea is to reprogram mature cells to turn them back into stem cells. This idea gave birth to iPS cells in which somatic cells can be reprogrammed by ectopic expression of a set of key reprogramming factors such as Oct4, Nanog, c-Myc, Klf4. Using this approach, the first iPS cells were generated from mouse skin fibroblasts (Takahashi and Yamanaka 2006), followed by human iPS cells from fibroblasts (Takahashi et al. 2007). The generated iPS cells showed similar characteristics of ES cells such as they have unlimited self-renewal ability, morphology appearance, expression of pluripotency markers, epigenetic state, and their capability to differentiate into all somatic cell types including the germ-line. Due to unique properties of iPS cells and difficulties in the generation of bona fide ES cells (lack of germline transmissions) from farm animals, iPS cell production techniques have been swiftly and widely adopted in livestock species such as cattle, sheep, goat, pig, horse, and buffalo, and generated stable pluripotent stem cells (for review, Kumar et al. 2015b; Kumar et al. 2015c; Ogorevc et al. 2016; Haridhasapavalan et al. 2019; Kumar et al. 2020a; Kumar et al. 2020b). After 6 years of iPS cell derivation, the first buffalo iPS cells have been generated from reprogramming of foetal fibroblasts using buffalo defined factors (Deng et al. 2012). Later, many reports are available on the derivation of buffalo iPS cells by different reprogramming approaches of somatic cells (Bag 2016; Mahapatra et al. 2017; Kumar et al. 2019; Rawat et al. 2019; Deng et al. 2019).

Buffalo is a multi-purpose and most valued animal among livestock species called "Black Gold" due to its significant contributions to the agricultural economies by providing milk, meat and hides along with draught power in many developing nations, especially in Southeast Asia. Due to their relatively high milk production and ability to thrive in extreme conditions, Buffalo may be considered to be the perfect candidate for the development of transgenic animals that express pharmacologically interesting proteins in milk. Furthermore, the consumption of buffalo milk is beneficial for health due to low cholesterol and high calcium and protein content than that of cow milk (Khan et al. 2019). Buffalo meat is also reported to be leaner with less fat and cholesterol than cow meat. Buffalo is a more efficient convertor of poor quality feeds and fibre than cattle. It is important to explore these beneficial inherent features of the species at cellular and molecular levels in order to develop buffalo as the best model for the development of humanized goods through genetic engineering. To achieve these goals, the availability of genuine pluripotent stem cells from buffalo would be an attractive biotechnology and biomedical research animal model that might enable for multifaceted genetic manipulations, including

knock-in and knock-out genes. Pluripotent stem cells could also be used as nucleus donors to improve cloning efficiency through somatic cell nuclear transfer (SCNT, Kou et al. 2010). In addition, efficient incorporation of genetic material for the production of transgenic buffalo producing therapeutic protein(s) in milk could be redefined. In addition to regenerative approach, buffalo pluripotent stem cell technology has a capable methodology for maintaining valuable genetic resources and sharing them efficiently within the biomedical community. Therefore, the goal of this chapter is to present the current updates on pluripotent stem cells generated from buffalo and their translational applications.

12.2 Embryonic Stem Cells from Buffalo

The mammalian development starts with the fertilization of an oocyte with a spermatozoon, which gives rise to the zygote (single cell embryo). The buffalo ES cells isolated from the ICM of pre-implantation embryos produced through IVF, NT, or PA process. These processes lead to a zygote formation followed by rapid mitotic divisions gives rise to a compact ball of cells called morula which eventually results in the blastocyst stage. The blastocyst having two cell compartments, the outer layer called trophectoderm and a mass of cells at inner side called ICM which further differentiates into epiblast and hypoblast. Trophectoderm cells and hypoblast give rise to the placenta and extra-embryonic tissue, whereas epiblast leads to the formation of the embryo proper which later develop into three germ layers, i.e. ectoderm, mesoderm, and endoderm. The resulting three layers are able to differentiate into any type of cell of the body and that is why epiblast is considered as pluripotent. Generally, mouse and human embryo develop into a blastocyst on day 3.5 and 7 respectively but in buffalo, it is somewhere between 7 and 9 days post-insemination and at this stage that embryos are used for the derivation of ES cells. Since the derivation of buffalo ES cells from the ICM of blastocysts in 2004, substantial progress has been made in this species (Table 12.1). The initial attempt was made to isolate pluripotent buffalo ES-like cells from the ICMs of IVF, PA, and NT embryos (Kitiyanant et al. 2004; Chauhan et al. 2005). The ICMs were mechanically removed from blastocysts and plated on mitocycin-c treated mouse embryonic fibroblasts (Kitiyanant et al. 2004) or buffalo foetal fibroblasts (Chauhan et al. 2005). These cells were attached on the culture dish and multiplied after sub-culture, expressed pluripotency related markers, able to formed embryoid bodies (Kitiyanant et al. 2004; Chauhan et al. 2005). Later, many reports are available on the derivation of buffalo ES cells from ICMs of IVF embryo (Verma et al. 2007; Huang et al. 2010; Anand et al. 2011; Kumar et al. 2011b; Sharma et al. 2011; Sharma et al. 2012; Singh et al. 2013; Shah et al. 2015; Deng et al. 2020), PA embryo (Sritanaudomchai et al. 2007; Muzaffar et al. 2012; Singh et al. 2012), or NT embryo (George et al. 2011; Muzaffar et al. 2012; Shah et al. 2015).

Apart from ICM, attempts have also been made to derive buffalo ES cells from 8-cells stage embryos (Kumar et al. 2012a, b), 16–32 cells stage embryos (Puri et al. 2010) and morulae (Verma et al. 2007; Huang et al. 2010) but these stages failed to

Table 12.1 Summary of the embryonic stem cells derived from buffalo

Source of embryo	Method for ICM Isolation	Formation of primary colony after (days)	Morphology of primary colony	Culture media and conditions	Survive up to	Expression of pluripotency markers observed through		Differentiation		Karyotype	Reference
						RT-PCR	Immuno cytochemistry	In vitro	In vivo		
PA- blastocysts	Mechanical	5–7	Tightly packed and cells having high nucleus/ cytoplasm ratio with prominent nucleoli	DMEM/F-12, FBS, L-glutamine, β – mercaptoethanol, BME amino acids, 5% CO_2 at 37 °C on MEF	>6M	NP	SSEA-4, TRA-1-81, Oct-4, SSEA-1 and TRA-1-60	EBs, chondrocytes	Teratomas	N	Sritanaudomchai et al. (2007)
IVF- blastocysts	Mechanical and enzymatic	2–5	Dome shaped	DMEM, FBS, mLIF, NEAAs, β-mercaptoethanol, L-glutamine, gentamicin sulphate, 5% CO_2 at 37 °C on BFF	P8	OCT-4	ALP	EBs, neuron and epithelium-like cells	NP	N	Verma et al. (2007)
IVF- blastocysts	Mechanical	8–10	Multicellular with distinct boundaries	DMEM, FBS, NEAAs, 2-mercaptoethanol, sodium pyruvate, mrLIF, hrbFGF, mrSCF, 5% CO2 at 37 °C on BFF	P8	OCT-4, NANOG, SOX2	AP, OCT-4, SSEA-1, SSEA-3, SSEA-4	EBs, epithelium, fibroblast and neuron-like cells	NP	NP	Huang et al. (2010)
IVF- blastocysts	Intact, mechanical and enzymatic	3–6	Compact with distinct boundary	DMEM, FBS, L-glutamine, mLIF, NEAAs and gentamicin sulphate, 5% CO2 at 37 °C on BFF	P7	OCT-4	AP, OCT-4, SSEA-4, TRA-1-60, TRA-1-81	NP	NP	N	Anand et al. (2011)
IVF-blastocysts	Mechanical	6–10	Compact with well-defined edges	KO-DMEM, KSR, L-glutamine, gentamicin sulphate, MEM-NEAAs, β-mercaptoethanol, mLIF, FGF2, 5% CO2 at 37 °C on BFF	>27M (P135)	OCT4, NANOG, SOX-2, REX-1, FOX-D3, NUCLEOSTAMIN, STAT-3	AP, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, OCT-4	EBs, neuron-, endodermal-, striated muscles-, epithelial-, fibroblast-like cell types	NP	N	Sharma et al. (2011)

(continued)

Table 12.1 (continued)

Source of embryo	Method for ICM Isolation	Formation of primary colony after (days)	Morphology of primary colony	Culture media and conditions	Survive up to	Expression of pluripotency markers observed through		Differentiation		Karyotype	Reference
						RT-PCR	Immuno cytochemistry	In vitro	In vivo		
IVF and HMC-blastocysts	Mechanical	5–10	Dome-shaped clump of cells	KO-DMEM, KSR, L-glutamine, MEM-NEAAs, β-mercaptoethanol, LIF, bFGF-2, gentamicin sulphate, 5% CO2 at 37 °C on BFF	P15	OCT-4, SOX-2, NANOG, NEUCLEOSTAMIN, STAT-3, FOXD-3	AP, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81	EBs, epithelial-, fibroblast-, neuron-like cell types	NP	NP	George et al. (2011)
IVF- blastocysts	Intact and enzymatic	3–5	Compact dome shaped with clear margin	DMEM, FBS, L-glutamine, rmLIF, NEAAs and gentamicin sulphate, 5% CO2 at 38.5 °C on BEF, SEF, GFF	P11	OCT-4, NANOG	ALP	NP	NP	N	Kumar et al. (2011a, b)
IVF- blastocysts	Intact and enzymatic	3–5 days	Compact dome shape with clear margin	DMEM, FBS, L-glutamine, rmLIF, NEAAs and gentamicin sulphate, 5% CO2 at 38.5 °C on BFF	P11	OCT-4, NANOG	ALP	EBs, neuron cell type	NP	N	Kumar et al. (2012a)
IVF- eight-cells	Enzymatic	8–12	–	KO-DMEM, FBS, α-glutamine, NEAA, β-mercaptoethanol, LIF, bFGF, SCF, IGF1, 5% CO2 at 37 °C on BFF	Days20	OCT-4, NANOG	AP, OCT-4, NANOG, SSEA-1, TRA-1–61, TRA-1–81	NP	NP	NP	Kumar et al. (2012b)
IVF, PA, HMC-blastocysts	Mechanical	8–15	Compact with distinct boundaries	KO-DMEM, KSR, NEAAs, L-glutamine, gentamicin, β-mercaptoethanol, LIF, bFGF-2, 5% CO2 at 37 °C on BFF	P53	OCT4, SOX2, NANOG, cMYC, FOXD3, STAT3, REX1, NUCLEOSTEMIN, TELOMERASE	AP, SSEA-4, TRA-1-60, TRA-1-81, OCT4, SOX2	EBs, epithelial-, muscle-, neuron-like cell types	NP	N	Muzaffar et al. (2012)
PA- blastocysts	Mechanical and enzymatic	5–8	Compact and dome shaped with well-defined boundaries	KO-DMEM, FBS, mLIF, FGF-2, NEAA, β-mercaptoethanol, and L-glutamine, 5% CO2 at 37 °C on BFF	Days150 (P30)	OCT-4, NANOG, SOX- 2, REX-1, FOXD-3, NUCLEOSTEMIN, TELOMERASE, STAT-3, c-MYC	AP, SSEA-3, SSEA-4, TRA-1-80, TRA-1-60, CD-9, CD-90	EBs, epithelial-, neuron-like cell types	NP	N	Singh et al. (2012)

IVF-blastocysts	Intact	7	Densely packed with clear border	DMEM, FBS, L-glutamine, β-mercaptoethanol, NEAA, ITS, gentamycin sulphate, LIF, bFGF, 5% CO2 at 38.5 °C on BFF or Matrigel	P15	OCT-4	AP, OCT-4, SSEA- 4 and TRA-1-60	NP	Teratomas formation	N	Verma et al. (2012)
IVF-blastocysts	Mechanical	8–10	Dome-shaped	KO-DMEM, KSR, bFGF, L-glutamine, rmLIF, NEAAs, gentamicin sulphate, 5% CO2 at 38 °C on BFF	>100P	OCT4, NANOG, SOX2, c-MYC, REX-1, STAT3, FOXD3, and TELOMERASE	ALP, OCT4, NANOG, SOX2, FOXD3, SSEA1, SSEA4, TRA-1-60, TRA-1-81, CD-90	EBs	NP	N	Shah et al. (2015)
SCNT-blastocysts	Mechanical	8–10	Dome-shaped	KO-DMEM, KSR, bFGF, L-glutamine, rmLIF, NEAAs, gentamicin sulphate, 5% CO2 at 38 °C on BEF	>100P	OCT4, NANOG, SOX2, c-MYC, REX-1, STAT3, FOXD3 and TELOMERASE	ALP, OCT4, NANOG, SOX2, FOXD3, SSEA1, SSEA4, TRA-1-60, TRA-1-81, CD-90	EBs	NP	N	Shah et al. (2015)
IVF-blastocysts	Mechanical	5–6	Round and tightly packed	High-glucose DMEM, KSR, NEAA, β-mercaptoethanol, pyruvate, L-glutamina, SCF, LIF, bFGF, streptomycin/ penicillin, BIO or CHIR99021, 5% CO_2 at 37 °C on BFF	P15	OCT4, SOX2, C-MYC, LIN28, E-cadherin, NANOG	OCT4, SOX2, SSEA-1, SSEA-4	NP	NP	NP	Deng et al. (2020)

IVF in vitro fertilization, *PA* parthenogenetically activated, *HMC* handmade cloned, *DMEM* Dulbecco's Modified Eagle Medium, *LIF* leukaemia inhibitory factor, *mrLIF* mouse recombinant leukaemia inhibitory factor, *hrbFGF* human recombinant basic fibroblast growth factor, *mrSCF* mouse recombinant stem cell factor, *IGF1* insulin-like growth factor 1, *bFGF* basic fibroblast growth factor, *NEAA* nonessential amino acids, *FBS* foetal bovine serum, *hrbFGF* human recombinant basic fibroblast growth factor, *mrSCF* mouse recombinant stem cell factor, *KO* Knockout, *KSR* Knockout serum replacer, *MEM* minimum essential medium, *SCF* stem cell factor, *ITS* insulin-transferring selenium, *SSEA* stage specific embryonic antigen, *TRA* tumour rejection antigens, *EBs* embryonic bodies, *MEFs* mouse foetal fibroblasts, *BFF* buffalo foetal fibroblasts, *GFF* goat foetal fibroblasts, *ALP* alkaline phosphatase, *KSR* knockout serum replacement, *N* normal, *NP* not performed

attach with culture dish and did not form primary colonies, indicating that the embryonic age could be a perilous factor for successful derivation of buffalo ES cells. This notion has been supported by the derivation of ES cells from eighth day hatched blastocysts and found superior over the ninth day expanded blastocysts (Kumar et al. 2012a, b), that could be because of healthier and more viable ICM cells (Verma et al. 2007; Huang et al. 2010; Anand et al. 2011; Kumar et al. 2011b). The buffalo ES cells generated from day 8 hatched blastocysts were able to maintain for up to 135 passages or more than 27 months in culture (Sharma et al. 2011). Furthermore, different methods for ICMs isolation were compared and mechanical isolation were found superior over enzymatic and whole embryo culture in terms of higher primary colony formation rate (Verma et al. 2007; Anand et al. 2011; Sharma et al. 2011). To the best of our knowledge, no study has been performed for derivation of buffalo ES cells from in vivo produced pre-implantation stage of embryos. However, the generated buffalo ES cells exhibited dome or round shaped, compact morphology with well-defined edges having high nucleus: cytoplasm ratio (Verma et al. 2007; Sharma et al. 2011; Shah et al. 2015).

Initially buffalo ES cells were cultured on DMEM or DMEM/F12 medium supplemented with 15–20% FBS, 1000 IU/mL leukaemia inhibitory factor (LIF), 1% nonessential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol and buffalo foetal fibroblasts as feeder layer which supported the growth up to 10–15 passages only (Verma et al. 2007; Huang et al. 2010; Anand et al. 2011; Kumar et al. 2011b). Later, the use of knock-out DMEM supplemented with knock-out serum replacer and addition of FGF2 along with LIF significantly improve the survival of buffalo ES cells colonies generated from IVF embryos and continued in the culture for more than 135 passages (Sharma et al. 2011). The recipe of culture media also supports the prolong survival (>40 passages) of buffalo ES cells derived from PA and NT embryos (Muzaffar et al. 2012; Singh et al. 2012; Zandi et al. 2014). These findings indicating that buffalo ES cells required both LIF and FGF signalling pathways to maintain pluripotency in vitro. Furthermore, an attempt has been made to improve survival of buffalo ES cells using rock inhibitor Y-27632 which support their pluripotency even in unfavourable conditions such as enzymatic dissociation to single cells or antibiotic-assisted selection after transfection (Sharma et al. 2013). Apart from that, the various signalling pathway also plays an important role in maintaining self-renewal and pluripotency of buffalo ES cells. In this context, Zandi et al. (2014, 2015) found that the signalling pathway of WNT3A is important both for the maintenance of pluripotency and differentiation of undifferentiated buffalo ES cells. More recently, Deng et al. (2020) observed that activation of Wnt/β-Catenin signalling pathway by glycogen synthase kinase-3 (GSK3) inhibitor promotes the colony formation and proliferation rate, increased expression of pluripotency related genes and maintain undifferentiated state of buffalo ES cells. These studies have shown steady progress in identifying the best conditions for the establishment of stable and competent buffalo ES cells. Chromosomal analysis of long term maintained buffalo ES cells showed normal karyotype (Sharma et al. 2011; Muzaffar et al. 2012). Buffalo ES cells expressed a number of surface markers evaluated through immunostaining such as alkaline phosphatase (ALP), SSEA-3,

SSEA-4, TRA-1-60, and TRA-1-81 and/or expressed pluripotency related genes analysed through PCR like Oct-4, Sox-2, and Nanog, Rex1, Telomerase, Nucleostamin, Stat-3, and FOXD-3 ((Verma et al. 2007; Huang et al. 2010; Anand et al. 2011; George et al. 2011; Muzaffar et al. 2012; Sharma et al. 2012; Zandi et al. 2014; Shah et al. 2015). Many researchers observed that the buffalo ES cells did not show the SSEA-1 expression (Anand et al. 2011; George et al. 2011; Sharma et al. 2011), whereas some are detected the expression of SSEA-1 (Kumar et al. 2012a, b; Sharma et al. 2012; Zandi et al. 2014), indicates that the SSEA-1 expression in buffalo ES cells is a variable and similar type of variability was also perceived in bovine ES cells (Munoz et al. 2008). The ES cells derived from humans and primates typically express SSEA-3 and SSEA- 4 but not SSEA-1 and during differentiation SSEA-3 and SSEA- 4 showed downregulation whereas observed upregulation of SSEA-1 expression (Draper et al. 2002; Wolf et al. 2004). The generated buffalo ES cells formed embryoid bodies and differentiated into various cell types in vitro which confirmed by lineage specific markers (Sritanaudomchai et al. 2007; George et al. 2011; Sharma et al. 2011; Muzaffar et al. 2012; Singh et al. 2013). Recently, it was demonstrated that buffalo ES cells differentiated in vivo to form teratomas having three germ layers (Sharma et al. 2012; Verma et al. 2012). However, formation of chimera and germ-line contribution has not yet been conveyed in this species.

12.3 Induced Pluripotent Stem Cells from Buffalo

ES cells have numerous applications in livestock to improve production traits with generation of transgenic and cloned animals, conservation of germplasm, disease modelling, regenerative medicine, etc. However, due to difficulties in their derivation, ethical consideration, epigenetic, and genetic alteration during culture, the potential of ES cells has not been completely exploited. Therefore, the recent approach has been adopted to reprogram the somatic cells into iPS cells, resembled with ES cells in morphology, expression of pluripotency markers, telomerase activity, teratoma formation, DNA methylation, formation of chimera, etc. and thus iPS cells have been designated an alternative to ES cells. Buffalo iPS cells were produced by reprogramming of foetal fibroblasts using buffalo origin Oct4, Sox2, Klf4, and c-Myc transcription factors mediated through retroviral vectors and cells survived up to 10 passages in culture (Deng et al. 2012). In principle, silencing of exogenous transgenes and activation of endogenous pluripotent genes are assurances of full reprogramming of somatic cells to iPS cells. Furthermore, the exogenous genes should not be integrating into the target cells genome or to be excised after completion of reprogramming. Deng et al. (2012) detected that the silence of the exogenous transcripts and expression of endogenous pluripotent factors in generated buffalo iPS cells after six passages of culture, indicating the complete reprogramming of somatic cells (Table 12.2). Thereafter, fibroblasts derived from buffalo foetal were reprogrammed to iPS cells using various combination of transcription factors such as Oct4, Nanog, Sox2, Klf4, c-Myc, and Lin28 (Bag 2016; Mahapatra et al. 2017; Kumar et al. 2019; Rawat et al. 2019). Previously, it was

Table 12.2 Summary of the derivation of induced pluripotent stem cells from buffalo

Cell type	Vector type	Reprogramming factors	Culture media with supplements and feeder cells	Survive up to passages	Expression of pluripotency-related markers		Differentiation		Chimera production	Chromosome status	Reference
					RT-PCR	Immunostaining	in vitro	in vivo			
Foetal fibroblasts	Retrovirus	Buffalo OSKM	DMEM, 20% ESC-FBS, 2 mM L-glutamine, 1% NEAA, 0.1 mM β-mercaptoethanol, 10 ng/mL bFGF, 10 ng/mL hLIF, penicillin-streptomycin, MEFs	10	Oct4, Sox2, Nanog, Stat3, Foxd3, GP130, E-cadherin, bFGF2, p53	ALP, SOX2, NANOG, SSEA-1, SSEA-4, E-cadherin	EBs	Teratomas	NP	Normal	Deng et al. (2012)
Foetal fibroblasts	–	–	KO-DMEM/F12, 20% FBS, 1% L-glutamine, 1% strepto-penicillin, 1% NEAA, 0.1 mM β-mercaptoethanol, 4 ng/ml hLIF, 4 ng/ml bFGF + chicken egg extract, BFFs	–	Oct4, Sox2, Nanog and FoxD3	ALP, OCT4, NANOG, SSEA-1, TRA-1–60 and TRA-1–81	NP	NP	NP	NP	Mahapatra and Bag (2014)
Foetal fibroblasts	Lentivirus	Murine OSKM	KO-DMEM, 20% FBS, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1% NEAA, 0.5% penicillin streptomycin, 10 ng/ml bFGF, 4 ng/ml hLIF, BFFs	20	Oct4, Sox2, Nanog, C-Myc, KLF4 and FoxD3	ALP, OCT4, NANOG, SOX2, SSEA1, TRA-1-60 and TRA-1-81	EBs	NP	NP	Normal	Bag (2016)
Foetal fibroblasts	Lentivirus	Murine OSKM	KO-DMEM/F12, 20% FBS, 1% L-glutamine, 1% strepto-penicillin, 1% NEAA, 0.1 mM β-mercaptoethanol, 4 ng/ml hLIF, 4 ng/ml bFGF + VPA, BFFs	18	Oct4, Nanog, Sox2, Klf4 and c-Myc	ALP, OCT4, NANOG, SSEA-1, TRA-1–60 and TRA-1–81	EBs	NP	NP	Normal	Mahapatra et al. (2017)

Foetal fibroblasts	Lentivirus	Human OSKM	KO-DMEM, 10% KSR, 2 mM L-Glutamine, 1% NEAA, 1000 IU/ml LIF, 5 ng/ml bFGF, 50 μg/ml gentamicin sulphate, BFFs	15	Oct4, Sox2, Klf4, c-Myc, UTF, Foxd3, STAT3, Rex1, telomerase, Nucleostamin	ALP, OCT4, NANOG and TRA1–81	NP	NP	NP	NP	Rawat et al. (2019)
Foetal fibroblasts	piggyBac	Human SOKMNL	DMEM/F-12, 20% KSR, 0.1 mM NEAA, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 ng/mL bFGF, 1000 U/mL hLIF, gelatin	15	Oct4, Sox2, Nanog, cMyc, Klf4, Lin28	ALP, SSEA-1, SSEA-4, SSEA-5, TRA-1-81, Oct4, Sox2, Nanog	EBs	NP	NP	Normal	Kumar et al. (2019)
Adipose tissue-derived mesenchymal stem cells	Retroviral	Mouse OSKM	DMEM, 20% FBS, 10 ng/ml bFGF, 10 ng/ml hLIF, 2 mM L-glutamine, 1% NEAAs, 0.1 mM βmercaptoethanol, 2 mM valproic acid, penicillin/streptomycin,	9	OCT4, NANOG	ALP, OCT4, NANOG, SSEA-4, and TRA-1-81	EBs	Teratomas	NP	NP	Deng et al. (2019)

OSKM Oct-4, Sox2, Klf4, and c-Myc, *SOKMNL* Sox2, Oct-4, Klf4, c-Myc, Nanog and Lin28, *ALP* alkaline phosphatase, *SSEA* stage specific embryonic antigen, *TRA* tumour rejection antigens¸ *EBs* embryonic bodies, *NP* Not performed, *DMEM* Dulbecco's Modified Eagle Medium, *LIF* leukaemia inhibitory factor, *hLIF* human leukaemia inhibitory factor, *bFGF* basic fibroblast growth factor, *NEAA* nonessential amino acids, *FBS* foetal bovine serum, *KO* knockout, *ESC* embryonic stem cell, *VPA* velporic acid, *BFFs* buffalo foetal fibroblasts, *MEFs* mouse foetal fibroblasts, *KSR* Knockout serum replacement

reported that the use of slight different combination of transcription factors improved the quality and efficiency of generated iPS cells in livestock (Kawaguchi et al. 2015; Talluri et al. 2015; Zhao et al. 2017). Similar, findings were observed for generation of buffalo iPS cells, additional Nanog and Lin28 factors or small chemical compounds and/or expression of Simian virus 40 large T antigen (SV40 T) are required (Deng et al. 2012; Kumar et al. 2019). It is surprising that either addition of a few genes or a combination of different genes leads to improve reprogramming efficiency indicating that each transcriptional factor plays a significant role in the cellular reprogramming of somatic cells. Subsequently, transduction of mouse Oct4, Sox2, Klf4, and c-Myc using lentivirus have been used to reprogram buffalo foetal fibroblasts into iPS cells which survived more than 20 passages in culture (Bag 2016). Later on, the same laboratory attempted to enhance cellular reprogramming efficiency using valproic acid, HDAC inhibitor, in fibroblasts culture media and transfected with lentivirus containing mouse Oct4, Sox2, Klf4, and c-Myc genes, but generated iPS cells were not survived beyond 20 passages (Mahapatra et al. 2017). Previous reports suppoerted that derivation and maintenance of iPS cells from domestic animals including buffalo has a problem mostly due to lack of an optimized culture media and conditions which is available for mice and human iPS cells (Liu et al. 2012; Cebrian-Serrano et al. 2013; Kumar et al. 2020c). Till date, the generated buffalo iPS cells were cultured in DMEM/KO-DMEM, β-mercaptoethanol, L-glutamine, non-essential amino acids, knock-out serum replacement, penicillin-streptomycin, FGF, and LIF supplemented media in 5% CO_2 in air at 37 °C. Under these conditions, generated cells were only maintained for 10–18 passages (Deng et al. 2012; Bag 2016; Mahapatra et al. 2017; Kumar et al. 2019). However, using similar culture media and conditions, bovine iPS cells were able to survive more than forty passages (Talluri et al. 2015), indicating the species specific requirement of cells in culture to maintain their pluripotency. Recent, report emphases that the protocols optimized for mouse and human somatic cell iPS cell generation do not effectively apply to bovine iPS cells, which display certain refractoriness to reprogramming that also influences sustenance (Pillai et al. 2019).

Research on the optimization of in vitro culture conditions for self-renewal and long-term proliferation of buffalo iPS cells should therefore be taken into account in future. A recent study provides a clue to optimize time period required to re-initiate reprogramming events in fibroblasts after introduction of exogenous genes which is time dependent events require specific culture conditions during reprogramming for efficient generation of iPS cells (Rawat et al. 2019). More recently, in hypoxic culture conditions (5% CO_2, 5% O_2, and 90% N_2 at 37 °C), adipose tissue-derived mesenchymal stem cells were reprogrammed efficiently and easily into iPS cells showed partial features of naive pluripotent stem cells such as packed dome morphology, active X chromosomes in female cells, packed colony formation after single-cell dissociation, dependency on LIF pathways, and expressed pluripotent markers OCT4, NANOG, REX 1, TRA-1–81, SSEA-1, SSEA-4, and KLF4 using pMX retroviral plasmids comprising mouse Sox2, Klf4, Oct4, and c-Myc (Deng et al. 2019). Commonly viral (lenti- or retro-) transduction of transcription factors has been used for derivation of iPS cells across the species including buffalo, but

associated with significant risks of genotoxicity and insertional mutagenesis, although having good reprogramming efficiency (Okita et al. 2007). It compels to use alternatives like non-integrating adenoviral vectors, plasmids, small molecules, recombinant proteins, and modified mRNAs, for derivation of iPS cell, but these approaches also have drawbacks like reduced kinetics rate, incomplete reprogramming, permanent genome modification, lack of reproducibility, and low efficiency (Ogorevc et al. 2016; Haridhasapavalan et al. 2019). Hence, DNA transposon systems extant promising alternatives to it above described strategies (Kumar et al. 2015b; Kumar et al. 2020a). In this direction, transposon mediated delivery of Oct4, Sox2, Klf4, cMyc, Nanog, and Lin28 resulted into generation of buffalo iPS cells from fibroblasts and maintained in feeder free culture system (Kumar et al. 2019). The generated buffalo iPS cells exhibited alike characteristics of the ES cells, such as morphological appearance, expression of ALP activity, and pluripotency related surface markers like Oct4, Nanog, SSEA-1, TRA-1–60, and TRA-1–81 and transcription based genes; Oct4, Sox2, Nanog, C-Myc, KLF4, and FoxD3, these cells showed normal chromosome number (Deng et al. 2012; Bag 2016; Mahapatra et al. 2017; Kumar et al. 2019; Rawat et al. 2019). These cells formed embryoid bodies in in vitro suspension culture which exhibited lineage specific marker expression indicating three germ layers (Deng et al. 2012; Bag 2016; Mahapatra et al. 2017; Rawat et al. 2019; Kumar et al. 2019) and also formed teratoma in in vivo condition (Deng et al. 2012, 2019), results represent advancements of iPS cells and showed an important step in the understanding of mechanistic nature of pluripotency in buffalo. All the information taken together, more studies are required to generate long term propagating stable buffalo iPS cells which could encourage the genetic alteration of buffalo genome and improve the production of transgenic animals through genetically modified iPS cells.

12.4 Translational Application of Buffalo Pluripotent Stem Cells

The pluripotent stem cells including ES and iPS cells derived from buffalo could be considered as valuable raw biomaterial for cell-based therapy, disease modelling, drug testing, breed conservation, and in vitro meat production due to its inherent properties of self-renewal and differentiation. A recent issue concerning animal health and rights has illustrated the importance of in vitro models (Andersen and Winter 2019) and now farm animals considered as most suitable for biomedical research (Andersson 2016; Selokar and Kues 2018; Hamernik 2019). Traditionally, laboratory animals (rodent) are used as animal models, due to their phylogenetic proximity to humans (Schroeder and DiPersio 2011). However, in rodent models, cell therapies do not always predict the actual response as it happens in human diseases due to their small size, limited life span, and high degree of inbreeding (Kehinde 2013; Ernst 2016). Therefore, large animal models including buffalo represent an extremely valuable potential biomedical model because of their similarity in anatomy, physiology, and health status to human being (Stanton et al. 2019; Cong et al. 2019; Kumar et al. 2020c). Recently, the sequence and analysis of water

buffalo genome have been completed (Low et al. 2019; Dutta et al. 2020). These findings will issue new identification of buffalo through genome manipulation and able to produce humanized milk and meat and also assist in exploring the possibility of trait based transgenesis and could be established as a model for the development of new cell-based therapies (Singh et al. 2020). Previous studies suggested that histological and ultra-structural characteristics of vitiligo skin of buffalo were found resemblance with human skin (Cerundolo et al. 1993; Singh et al. 2016). Vitiligo is a disease in which the skin loses its pigment cells (melanocytes), resulted into appearance of discolouration patches in different areas of the body, including the hair, skin, and mucous membranes. This indicates that buffalo could be a potential model to study human skin depigmentation disorder and know-how the etio-pathology of human skin vitiligo disease. However, the availability of pluripotent stem cells from buffalo may lead to develop as alternative animal models and contributed to the translation to the human clinic. Furthermore, pluripotent stem cells derived from the species could improve efficiency and reduce costs in various fields, such as transgenic animal production and drug development, conservation of biological diversity, and in vitro meat production. For the first time, produced meat in vitro using bovine stem cells offer the clean and alternative to slaughtering animals (Slade 2018).

In this regards, production of buffalo meat using pluripotent stem cells would be beneficial for health point of view due to the presence of more iron, vitamins, minerals, and protein and less fat than cattle meat (Tamburrano et al. 2019). The in vitro high-quality meat production depends on the types of cells; source of ingredients and its composition. Among cells, myoblast or satellite cells, and pluripotent stem cells are most important due to its differentiation ability (Arshad et al. 2017). Several studies demonstrated that the buffalo ES cells have ability to different into cells of skeletal myogenic lineage (Singh et al. 2013; Zandi et al. 2014). Apart from that many studies showed that buffalo ES cells differentiated into hepatocyte-like cells, neuron-like cells, and epithelial-like cells indicate the suitability of the species for regenerative studies (Singh et al. 2012; Zandi et al. 2014). Theoretically, pluripotent stem cells can be differentiated into any type of cells, but differentiation into functional germ cells is a long-standing goal of the researchers. The ultimate application of the generated germ cells will be the production of sexually recombined genotypes through IVF that improve the breeding progress in animals and help to treat infertility problems (Goszczynski et al. 2019). Shah et al. (2017) revealed that under appropriate in vitro culture conditions, buffalo ES cells are able to differentiate into oocytes as well as spermatocytes. Such types of studies would be helpful for understanding biochemistry, epigenetics, and genetics of spermatogenesis and gametogenesis for the production of designer gametes and that improve the breeding scheme. These studies describe several novel uses of buffalo to gain a better understanding of the mechanisms underlying human diseases. Hence, the generation of pluripotent buffalo stem cells will likely serve as a bridge between well-established rodents and poorly defined human pluripotent cells, facilitating the translation from experimental studies to curative treatments of ground-breaking cell therapies. Furthermore, development of new technologies will likely allow using of buffalo as additional biomedical models in the future.

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Domesticated Buffalo: A Model for Human Biomedical Research

13

Vijay Pal Singh and Sujoy Khanna

Abstract

Domesticated buffalo (*Bubalus bubalis*) since its domestication in the initial Neolithic period have proved to be very economical and multipurpose animal amongst other domesticated livestock species. Buffaloes have played a major role in various techniques related to assisted reproduction like editing of genome, genetic engineering, cryo-conservation of embryos and sperm, embryo transfer, somatic cell nuclear transfer, in vitro fertilization, etc. Over recent years, stem cell biology has made quick progress in buffalo species. Bubaline extra-embryonic stem cells in addition to embryonic stem cells have become the point of interest of the scientific community, leading to the establishment of different sources of bubaline extra-embryonic stem cells. Notably, therapeutic regenerative medicine and experimental biology may gain a lot from the success achieved in bubaline extra-embryonic stem cells. The utility of multipotent non-embryonic stem cells in basic and applied research is also currently investigated. Bubaline extra-embryonic cells along with buffalo amniotic mesenchymal stem cells might serve as valuable assets for further improvement in assisted reproduction.

Keywords

Domesticated buffaloes · Stem cells · Extra-embryonic · Vitiligo

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13.1 Domestication of Buffaloes

Ever since the domestication of the first animal on this planet, humans have tirelessly tried to change the gene pools of different species by selection and domestication. Those animals were selected that could acclimate better and reproduce in confinement, provide traction for agricultural activities, transportation and other essential goods to humans such as milk, meat, skin, fur and companionship. Likewise, domestication of buffaloes was an evolutionary process making the animals fit to contribute to the development of mankind (Ahmad et al. 2020).Buffaloes were domesticated during the agronomical surge of the initial Neolithic period, approximately dating back to 12,000–14,000 years, when the humans have just started cultivating major crops (Wang et al. 2014). Contrarily, modern breeding in buffaloes started just 300 years ago. Breeding animals were selected based on the demand by humans (Crowley and Adelman 1998). Controlled breeding and continual practices related to animal husbandry resulted in morphological and behavioural differences of domesticated buffaloes from their wild correlatives, leading to adaptation of different bubaline species to agricultural environment of that time. In this process of domestication, taurine cattle were domesticated first, followed by swamp and riverine buffaloes (Barker 2014). It was a multistage and complex process that led to alteration of behaviour, morphology and physiology of the domesticated animals in relation to their wild ancestors (Trut et al. 2009). In this domestication process, gene variants with favourable phenotypic effects were enriched.

13.2 Farm Animals as Animal Research Model

Farm animals along with rodents and nonhuman primates are validated model animals used for testing various procedures of surgery, pain management drugs, treatment of blood diseases and shock trauma, gene therapy, cancer treatment, stem cell therapy, tissue transplant, organ transplant, etc. (Selokar and Kues 2018). Rodents are the most easily available and economical animal model with certain limitations. They have a very short gestation period (Madeja et al. 2019). Inflammatory and pharmacological reactions in rodents also differ distinctively, since the rodent metabolism is much agile in comparison to humans. Thus, they mimic poor proteomic response to the stress caused by inflammatory and pharmacological reactions (Seok et al. 2013). Nonhuman primates are physiologically and pathophysiological closer to humans; but their usefulness is limited by their limited availability, high cost, ethical issues and need for sophisticated equipment for experimentation (Havel et al. 2017). Farm animals play a significant role as research models. Their average size is larger than the laboratory species, and larger volumes of blood can be collected for analysis. Frequency of sample blood collection can also be high with no significant changes in the blood biochemistry and tissue biopsies can be carried out more frequent. This enables the study of changes in metabolites, hormones, cellular components or immune factors to be conducted in same animal over the period of time. The physiology as well as genome sequence of farm animals

is more meticulously related to that of humans than to the rodent species (Ireland et al. 2008; Chu et al. 2010). Thus, they may be preferred models for carrying out study on many genetic diseases of humans.

Farm animals have a major contribution in advanced as well as basic biomedical research of stem cell biology, reproduction and translational research for humans (Humphray et al. 2007; Tellam et al. 2009). The diversity in farm animals plays a major factor in these research fields in various species, and also helps in identifying conserved and species-specific mechanisms. The long history of their strong phenotypic selection makes them unique models for the biomedical research. The novel mutations contribute to this phenotypic evolution which resulted from thousands of years of selection of these farm animals. Characterization of such novel mutations also provides an insight of various biological mechanisms and gene functions of species. Deletions, duplications and inversions cause structural changes in the DNA contributing significantly to evolution of the phenotypic diversity in various domestic animals (Andersson 2016).

13.3 Domesticated Buffalo as Animal Research Model

Domesticated buffalo (Bubalus bubalis) was traditionally bred in Asia for milk, meat, agricultural and transportation traction purposes (Singh et al. 2016a, 2009). It has recently emerged as an unconventional animal research model, which is now bred worldwide (Singh et al. 2016a), gaining considerable interest of scientific community as a large animal research model for basic and applied sciences, and particularly in stem cells research, potential translational approaches and developmental phenomena (Singh et al. 2009; Yadav et al. 2012; Ghosh et al. 2016). In order to meet the growing demand of production, its conservation and disseminating valuable germplasm of buffalo, several Assisted Reproduction Techniques (ARTs) such as editing of genome, genetic engineering, cryo-conservation of embryos and sperm, embryo transfer, somatic cell nuclear transfer, in vitro fertilization, etc. have successfully been developed.

Sequencing of the buffalo genome provided valuable information for developing various strategies for improvement of breed, and may prove to be an important asset in studies related to biomedical and veterinary sciences. Scientists across the globe are constantly working towards creating an understanding regarding the extra-embryonic stem cells, transgenic buffaloes, buffalo milk, bubaline placenta, and may be extremely useful in genetic engineering, nuclear transfer cloning and regenerative medicine (Singh et al. 2020).

13.3.1 Role of Buffalo in Stem Cell Research

Stem cells research plays an important component for treatment of cancer, autoimmune, degenerative and genetic disorders of humans (Marmotti et al. 2017; Blau and Daley 2019). They play a major role in human regenerative medicine as well as in

regenerative medicine and veterinary genetic engineering (Yadav et al. 2012; Garrels et al. 2012; Nazari et al. 2016). Buffaloes or other farm animals mimic human disease phenotypes better than rodents, making them a better model for research (Vandamme 2014). Injuries related to bone, ligaments and tendons are also very common in farm animals. Hence, they could be explored as a model for these pathologies of humans (Yadav et al. 2012; Marmotti et al. 2017; Plews et al. 2012). The stem cells technology has varied applications in large ruminants' research. In future, it may open up new ways to model diseases of humans in buffaloes as well as for carrying out investigation of basic ontogenetic mechanisms. After the first study of bubaline embryonic stem cells (ESCs) derived from IVF-derived embryos (Verma et al. 2007), various related studies have revealed the diverse nature of stem cells in this species (Sritnaudomchai et al. 2007; Zandi et al. 2014; Ghosh et al. 2015; Deng et al. 2018; Kumar et al. 2018).

13.3.1.1 Extra-Embryonic Stem Cells

Extra-embryonic stem cells are of great use in regenerative medicines of humans as well as animals. Reprogramming of these versatile cells can be carried out efficiently for differentiation into different tissue types such as bones, muscles, skin and hepatic tissues. Non-embryonic mesenchymal stem cells (MSCs) could potentially make it possible to preserve as well as tweak the genetic potential of various livestock species. Reprogramming can be carried out using fibroblasts and epithelial cells obtained from buffalo foetuses (Moraghebi et al. 2017). Foetal adnexa is a reliable source of multipotent stem cells in case of buffaloes. The capacity to reprogramme in foetal stem cells is displayed by using these as donor nuclei to produce clones of buffalo embryos (Em et al. 2016a, b; Sadeesh et al. 2016). Cord blood stem cells, buffalo amniotic mesenchymal stem cells (bAMSCs), and Wharton's jelly stem cells have stem cell-like features. In vitro steady proliferation of the bAMSCs have fibroblastic-like morphology. They express MSC specific pluripotent markers and stemness markers. bAMSCs are very useful as cell resource for production of transgenic donor cells for generation cloned transgenic animals and other genome reprogramming studies, because of their ability to differentiate into osteogenic, chondrogenic, adipogenic and neural lineages (Deng et al. 2018).

The structure of cells in amniotic fluid of buffaloes is heterogeneous in nature and has the similar abilities to differentiate into osteoblasts, adipocytes, and fibroblasts-like different cell lineages (Dev et al. 2012a, b). Buffaloes have a variety of multipotent stem cell-like cells which are heterogeneous in nature (Ghosh et al. 2015). The epithelial cells of buffalo derived from amniotic membrane, also known as amniotic epithelial cells (AECs).They have a polygonal shape and are isolated from the placenta. In vitro differentiation of AECs into osteogenic, chondrogenic and adipogenic lineages could also be induced (Ghosh et al. 2015).

13.3.1.2 Reprogrammed Stem Cells

The somatic cells are reprogrammed to pluripotency by transducing the fibroblasts with specific reprogramming factors (Takahashi and Yamanaka 2006). It has potential to open up various innovative therapeutic approaches. The technology to

transform the somatic cells into pluripotent stem cells has raised the hopes towards research and development in the field of regenerative and personalized medicine (Roberts et al. 2009; Talug and Tokcaer-Keskin 2019; Yumlu et al. 2019). The risk of mutations and genetic modifications is an impediment associated with this method (Kumar et al. 2018). Epigenetic modifications, epigenetic state of target somatic cells, pluripotency inhibition, cell senescence and micro RNAs are major obstacle towards successful induced pluripotency (Haridhasapavalan et al. 2020). However, development of optimized reprogramming methods leads to minimal effect on the genome along with generating iPSCs for clinical as well as veterinary applications (Liu et al. 2020). Genetically modified animals for research and production of recombinant drugs may be developed using reprogrammed stem cells technology. (Lu et al. 2018; Semak et al. 2019).

13.3.2 Transgenic Buffaloes and Their Prospects

Genome editors are used to modify or edit the genome of buffalo in order to produce transgenic animals. It may be used to study various biological, genetic and developmental phenomena (Schatten and Mitalipov 2009). In addition, they might also act as live bioreactors for production of recombinant therapeutic proteins (Bertolini et al. 2016) and as model experimental animal for studies related to xenotransplantation. (Singh et al. 2019).The transgenic embryos are generated through microinjection of gene constructs into the zygotes (Bosch et al. 2015). Transfer of these embryos to the synchronized surrogate animals is then carried out for production of genetically engineered offsprings (Meng et al. 2015; Mehta et al. 2019). and modern clinical investigations also make use of the genetic modifications, genome-editing of EESCs and transcriptional analyses in the corpus luteum of bubaline placenta (Paul et al. 2019).

13.3.3 Buffalo Milk

Buffalo milk serves as the bioreactors for producing recombinant proteins because of the high fat and protein contents. The mammary glands of buffaloes are a reliable source for large-scale synthesis of human insulin. The promising results are shown by cloning of transgenic embryos from foetal fibroblasts of buffalo which have the capacity to express the human insulin gene under bubaline b-lactalbumin promoter (Mehta et al. 2017).

13.3.4 Bubaline Placenta

Cotyledonary placenta of domesticated buffalo consists of parenchyma which is rich in extracellular matrix. It favours the development of embryo and foetus (Schmidt et al. 2006). Histo-morphology, reproductive physiology and anatomy of the uterus

of buffalo species differs from that of cattle and African buffalo (Schmidt et al. 2006). The endometrium of domesticated buffaloes consists of ovoid or rounded, gland-less and dome shaped caruncles. The buffalo placenta meal so possesses slightly conical villi branching and also does not develop caruncular stalk (Schmidt et al. 2006).

13.3.5 Animal Research Model of Vitiligo

Vitiligo is a spontaneously acquired, multifactorial, depigmenting disorder. Most of the vitiligo animal models are either induced or genetically programmed while in case of humans, there is a spontaneous loss of functional melanocytes triggered by multiple etiological factors. Bubaline cells are made to acquire depigmentation to recapitulate histological, molecular, immunohistochemical and ultrastructural changes as is observed in human vitiligo, and therefore serves as the best model to study vitiligo pathogenesis facilitating the development and discovery of efficient therapies for its treatment (Singh et al. 2016b). Buffalo may also be used as an animal model for development and testing of the topical medications, surgical transplants and other pharmaceuticals to alleviate the vitiligo (Singh et al. 2016b).

13.4 Challenges

During recent years, stem cell applications in human as well as veterinary regenerative medicine have increased manifold. The use of domesticated buffaloes for in vitro cellular proliferation and stem cells technology have increased manifold. Intense further studies are further required for better development of genetically modified stem cells and their differentiation into other types of cell. In future buffalo will surely attract massive attraction of researchers and scientific community because of its genetic capability to adapt to various climatic zones, resistance to diseases and climatic stress and its genetic proximity to human genome.

Since its domestication, buffalo has always been an important species for agricultural and livestock farming. It may be concluded that the role of buffalo is not only limited to milk and meat production but may prove to be a model for better understanding of cell differentiation, cellular reprogramming, and for the development of new cell-based therapies for human and veterinary medicine.

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Part III

Reproductive Biotechnologies

Advances in Embryo Production in Buffaloes: In Vivo Versus In Vitro Procedures

14

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Abstract

The popularity of buffalo is increasing day by day due to its better quality of milk, meat, and powerful body structure. These characteristics make this species an economically important animal. Maximum diffusion of genetically superior dams and sires can be possible by using reproductive biotechniques. Given the low rate of obtaining embryos by MOET (multiple ovulation and embryo transfer), the combination of ovum pickup (OPU) with in vitro embryos production (IVEP) in buffalo is the best alternative for the genetic improvement of the herd and to increase the productive indices. The OPU-IVEP in buffalo reached the commercial stage through continuous scientific efforts but the cost of production is still high. In this chapter, we discuss the advances in the production of in vivo and in vitro buffalo embryos.

Keywords

Buffalo · Embryo · MOET · Ovum pickup

Otávio Mitio Ohashi was deceased at the time of publication.

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14.1 Introduction

The first buffalo male calf, named Herman, was born as the result of an embryo transfer (ET) back in 1983 (Drost et al. 1983). To produce the Herman, a 7-day blastocyst was nonsurgically collected and transferred to a recipient buffalo. This fact caused some surprise because the technique of ET was used in the USA, a country with no tradition of buffalo breeding. Furthermore, at that time, the knowledge about estrus detection, follicular dynamics, follicular wave emergence, and super stimulatory protocols using gonadotrophins—FSH, eCG (PMSG) was scarce. Also, buffaloes were known to have smaller ovaries, a lower number of primordial follicles as well as a higher rate of follicular atresia as compared with *Bos taurus* and *Bos indicus* cow (Vale et al. 1982; Danell 1987). Danell (1987) reported 12,636 and 10,132 primordial follicles in cyclic and noncyclic buffalo heifers, respectively; whereas a report of Carvalho et al. (2007) found a mean number of 15,449 morphologically normal preantral follicles per ovary in prepubertal, pregnant, and nonpregnant buffaloes. Thus, the evaluation of follicular dynamics, superovulation response, and embryo recovery following super stimulatory treatment initiated at estradiol-17β induced follicular wave emergence and its comparison with conventional super stimulatory protocol in buffaloes need a basic knowledge for the use of ET in buffaloes (Carvalho et al. 2011).

Misra et al. (1990) conducted a study to identify the most effective dosing level of FSH for superovulation in buffaloes. Eighty-three buffaloes were treated with prostaglandin. A functional corpus luteum (CL) was palpated in only 73 buffaloes one day before superovulation treatment was initiated. Eight treatments were used in buffalo: Protocol I ($n = 8$), 9 mg Folltropin (Porcine Pituitary Extract) on Days 9–12 of the cycle; Protocol II ($n = 10$), 18 mg Folltropin on Days 9–12 of the cycle; Protocol III ($n = 9$), 18 mg Folltropin on days 13–15 of the cycle; Protocol IV ($n = 9$) 21.6 mg Folltropin on days 9–12 of the cycle; Protocol V ($n = 9$) 21.6 mg Folltropin with GnRH on days 9–12 of the cycle; Protocol VI ($n = 10$) 25.2 mg Folltropin on days 9–12 of the cycle; Protocol VII ($n = 9$) 28.8 mg Folltropin on days 9–12 of the cycle; Protocol VIII ($n = 9$) 36 mg Folltropin on days 9–12 of the cycle. The highest ovulation rate was observed in Protocol VI ($x = 5.3 \pm 0.79$), which is significantly higher ($P < 0.01$) than in other protocols. Maximum embryos ($x = 3.7$) were recovered using Protocol III. Whereas the largest number of transferable embryos ($x = 2.2$) was recovered from Protocol V. The use of GnRH and superovulation treatment on days 13–15 has no advantageous effect on the ovulation rate. Overall, 41 embryos were transferred to 35 recipients: nine buffaloes became pregnant; 59 embryos were frozen; 12 were thawed; nine good frozen embryos were transferred to eight recipients, three of which were diagnosed pregnant.

14.2 In Vivo Embryo Production

14.2.1 MOET

Multiple ovulation and embryo transfer (MOET) is one of the repro-biotechnologies, which is utilized in the world to produce a high number of in vivo embryos (Neglia and Bifulco 2017). MOET optimizes the female contribution to genetic progress and it increases genetic gain by 63 to 70% per year from juvenile and adult buffalo compared to progeny testing (Gandhi and Singh 1994). In the buffalo species, the application of this technology meets several difficulties, and the embryo recovery rate is lower than that recorded in cattle (Neglia and Bifulco 2017). Buffaloes have some reproductive drawbacks include delayed puberty, higher age at first calving, long postpartum anoestrus period, long inter-calving period, poor detection of heat, and low conception rate (Misra and Tyagi 2007). In buffalo, generally, the recovery rate is 2.5–3.0 viable embryos per flushing and more than 4 in some cases (Misra and Tyagi 2007). The pregnancy rate following embryo transfer is 30–40%. Some authors reported satisfactory responses to superovulatory treatment (Baruselli 1997), while others reported poor responses. The recovery rate of embryos in buffalo is less as compared to bovine (Baruselli et al. 2000; Misra and Tyagi 2007). The other authors have also been reported a lower number of flushing embryos (Karainov 1986; Madan 1990; Drost 1996; Zicarelli 1997). In buffaloes, only 34.8% of embryos were recovered through super stimulated follicles (Baruselli et al. 2000) which is much lower than bovines (63–80%) (Adams 1994). The reason for this imparity is the failure of oocytes to enter the oviduct after super stimulation treatment (Baruselli et al. 2000). In addition, a morphometric study revealed that buffaloes have a higher number of anovulatory follicles, a more rigid ovary-mesovarium connection, and a thicker infundibulum muscle layer than cows (Baruselli et al. 2013). Therefore, all these causes make this technique less efficient in buffaloes. The in vivo embryo production involves the steps shown in Fig. 14.1.

14.2.1.1 Selection of Animals (Donors and Recipients)

The animals should be healthy, disease-free, have normal reproductive organs, routinely dewormed and vaccinated. Animals should be between second to sixth lactation. Donors must be genetically superior. At the time of selection, if buffalo has more than 10 antral follicles per ovary is desirable (Ohashi et al. 2017). It lowers the cost of embryos production because available of a greater number of follicles for aspiration. Recipients are genetically inferior and normal cyclic animals with good mothering ability.

14.2.1.2 Superovulation

The bovine superovulation protocols are used in buffalo (Singla and Madan 1990). The protocol starts on the mid-cycle of estrus (days 9–14) in the breeding season (Smith 2009). Descending doses of FSH for 4 days are used, followed by injection of prostaglandin-F2α with the seventh and eighth injection of FSH (Seidel and Seidel

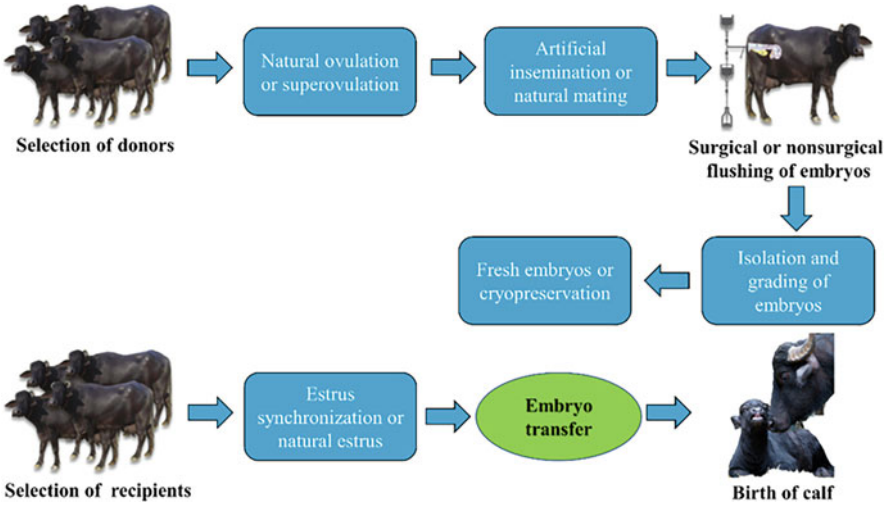
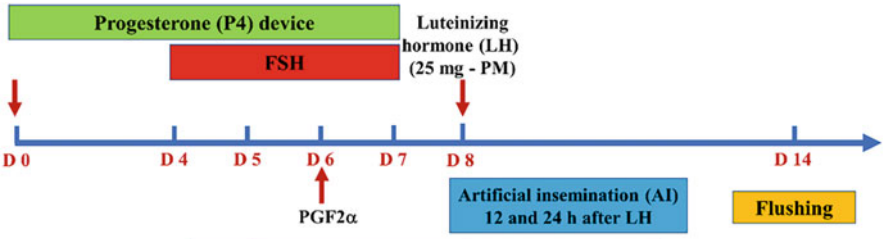


Fig. 14.1 Schematic representation of in vivo embryo production in buffaloes



Day	AM	PM
0	Insertion P4 device + estradiol benzoate (2 mg)	-
4	FSH	FSH
5	FSH	FSH
6	FSH + PGF2α	FSH + PGF2α
7	FSH	FSH + removal P4 device
8	-	LH
9	Fixed time AI	Fixed time AI
14	-	Flushing

Fig. 14.2 Different steps for estrus synchronization and superovulation procedures in buffaloes

1991). In Fig. 14.2, it is summarized the different hormonal compounds and procedures for superovulation in buffaloes.

14.2.1.3 Artificial Insemination or Natural Mating

The breeding bulls or semen straws should be of high genetic merits.

14.2.1.4 Flushing of Embryos and Transfer

The nonsurgical flushing of embryos is more commonly used and the procedure is similar to that of cattle. In buffaloes, the embryos are more rapidly developed than cattle. Therefore, it is recommended that the uterine flushing should be done on day 5 or 6 (Drost 1991) as compared to day 7 in cattle. On day 6, the flushed embryos are morula to hatched blastocyst stages (Misra 1993). After flushing, the embryos are classified and grades I and II are used for the transfer or go for cryopreservation depending upon the necessities availability of recipients.

14.2.1.5 Classification or Grading of Embryos

Quality grades or codes of bovine embryos range from 1 to 4 and the grades are nominated based on the morphological integrity of embryos (Bó and Mapletoft 2013).

Grade 1 (Excellent or Good): The embryos have spherical and symmetrical mass and with each blastomere that is uniform in color, size, and density. At least a minimum of 85% of the cellular mass of embryos must be intact and viable. The zona pellucida should be smooth. Concave or flat surfaces are not desired that may cause the embryo to adhere to a Petri dish or a straw. Grade 1 embryos are also known as "freezable embryos" because they survive well after the freezing/thawing process.

Grade 2 (Fair): These embryos have moderate irregularities in the overall shape of the embryonic mass or size, color, and density of each blastomere. At least a minimum of 50% of the cellular mass of embryos must be intact. The survivability rates of these embryos are lower than Grade 1 embryos after the freezing/thawing process. Grade 2 embryos are also known as "transferable not freezable" because adequate pregnancy rates are obtained after the transfer of these embryos as fresh.

Grade 3 (Poor): These embryos have major irregularities in the shape of the embryonic mass or size, color, and density of each blastomere. At least a minimum of 25% of the cellular mass of embryos must be intact. These embryos do not survive after the freezing/thawing process and the pregnancy rates are also lower than those obtained from Grade 2 when fresh embryos are transferred.

Grade 4 (Dead or degenerating): These structures could be embryos, oocytes, or one-cell embryos. They are nonviable and should be cast away or disapproved.

14.3 In Vitro Embryo Production

Given the low rate of obtaining embryos by MOET, the combination of ovum pick-up (OPU) with in vitro embryos production (IVEP) in buffalo is the best alternative for the genetic improvement of the herd and to increase the productive indices (Kumar et al. 2020). The oocytes recovery through OPU must be done in females of genetic superior grade as well as the in vitro fertilization (IVF) procedure must use semen of a genetic superior sire. The in vitro culture (IVC) of embryos takes 6 days when the embryo according to their quality will be transferred to recipients or frozen (Fig. 14.3). The OPU-IVEP is used commercially in buffalo but the efficiency is

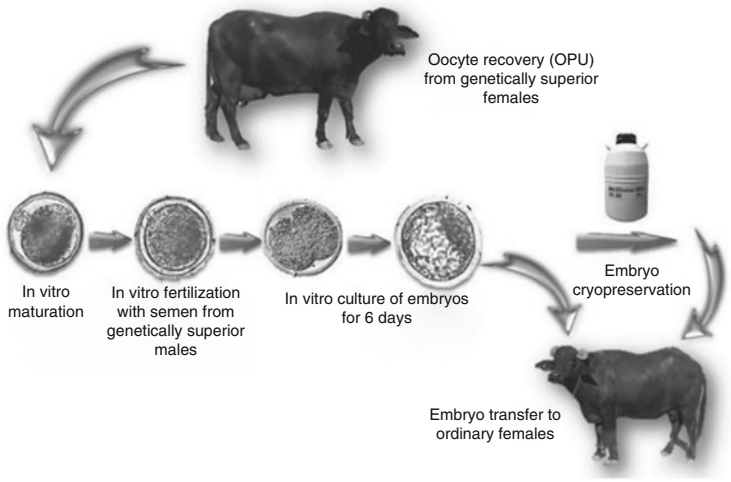


Fig. 14.3 Schematic presentation of in vitro embryo production in buffaloes (adapted from Kumar et al. 2020)

lower than bovine (Lonergan and Fair 2008). The lower efficiency in buffalo is due to two main biological problems. The first is lower numbers of available follicles for aspiration on the ovary, which results in a lower number of blastocysts. The second is buffalo oocytes have more fragile zona pellucida (Mondadori et al. 2010) and a weaker association between cumulus cells and the oocyte (Ohashi et al. 1998; Gasparrini 2002). IVEP involves a series of unified consecutive steps: (1) collection of oocytes by retrieval from abattoir-derived ovaries or by ovum pick-up from live donors; (2) selection of developmentally competent oocytes and in vitro maturation (IVM) of the selected oocytes; (3) sperm capacitation and in vitro fertilization (IVF), and (4) in vitro culture for embryo development (IVC).

14.3.1 Ovum Pick-Up (OPU)

The method of oocytes recovery is a primary and important step because the quality of oocytes directly affects the quality and quantity of embryos during IVEP (Nandi et al. 2002). However, oocytes obtained from abattoir-derived ovaries are cheaper and more in number than live animals (Das et al. 1996). For commercial purposes, live animals are the preferred source for oocytes recovery. Therefore, transvaginal ovum pick-up (TVOPU) and laparoscopic ovum pick-up (LOPU) methods to the buffalo's IVEP industry. The TVOPU is widespread in several countries, but Brazil has achieved a prominent position because of the surprising number of embryos produced by this technique in the country (Stroud and Callesen 2012). This technique needs one ultrasound with the vaginal probe, probe holder, OPU needle, suction pump, and OPU tube system (Fig. 14.4). Generally, in buffaloes, a

Fig. 14.4 TVOPU method: equipment required for TVOPU (1), A-Ultrasound equipment; B-Vaginal probe; C-Probe holders; D-Suction pump; E-OPU tube system; F-OPU needle. Follicles aspiration (2). Ultrasound image of the superstimulated ovary (3)

5–6.5 MHz vaginal probe is used. The OPU needle varies from 17 to 20 G and is connected to a vacuum pump regulated from 40 to 115 mmHg (Manjunatha et al. 2008). The follicular aspirate is collected in a tube with a recovery medium, which is kept warm in a heating block (Galli et al. 2014). The hormonal stimulation of ovaries with FSH before TVOPU has been successfully used in IVEP programs in cattle, increasing the total embryos produced per session (Vieira et al. 2014). In buffalo, Carvalho et al. (2019) was also observed greater blastocyst rates and embryo yield after the stimulation of ovaries with FSH per TVOPU session. In buffalo, the FSH increases the proportion of large and medium-sized follicles available (Carvalho et al. 2019).

However, it is not repeatable in the long term because of possible side effects (Galli et al. 2001). Baldassarre et al. (2017) conducted a study in which they used three groups of buffalo calves aged between 2 and 6 months (<120 days old, 120–150 days old, and> 150 days old) receiving two different treatments with gonadotropin stimulation. They did not observe any significant ($P > 0.05$) difference of recovered cumulus–oocyte complexes between the age and treatment groups. They aspirated a total of 903 antral follicles, obtaining an oocyte recovery rate of 85.7%.

A comparative study (Silva et al. 2017) for in vitro production of embryos in buffalo was conducted. A group of calves (2–4 months of age) was compared with prepubertal heifers (13–15 months of age) and lactating adult buffalo cows. LOPU was performed for calves and TVOPU for other groups, after hormonal stimulation protocol. Among the females categories, there was no significant statistical difference in the number of viable oocytes (calves = 7.63 ± 2.69, heifers = 6.20 ± 1.55, buffalo cows = 3.20 ± 0.90, $P = 0.1033$), cleaved structures (calves = 2.75 ± 0.86; heifers = 3.10 ± 0.67; buffalo cows = 2.10 ± 0.43, $P = 0.5492$) and embryos produced (calves = 1.00 ± 0.57; heifers = 1.50 ± 0.34; buffalo cows = 1.10 ± 0.38, $P = 0.3621$). In contrast, a significant statistical difference was observed in the total oocytes retrieved (calves = 10.88 ± 3.25; heifers = 15.50 ± 2.07; buffalo cows = 5.80 ± 1.29, $P = 0.0129$) and in structures conducted for IVC (calves = 10.38 ± 3.06; heifers = 15.30 ± 2.06; buffalo cows = 5.70 ± 1.30,

$P = 0.0110$). The aspirated follicles and oocyte recovery rates by TVOPU in different studies are presented in Table 14.1.

14.3.1.1 Grading of Oocytes

The aspirated oocytes are classified or graded (Fig. 14.5) based on surrounding cumulus cell layers and the homogeneity of ooplasm (Gordon 1995).

Grade A (1): more than three layers of compact cumulus cells and a homogeneous ooplasm.

Grade B (2): one to three layers of compact cumulus cells with homogeneous ooplasm having a coarse appearance and a darker zona pellucida.

Grade C (3): less compact cumulus or partially denuded with irregular ooplasm containing dark clusters.

Grade D (4): completely denuded oocyte or expanded cumulus cells and the ooplasm is irregular with a jelly-like matrix.

14.3.2 In Vitro Maturation (IVM)

The IVM is an important step and enables immature oocytes for in vitro fertilization (IVF). It is characterized by the resumption of meiosis, nucleus morphology changes, perivitelline space growth, and expansion of cumulus cells (Mondadori et al. 2010). The IVM rate in buffalo is approximately 80%, similar to that obtained in bovine (Santos et al. 2002; Hammam et al. 2010). However, the maturation rate is 40–50% in female buffalo of 2–6 months of age, which is lower than those reported in adult animals (Baldassarre et al. 2017). The maturation rate and embryonic development following IVF are influenced by the culture medium, protein supplements, and hormones (Mahmoud and El-Naby 2013). The different culture media have been tested for IVM of buffalo oocytes, such as Ham's F-10 (Totey et al. 1993a), minimum essential medium—MEM (Ravindranatha et al. 2001), and TCM199 (Pandey et al. 2010). However, TCM199 gives better results may be due to the presence of essential amino acids and glutamine that stimulate DNA and RNA synthesis and enhance cell division (Gordon 2003). Schroeder et al. (1990) revealed that the addition of fetal calf serum in the IVM media improves the fertilization capacity of oocytes because it contains fetuin, a major glycoprotein that can prevent hardening of the zona pellucida during IVM. The hormones supplemented with IVM media are follicle-stimulating hormone (Hegab et al. 2009), equine chorionic gonadotrophin (Gupta et al. 2001), luteinizing hormone, and estradiol (Nandi et al. 2002).

The time required for in vitro nuclear maturation i.e., arrival to metaphase II (MII) stage, is from 18 to 24 h (Gasparrini et al. 2008). Furthermore, an increase in the duration of IVM is related to a decrease in blastocyst rates (Oba and Camargo 2011). Thus, a maturation period of more than 24 hours may result in improper chromatin configuration, oocyte aging, and a decrease in oocyte capacity for growth (Kumar and Anand 2012). Moreover, heat stress affects the reproductive efficiency of buffalo in vivo (Vale et al. 2019) as well as in vitro (El-Sayed et al. 2018). The quality and quantity of aspirated oocytes are decreased during heat stress (Nandi

Table 14.1 Oocyte recovery rates by transvaginal ovum pick-up and subsequent cleavage/blastocyst rates obtained by in vitro embryo production in buffaloes (adapted from Kumar et al. 2020)

Aspirated follicles[a]	Recovered oocytes[a]	%	Aspiration frequency	Cleavage rate (%)	Blastocyst rate (%)	Blastocyst/buffalo/session	References
6.8 ± 0.3	4.1 ± 0.5	57.7	Twice a week (control)	41.7	26.0	1.2 ± 0.2	Sá Filho et al. (2009)
9.1 ± 0.6	5.2 ± 0.5	54.5	Twice a week (bovine GH)	46.2	19.7	1.3 ± 0.6	
4.8 ± 0.3	2.3 ± 0.1	53.6	Twice a week (mid-winter)	52.7	8.6	–	Di Francesco et al. (2012)
4.7 ± 0.2	2.2 ± 0.1	49.3	Twice a week (spring–summer)	59.8	6.4	–	
4.2 ± 0.2	2.2 ± 0.2	57.4	Twice a week (autumn)	65.6	23.2	–	
5.3 ± 0.2	2.7 ± 0.2	50.0	–	53.5	11.5	0.3 ± 0.1	Gasparrini et al. (2014)
14.8 ± 0.8	9.9 ± 0.6	73.6[b]	14-day interval	32.7	19.5	1.7 ± 0.4	Ferraz et al. (2015)
11.3 ± 0.5	7.6 ± 0.4	69.3[b]	7-day interval	33.4	18.6	1.3 ± 0.2	
17.3 ± 1.0	10.1 ± 0.7	58.5[b]	14-day interval + bovine GH	26.0	13.4	0.8 ± 0.2	
14.3 ± 0.6	9.3 ± 0.4	67.4[b]	7-day interval + bovine GH	35.1	9.6	0.7 ± 0.1	
273 (total)	4.5 ± 0.5[b]	51.0[b]	14-day interval	64.0[b]	28.0[b]	–	Konrad et al. (2017)
266 (total)	2.8 ± 0.5[b]	31.5[b]	7-day interval	44.0[b]	6.0[b]	–	
13.5 ± 5.6	10.2 ± 6.5	0	7-day interval	–	21.8[c] 23.0[c] 17.0[c]		Marin et al. (2019a)

[a]Buffalo/session
[b]$P < 0.05$
[c]Bull 1, 2 and 3, respectively

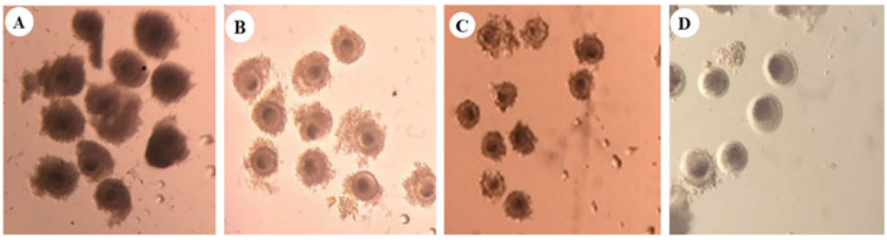


Fig. 14.5 Classification of buffalo oocytes; A (grade 1), B (grade 2), C (grade 3), and D (grade 4)

et al. 2001). The capability of oocytes to mature in vitro is decreased by high ambient temperature and humidity (Zoheir et al. 2007; El-Sayed et al. 2018). The IVM is affected by the size of follicles from which oocytes are aspirated (Yousaf and Chohan 2003). The same authors observed 32 and 32.7% IVM rates for oocytes aspirated from 2–3 mm and from 3–4 mm follicles, which were significantly lower than 67.1 and 79.1%, for oocytes aspirated from 4–6 mm and 6–8 mm follicles, respectively. The presence of cumulus cells plays an important role in vitro cytoplasmic and nuclear maturation of oocytes (Mahmoud et al. 2010). They prevent the hardening of the zona pellucida, produces cytoplasmic maturation factors, provide energy for oocyte maturation, and help in fertilization (Tanghe et al. 2002).

14.3.3 In Vitro Fertilization (IVF)

For IVF, frozen semen can be used because it assures the homogeneity of the experiment and commercial desired. The cleavage rate is lower in buffalo (~ 45–50%) (Suresh et al. 2009) than bovine (~ 70%) (Sales et al. 2015) after IVF. In buffalo, different studies revealed that frozen semen used from the different bulls may be a responsible factor for the variation of the cleavage rates (Aoyagi et al. 1988; Shi et al. 1990; Galli et al. 2001). In contrast, Marin et al. (2019a) did not observe any significant difference by using frozen semen from two different buffalo bulls. In addition, buffalo bull semen is also influenced by the variation of the season (Gupta et al. 1978; Manik and Mudgal 1984). The buffalo bull semen has a higher content of calcium and phosphatases, and lower content of citric acid, hyaluronidase, and fructose, than bovine semen (Agarwal and Tomer 1998). All these factors make the freezability and fertility of buffalo semen poor as compared to bovine bull semen (Muer et al. 1988; Andrabi 2009).

The protocols for the preparation of buffalo bull semen for IVF are the same as those used for bovine (Fig. 14.6), including washing and capacitation (Parrish 2014). Most of the described protocols have used heparin for in vitro sperm capacitation (Totey et al. 1993b). However, alternative substances such as nitric oxide donors (Jagan Mohanarao and Atreja 2012), methyl-β-cyclodextrin (Gasparrini et al. 2014), or osteopontin (Boccia et al. 2013) have shown potentially useful results. In buffalo, heparin is used at the concentration of 10 μg/ml (Totey et al. 1992; Chauhan et al.

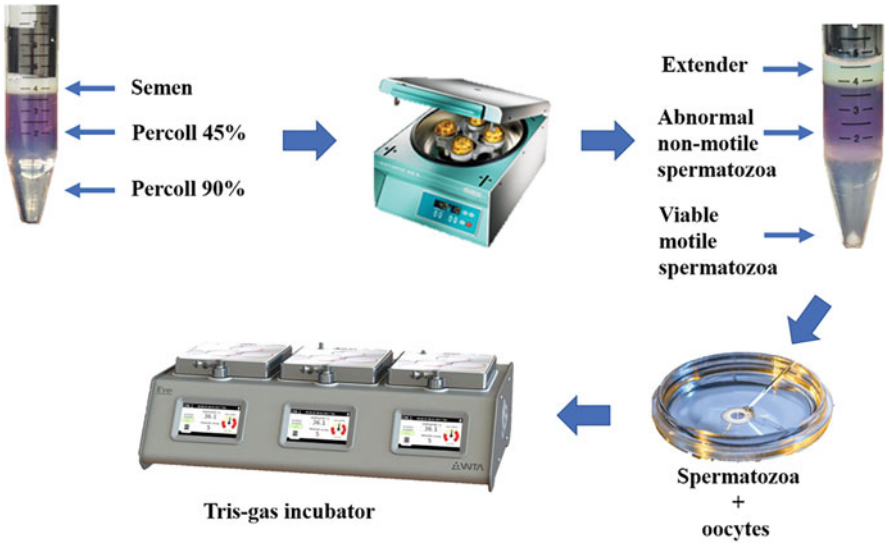


Fig. 14.6 The different steps for buffalo semen manipulation before in vitro fertilization

1997). Sperms can be preincubated with heparin (Kumar et al. 1994; Pal and Dhanda 1994) for a time of 4– 6 h (Chauhan et al. 1997, 1999; Nandi et al. 1998) or added directly to the fertilization medium (Totey et al. 1992, 1996).

The incubation time and sperm concentration should be adequate to obtain better results during IVF (Marin et al. 2019b). Sufficient incubation time is necessary to enable correct induction of plasma membrane vesiculation and complete acrosomal reaction. The recommended concentration of sperms for IVF is two million sperms/ml (Totey et al. 1992), however, 0.7 million sperms/ml (Chungsoongneon and Kamonpatana 1991) and eight to ten million sperms/ml (Nandi et al. 1998) have also been reported. A minimum time for co-incubation of 4–6 hrs is proposed. However, a better blastocyst rate was observed when this co-incubation time was extended at least by 16 hours (Kumar and Anand 2012). It was reported that if the incubation time is greater than 20 h, increased the incidence of polyspermy that reduced the blastocyst production rates (Gasparrini et al. 2008). The cleavage rates after IVF from different studies are presented in Table 14.1.

14.3.4 In Vitro Culture (IVC)

For IVC, various culture media have been tested such as culture in sheep oviduct (Galli et al. 2001), somatic cell coculture systems (Dantas 2002), culture media supplemented with blood serum, or semi-defined media supplemented with bovine serum albumin (Wadhwa et al. 2009), and recently, synthetic oviductal fluid medium supplemented with 5% fetal bovine serum (Marin et al. 2019a). However, irrespective of the media used, the average blastocyst rate is 22% (Suresh et al.

2009; Baruselli et al. 2018), which is lower as compared to cattle (~ 40%) (Sales et al. 2015). The blastocyst rates in IVEP studies are presented in Table 14.1. Insufficient information about the metabolic and biochemical needs of buffalo embryos is a major challenge to developing culture media suitable for this species. Gasparrini (2013) stated that the improvement in blastocyst production rates may be mainly due to changes in IVM and IVF systems, rather than changes during the IVC period. In addition, buffalo embryos develop 12–24 h more rapidly than bovine embryos in both in vivo and in vitro conditions, indicating that there are specific features of metabolism in this species (Galli et al. 2001).

Preis et al. (2005) were reported that supplementing glucose to the culture medium plays an important role in oocyte and embryo culture in many species. Studies revealed that during earlier culture stages of buffalo embryos require sufficient concentration of glucose for proper development (Suárez Novoa et al. 2011; Kumar and Anand 2012). Around day 4 of culture, buffalo embryos require relatively high glucose concentrations (1.5 mM), while a decreased concentration or even absence of glucose in the late culture stages did not show any deleterious effects (Gasparrini 2013). Furthermore, Kumar and Anand (2012) observed encouraging results when the culture media was supplemented with high glucose concentrations (5.6 mM) during IVM and IVC in buffalo.

14.4 Conclusion

In buffalo, various factors affect the success rate of IVEP such as (1) age, body condition scoring, and health condition of donors; (2) retrieved quality oocytes; (3) pH and osmolarity of the IVC medium, (4) IVC conditions (temperature, humidity, and gas). These factors directly affect the in vitro maturation, cleavage, blastocyst rate, and quality of embryos. The pH range of the IVC medium must be 7.1–7.4. The osmolarity of the IVC medium should be 275–285 mOsm. If the osmolarity is higher than these ranges, oocytes and embryos will shrink or vice versa. Both the situations are detrimental for oocytes and embryos. During all the in vitro process handling or manipulation of oocytes or embryos under the laminar airflow should be fast and temperature should be maintained at 37 °C. Ultraviolet rays, pesticides, the smell of detergents and perfumes are also detrimental for oocytes and embryos.

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Application of Fixed-Time Artificial Insemination in Water Buffaloes

15

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Abstract

To date, no reproductive biotechnology has had such a massive and significant impact on herds' genetic progress and technological transformation as Artificial Insemination (AI). Nonetheless, the water buffalo is characterized by a low intensity of estrus signs, affecting the detection of animals in estrus, compromising the success of conventional artificial insemination on a large scale, thus, limiting genetic progress through this technique. Furthermore, low reproductive performance in water buffalo has also been described, mainly attributed to a late onset of puberty, reproductive seasonality, long calving intervals, and estrus' low expression. Therefore, several protocols have been developed for fixed-Time Artificial Insemination (TAI) to improve water buffalo's reproductive performance and omit the need for heat detection. These hormonal treatments allow controlling follicular dynamics and luteal function, synchronizing estrus and ovulation, and, most importantly, avoiding the complicated detection of estrus in this species. Herein, we provide the basics for fixed-Time Artificial Insemination (TAI) application in water buffaloes, including the fundamental knowledge and aspects of applying the technique successfully. Basic concepts, advantages, disadvantages, physiological mechanisms of the hormonal protocols to

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synchronize and induce the ovulation, as well as a brief description of factors affecting the efficiency of the fixed-TAI programs, are included in an easy way fashion for clinicians, technicians, and veterinary or animal sciences students.

Keywords

Buffalo · Time Artificial Insemination · Estrus · Ovulation

15.1 Introduction

So far, no reproductive biotechnology has had such a massive and significant impact on herds' genetic progress and technological transformation as Artificial Insemination (AI). The genetic progress and production levels achieved in the dairy industry through AI seem to have no limits, yet AI has still not been widely used globally by the water buffalo's industry worldwide. Even though buffalo's reproductive biology is more likely similar to that of cattle, there are unique characteristics and meaningful differences in applying this biotechnology to improve buffalo's productivity effectively.

In the livestock industry for both cattle and water buffalo, to obtain optimal productivity and profitability, it is essential to achieve optimal reproductive performance. To accomplish this goal, factors like the environment, nutrition, health, and general herd management should be controlled. Some authors have described lower fertility in water buffalo (*Bubalus bubalis*) than cattle (*Bos indicus* and *Bos taurus*). Low reproductive performance in water buffalo is mostly attributed to a late onset of puberty, reproductive seasonality, long calving intervals, and estrus' low expression (Drost 2007). Reproductive seasonality observed in water buffalo prolongs the postpartum anestrus periods, compromising their reproductive performance (Nava-Trujillo et al. 2019a). This seasonality has been attributed to environmental factors, primarily associated with the photoperiod, generally negatively affected by the increase in day length (Presicce et al. 2005). Besides, the high temperature-humidity index during summer favors the appearance of thermal stress due to the restricted cooling mechanism in this species. Limited thermoregulation through skin evaporation in the water buffalo is linked to sweat glands' low density (Das and Khan 2010).

Furthermore, the water buffalo is characterized by a low intensity of estrus signs, affecting the detection of animals in estrus, compromising the success of conventional artificial insemination on a large scale, thus, limiting genetic progress through this technique. Some factors associated with the low expression of estrus signs in water buffalo are nocturnal behavior (Das and Khan 2010), infrequent homosexual behavior (Perera 2011), variable duration of estrus (5–27 h), and variable occurrence of ovulation amongst animals (24–48 h: mean 34 h) after the onset of heat (Perera 2011). Several protocols have been developed for fixed-Time Artificial Insemination (TAI) to improve water buffalo's reproductive performance and omit the need for heat detection. These hormonal treatments allow to control follicular dynamics and luteal function, synchronize estrus and ovulation, and, most importantly, avoid the

complicated detection of estrus in this species (Carvalho et al. 2016; Monteiro et al. 2016; Gutiérrez-Añez et al. 2018).

Protocols used in water buffalo are adapted from TAI protocols in cattle. The application of cattle ovulation synchronization protocols to water buffaloes have resulted in lower pregnancy rates (De Rensis and López-Gatius 2007; De Rensis et al. 2005; Karen and Darwish 2010; Rossi et al. 2014), when are compared to those obtained in cattle (Pursley et al. 1997a; Sá Filho et al. 2011; Bó and Baruselli 2004; Wiltbank and Pursley 2014). Substantial evidence of lower pregnancy rates after fixed TAI in water buffalo than cattle is observed when those protocols are applied in buffaloes during seasonal anestrus (De Rensis and López-Gatius 2007; Karen and Darwish 2010; Rossi et al. 2014). However, new protocols considering buffalo's physiology for ovulation synchronization and fixed TAI in this species have resulted in a high pregnancy rate index. In that sense, in this chapter, we summarized some of the significant advances on fixed-TAI protocols in water buffalos for both breeding and non-breeding season.

15.2 Overview of the Water Buffalo's Estrous Cycle and Reproductive Performance

As in the cow, the estrous cycle in the buffalo is divided into two phases, 1. Estrogenic phase, also known as the follicular phase, and 2. The progestational phase is also referred to as the luteal phase. The follicular phase is divided into proestrus and estrus, while the luteal phase is divided into metestrus and diestrus. The estrous cycle duration ranges from 16 to 33 days, with the highest concentration between 21 and 24 days. Unlike cows, the duration of heat in water buffalo could vary from 8 to 32 h, and heat symptoms are less pronounced than in cattle (Drost 2007). They are also characterized by nocturnal sexual behavior, with little or rare homosexual activity and slight mucus discharge, which makes it difficult to correctly identify females in heat, bringing as a consequence a high silent ovulation rate, also referred to as silent heat when compared to cattle (Perera 2008). A particular estrus sign known as tail curling when exerting pressure on the pelvic area is a secondary sign present in this species that is not present in cattle. This maneuver could also be considered a practice in animals under suspicion of heat in heat detection programs.

Water buffaloes can be defined as short-daylight seasonal polyestrous animals, but under some special conditions, they can reproduce throughout the year (Perera 2011). Although in tropical climates, the length of photoperiod during a day is relatively constant (~12 h of light and 12 h of dark), other factors, such as the rainfall changes, seem to influence the cyclical reproductive pattern, according to the availability and quality of forage (Perera 2011). Nevertheless, one has to consider that seasonal reproductive activity in water buffalo (*Bubalus bubalis*) is defined by exogenous (photoperiod, climate, nutrition, management) and endogenous (hormones, genotype) factors (D'Occhio et al. 2020).

In Venezuela, a tropical-located country, the water buffalo's reproductive pattern is characterized by seasonal breeding, with a reproductive activity that could cover

mostly from September to February, followed by a calving season markedly from September to December, with strong peaks during October and November (Nava-Trujillo et al. 2019a, b). This seasonal behavior could promote prolonged anestrus periods during the unfavorable season (March, April, May, June, July) until the resumption of ovarian activity in the next favorable season, reducing their reproductive performance (Gutiérrez-Añez, Data not published). Similar effects of climate and nutrition have been revised by Perera (2011) on the reproduction patterns in India, In the Amazon region of Brazil, and even also in Italy, a country with a temperate climate, where buffalos are farming under intensive or semi-intensive systems under a constant balanced diet throughout the year, a seasonal reproductive pattern, is also observed. Causes of anestrus in the water buffalo during summer in temperate climates or under heat stress in tropical and sub-tropical could be associated with elevated blood prolactin concentrations (hyperprolactinemia), which in turn reduced the gonadotropin secretion (Roy and Prakash 2007; revised in D'Occhio et al. 2020), and the progesterone profile (Roy and Prakash 2007). In temperate climates, reproductive activity is influenced by photoperiod and mediated by melatonin secretion (Borghese et al. 1995) and melatonin receptor 1A (MTNR1A) gene expression (Carcangiu et al. 2011; D'Occhio et al. 2020). Reduced oocyte quality and developmental competence in Italian Mediterranean buffaloes submitted to in vitro embryo production (IVEP) during spring and summer are observed compared to autumn and winter (Di Francesco et al. 2011). Additionally, the resumption of postpartum ovarian activity, and subsequent conception, can be affected by various factors such as breed, nutrition plan, milk production, lactation, uterine involution, delivery season, or seasonality (Barile 2005).

15.3 Conventional Artificial Insemination Vs. Fixed-Time Artificial Insemination

The deficiency of finding an acceptable method for estrus detection in the water buffalo, accompanied by the high variability in the duration of the heat that influences the timing of ovulation amongst animals, makes the conventional AI in this species suboptimal when using frozen semen. Buffalo sperm, subjected to freezing and thawing, appear to have a shorter fertile lifespan within the female tract than fresh semen (Moioli et al. 1998). These aspects could be a reason for the low conception rates found in this species in AI programs under natural or spontaneous heat.

The commercial application of fixed TAI in buffalo herds has been possible thanks to understanding the ovarian follicular dynamics, studied through ultrasound (Baruselli et al. 1997), and knowledge of endocrine control and hormonal profiles during the estrous cycle in this species (Terzano et al. 2012). These protocols for the treatment of anestrus and oestrus synchronization have had various success rates, providing effective pregnancy rates, ranging from 35% to 60%, comparable and in some cases higher than the parameters achieved in buffaloes reproduced under natural estrus.

15.4 Fixed-Time Artificial Insemination (TAI) Programs

15.4.1 Definition

In simple and practical terms, the fixed TAI, also known as timed AI after synchronization of ovulation (Wiltbank and Pursley 2014), is a technology that allows pre-established Artificial Insemination (AI) without the need for heat detection. Generally, the fixed TAI is implemented in batches of animals simultaneously under a pre-planned scheme or schedule. It translates into a convenient way to decide the number of animals to inseminate and choose the most appropriate time to do it (season, month, day, and hour). Fixed TAI is possible thanks to implementing a hormonal protocol for ovulation synchronization.

From the technical point of view, the fixed TAI could be defined as reproductive biotechnology that, based on the physiological knowledge of the female's estrous cycle, allows combining several hormones, which will be administered at a fixed or pre-established regime, allowing synchronizing the follicular development and the ovulation in more than 85% of treated females. The result is the programmed or systematic insemination carried out in a group of animals in a previously defined time, without the need for heat detection.

15.4.2 Advantages

- Eliminates the need for imprecise heat detection in buffalo species.
- Optimizes the use of semen and makes the AI technique more efficient.
- It allows to massively increase the use of AI, thereby accelerating the genetic progress of the herds.
- Fixed TAI favors individualizing the crossing of each female with unrelated bulls, thus minimizing the risk of consanguinity.
- It allows controlling reproductive seasonality, thus promoting the possibility of stabilizing milk/cheese production and economic income of the farm by achieving a convenient distribution of calvings throughout the year.
- It is helpful for the treatment of postpartum anestrus.
- It helps reduce days open and increases the reproductive performance of the herds.
- In the case of heifers, it allows advancing their reproductive seasonality (30–60 days), minimizing the risk that this category of animals after the first calving will remain open in the following season, considering that in primiparous buffaloes, the resumption of ovarian activity is dramatically affected.
- It improves reproductive controls since the TAI enters as a technological package that requires management improvement of the farm (records, management, sanitary program, food, among others).
- It enhances the productivity and profitability of the livestock business.

15.4.3 Disadvantages

- Drug cost.
- Technical staff requires basic training in the application and management of hormones.
- Big batches of animals (over 50) require well-organized and planned logistics, with high time-consuming and challenging work. Enough supporting staff is required.
- Three to four activities are implemented chronologically, including reproductive status checking and animal selection, hormone administration, and artificial insemination, necessary to perform the whole program. Climatic problems and logistics issues could affect the rigid schemes of activities.

15.5 Fixed-TAI Protocols During the Reproductive Season

15.5.1 GnRH and $PGF_2\alpha$ Based Protocols (Ovsynch)

One of the most critical constraints of using Prostaglandin F2-alpha (PGF2α) in reproductive control is high estrus and ovulation interval variability. There is not a proper synchronization because prostaglandin only regulates the lifespan of the corpus luteum and not the follicular development (Pursley et al. 1997b). The Ovsynch protocol, originally designed for cows (Pursley et al. 1995), consists of combining Gonadotropin-releasing hormone (GnRH) and PGF2α to control the lifespan of the corpus luteum, the follicular development, and the ovulation. This protocol has been adopted to buffaloes and currently is the most used protocol for the synchronization of ovulation and fixed TAI in water buffaloes, and numerous studies have been published (De Araujo Berber et al. 2002; Baruselli et al. 2003; Neglia et al. 2003; Campanile et al. 2005; De Rensis et al. 2005; Ali and Fahmy 2007; Karen and Darwish 2010; Oropeza et al. 2010; Derar et al. 2012; Di Francesco et al. 2012; Ghuman et al. 2012; Rossi et al. 2014; Ghuman et al. 2014; Hoque et al. 2014; Gupta et al. 2015; Waqas et al. 2016; Hussein et al. 2016; Neglia et al. 2016; Arshad et al. 2017; Ramoun et al. 2017; Rathore et al. 2017; Sharma et al. 2017).

15.5.2 Mechanism of Action of Ovsynch-Based Protocols

The physiological mechanism of Ovsynch-based protocols is schematically shown in Fig. 15.1. The Ovsynch protocol could be considered the precursor of the fixed TAI programs in cattle. It consists of two GnRH applications and one PGF2α. The first application of GnRH (day 0) induces ovulation of the dominant follicle present at this moment, followed by the further formation of a new corpus luteum and or the atresia of small follicles, causing the emergence of a new follicular wave. The application of PGF2α (day 7) causes luteolysis and a fall in progesterone levels. Forty-eight hours later, the second application of GnRH on day 9 causes ovulation of

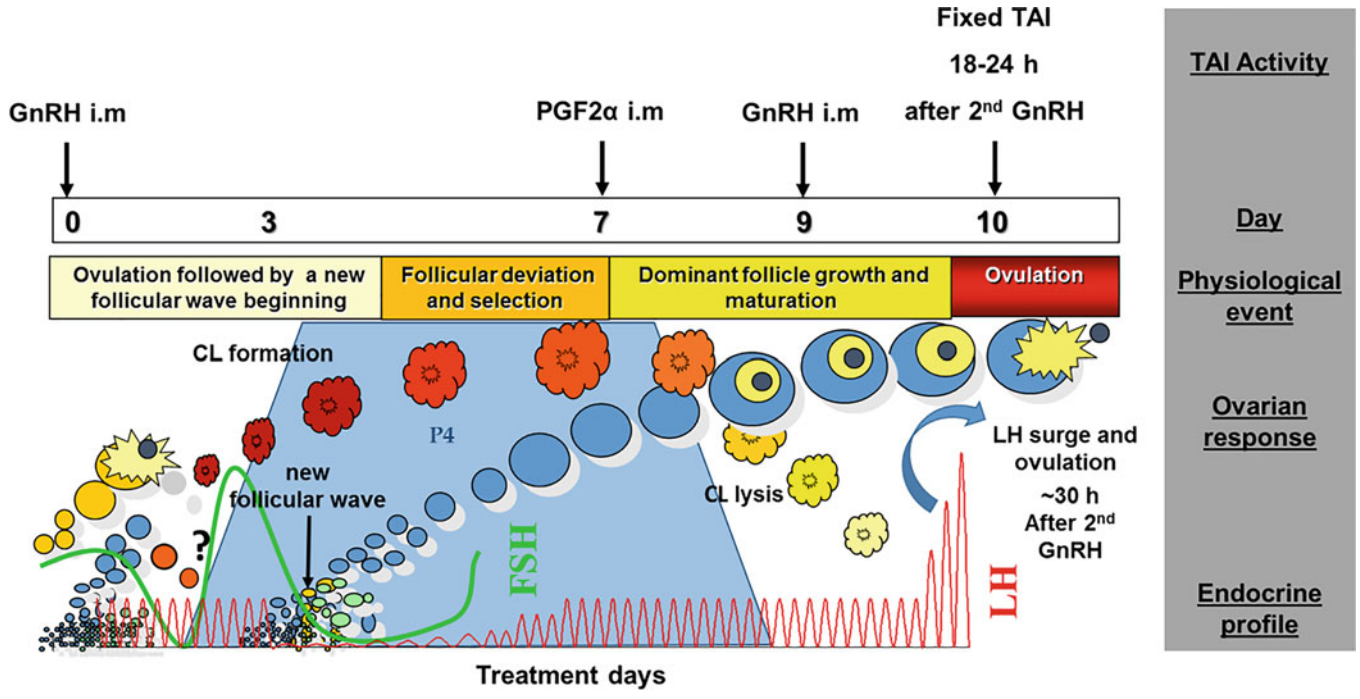

Fig. 15.1 Schematic representation of the physiological mechanism of action of Ovsynch protocol for fixed-time artificial insemination in water buffaloes. Application of gonadotropin-release hormone (GnRH) on day 0 induces the ovulation of a dominant follicle or luteinization of subordinate follicles (question mark). After ovulation, a CL (corpus luteum) starts to form, followed by a new follicular wave starting ~ on day 3. On day seven (day 7), Prostaglandin F2-alpha (PGF2α) administration induces the lysis of the CL formed, thereby decreasing P4 concentrations. On day nine (day 9), the second GnRH doses induce a uteinizing hormone (LH) surge, followed by ovulation ~30 h after. Fixed-Time Artificial Insemination (TAI) is performed 8–12 h prior to ovulation, allowing sperm transport and capacitation

the dominant follicle originated after the first GnRH injection, with insemination taking place at day ten (10), 18–24 h after the second GnRH, and without the need for estrus detection (Pursley et al. 1995).

The efficiency of the original Ovsynch protocol in buffalo in anestrus has been low; however, modern Ovsynch-based protocols have been developed; improving its efficiency in both cyclic and anestric buffalo, representing an additional alternative to progestin treatments. Some modifications of the original Ovsynch protocol will be discussed in this chapter.

15.5.3 Hybrids-Ovsynch Protocols in Water Buffalo

Some modifications of the Ovsynch have allowed obtaining acceptable results when used in acyclic buffaloes or during the low reproductive activity season. However, there are still scarce published studies on these, and therefore more research is necessary.

15.5.4 Cosynch

The Cosynch modification consists of artificial insemination on day nine (9) simultaneously with the second GnRH injection, reducing 1 day of labor (Geary and Whittier 1998). Conception rate of 62.5% and up to 75% when buffaloes received a dose of 400 IU of equine chorionic gonadotropin (eCG) 3 days before the first injection of GnRH (Cosynch-Plus) when applied to postpartum multiparous buffaloes during the reproductive season has been reported (Kumar et al. 2016). Recently, a modification of the Cosynch-Plus protocol applied to multiparous buffaloes in anestrus and during the low reproductive activity season presents encouraging results. This modification included the administration of 400 IU of eCG 3 days before the first application of GnRH, with or without intravaginal progesterone supplementation for 7 days. On day seven, the correspondent dose of PGF2α was injected. The protocol was completed with a dose of 2000 IU of human chorionic gonadotropin (hCG) on day 9 (replacing GnRH) at the time of the first insemination and the second insemination was performed 24 h later (day 10). When intravaginal progesterone was not inserted, 53.8% of the conception rate was observed, whereas it fell to 33.8% when administered. After subsequent insemination, the pregnancy rates were 69.2% and 86.6%, respectively (Dhaka et al. 2019).

15.5.5 Double Ovsynch

The Double Ovsynch is a pre-synchronization and consists of repeating a second Ovsynch 7 days after the first one is finished, inseminating 16 h after the fourth GnRH injection. Hoque et al. 2014, evaluated this protocol and reported higher

ovulation and conception rates than traditional Ovsynch (83.3% and 44.4% vs. 72% and 28%, respectively; $P < 0.05$).

15.5.6 Ovsynch Plus

Ovsynch Plus includes a dose of eCG (500 IU) applied 3 days before the Ovsynch protocol. When it was applied to acyclic buffaloes during the low reproductive activity season, only a numerical improvement in the conception rate was observed compared to the traditional Ovsynch (34.5% vs. 23.1%, $P > 0.05$) (Sharma et al. 2017). Rathore et al. 2017 reported similar results during low reproductive activity season (Ovsynch Plus: 28% vs. traditional Ovsynch: 24%, $P > 0.05$).

15.5.7 Double Synch

Mirmahmoudi and Prakash 2012, applied the Double synch protocol (a dose of PGF2α 48 h before the first GnRH injection of Ovsynch) during the low reproductive activity season (April–May, in India). The authors reported an ovulation rate of 100% after the second GnRH injection, an interval to ovulation of 23.2 ± 1.0 h (range 20–28 h), and a conception rate of 58.1% ($P < 0.05$); this latter was higher than in buffaloes inseminated under spontaneous estrus (27.3%).

15.5.8 G6G

This protocol consists of the application of a dose of PGF2α and GnRH 8 and 6 days before starting the Ovsynch protocol respectively and allowed to increase the progesterone levels on Ovsynch day seven; subsequently, the diameter of the ovulatory follicle at the moment of insemination (12.5 ± 0.3 mm, $P = 0.04$) and ovulation (14.8 ± 0.3 mm, $P = 0.02$) compared to the traditional Ovsynch (11.6 ± 0.3 and 13.7 ± 0.3 respectively), additionally a tendency to improve the conception rate was observed (56% vs. 32%, $P = 0.08$) (Waqas et al. 2016).

15.5.9 Melatonin and Progesterone Supplementation

Treatment with melatonin in the form of subcutaneous implants (18 mg / 30 Kg) before Ovsynch in anestrus buffalo heifers and during the low reproductive activity season increased the conception rate at first insemination (30% vs. 0%) and accumulated pregnancy (50% vs. 20%) (Kavita et al. 2018).

Progesterone supplementation through an intravaginal device (PRID) between days 0 (first application of GnRH) and 7 (application of PGF2α) of Ovsynch in postpartum buffaloes and during the long photoperiod season improved the conception rate regarding control (46.51% vs. 27.71%) (De Rensis et al. 2005).

Furthermore, progesterone supplementation would be ideal for the treatment of acyclic buffaloes during the long photoperiod season since a higher conception rate was reported in this group compared to traditional Ovsynch (30% vs. 4.7%, $P = 0.04$), and this effect was not observed in cyclic buffaloes (51.5% vs. 35.7%, $P = 0.077$) (De Rensis et al. 2005). These results coincide with the 50% pregnancy recently reported in Egypt in cyclic multiparous buffaloes during the season of low reproductive activity (April–October) (Ramoun et al. 2017).

15.6 Fixed-TAI Protocols During Both the Reproductive and Non-reproductive Season

15.6.1 Progesterone (P_4)-Based Fixed-TAI Protocols

Progestogens constitute a group of hormonal products primarily used mainly to synchronize the heat in cattle. Initially, for more than four decades, the route of administration of progestogens was restricted to repeated intramuscular injections for several days. Later, other routes of administration such as oral (Melengestrol Acetate), subcutaneous implants (Norgestomet), and intravaginal Progesterone (P4) devices were used. So far, the intravaginal route has been the most widely disseminated and preferred in both cattle and buffalo. Various intravaginal devices impregnated with P4 or progestogens used in different countries across the world; thus, some includes the CIDR (Controlled Internal Drug Release; Zoetis, Zoetis Animal Health, Parsippany, NJ, USA), PRIDDelta (Progesterone Releasing Intravaginal device; CEVA-Santé Animale, France), DIB [Dispositivo Intravaginal Bovino (Bovine Intravaginal Device, Sintex, Argentina), and PregnaHeat-E (Viateca, Venezuela).

The P4-based protocols in the buffalo species have the advantage of being efficient during both reproductive season (favorable) and non-reproductive season (not favorable). Progesterone devices for ovulation synchronization are combined with Estradiol (E2) (Carvalho et al. 2014; Monteiro et al. 2016), gonadotropin-releasing hormone (GnRH) (Gutiérrez-Añez et al. 2018), Prostaglandin F2-alpha (PGF2α), and equine chorionic gonadotropin hormone (eCG) (Carvalho et al. 2014; Monteiro et al. 2016, Gutiérrez-Añez et al. 2018). Most of these programs derived from the adaptations made to the hormonal protocol in cattle, based on the similarities in the waves of follicular dynamics (Perera 2008; Baruselli et al. 1997) and hormonal profiles in the course of the estrous cycle as described in cattle (Presicce 2007).

Various variants and modifications of the protocols have been evaluated based on the days of the permanence of the device (seven, 8 and 9 days), the concentration of P4, hormones to control follicular development (E2-P4, GnRH, eCG, FSH), luteal phase (P4, PGF2α) and ovulation [E2, GnRH, porcine luteinizing hormone (pLH)] (De Rensis et al. 2005; Carvalho et al. 2014; de Araujo Berber et al. 2002; Monteiro et al. 2016; Carvalho et al. 2017; Gutiérrez-Añez et al. 2018). Other variables, such

as hormonal dose and time of insemination after removing the device, have also been studied.

15.6.2 Mechanism of Action of P_4-Based Protocols

The physiological mechanism of P4-based protocols for ovulation synchronization and fixed TAI is schematically shown in Fig. 15.2. These products act like an artificial corpus luteum, inhibiting the pulsatile secretion of luteinizing hormone (LH) and, therefore, ovulation. However, the hypothalamus continues the synthesis of GnRH, consequently accumulating it. When the source of P4 is removed, the progesterone blockade on the hypothalamus ceases, triggering the massive release of GnRH and gonadotropins, initiating a normal, ovulatory, and a potentially fertile new cycle.

When estrogens and progesterone or progestogens are combined, the following occurs: estrogens in the hypothalamic-pituitary axis inhibit the release of a follicle-stimulating hormone-releasing hypothalamic hormone (FSH-RH, or FSH-GnRH), suppressing it at the level of the pituitary the synthesis and secretion of follicle-stimulating hormone (FSH). On the other hand, controlled progesterone secretion by the device in the hypothalamic-pituitary axis inhibits the release of luteinizing hormone-releasing hypothalamic hormone (LH-RH, or LH-GnRH); suppressing at the level of the pituitary gland the synthesis and secretion of luteinizing hormone (LH). Consequently, the suppression of the secretion of gonadotropins (FSH and LH) forces that the follicle (s) that is (are) present at the beginning of the protocol (day 0), either in the recruitment or selection stage (dependent on FSH), deviation or dominance (mainly dependent on LH), initiate the atresia due to the absence of the necessary hormones (gonadotropins) that stimulate and maintain their growth.

Once the estrogen administered exogenously is metabolized by the animal (generally 48–72 h in the case of EB), the inhibition for FSH release at the pituitary level ceases. Likewise, follicle atresia will result in a progressive decrease in the levels of endogenous inhibin and estradiol produced by the dominant follicle. The decrease in inhibin and endogenous estradiol (both inhibitory factors of FSH secretion) results in their inhibitory action cessation. It systematically allows the generation of FSH pulses and the restart of a synchronized new follicular wave 3–4 days later in treated animals, ensuring the emergence and ovulation of a new and more viable follicle containing a competent ovum at the device removal time. Generally, the device is kept for 8–9 days, allowing the development and maturation of a dominant preovulatory or Graff follicle until the time of device removal.

The incorporation of equine chorionic gonadotropin (eCG) at the end of the TAI protocol produces an increase in the follicular growing rate, stimulating final follicular growth and oocyte maturation, increasing the ovulation rate, progesterone concentrations, and subsequent pregnancy rate. Application of PGF2α on P4-based protocols allows the lysis of the luteal tissue (corpus luteum or luteinized follicles), avoiding the negative feedback that luteal tissue and basal levels of P4 may

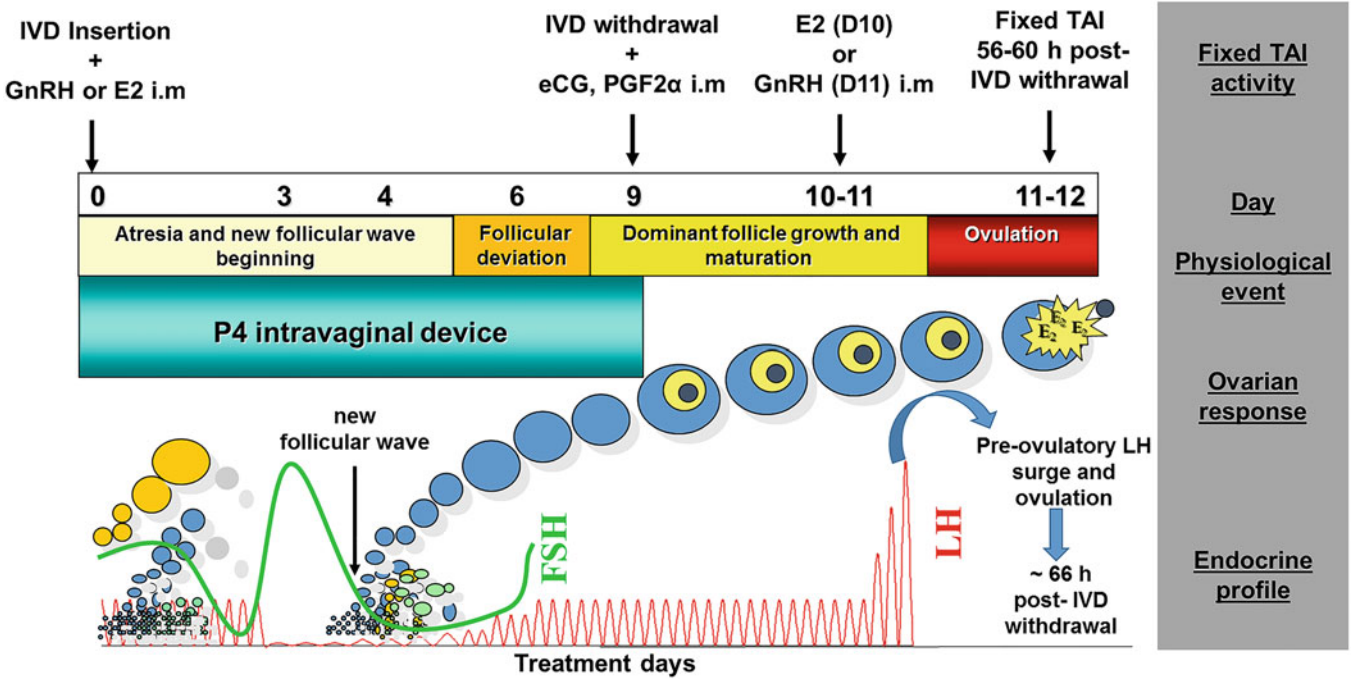

Fig. 15.2 Schematic representation of the physiological mechanism of action of progesterone-based protocol for fixed-time artificial insemination in water buffaloes. On day 0, the insertion of progesterone (P4) intravaginal device (IVD) plus the application of gonadotropin-release hormone (GnRH) or estradiol (E2), usually 2 mg of estradiol benzoate (EB), induces the atresia of the follicles present. A new follicular wave begins 3–4 days after. On day nine (day 9), the administration of equine chorionic gonadotropin (eCG) favors the maturation of the dominant follicle. At the same time, Prostaglandin F2-alpha (PGF2α) induces the lysis of any luteal tissue (luteinized follicles or corpus luteum), thereby a decrease in P4 concentrations, which could interfere with the preovulatory surge of luteinizing hormone (LH). On day ten (D10), the application of E2, usually 1 mg of EB, or on day eleven (D11) administration of GnRH, induces a

surge of LH, followed by ovulation ~66 h after the IVD withdrawal. Fixed-Time Artificial Insemination (TAI) is performed 8–12 h prior to expected ovulation, allowing sperm transport and capacitation. All treatments begin with the insertion of the P4 intravaginal device (day 0), accompanied by the simultaneous injection either of an estrogen ester, usually estradiol benzoate (EB), or gonadotropin-releasing factors (GnRH). These hormones cause atresia or ovulation of the dominant follicle [atresia in the case of estradiol, or ovulation (preovulatory follicle) or atresia (small follicle) in the case of GnRH], thus preventing the possible persistent or aging follicles from ovulating, which can negatively interfere with fertility

exert in the pulses of LH surge during the final maturation of the follicle or during ovulation.

Finally, an ovulation inducer is administered after removal of the device (Estradiol or GnRH), which stimulates the preovulatory surge of LH and the synchronization of ovulation; allowing in this way the establishment of a single moment to carry out the AI without the detection of heat in all treated animals. A study carried out by Carvalho et al. (2017), indicated that both estradiol benzoate administered either 24 or 36 h and GnRH at 48 h induced comparable follicular responses, ovulation, and pregnancy rates in the buffalo cows and heifers.

15.6.3 P_4/Estradiol (E_2)-Based Fixed-TAI Vs. P_4/GnRH/$PGF_2\alpha$-Based Fixed-TAI

The protocols which combine estradiol (E2) and progesterone (P4) have been used effectively with adequate pregnancy rates during both breeding and the non-breeding season in water buffalos under tropical conditions (Carvalho et al. 2013, 2016; Monteiro et al. 2016). Nevertheless, it is essential to consider that the uses of oestradiol-17β, as well as its related esters, aimed to estrus synchronization of food-producing animals, have been prohibited due to public health regulations in the European Union (EU) (Lane et al. 2008), and are omitted in the list of medications approved by the Food and Drug Administration of the United States (FDA, USA 2017).

Under the mentioned restriction above, an alternative to substitute the estradiol-related compounds in the synchronization protocols must be considered. The estradiol substitution by GnRH in the P4-based protocols is an alternative that allows synchronizing the follicular waves in both cattle and buffaloes. The use of fixed-time artificial insemination (TAI) protocols could adapt to the particular conditions of each country or region and respond to farmers' needs, but at the same time, it must consider and ensure the health of consumers of animal products as a prevailing condition regarding its uses.

In dairy buffalos subjected to E2-P4-based fixed-time artificial insemination has been a reported high pregnancy rate (64.0%) during the breeding season (Monteiro et al. 2016), and over fifty percent (55.9% and 52.7%) during seasonal anestrous (Carvalho et al. 2013, 2014), comparable with the results observed in a trial conducted with GnRH (Gutiérrez-Añez et al. 2018). In those experiments mentioned above, the devices were maintained for 9 days, followed by GnRH administration to induce ovulation 48 h after device withdrawal (Day 11). However, in those studies, Estradiol Benzoate (EB) was injected on day 0 instead of GnRH, and the fixed-time artificial insemination was performed 16 h post-GnRH injection (day 12), instead of 8–12 h post-GnRH administration as was done in the experiment by Gutiérrez-Añez et al. (2018).

In the last years, our research group has been focused on assessing the efficacy of a novel ovulation synchronization and TAI protocol (Fig. 15.3) based on the combination of P4, GnRH, and PGF2α on pregnancy rate in water buffalo cows

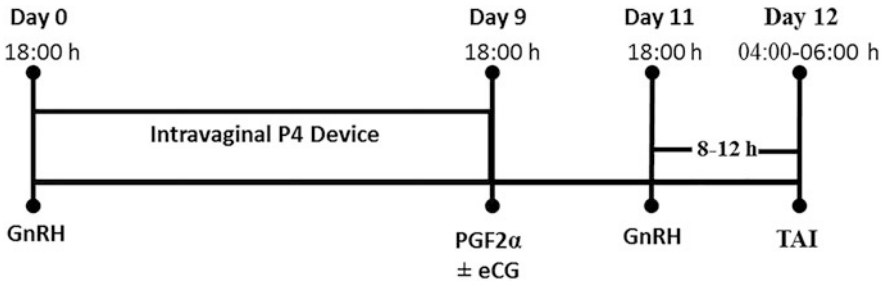

Fig. 15.3 Schematic diagram showing the P4 + GnRH/PGF2α/GnRH-based protocol for ovulation synchronization and fixed-time artificial insemination (TAI) during the non-breeding and the breeding season in dairy buffalo cows (Adapted from Gutiérrez-Añez et al. 2018). GnRH: gonadotropin-release hormone; eCG: equine chorionic gonadotropin; PGF2α: prostaglandin F2-alpha. Application of eCG at the time of intravaginal device withdrawal could be optional, but it is recommended its application under low body condition score, profound anestrous, and in primiparous cows

treated during both breeding (October to December) and non-breeding season (June to August), under tropical conditions.

Differences in pregnancy outcomes among similar protocols based on P4 intravaginal devices, combined with E2 or GnRH and PGF2α, could be attributed to many factors, including P4 concentration, protocol length, and device permanence, time for TAI after device removal or after induction of ovulation by GnRH, semen quality, management, environmental conditions, breeds, and cyclicity, among others, are discussed in this chapter.

15.6.4 Addition of Equine Chorionic Gonadotropin (eCG)

Incorporating equine chorionic gonadotropin (eCG) into ovulation synchronization and TAI protocols increases the maximum diameter of dominant follicles, ovulation rate, corpus luteum diameter, P4 concentrations, and pregnancy rate of buffaloes during the non-breeding season (Carvalho et al. 2013). Usually, the eCG is administered at the moment of the device withdrawal in a dose of 400–500 UI (Fig. 15.3).

In one study performed in non-cyclic and cyclic multiparous Murrah buffalo cows, the treatment with eCG within a CIDR-based protocol was able to increase the ovulation rate (eCG: 84.4% vs. control: 57.6%) but did not improve pregnancy rate in non-cyclic (eCG: 38.1% vs. control: 21.1%) and cyclic cows (eCG: 35.7% vs. control: 45.5%) (Murugavel et al. 2009). Perhaps the absence of statistical differences in pregnancy rate in that study might be due to the small number of animals per group. Likewise, in another study currently performed by our research group (data unpublished), the addition of the eCG could not increase the pregnancy rate significantly, including in the non-breeding season. This result suggests that the protocol based on P4, GnRH, and PGF2α was efficient in controlling both ovarian

Table 15.1 Pregnancy rates in dairy water buffaloes synchronized with a protocol based on one P4 intravaginal device combined with GnRH, PGF2α, and either with or without eCG (P4 + GnRH/PGF2α ± eCG/GnRH) during both breeding and non-breeding season (Gutiérrez-Añez et al. data unpublished)

Effect	N	Pregnancy rate (%)	*P* value
Non-breeding season			
+eCG	32/51	62.74	0.65
-eCG	31/55	56.31	0.68
Subtotal	**63/106**	**59.4**	**0.72**
Breeding season +eCG	33/53	62.26	0.70
-eCG	29/48	60.41	0.75
Subtotal	**62/101**	**61.3**	**0.60**
Total	**125/207**	**60.39**	

+eCG: With equine chorionic gonadotropin (eCG). *–eCG*: without equine chorionic gonadotropin (eCG). *NS*: No significant differences within season and groups ($P > 0.05$)

follicular dynamics and ovulation, obtaining a satisfactory pregnancy rate in lactating buffalo cows during the breeding and the non-breeding seasons (Table 15.1).

We assumed that possibly the absence of effect of eCG on the pregnancy rate in such experiment was due to the homogeneous and good body condition score (BCS) in all groups of buffalos treated (3.2 ± 0.2), as well as to the advanced postpartum period (147.9 ± 23.4) and parity (3.5 ± 0.6). In cattle, it has been documented that the effects are not always observed in cyclic animals but are evident in animals by which the LH secretion and ovarian activity is compromised; for instance, during the early postpartum period, under seasonal heat stress, or in animals with a low body condition score or in anestrous (De Rensis and López-Gatius 2014). On the other side, Sales et al. (2016) found that the eCG treatment increased final follicular growth, ovulation rate, and fertility in *Bos indicus* cows submitted to TAI protocols, especially in primiparous cows.

15.6.5 Progesterone Concentration and Reuse of Intravaginal Devices

High circulating P4 concentrations, either from endogenous or exogenous sources, decrease LH pulse frequency (Bergfeld et al. 1995; Carvalho et al. 2014). These reports suggest that high levels of P4 in protocols could negatively affect the ovulation and pregnancy rates in both cattle and buffalos. In a recent study performed by Gutiérrez-Añez et al. (2018) using the mentioned protocol above (Fig. 15.3) with intravaginal devices containing different P4 concentrations combined with GnRH and PGF2α was found a higher pregnancy rate in buffalo cows treated with 1.0 g of P4 (DIB, 62.7%), compared to those cows treated with 1.34 g of P4 (CIDR, 40.0%). These differences could be attributed to the difference in P4 concentrations, chemical composition, or the rate of P4 absorption, suggesting that

1.0 g of P4 was sufficient to control follicular development and ovulation, which resulted in an adequate pregnancy rate (Carvalho et al. 2016; Gutiérrez-Añez et al. 2018). Furthermore, in the experiment by Gutiérrez-Añez et al. (2018), reusing the intravaginal device (two and three times) did not negatively affect the pregnancy rate. Likewise, Carvalho et al. (2014) stated that low circulating P4 concentrations observed in buffaloes treated with used P4 devices (once or twice) are sufficient to promote adequate ovarian follicular growth and pregnancy outcomes as observed in buffaloes treated with new 1.0 g P4 devices.

15.6.6 Protocol Length and Device Permanence

Lower pregnancy rate (27–45%) in both Mediterranean (Neglia et al. 2003; De Rensis et al. 2005) and Murrah buffaloes (Murugavel et al. 2009), using a combined P4 for 7 days, GnRH and PGF2α protocol (PRID: 1.55 g of P4 and CIDR: 1.38 g of P4, respectively) have been observed. Apparently, in water buffalo, the protocols which maintaining the intravaginal progesterone devices for 9 days, accompanied by a GnRH injection 48 h later, and performing the fixed TAI 8–16 h post-GnRH (Gutiérrez-Añez et al. 2018; Monteiro et al. 2016; Carvalho et al. 2014), have shown to be more efficient to control the follicular dynamics, to synchronize the ovulation and produce higher pregnancy rates than the 7-day P4 protocols (Neglia et al. 2003; De Rensis and López-Gatius 2007; De Rensis et al. 2005; Murugavel et al. 2009; Bhat et al. 2015).

This theory could be physiologically reinforced, considering that in P4-based protocols, since the follicular wave emergence (3–5 days) after starting the hormonal treatment, until the ovulation should be necessary from 8 to 10 days approximately. Baruselli et al. (1997) have informed diameter of ovulatory follicles (1.55 ± 0.16 and 1.34 ± 0.13 cm), the duration of the growing phase (7.39 ± 1.55 and 5.50 ± 1.22 days), the static phase (6.88 ± 2.37 and 5.30 ± 1.34 days), and follicular linear growth of the ovulatory follicle (0.131 ± 0.01 and 0.172 ± 0.02 cm/d) for buffalo cows of 2- and 3-wave cycles, respectively. Application of GnRH 48 h after the device withdrawal (day 11) has been documented to induce ovulation between 25 and 28 h later (73–76 h after device removal (Baruselli et al. 2003; Carvalho et al. 2016). These observations suggest that artificial insemination should be performed 60–64 hours after the intravaginal device's withdrawal (8–12 h before the ovulation), which provides the necessary time for sperm transport and capacitation process (Fig. 15.2).

15.7 Evaluation and Factors Affecting the Efficiency of the Fixed-TAI Programs

When implementing a fixed-TAI program, several factors such as season (Favorable-Unfavorable), parity, postpartum period, body condition score, semen quality, general health status, and the reproductive tract condition should be considered.

A higher conception rate was reached during the season of high reproductive activity (48.80% vs. 6.90%, $P < 0.05$); in buffaloes with a body condition score ≥ 3.5 (on a scale of 1 to 5) compared to those with a body condition score < 3.0 (54.03% vs. 31.1%; $P < 0.05$); and in multiparous (51% vs. 35.5%, $P < 0.05$) (Baruselli et al. 2003). These results agree with those reported by de Araujo Berber et al. 2002, with a conception rate of 61.7% for multiparous and 30.8% for primiparous ($P < 0.05$). Hoque et al. 2014 observed that both ovulation and conception rates were higher in multiparous in comparison with primiparous (83.3% and 33.3% vs. 42.8% and 14.3%, respectively) and buffaloes with a body condition score $\geq$ of 3.5 (on a scale from 1 to 5) in comparison with those with a body condition score < 3.0 (79% and 31.6% vs. 50 and 16.7%, respectively). Besides, in Italy, a higher pregnancy rate was observed during the high reproductive activity season (58%) in comparison with the low reproductive activity season (45.6%) (Di Francesco et al. 2012). On the contrary, Rossi et al. (2014), when analyzing the fixed-TAI programs carried out for three consecutive years in Italy, did not find significant differences in the pregnancy rate according to the body condition score, days in milk or days postpartum, milk production, age (parity or number of calvings) and year of implementation of the TAI program.

Ovarian structures on day 0 could affect the results. Baruselli et al. 2003 observed that a high ovulation rate after the second dose of GnRH was related to a follicle with a larger diameter at the first GnRH (day 0) and high levels of progesterone at the moment of PGF2α dose (day 7). De Rensis et al. 2005 observed that buffaloes with follicles $\geq$10 mm at day 0 had a higher conception rate than those with $\leq$10 mm. Ghuman et al. 2014 observed that buffaloes becoming pregnant had higher progesterone levels at day 0 (2.23 ± 0.29 ng/mL) than non-pregnant (0.55 ± 0.24 ng/mL, $P < 0.05$). Souza et al. 2015 observed that buffaloes with corpus luteum at day 0 had a larger follicle on day 10 (13.9 ± 0.3 mm vs. 12.3 ± 0.3 mm, $P < 0.01$), higher ovulation rate (87.8% vs. 52%, $P < 0.01$) and higher conception rate (65.3% vs. 20%, $P < 0.01$) than buffaloes without corpus luteum. While Neglia et al. 2016, observed that buffaloes ovulating after the first GnRH had a higher conception rate (75.7%) compared with those buffaloes that did not ovulate (30.4%, $P < 0.05$) and in the same way, a difference was observed in progesterone levels at day 0 between pregnant and non-pregnant buffaloes (1.90 ± 0.2 vs. 1.40 ± 0.2, ng/mL; $P < 0.05$), which coincides with that reported by Souza et al. 2015. Besides, the selection of buffaloes at the moment of insemination according to the ovulatory follicle diameter could also improve the conception likelihood. Campanile et al. 2005 synchronized 243 postpartum multiparous buffaloes during the transition to the season of low reproductive activity. At the moment of insemination, 34 buffaloes

were excluded by not having a follicle >10 mm of diameter, and only 209 buffaloes were inseminated; a pregnancy of 63% at day 26 post-insemination was reached, however, given that the trial was carried out during the transition to long days, pregnancy decreased to 34% at day 40, which represents late embryo mortality of 45%. Late embryo mortality is one of the major limitations of reproductive efficiency in buffaloes inseminated during the transition to long days (Nava-Trujillo et al. 2019c). Therefore, the traditional Ovsynch protocol could be an excellent choice to apply during the short photoperiod season, when anestrus is low, in multiparous buffaloes, which has lower anestrus incidence, and those buffaloes with good body condition score.

On the other hand, the establishment of pregnancy results from the sum of the effects of various factors; some are not even evident and are not considered, as is the case of mastitis. Clinical mastitis before the first service increased the interval to conception (148.79 ± 12.66 vs. 76.1 ± 2.89 days), with longer intervals when mastitis occurred between the first service and the pregnancy diagnosis (232.47 ± 17.96 days, $P < 0.05$) (Manimaran et al. 2014). A positive genetic and phenotypic correlation between somatic cells score and age at first calving, calving interval, number of services per conception, and days open has been reported (de Camargo et al. 2015). In addition, clinical and subclinical mastitis affect conception rate after estrus synchronization. Conception rates at day 25 and 45 post-insemination were lower in buffaloes with clinical (28% and 16%, respectively) or subclinical mastitis (55.56% and 44.45%, respectively) compared to healthy buffaloes (69.57% and 60.87%, respectively, $P < 0.05$) (Mansour et al. 2017). Similarly, a reduction of 16.11 and 36.2 percentual points in conception rate at 40–45 days was observed by consequence of subclinical and clinical mastitis in comparison with healthy buffaloes (55%, $P < 0.05$) (Mansour et al. 2017).

These consequences could be due to the reduction in the diameter of preovulatory follicle observed in buffaloes with clinical and subclinical mastitis (12.40 and 10.25 mm, respectively) in comparison with healthy buffaloes (14.35 mm, $P < 0.05$), and consequently a reduction in estradiol level at the day of estrus and a reduction of corpus luteum diameter and progesterone level was observed in buffaloes with clinical and subclinical mastitis (Mansour et al. 2017). Also, the worst impact of mastitis occurred when it is present between 15 days before and 30 days after insemination, and this could be a consequence of the reduction in the diameter of the corpus luteum and the lower production of Progesterone (Mansour et al. 2017). The negative impact of mastitis on fertility could be related to the higher cytokines (TNF-α, IL-6, IL-1β, and IFN-γ) observed in buffaloes with clinical and subclinical mastitis (Mansour and Zeitoun 2019). Therefore, when planning a fixed-time insemination program, it is necessary to consider the health of the udder at the beginning of the hormonal treatment and on the day of insemination to avoid mastitis's adverse effects reducing the cost for each pregnancy. Furthermore, given the few published works in this area, further research in this field is required.

15.8 Conclusions

Water buffalo production systems have significant challenges, one is a genetic improvement, and the second is reducing non-productive days. Advanced age at first calving and prolonged calving to conception interval are attributed to this species. Implementing a reproductive control program, including fixed-TAI programs, is a fundamental key to reducing non-productive days, increasing reproductive efficiency in the short term, accelerating genetic progress, and ultimately increasing the buffalo systems' profitability. This chapter has detailed the recent advances in protocols for synchronization of ovulation and fixed TAI. We also have shown different possibilities to start and maintain such programs in different scenarios according to the buffaloes' characteristics, the season of the year, and the system's productive purpose.

We also have described that the establishing protocols that combine P4, GnRH, PGF2α, and eCG out or during the buffalo breeding season generate satisfactory results. However, there is no "magic" and "perfect" protocol. Each situation and condition deserve certain adjustments that should always consider the possible factors that affect the fixed-TAI programs' efficiency, including season, animal health status, nutritional situation, and cost, considering that assisted reproductive technologies supplement good management and not replace it. Finally, basic knowledge on buffalo's reproductive physiology combined with recognizing the different factors that affect the fixed-TAI programs' efficiency reinforces either the likelihood to obtain good fixed-TAI outcomes or gives a better insight when the results were not as we were expecting.

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Semen Sexing in the Buffalo (*Bubalus bubalis*)

16

Giorgio A. Presicce

Abstract

The efficiency in reproductive performances together with the increase in genetic merit with regard to milk and meat productivity in the buffalo, has to be linked to the implementation of AI and the use of semen from the top elite bulls. This has been accomplished with difficulty and over a number of decades, and its full exploitation is still undergoing. In addition, when thinking on the application of the most recent reproductive technologies in the buffalo, the thought must go to the two subspecies considered, the river and the swamp type, and their different countries of origin, environment and production expectations. The idea of enhancing the yearly genetic gain and the management of buffalo farms through the use, more recently, of sexed semen has gained attention from the most competitive farms and livestock entrepreneurs. In this chapter, a review on the early attempts and recent developments in the use of sexed semen in the buffalo species will be given, together with an outlook on the possible scenarios deriving from the full exploitation of this technology.

16.1 Introduction

The terms sperm cell and semen define a different physiological concept, the first being the single cell or spermatozoon responsible for fertilization and the second the mixture of the multitude of sperm cells and secretions produced inside the male reproductive tract and by the accessory sexual glands. They are both generally used though, to describe and refer to the technology which is the focus of this chapter. The

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quest for the control of sex in offsprings dates back to Hippocrates and the early Greek philosophers. Following Hippocrates, Aristotle (384–322 B.C.) described the method on how to obtain male progeny by stating that *"the woman wishing a male child should lie on her right side during intercourse, where the highest generative heat exists"*. From those early times, passing through the discovery of spermatozoa by the dutch Leewenhoeck (1677), and the first attempts of artificial insemination by Lazzaro Spallanzani in frogs in 1777 and later bitches in 1780, the discussion and technology for sexing semen took roots and consistency in the early 1970s of the twentieth century, when deeper studies were conducted on the various components of sperm cells, and a number of possible strategies were thought, developed, and tested.

Every possible difference between X- and Y-bearing sperm cells had been individually considered in its efficiency at the time of the study, and the possibility to use multiple tests anticipated as well, with the final goal to reach a standardized production system. Schröder (1932) was probably the first to effort the separation of X- and Y-bearing spermatozoa by electrophoresis on the assumption that the two cell types may harbor different charges on their surfaces, and therefore able to migrate to the opposite electrodes. The supposed difference in mass between the two cell types then, was also thought to be the key for a separation by centrifugation (Lindahl 1958). Later on, the fluorochrome quinacrine was used by Zech (1969) to stain the Y-chromosome and seemed promising at that time. Another possibility taken into consideration was the development of methods for the detection of post-meiotic gene expression and the identification of different gene products of the two sex chromosomes based on physical or biochemical separation. It was soon clear though, that little hope had to be placed on the use of X- or Y- linked gene products or the use of H- Y antigen as a means for separation of X- from Y-chromosome bearing sperm cells (Ohno 1982), although it was thought earlier that specific immunological differences between X- and Y-bearing sperm cell surfaces could bring to a reliable separation of the two classes of spermatozoa (Edelman et al. 1971). On the ground of physical variability existing between the two classes of spermatozoa bearing the X- or the Y-chromosome, differences in membrane components were also thought to provide a good start for establishing possible tests. X- and Y-bearing sperm cells in fact may have different surface characteristics at spermiation from the germinal epithelium. During epididymal transit though, and upon mixture with seminal plasma, sperm cells are coated by antigenic material which modifies the plasma membrane, masking any possible inherent difference between the surface characteristics of X- and Y-sperm cells (Nicolson 1982). Finally, density gradient chromatography (Erickson et al. 1973) and fluorescent activated cell sorting (Gledhill et al. 1979), reported an enrichment of X- and Y-bearing sperm cells. Overall, the separation of X- and Y-sperm cells based on physical methods has been tried in the past with well-designed experiments, although the most important obstacle to the reliability of such tests was the inability to reproduce results, possibly due to the variability in handling semen among scientists. It was also thought that the low degree of resolution of the physical methods employed in the past, dictated the difficulties encountered (Meistrich 1982).

16.2 The Technology for Semen Sexing Today

We have quickly seen above the physiological basis for testing early ideas with the goal of sexing sperm cells in the past, and the technologies behind it. It was clear that none of them could be considered completely reliable, alone or in combination with other tests. In addition, it was also clear that for a system to work reliably had to be consistent over time and across laboratories and had to give a high enough rate of purity of sexed samples, if a commercial application had to be foreseen in the arena of animal production. We have also highlighted the importance on the scene of the development of cell sorter machines, designed to individually analyze single cells for a variety of signals: from cancer to metabolic disturbances (Voronin et al. 2020). Together with the use of vital dyes such as Hoechst 33342, the improvement in efficiency and speed of cell sorting machines has determined the initial commercial application of this technology in production animals.

Therefore to date, the selection of X- and Y-bearing spermatozoa is accomplished by flow cytometry, the only currently used technique able to determine the sex of future progeny by measuring DNA content of individual sperm cells, and by maintaining their viability. The technology was developed by Johnson et al. (1999) and became known as the Beltsville Sperm Sexing Technology. This technology lies its foundations on the intrinsic difference in DNA content between individual X- and Y-bearing sperm cells, which in cattle and buffalo is around 3.8% (Johnson 2000; Presicce et al. 2005a). With regard to the identification of X- and Y-bearing sperm cells by the evaluation of DNA content, the starting assumptions was that the value of dry mass of sperm cells is proportional to DNA content (Sumner and Robinson 1976), and that the fluorescent intensity is proportional to cellular dye content (Kerker et al. 1982). To ensure such proportionality though, certain aspects have to be taken into consideration. In fact, DNA measurement in sperm cells is complicated by the highly condensed sperm nucleus which makes the quantitative fluorescent staining difficult by causing a high refractive index, in addition to the flatness of the sperm cell head, making the fluorescent measurement highly sensitive to cell orientation with respect to the excitation given by the light source and fluorescent detectors (Gledhill et al. 1976). A number of DNA dyes have been used in the past coupled to cell sorting, such as quinacrine, a medication also known as mepacrine, related to chloroquine and used as an antiprotozoal, antirheumatic, and an intrapleural sclerosing agent (Zech 1969), propidium iodide (Grogan et al. 1981), or ethidium bromide plus mithramycin (Gledhill et al. 1982). In the course of the very first attempts at sexing sperm cells with the aid of cell sorters, the cell membranes of spermatozoa had to be removed by the use of the membrane impermeant fluorescent dye DAPI (4′ − 6-diamindino-2-phenylindole), with the net results of killing the cells (Garner et al. 1983).

More recently, fluorescent in situ hybridization techniques (Kawarasaki et al. 1998), gold nanoparticles binding to Y-chromosome-specific sequence (Rath et al. 2013), and semen separation by swim-up method with validation by real-time PCR (Asma-ul-Husna Awan et al. 2017), have provided alternative solutions for sperm cell sexing, although without the possibility today of a commercial opportunity.

Therefore, to date, the only commercially feasible technology available is represented by the current Beltsville technology. This technology requires the adoption of a high-speed cell sorter machine for flow cytometric sorting, through which semen is labeled with the fluorescent vital and membrane permeant dye Hoechst 33342, which binds to the DNA of sperm cells and enable them to remain intact after sorting (Johnson et al. 1987). A greater amount of dye will be taken by the sperm cell bearing the X-chromosome, due to its larger size compared to the Y-chromosome. When passing through the cell sorter apparatus, each sperm cell is encased into a single droplet of fluid and then exposed to UV light. The fluorescence emitted, and registered by the apparatus, will be brighter depending on the amount of DNA and therefore by the passing of either X- or Y-bearing spermatozoa. Within the continuous stream of droplet-encased sperm cells, each single droplet of fluid is assigned with an electric charge corresponding to its chromosome status, being positive and negative for, respectively, X- and Y-bearing sperm cells. The stream of X- and Y-droplets is then separated by means of electrostatic deflection and collected into separate collection tubes for further processing.

16.3 The Backbone of Reproductive Physiology in the Buffalo

It is essential, before providing the results derived from the utilization of the semen sexing technology in the buffalo, to give an account of the most important features of male and female reproductive physiology in this species. As amply documented, buffaloes are intrinsically seasonal animals and both male and female buffaloes may be affected or even altered by seasonal influences in their reproductive physiology and performances (Presicce 2007). Buffalo bulls have been tested and selected in order to provide semen for AI only recently, just in the last few decades, if we compare it with cattle bulls. In fact, the latter have been the first to be the target of intensive selection, by choosing top bulls for genetic improvement and selecting them, along the line, also for semen quality and freezability. Despite the efforts in the last decades to select elite buffalo bulls for enhancement of the yearly genetic merit, a large variability still remains in terms of semen quality. This variability may affect heavily the utilization of the best bulls if poor semen is found and proven unusable for freezing/thawing processing and AI. For example, semen of buffalo bulls may be more prone to oxidative stress due to a higher lipid peroxidation probably linked to a reduced activity of antioxidant enzymes (Nair et al. 2006). This wide variation in semen quality is of course inevitably caused by the lack of long time selection in the riverine buffalo (Saeed et al. 1990; Presicce et al. 2003), and even more in the swamp buffalo, typical of east-Asian countries, where local and social conditions together with the characteristic family management of these animals, do not favor a modern genetic selection. In river buffaloes, more intensively managed in competitive farms when compared to the swamp counterpart, the use of AI has also been characterized until recently by poor results, and a consequent general disbelief by the farmers themselves in the good use of this technology for the betterment of buffalo reproductive management and genetic improvement. This is another element that has been

the cause for a slow introduction of AI in the management of the best conducted buffalo farms. This is true even more in the swamp counterpart, where usually animals are owned in small numbers by farmers, making the task of genetic selection and improvement by semen processing and AI more difficult, slower at the best, and today possibly unrealistic.

Female buffaloes, although similarly equipped to cattle with two- to three-waves of follicle development within each estrous cycle (Taneja et al. 1995; Presicce et al. 2004), are characterized by a tenfold lower number of primordial follicles at birth (Danell 1987). This is reflected then, in the lower available count of antral follicles at each cycle and their potential use in the course of other assisted reproductive technologies such as in vivo (MOET) and in vitro embryo production and transfer, or storage by cryopreservation methods (Gasparrini 2002; Presicce 2007). The season, and in particular the lengthening of daylight hours, is responsible for a negative differential reproductive performance in female buffaloes when compared to the other part of the year characterized by reduced daylight hours (Singh and Lal 1994; Zicarelli 1997). The most important signal indicating a clear sensitivity to the photoperiod in buffaloes is given by the nocturnal rise, although very variable among animals, in melatonin levels (Parmeggiani et al. 1992). Among female buffaloes though, older animals are more sensitive to such photoperiodic alternance in reproductive efficiency, younger animals such as heifers being characterized by a more uniform reproductive function (Zicarelli 2017). In fact, any disturbances in the reproductive organs when appearing at the end of the good reproductive season, between the end of summer and the end of the subsequent winter and until spring at latitudes above the equator, will lead especially older animal into anaetrus (Esposito et al. 1992; Campo et al. 2002; Zicarelli 2007). In countries such as Italy, where the highest demand for milk production coincides typically with the season characterized in buffaloes by the lowest reproductive efficiency and pregnancy rate, a strategy has been devised that offsets such condition. A strategy which has been termed OBSM (out-of-breeding-season-mating technique), in which bulls are removed from the herd in the most optimal months for reproductive function, and reintroduced between spring and summer in order to have most pregnancies the following end of winter and beginning of spring (Zicarelli 2017). This technique confirms the possibility to "guide" the reproductive function of female buffaloes in order to obtain pregnancies and calving when they are mostly needed, according to commercial request. This is usually accomplished at the price of a reduced income by the farmers when this technique is firstly introduced in the farm, but with a profit largely regained though in the following seasons and years.

The possibility to choose the best time for achieving pregnancies in buffalo farms without compromising farmers' income has been amplified by the introduction and implementation of AI, especially in the most competitive farms, by combining the use of the best bulls with the refinement of protocols for the synchronization of ovulation. In fact, given the unpredictability of estrous display in female buffaloes, the use of behavioral components such as length and signs is unreliable when AI has to be performed (Baruselli et al. 1994). As above just anticipated, a breakthrough has come with the introduction of protocols for the synchronization of ovulation among

animals, derived mainly from the application of the same in cattle, such as Ovsynch (Pursley et al. 1995). Although at times customized to the buffalo, given some physiological differences in the release of reproductive hormones and their timing (Neglia et al. 2003; Oropeza et al. 2010; Carvalho et al. 2013), these protocols have provided the possibility to obtain acceptable to good rate of pregnancies in both heifers and pluriparous buffaloes when using ordinary unsexed semen (Rossi et al. 2014).

16.4 Early Attempts in the Buffalo

In 1988, the first live offspring in cattle and rabbit were born following use of sex-sorted semen (Morrell et al. 1988). A decade went by until the technology became feasible for its commercial exploitation, thanks especially to the development and availability of high-speed flow cytometry sorters (Seidel et al. 1999).

More or less two decades, since the first viable offspring generated by the use of sexed sorted semen, passed before the birth of the first buffalo calves thanks to the same technology (Presicce et al. 2005a). In that very first trial, young bulls in the course of training to serve the artificial vagina were tested, until one of them was selected for initial semen processing in a farm in the south of Italy, and shipment within the same day in a cooled box to the lab in Germany for further processing, sexing and freezing. The sexed samples in straws were then shipped back to Italy, where they were used for AI following implementation of a protocol for synchronization of ovulation (Ovsynch) in pluriparous buffaloes. A special instrument, named Ghent insemination device developed for optimizing the number of sperm cells to be used for AI (Verberckmoes et al. 2004), and successfully also tested in buffaloes (Senatore et al. 2004), was at that time used for deposition of sexed semen at the utero-tubal junction of the horn ipsilateral to the ovary bearing the dominant ovulating follicle. The results from that first trial showed that the purity reached after cell sorting in the young buffalo bull was similar to what reported in cattle (Fig. 16.1), and that pregnancy rates between unsorted and sorted semen following AI was very satisfying and similar.

In addition, calves born by the used of sorted semen showed no difference in any of the parameters used to evaluate calf viability, when compared with calves born by the use of unsorted semen (Presicce et al. 2005b). That successful initial trial, opened a new era in the management of buffaloes, adding a new important tool for the improvement of genetic selection in this species and for the reproductive management across farms throughout the world. Since then, companies in Italy and in the rest of the world have been commercialized buffalo sexed semen, in line with conventional and more modern procedures for the identification of the best and top elite bulls.

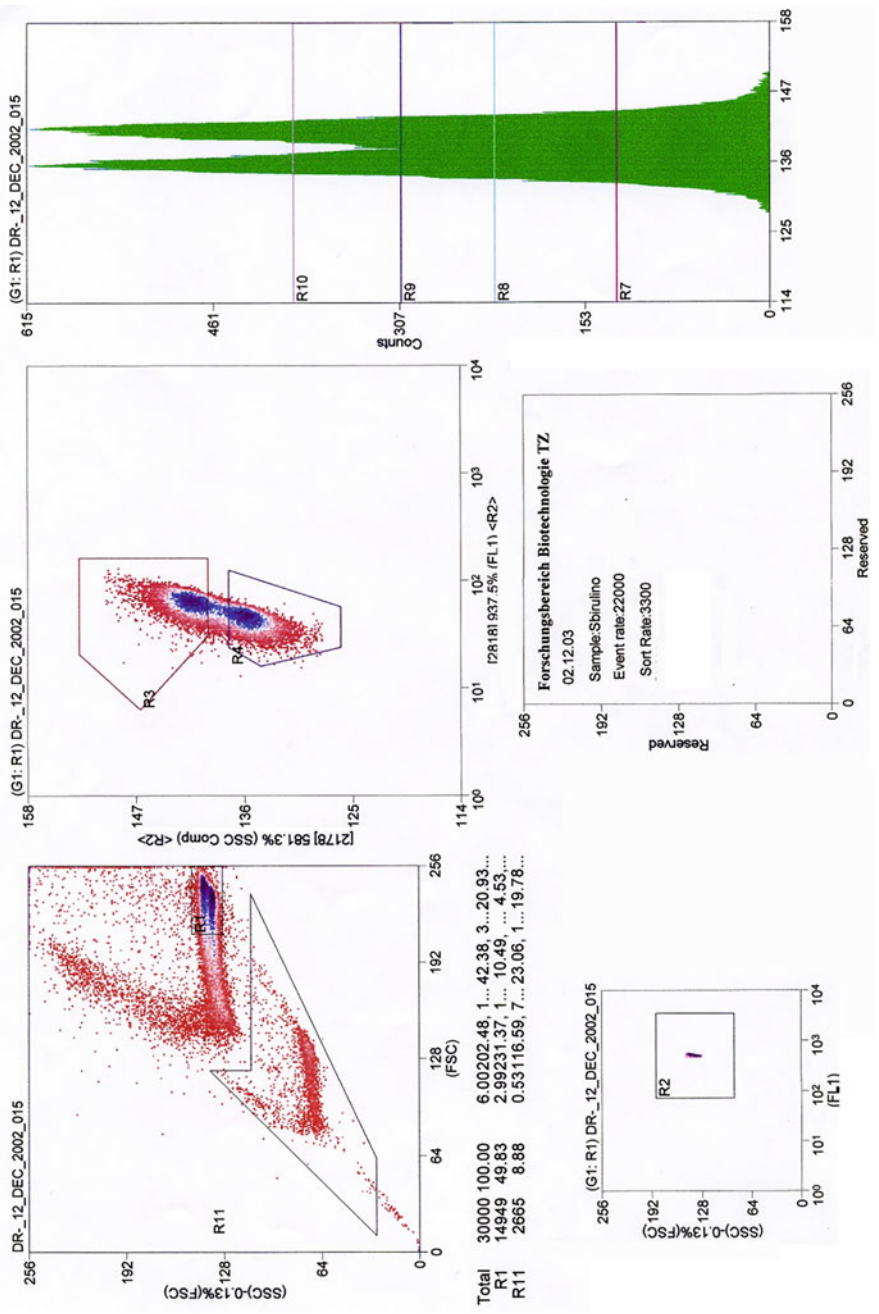

Fig. 16.1 Sorting flow chart of the first buffalo bull ever used for AI with sexed semen

16.5 More Recent Developments

Since the first results derived from the adoption of sorted semen and AI in buffaloes, other research groups have delved into the same topic. A first significant improvement was reached when it was reported and demonstrated in heifers that it is not necessary to use a special device for deposition of sexed semen in the utero-tubal junction in order to have satisfactory pregnancy rates. In fact, in that study (Campanile et al. 2011) an ordinary insemination gun was used and the results obtained, confirmed that pregnancy rates were similar between animals inseminated with unsorted or sorted semen. In addition and similarly important, was the realization that insemination into the body of the uterus as opposed to insemination into the horn of the uterus ipsilateral to the ovarian site of ovulation, was even more effective and gave higher rate of pregnancy. These somewhat surprising results supported the idea that from that moment on, sexed semen could be easily deposited into the body of the uterus as ordinarily done with unsorted semen, bypassing also the need of a special technical expertise to inseminate buffaloes with sorted semen.

The combined use of sexed semen and AI in pluriparous buffaloes was also tested to determine if pregnancies could be affected in terms of early embryonic mortality, and if a season effect was in store, between the transitional and the high breeding seasons (Campanile et al. 2013). In that follow-up study, once again it was ascertained that both unsorted and sorted semen give similar results in terms of pregnancy rate. In addition, the use of sexed semen did not alter progesterone production nor increased embryonic mortality, when confronted with the opposed unsexed semen.

In between the first study on the detection of water buffalo sex chromosomes in spermatozoa by fluorescence in situ hybridization (Revay et al. 2003), and the first (Presicce et al. 2005a, 2005b) and last reports (Campanile et al. 2013) on the successful use of sexed semen in buffalo in conjunction to AI, other studies have been performed by other authors on sorting of X- and Y-bearing sperm cells in Murrah and Nili-Ravi buffaloes (Lu et al. 2006), on the use of sexed spermatozoa in the course of in vitro fertilization (Lu et al. 2007; Liang et al. 2008), and again on the combined use of AI and sexed semen in Mediterranean buffaloes (Gaviraghi et al. 2013).

As already reported in this review, prior to the establishment and effectiveness of cell sorting machines and vital staining with Hoechst 33342 for evidence of differential DNA content in sperm cells, attempts at selecting the two population of X- and Y-bearing sperm cells had been made by addressing attention to other constituents of the sperm cells themselves. Recently, a totally different system has been proposed by an American-based company (EmLab Alpha Genetics, Chicago, USA, 2016), where a putative agent is supposed to selectively activate and increase fertility of X-bearing spermatozoa (HeiferPlus™), ensuring thus an increase of female calves up to 20%. The fertilizing capacity of the X-bearing sperm cell would be enhanced in the reproductive tract of the female following a short period of external co-incubation of semen and the product itself prior to AI. No additional information are provided by the Company, and the active principle of the commercialized product is

undisclosed, although it is possible that it could consist in some chemical substance enhancing sperm motility. In addition, in cattle it is claimed that not only a higher percentage of female calves are born by this method, but also an increase in the overall fertility of the animals treated with such a product, is reported. This product, following closely the instructions given, was also initially tested in buffaloes with only the results on conception rates reported, found anyway lower when compared to conventionally treated semen for AI (Barile et al. 2013).

More recently, the same product was tested in another buffalo farm, with the intention to verify the efficacy in terms of conception rates and possible skewed rate of female vs male born calves. The trial involved both heifers and pluriparous buffaloes and with both category of animals, significant or at times nonsignificant higher conception rates were reported in control animals as opposed to those receiving the HeiferPlus™ treatment. In addition, when it comes to the ratio of female vs male calves born following AI, it seemed that for both categories of animals the treatment did not favor the fertilization of oocytes by X-bearing spermatozoa, as claimed by the Company. This was verified by fetal sexing at 60 to 65 days following AI and confirmed at birth (Presicce, unpublished). In conclusion, this last approach of enhancing the fertility of the X-bearing spermatozoa by an undisclosed mechanism, in buffalo did not prove true in our hands.

16.6 Future Expectations

Probably, already 40 years ago the most competitive cattle breeders in North America would have paid more than 100 $ extra per sexed calf produced, mostly for females but also, and possibly even more, for males from the top elite population. It was predicted that a contained surcharge for the sale of sexed semen, would have easily produced a market for production of males for fattening or veal from less valuable cows, and female offspring from superior genetic females (Seidel and Amann 1982).

There is general agreement that conception rates with pluriparous milking cows are lower with sexed semen than unsexed semen, and that maiden heifers give a higher pregnancy rates with the use of sexed semen when confronted to cows (DeJarnette et al. 2008). The market of sexed semen in the dairy cattle industry is today well established, and since there is an increasing demand for dairy and beef products across the globe, production efficiency has to be improved. The technology of sexing semen comes to a rescue in this regard in dairy farming; in fact there is a surplus production of unwanted male calves and therefore sexed semen can be used to generate herd replacements. In addition, this technology ensures that more heifers for herd expansion at a faster rate from within the herd can be produced, thereby minimizing biosecurity risks associated with bringing in animals from different herds.

In this review we have already highlighted the difficulties encountered in the buffalo production system, to include not only the most sophisticated and latest

Table 16.1 Ideal buffalo farm whose reproductive management is conducted, other than natural mating, with the use of AI and both unsexed and sexed semen

REPRODUCTIVE AND PRODUCTIVE PARAMETERS IN LACTATING BUFFALOES		
farm turnover 20%; bull effect = 0; mortality = 0; mean farm milk yield = kg 2.550		
	semen	
	sexed	non sexed
to get	20 ♀	20 ♀
% conception	40	45
% top buffaloes to AI	50	90 (*)
mean milk (kg) of inseminated buffaloes	2800	2550
selective differential kg	250	50
hereditability (30%)	75	15
* half of them will be ♂		

reproductive technologies, but also the very first and most basic approaches like AI, be it with unsexed or sexed semen. The benefits received by the implementation of AI with sexed semen, given the unavoidable cost surcharge involved, can be considered only for those most competitive farms and farmers. This is further restricted to those countries where economic and environmental conditions may favor the adoption of more costly attempts at improving the genetic makeup of animals, especially in the river subspecies such as Mediterranean, Murrah and Nili-Ravi. We have recently proposed a tentative prospect on the likewood of improvement in milk production in Mediterranean buffaloes, following use of sexed semen and AI. From a given average in milk production within a buffalo population, we have considered some realistic assumptions in the buffalo farm management, and at the same time removing from the possible causes of increased or decreased productivity two important elements, such as bull effect and calf mortality. From these starting points we have then considered animals to be inseminated with either sexed or unsexed semen (Table 16.1), even granting some slighter higher conception rates from the use of unsexed semen.

From these parameters, a projection of increased productivity and income has been envisaged over the following decade (Table 16.2).

As we can see, a clear tendency in the increase of both productivity and income is foreseen when sexed semen is employed within well managed buffalo farms. This is so even when we indulge in granting some higher conception rate with the use of unsexed semen. Evidence though, from the trials run so far with the combined use of

Table 16.2 Milk production and economic return from the use of both unsexed and sexed semen over the years

difference from starting mean (kg 2.550)					
	semen			difference (sexed vs non sexed) gain	
After … years	sexed	non sexed	milk difference	X head	X 10 heads
	(kg)	(kg)	(kg)	€	€
4	175	163	12	15	150
5	324	289	35	43	434
6	442	377	66	82	821
7	528	426	102	127	1274
8	579	436	143	178	1782

AI and sexed semen, suggests that no significant differences are probably expected when confronted with the use of unsexed semen, in both heifers and pluriparous buffaloes.

In conclusion, the use of sexed semen has entered into the reproductive management of the best conducted and productive buffalo farms in many countries of the world. Although still marginal, its use coupled to the most recent technologies for the selection of the best animals, will undoubtedly pave the road for a faster genetic gain and enhancement of productive parameters, both in quality and quantity.

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Advances in Cryopreservation of Buffalo Semen

17

William Gomes Vale, Samia Rubielle Silva Castro, Aluízio Otávio Almeida-Silva, Juan Carlos Gutiérrez-Añez, and Pawan Singh

Abstract

The domestic buffalo, also known as the Indian river and dairy buffalo, originates from the Indian subcontinent—India, Pakistan, and further west to the Balkans, Egypt, Italy, and Mediterranean countries. While the swamp buffalo originates from Southeast Asia and China, both buffalo types are found in Australia, North and South America, Europe, and African countries. The two existing types of buffalo are recognized based on morphological features of the chromosome. The river buffalo type has $2n = 50$ chromosomes and the swamp buffalo type has $2n = 48$. These two types produce a hybrid buffalo with $2n = 49$ chromosomes. This hybrid can be crossed with a river or swamp buffalo resulting in a product with $2n = 48$ or $2n = 50$ chromosomes that receives the normal number of chromosomes characteristic of a dairy type. According to FAO, the estimated global population of water buffalo is more than 230 million, distributed in more than 50 countries. Interestingly, the population continues to grow and reminds us of the famous first line of the textbook *The Husbandry and Health of the*

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Domestic Buffalo written by Cockrill (1974). It was that "among the animals which science has neglected the domestic water buffalo serves as an outstanding example." Nowadays, buffaloes are recognized in many countries because of their dairy products such as milk, *mozzarella*, *burrata,* and other high-quality cheeses. There is also an increasing demand for buffalo meat, which has transformed India (2016) into the world's largest exporter of buffalo meat, with annual exports equal to US$4 billion. Besides the meat exports, another major contribution of buffalo in the world market from India is leather. A significant proportion of buffalo heads used for hiding in small industries generates employment opportunities for millions of people. In addition, this animal plays a role in the draft, manure, religious rituals, entertainment, and sports. Therefore, buffalo farming is an emerging economic activity.

Thus, this chapter aims to analyze some concepts on reproductive management, semen evaluation and cryopreservation for male buffalo. Also, to point out how to use the reproductive potential of genetically elite males for the genetic improvement of this species.

Keywords

Buffalo · Frozen semen · Spermatozoa · Semen preservation

17.1 Introduction

Looking back over more than seven decades a research paper about artificial insemination (AI) of buffalo did not exist although the Allahabad Agricultural Institute in India claimed to have done the first artificial insemination (AI) in buffalo producing a calf on August 21, 1943 (Bhattacharya 1974). Undoubtedly an important brand, however, the information on the methodology used has not been described, leading to the assumption that AI was carried out with fresh or diluted semen. Even though, in many ways, the physiology of male buffalo reproduction was treated similarly to the bovine, various aspects of reproduction between these two species are quite different. To achieve the best results, the knowledge of AI gathered from the bovine bull cannot, therefore, be applied exactly as in buffalo species without any modifications (Bhattacharya 1962, 1968).

Further on, the use of frozen semen as a tool for the genetic improvement of buffalo was initiated by Bhattacharya and Srivastava (1955) in India, when AI was first performed with relative success, using frozen semen in pellets and ampules. Immediately, several workers continued to work not only in India but in other countries also (Roy et al. 1956; Basirov 1964; Sahni and Roy 1972). Although more than 95% of the buffalo population was located in Asia, the production of buffalo has spread extensively in Latin America and the Mediterranean area (Andrabi 2009). The unfamiliarity with information resources, inadequate technology applying to buffalo semen, concerns about pattern extenders, glycerol percentage, equilibrium time, freezing methods, and lack of standardization are leading to

poor and variable results. For these last reasons the use of deep freezing of bovine semen methodology applied to buffaloes failed, and it was difficult to set up a standardized use of own technology for buffalo species, which led to poor and inconclusive results when semen was tested at field level. For some authors, the low resistance of the male buffalo spermatozoa would be an intrinsic factor of the specie due to its difference in composition when compared to bovines. As a result, buffalo spermatozoa generate poor results when bovine extenders are used (Bhattacharya 1962, 1968; Vale et al. 1984).

17.2 Management of Young Buffalo Aiming Its Use as a Future Semen Donor

The calving percentage in buffalo herds is one of the main factors influencing success in the farming operation. This calving percentage influences economic returns and also the genetic improvement of the herd. When the farm uses the practice of intensive rearing with the aim of genetic improvement, the calves of the top-level dams, which are milking, the following methods of feeding suckling buffalo calves are generally practiced:

- Mother in a pasture during the day, with the offspring separated from the mother at night; mothers are milked once or twice daily;
- Suckling two times a day, access to concentrates in a creep feeder or in pasture;
- Controlled ingestion of milk and free access to pasture, silage, or concentrate.

Though little improvement in Body Condition Score (BSC) is possible before puberty, the use of males with lower or higher BCS should be corrected before putting them in breeding; BSC should not be <2.0 or >4.5 on a 1–5 point scale. Both undernourishment and obesity are not good for a neonatal, post-weaning, and reproductive performance life. In addition to assuring appropriate BSC, a balance of energy and protein is important, and this assessment of nutrients in the feeding diets of the animals must be assured (Fig. 17.1).

Minor key nutrient intake should be rectified before the start of the breeding season. An appropriate offering of mineral mixture adequate for growing young animal's maintenance is recommended. In addition, young males selected for semen production should be free of genetic abnormalities, including those combined with gonadal hypoplasia, the arrest of development of mesonephric ducts, and must have adequate health management, which includes a deworming and vaccination program against the principal infectious and parasitic diseases (Vale et al. 1988; Ohashi et al. 2011).

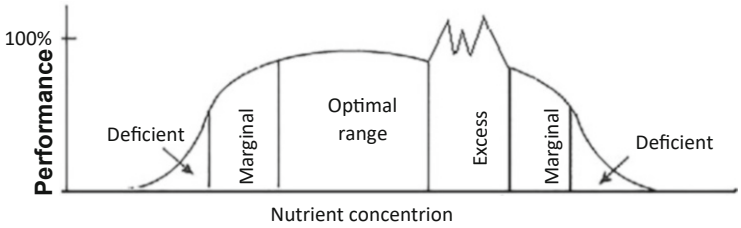

Fig. 17.1 Effect of nutrient amount on physiological performance of domestic animals

17.3 Pre-breeding Management of Young Buffalo

17.3.1 Onset of Puberty

First of all, to start the buffalo semen production, it is imperative to be familiar with his ancestor's history about milk or meat production, since there is a lack of progeny tested buffalo males in America so far. Anyway, during the pre-breeding time, puberty must be observed and referred to as the average age at which the buffalo male shows sperm cells in the lumen of seminiferous tubules, which occur around 9 months of age (Ohashi 1993). However, for semen collection, the age recommended to start is 18 months when the animal seems to be ready to produce a normal ejaculate with suitable volume, sperm motility, concentration and total number per ejaculate, and percentage of live spermatozoa (Ahmad et al. 1984, 1989). Thus, breed, age, size, feed intake, and probably other factors each play a part in bringing on puberty in buffalo male. In the literature, reports indicate that puberty can be reached at 12 months of age (Table 17.1). Usually, before the onset of puberty, around 12 months of age, the male begins to mount on other young males, indicating the onset of sexual interest (Fig. 17.2). This behavior emerges due to the hormone secretion initiated by the hypothalamic-pituitary axis that influences all animal's metabolic activity (Vale et al. 2008). Such behavior is very important and has a marked influence on the reproductive capacity of the animal as a semen donor.

The mammalian animal's reproductive cycle in males and females is regulated through the hypothalamus, anterior pituitary, and gonadal hormones and their interaction. The secretion of anterior pituitary hormones both in male and female animals is controlled by the hypothalamus. During the period from 6 to 10 months of

Table 17.1 Age of the first appearance of some sexual characteristics among well-feed and well-managed buffalo male

First protrusion of penis during mounting (month)	7–8
Complete separation of penis and sheath (month)	9–10
First spermatozoa detected in ejaculate (month)	7–10
Mean age of puberty (month)	12
Mean body weight at puberty (kg)	300–350
Scrotal circumference (cm)	22–25

Fig. 17.2 Mounting of young bull on another. (Courtesy of NDRI, Karnal, India)

age in bovine and probably in buffalo, the hypothalamus sends a gonadotropin-releasing hormone (GnRH) to the anterior pituitary causing the release of luteinizing hormone (LH) from the anterior pituitary into the blood which will stimulate the Leydig cells in the testis to secrete and release testosterone. Then testosterone starts to be secreted between 5 and 6 months of age; body development and puberty start to reach with the secretion of testosterone from Leydig cells and adrenals glands release. Although FSH and LH are named for their functions in female reproduction, they are produced in both sexes and play important roles in controlling reproduction; however other hormones have specific functions in the male and female reproductive systems, such as leptin, ghrelin, IGF, and insulin (Fig. 17.3). These hormones play important roles in regulating body weight, eating behavior, and reproduction, acting on the central nervous system and targeting reproductive organs. As a marker of adequate nutritional stores, these hormones can act on the central nervous system to initiate the complex process of puberty and maintain normal reproductive function (Squires 2010).

The buffalo species is among domestic animals, undoubtedly the one that uses the five senses with greater accuracy. Hence the five senses, sight, hearing, smell, taste, and touch, are well expressed during one set of sexual development. These senses can be identified and understood as sexual behaviors. In domestic buffalo, the smell seems to be the greatest signal through the action of pheromones in genital secretions

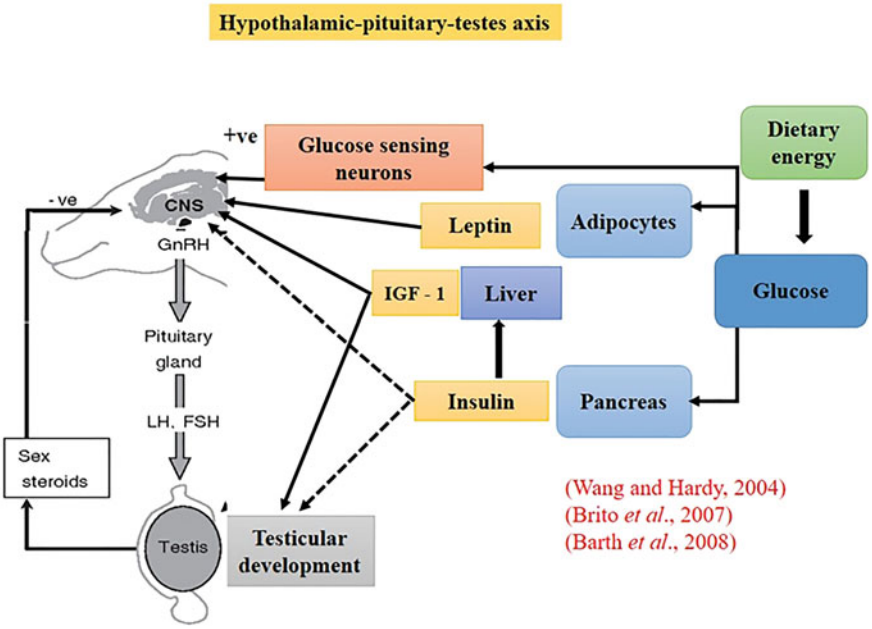

Fig. 17.3 Hypothalamo-pituitary-testis axis and other subjects which regulate the age at puberty in bulls. (Adapted from different authors)

and urine, promoting a series of actions that stimulate sexual activity, on the vomeronasal area. Therefore, it seems obvious that by the analysis of Fig. 17.3, there are interactive conjugations among these five senses and the CNS, hypothalamus, and adenohypophyses in the signaling release factors (RF) and the hormone LH which act directly on the male function producing testosterone by the Interstitial Cells Stimulating Hormone (ICSH) or Leydig cells, as well as anterior pituitary gland producing TSH and FSH which starts the production of spermatozoids by the seminiferous tubule (Squires 2010) (Fig. 17.4).

17.3.2 Relationship of Age, Body Size, and Scrotal Circumference to Semen Production

Although different authors assumed that buffalo males are low precocious regarding the reproductive performance, our experience in Brazilian conditions and reports from other countries have demonstrated to be contrary to such affirmation and that the main cause of delayed puberty and sexual maturity seems to be related to errors which occurred just after calving throughout weaning and post-weaning. Just after the birth, the testis has few functional Leydig cells with the seminiferous tubules containing the Sertoli cells, which are the sustentacular base for the gonocytes,

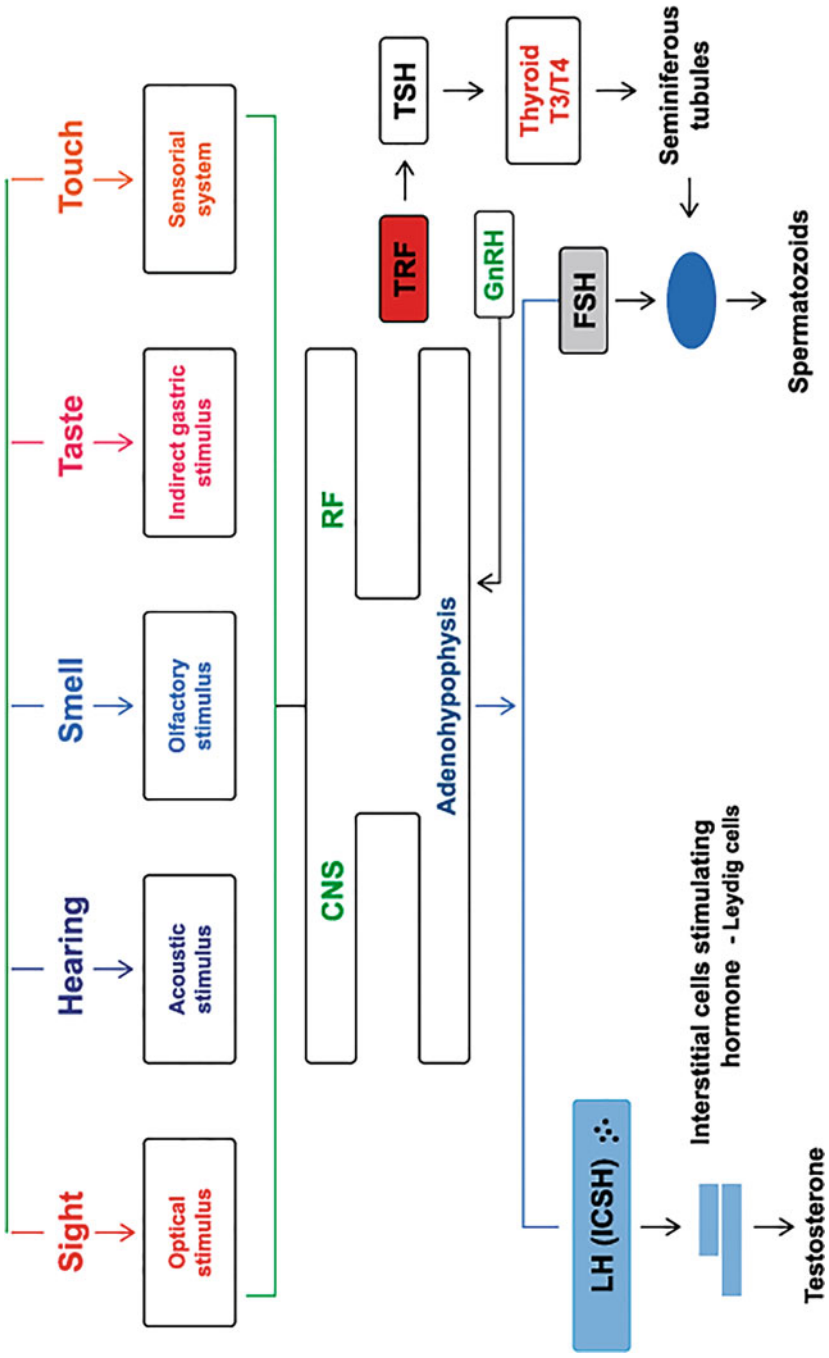

Fig. 17.4 Integration of the biological five senses and reproduction in buffaloes

which will be transformed in spermatogonia. After this initial phase, the young male goes through the infertile period reaching the weaning time which will occur between 6 and 8 months, when the hypothalamus starts secreting GnRH and consequently the anterior pituitary initiates a progressive secretion of gonadotrophin FSH and LH with the beginning of the secretion of testosterone by the Leydig cells of the testis and starting the process of spermatogenesis which occur in well-managed bovine and buffalo males at the age of 12–14 months (Ohashi 1993; Brito et al. 2007). Once the buffalo is selected, it is important to know the animal's history, concerning genetic potential records and management to which he is submitted if he has received normal nutrition that meets all the needs regarding energy, protein, and minerals, which will meet their body development which will be classified through the body score condition (BSC) (Vale et al. 2008).

Thus, puberty in the buffalo male should be considered the period that the animal starts producing viable sperm cells. Consequently, the beginning of the spermatogenic process starts with the lumination and growth of the diameter of the seminiferous tubules and their lumen. In this phase also occurs a quick growth of the whole genital system when the animals attempt to mount females or even males and successful reproduction does occur if the male crosses a female in estrus. After this period follows the period of sexual maturity when the genital system reaches the full production of spermatozoa in the ejaculate and hormones. In animals well managed this period can be achieved at 22–24 months. At this time it is quite common for male buffaloes to produce an ejaculate with the optimal characteristics regarding volume, concentration, vigor, motility, and normal sperm morphology with a compatible freezing process (Vale et al. 2008, 2014).

The influence of age, body size, and circumference of the scrotum on the ability of the buffalo bull to produce semen has been recognized for a long time, but unfortunately, it has not been studied extensively. The body mass and scrotal circumference (SC) significantly influence the reproductive efficiency of a male and have a positive correlation with semen production in young buffalo bulls (Ahmad et al. 1989) and bovine bulls over 18 months of age (Carter et al. 1980; Brito et al. 2007). The SC determination is an integral part of breeding soundness examination and possesses greater value as an indicator of puberty, total sperm production, semen quality, pathological conditions of the testes, as well as the subfertility or infertility of a bull (Ahmad et al. 1989; Vale et al. 2008, 2014). Furthermore, SC is highly inheritable and is associated with the age of puberty in daughter and half-sib heifers and their future productive and reproductive performance (Brinks et al. 1978). Season influences both body mass and SC in bovine bulls (Javed et al. 1998; Vale et al. 2008) (Fig. 17.5).

Earlier studies revealed that the genetic makeup of the animal largely influences the sexual desire/behavior in bovine (Hultnas 1959) and in buffaloes (Yassen and Mahmaoud 1972; Bongso et al. 1984). Nevertheless, factors such as age, environment, and management are also held accountable for variation in sexual behavior (Vale 1994, 1997).

Furthermore, Vale (2011) suggested for the mature Murrah buffalo males with 30–36 months of age the scrotal sac must have a circumference >30 cm. In a survey

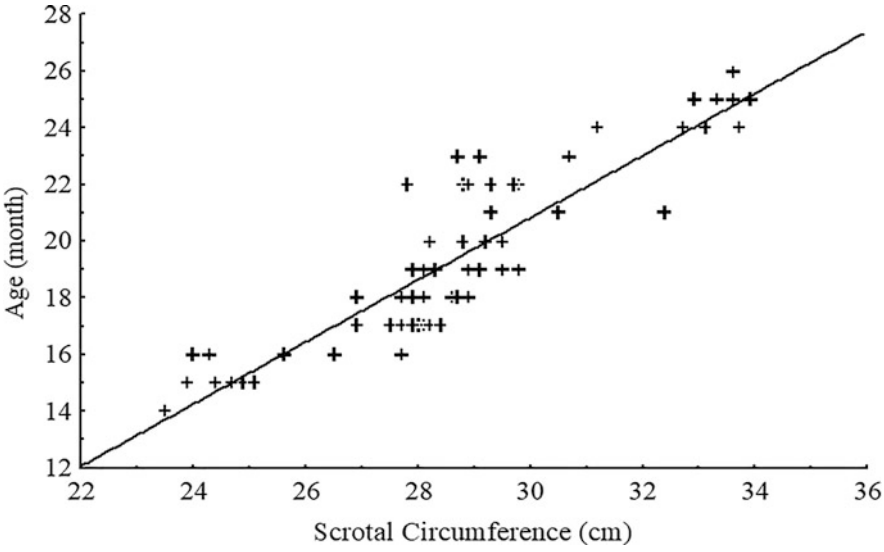

Fig. 17.5 The relationship between age (month) and scrotal circumference (cm) in Murrah buffalo bulls. (SC = 14.566 + 0.728*Age; $R^2 = 0.79$)

involving more than 180 Murrah buffalo males, an average of the scrotal circumference of 30 ± 3.6 cm was found (range 24.4–31.0 cm). Besides in the same survey an increased linear growth and correlation among scrotal circumference, body weight, and animal age was found.

Therefore, reports from Australia, Brazil, and Malaysia found that for swamp buffalo puberty and sexual maturity are delayed when compared with the river type. In Australia, it was reported that scrotal circumference and body weight were variable according to the year season (McCool and Entwistle 1989), and this phenomenon is also observed in male buffaloes on the island of Marajó, Brazil, during the dry season; when the growth and bodyweight of the animals were suppressed due to the undernourishment, the testis parenchyma and length were decreased (Vale, unpublished data).

On the other hand, it was observed that the testicles were found to have achieved the total growth at the age of 2.77 ± 0.09 years, with a body weight of 275.6 ± 8.5 kg, and a scrotal circumference of 20–21 cm. For the Mediterranean breed raised in Brazilian conditions, it was found that puberty and sexual maturity were achieved at the age of 10–14 and 24 months of age, as well as the scrotal circumference was found to have 21.7 ± 1.9 cm and 31.1 ± 2.9 cm, respectively (Ohashi 1993). Therefore for the Murrah breed at the puberty 12–17 and beginning of sexual maturity at 18–23 months, the scrotal circumference was found to be 21 ± 3.3 and 25 ± 3.2 cm, respectively (Table 17.2) (Vale 2011).

Table 17.2 Classification of scrotal circumference in buffalo males of Murrah breed, in Brazil (Vale et al. 2004)

Age (months)	Average (cm)	Very good (cm)	Good (cm)	Questionable (cm)
12–17	21 ± 3.3	>23	23	>19
18–23	25 ± 3.2	>26	25	>21
24–29	27 ± 2.8	>29	28	>23
30–35	29 ± 3.5	>30	29	>25
36–41	32 ± 3.1	>33	32	>28
42–47	34 ± 2.9	>34	33	>30
48–53	36 ± 3.5	>36	34	>31
54–60	38 ± 3.6	>39	36	>32

17.4 The Breeding Soundness Examination (BSE)

Before a general clinical examination itself, it is necessary to assess the development and body configuration of the physical state and the sexual characteristics. The general clinical examination is carried out of the genital tract in order to assess the condition and or body development and Breeding Soundness Examination (BSE), a general health examination based on the prevention of hereditary, genital, infectious, and parasite diseases (Table 17.3) (Vale et al. 2014).

Furthermore, to obtain a better quality ejaculate to be deep frozen, a bath before semen collection with cold water improves the semen quality. Moreover, buffalo semen collection must be performed throughout an AV with a temperature between 44 and 46 °C, using a female or a male buffalo as a dummy. It is important to do false mounts before each collection (2 or 3 false mounts) to improve the quality of subsequent ejaculates and then a first and second ejaculate within 30 min intervals. It is recommended to use AV with a short length in young buffaloes (Fig. 17.6). After collection, the semen should be evaluated for the initial parameters and then placed in a water bath at 37 °C (Vale 1994, 1997). Due to the buffalo usually exhibiting best sexual behavior during the nighttime, the best time to harvest buffalo

Table 17.3 Presentation of the procedures for Breeding Soundness Examination (BSE) in buffalo bull

Summary of the breeding soundness examination in buffalo male: andrological examination
The following points should be considered during BSE (Merkt and Krause 1983). I. *Hereditary health* (the male buffalo must be free of hereditary diseases and disorders/abnormality in the genital tract that limit its use as a sire) II. *General health* (the male buffalo must be free of extragenital diseases that limit its use as a sire) III. *Normality of the genital tract and sexual health* (the animal must be free mainly of functional disturbs/abnormality and infectious contagious diseases) IV. *Serving capacity*—"*Potentiacoeundi*" (be able to breed a female without problem and interruption) V. *Fertilizing capacity*—"*Potentiagenerandi*" (produce sperm cells with capacity to fertilize an ovum throughout the production of normal spermatozoa—normospermia)

Fig. 17.6 Semen collection in a buffalo

semen is early in the day (still dark) or late in the evening, with the need of the time of semen collection, a quiet environment, without noise or another form of disturbance that could cause discomfort of concentration on the buffalo who will donate semen (Vale et al. 2008).

17.5 Libido, Semen Collection, and Evaluation

Although young buffalo that have reached puberty can breed, however reproductive capacity increases as the male continues to mature. This aspect is directly related to the management, age, body weight, and testicular weight. It is important to note that poorly fed animals, without adequate hygienic-sanitary care and adequate animal comfort, present changes in their initial sexual behavior (Vale et al. 1988). Buffalo generally attain mating ability about 6 weeks after puberty for which they need to manifest libido and have sufficient penile development to permit normal ejaculation. In males, puberty is the earliest age at which they can impregnate females or serve the artificial vagina. The age at which androgens begin to be secreted in higher quantities by the testes and rapid rise in fructose content in the seminal vesicles approximately coincide with the time at which it first becomes possible to collect a buffalo ejaculate.

At puberty, buffalo males show a weak libido when compared to *Bos taurus* although they are more easily trained to serve artificial vagina and many will serve at

Table 17.4 Assessment basis of libido and mating behavior in males (Javed et al. 1998)

		Observations	Rank value
Libido	Approach to the teasing site	Indifferent	0
		Lethargic	1
		Keen	2
	Erection of penis	Absent	0
		Within sheath	1
		Outside sheath	2
Matting behavior	Approach to teaser	No interest	0
		Sluggish	1
		Eager	2
	Mounting	Without protrusion	0
		Incomplete protrusion	1
		Complete protrusion	2
	Copulatory thrust	No	0
		Weak	1
		Strong	2

the first attempt. Through the observation of buffalo males at AI centers for libido and mating ability it is clear that for this species those characteristics are little pronounced than in bovine.

In a study conducted on the libido and mating behavior of Nili-Ravi bulls, monitored during the time of semen collection by allocating a numerical value as described by Javed et al. (1998), the results of analysis of two ejaculates collected from each buffalo bull and the period between two ejaculates are expressed in Table 17.4. According to Vale et al. (2014), when young buffalo males are put together in a group, showed a good interaction and start a teasing behavior like beating the rear rump, smelling and licking the anal and inguinal region, smelling the prepuce and urine, and showing Flehmen reflex (Figs. 17.2 and 17.7). However, they attempt incomplete mount by exposing the penis, but do not perform ejaculation (Fig. 17.8). The same behaviour is shown when they are presented to a restrained dummy female or one female in estrus. They begin to sniff and lick the vulva, the Flehmen reflex, incomplete mating (the animal exposes the penis, but does not ejaculate), mounting without penile exposition, intermittent ejaculation of the penis before the mount. In addition, rest the chin on the female rump and tease the female body by the head (Vale et al. 2008, 2014).

These behaviors were also reported in studies by Anzar et al. (1993) and Samo et al. (2005). Johari (1960) draws attention to the occurrence of Flehmen reflex and repetitive small penile movements with a projection of the penis by a few centimeters out of the preputial ostium (Figs. 17.7 and 17.8).

Fig. 17.7 Buffalo showing "*flehmen,*" a typical olfactory stimulus during the courtship with a female during the time of mating behavior

Fig. 17.8 Young buffalo bull's mounting frequency on fellow males increased during puberty. (Courtesy of NDRI, Karnal, India)

17.6 Assessment of Buffalo Semen Characteristics

The evaluation of buffalo semen is similar to that for bovine and divided into physical and morphological characteristics, as stated in Tables 17.5, 17.6, and 17.7 and Fig. 17.9, and according to Merkt and Krause (1983) used for different domestic animal species.

Table 17.5 Characteristics of a normal buffalo ejaculate collected through an artificial vagina, according to Vale et al. (2014)

Semen pattern	Characteristic
Color	White, milk white, with light blue tinge
Volume	3 ml (2–8)
Wave or swirl motion	>3
Motility (%)	>70
Vigor (individual motility)	>3
Concentration	6×10^5 to 12×10^5
Live sperm (%)	>70
Abnormal sperm (%)	<70
pH	6.7–7.5

Table 17.6 Descriptive and numerical scales for evaluation of microscopic waves pattern of semen from buffalo, according to Vale et al. (2014)

Description scale	Numerical scale	Appearance pattern
Excellent	5	Waves are very dark, more waves in all directions
Very good	4	Waves are dark, separate, and with rapid motion
Good	3	Waves apparent; moderate motion
Fair	2	Barely distinguishable waves in motion
Poor	1	No waves; sperm cells have static movement
Very poor	0	No wave; sperm cells immotile

Table 17.7 Descriptive and numerical scales for evaluation of microscopic motility of sperm cells from buffalo, according to Vale et al. (2014)

Motile cells (%)	Descriptive value	Numerical value
0–20	Very poor	1
20–40	Poor	2
40–60	Fair	3
60–80	Good	4
80–100	Very good	5

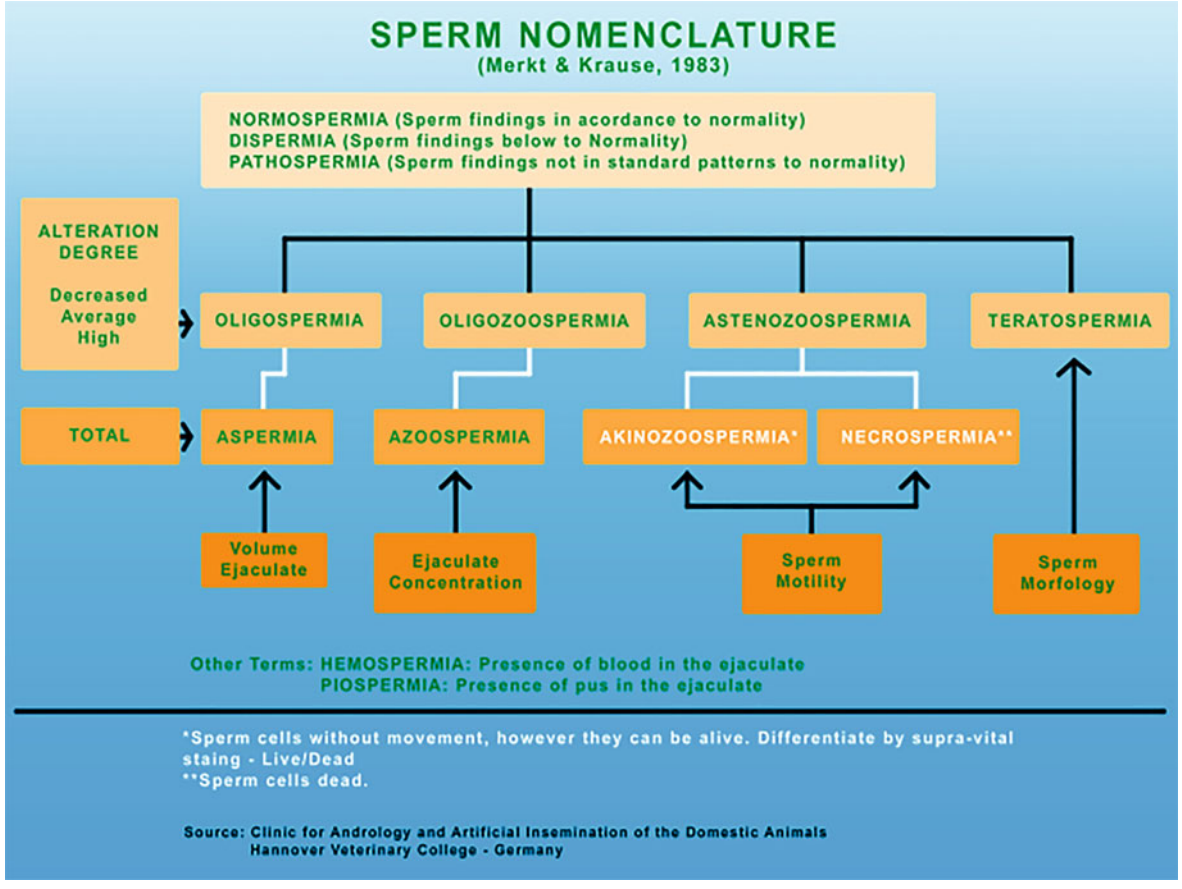

Fig. 17.9 Assessment of buffalo ejaculate according to physical and morphological characteristics (Merkt and Krause 1983)

17.6.1 Color

The color of the ejaculate depends upon the concentration of the sperm cells present in the semen; therefore the degree of color reflects the sperm density in the ejaculate. In general normal color of buffalo, ejaculate varies from light milky, milky, and creamy with a light touch of blue color.

17.6.2 Volume

Ejaculate volume in buffalo bulls varies with age, season, diet, and general management. In our previous experience, young bulls when they just start semen donation around puberty produce about 1.0 ml of semen that increases to about 4 ml after they attain sexual maturity. The heat stress causes testicular degeneration, and reduction in ejaculate volume and other semen attributes. The ejaculate volume is measured immediately after semen collection in the graduated collection tube itself. Generally, buffalo bulls ejaculate less semen volume than cow bulls. Young bulls produce less volume of 1–3 ml per ejaculate whereas old bulls can ejaculate up to 6 ml or more (Fig. 17.10).

Fig. 17.10 First and second ejaculate, both high volume and concentration

17.6.3 Wave Motion

A thick drop of the ejaculated semen is put on the clean and dry glass slide maintained at 37 °C under 10× in a light phase-contrast microscope. The wave or swirl motion of semen is observed. The semen drop is observed for the waves and eddies which is formed by the movement of seminal plasma and sperm cells movement. These swirls emerge and then disappear, or mass motility of semen generally is graded into a score of 0–5 (Table 17.6). A good quality buffalo bull ejaculate generally shows the mass motility of >3, which is equal to 60% or more of mass activity.

17.6.4 Concentration

Buffalo ejaculate sperm concentration normally varies from 600 to 1200 × 10^6 per ml, and sperm concentration is influenced by the season, diet, age, and scrotal circumference of the bulls (Vale 2007).

Sperm concentration is the density of spermatozoa generally measured per mm^3 of the semen volume. Various methods are employed to estimate the sperm concentration among which hemocytometer and photoelectric calorimeter methods are commonly being used (Figs. 17.11 and 17.12). The following procedure is used to

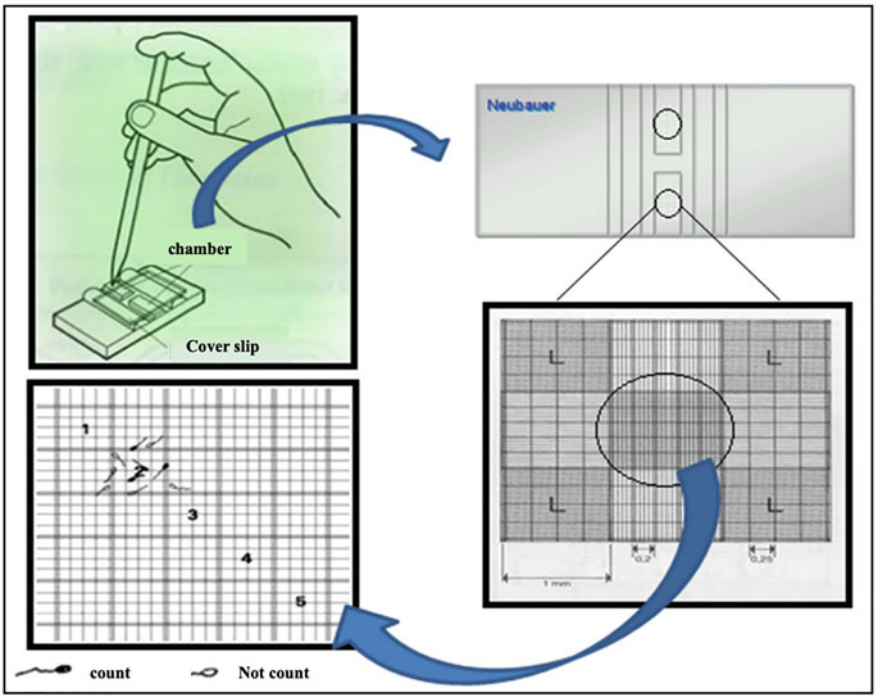

Fig. 17.11 Picture showing that how the sperm concentration of an ejaculate is determined by 961 with a hematimetric chamber model Neubauer

Fig. 17.12 Photometer SDM 6 for determination of sperm cell concentration and dose calculation of different species used in buffalo semen processing laboratories, Minitüb GmbH, Germany

accurately estimate the sperm concentration with the help of a hemocytometer: 20 µl of semen is drawn into a glass Sahli pipette or an Eppendorf automatic pipette and is diluted in 4 ml of 1% Hancock buffered formalin saline or 1% sodium citrate buffered solution, making a final dilution of 1:200. The diluted semen sample is filled into a hematimetric Neubauer chamber similar to blood cell counting. Each spermatozoon in the five squares—the entire ruled central square and four corner squares—is counted. The obtained value is multiplied by 10,000 to arrive at a total number of sperm (Vale et al. 2014).

17.6.5 Sperm Motility

Sperm motility is a rapid way of evaluating the grass motility of sperm. On a clean and dry glass slide maintained at 37 °C, a small drop of semen is placed and a coverslip is put on it. Further, it is assembled in the thermoregulated stage and examined at 40 or 100× magnification.

Season and frequency of collection affect the initial motility. Generally, when semen is collected twice a week, both first and second ejaculate show good motility (>3), with more than 60% of the spermatozoa showing rectilinear progressive motility.

17.6.6 Vigor

Individual motility or vigor is observed in the field having the highest number of motile spermatozoa. One individual sperm must be tracked until it vanishes from the area of observation, and this operation has to be repeated several times. The ejaculate with more than 60% of individual motility is considered a good ejaculate.

17.6.7 Live and Dead

Determination of viability or live and dead sperm helps predict the quality of ejaculate. Semen samples exceeding 30% initial dead sperm may not be good for freezing. If the viability of spermatozoa drops below 50%, then the fertility of the semen sample may be questionable even through natural breeding. In the second ejaculate, the live sperm percentage may increase and semen is collected twice a week at regular intervals. For counting live and dead spermatozoa differential staining techniques have been employed and smears may be prepared for instantaneous use (Fig. 17.13). The smear is prepared on a clean glass slide pre-heated to 37 °C and stained with 1% eosin solution. 200 sperm cells should be counted, and live and dead cells are expressed in percent.

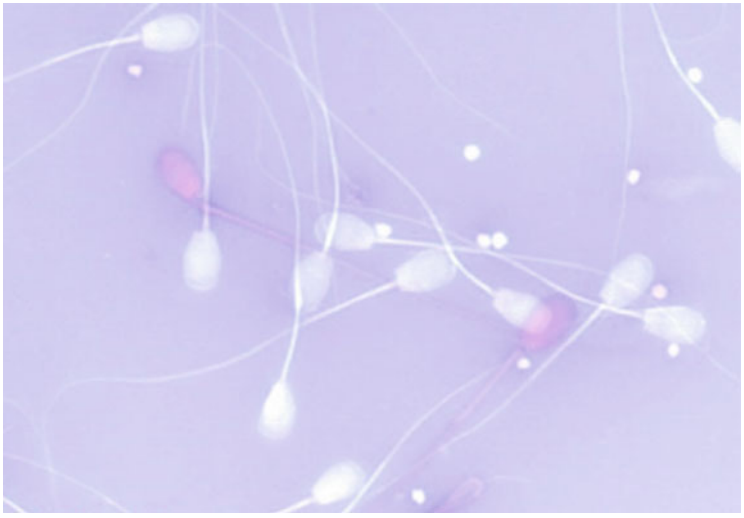

Fig. 17.13 Live and dead sperm cells. Note two sperm cells stained with red color by eosin, whereas the normal sperms have a whitish-blue color, 400×

17.6.8 Sperm Morphology

While going for freezing of buffalo semen, the percentage of sperm abnormality is an important point to be considered. Smears should be prepared from fresh ejaculate to study the morphology of spermatozoa (Figs. 17.14, 17.15 and 17.16).

The prepared smears should then be stained through appropriate staining techniques. The Cerosky carbo-fuchsine-eosin stain method has been used successfully. Different kinds of structural abnormalities related to head, midpiece, and tail are noted down. The sperm abnormalities may either be hereditary or be of developmental and of environmental nature (Fig. 17.17).

Sperm abnormalities can be categorized according to their nature and source. Under the oil immersion objective (×1.000) of the microscope stained smears are examined and 200 spermatozoa are counted. Alternatively, a wet preparation of fresh semen with the sperms fixed in a buffered formalin saline solution and a drop of the fixed material will be mounted in a slide and a coverslip and observed under an interference or phase-contrast microscope (Figs. 17.17 and 17.18).

Major defects: 1. Underdeveloped cells; 2. double forms; 3. acrosome ("knobbed sperm") defect; 4. diadem defect; 5. decapitated sperm defect (the tails appear active); 6. pear-shaped heads; 7. heads that are narrow at the base; 8. heads with an abnormal contour; 9. small abnormal heads; 10. free (detached) abnormal heads; 11. the "corkscrew defect" of the midpiece; 12. other midpiece defects; including the "tail-stump" defect and accessory tails; 13. proximal cytoplasmic droplet; 14. pseudodroplet and other thickened midpieces; 15. coiled or strongly folded tails (including "Dag defect"). Other cellular elements that may also be present include: a. epithelial cells; b. erythrocytes; c. medusa formations; d. boat cells; e. mononuclear cells; f. neutrophils	
Minor defects: 16. Narrow heads; 17. small, normal heads; 18. giant and short, broad heads; 19. detached normal heads; 20. detached acrosomal membranes; 21. abaxial implantation of the tail; 22. distal droplet; 23. simple bent tail; 24. terminally coiled tail. Other cellular elements that may also be present include: a. epithelial cells; b. erythrocytes; c. medusa cells; d. boat cells; e. mononuclear cells; f. neutrophils	

Usually, the freshly collected buffalo semen has a wide range of pH (6.5–7.2). Though the concentration of sugars in buffalo semen is lower, the rapid breakdown leading to the formation of lactic acid can reduce the pH of semen. Concurrently, environmental factors have a strong effect on the morphological and biochemical characteristics of semen. It has been revealed that better quality of semen is produced during winter and spring in temperate regions and deterioration of semen quality during autumn and summer (Vale 2007). Contrarily, better quality semen is produced during the rainy season in tropical regions. The best time for cryopreservation of semen in the warm and humid tropical Amazon region is between January and June (Vale et al. 2014).

Semen quality tends to decline during the summer as buffaloes are more sensitive to heat stress. Improvement measures such as the sprinkling of water on animals during the hotter part of the day, allowing the animals to wallow, optimum

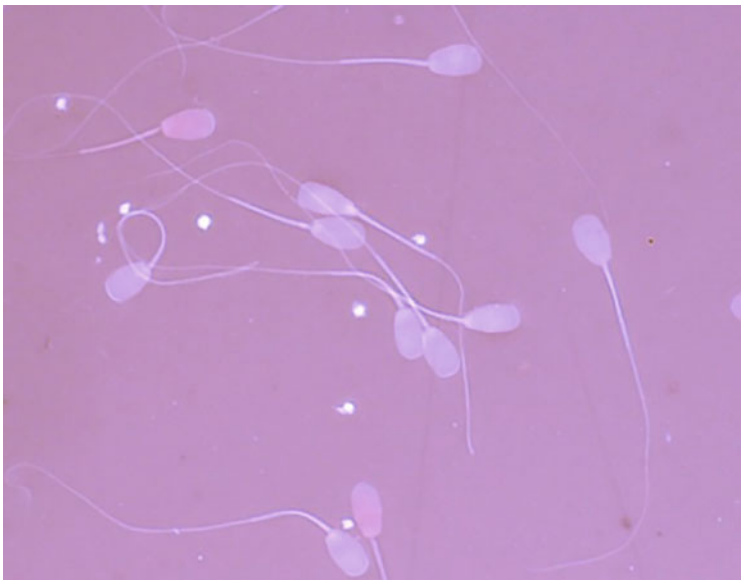

Fig. 17.14 Normal sperm in a smear stained by Giemsa, 200×

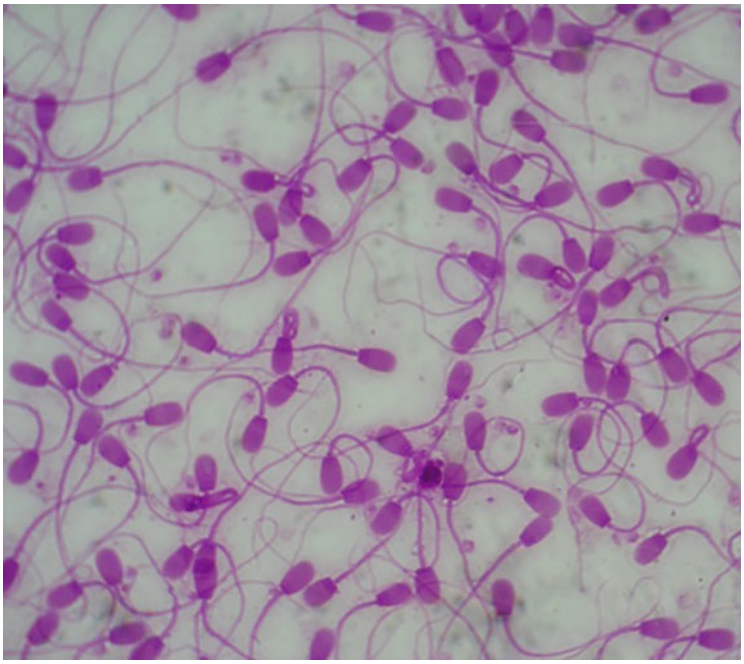

Fig. 17.15 Normal sperm in a smear stained by Cerosky, 100×

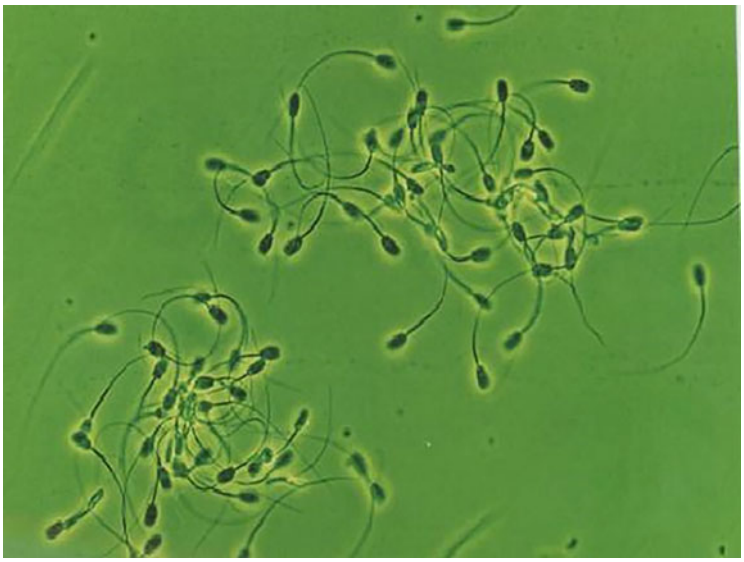

Fig. 17.16 Normal semen in humid preparation for phase-contrast 100×

ventilation, and protecting the animals from hot wind and radiation exchange may overcome the problem of deterioration of semen quality during thermal stress.

Buffaloes possess a poorly developed thermo-regulatory mechanism and are more prone to heat stress during the summer season. Thus, free access to shadow and water is essential. In tropical regions, such as Northern Brazil (Amazon region) it is highly essential to incorporate better management to lessen the burden of heat stress (Vale 2007; Castro et al. 2017). The use of adequate protection against sun rays, use of showers, or access in a water pond/swimming pool is the key for the year around semen frozen production.

17.7 The Establishment of Suitable Extenders for Deep Freezing Buffalo Semen

After the Seminar on Reproduction and Artificial Insemination in Buffalo, promoted by FAO and the Swedish government, held in Karnal, India, 1978, several advances were obtained in different laboratories around the world, culminating in obtaining fertility rates higher than 65% of birth (Vale et al. 1984; Sengupta and Sukihija 1988; Vale et al. 1991a, b; Dhami et al. 1996). Regarding this, several problems related to extenders were outlined, mainly after the use of buffer substances TRIS (Hydroxy-methyl-amino-methane), TES (Hydroxy-methyl-amino-ethan), skim milk, Laiciphos 478, milk-citrate-lactose, lactose, citrate, and citric acid-buffalo whey, specifically for buffalo semen (Roy et al. 1956; Pavithran et al. 1972; Anand 1979;

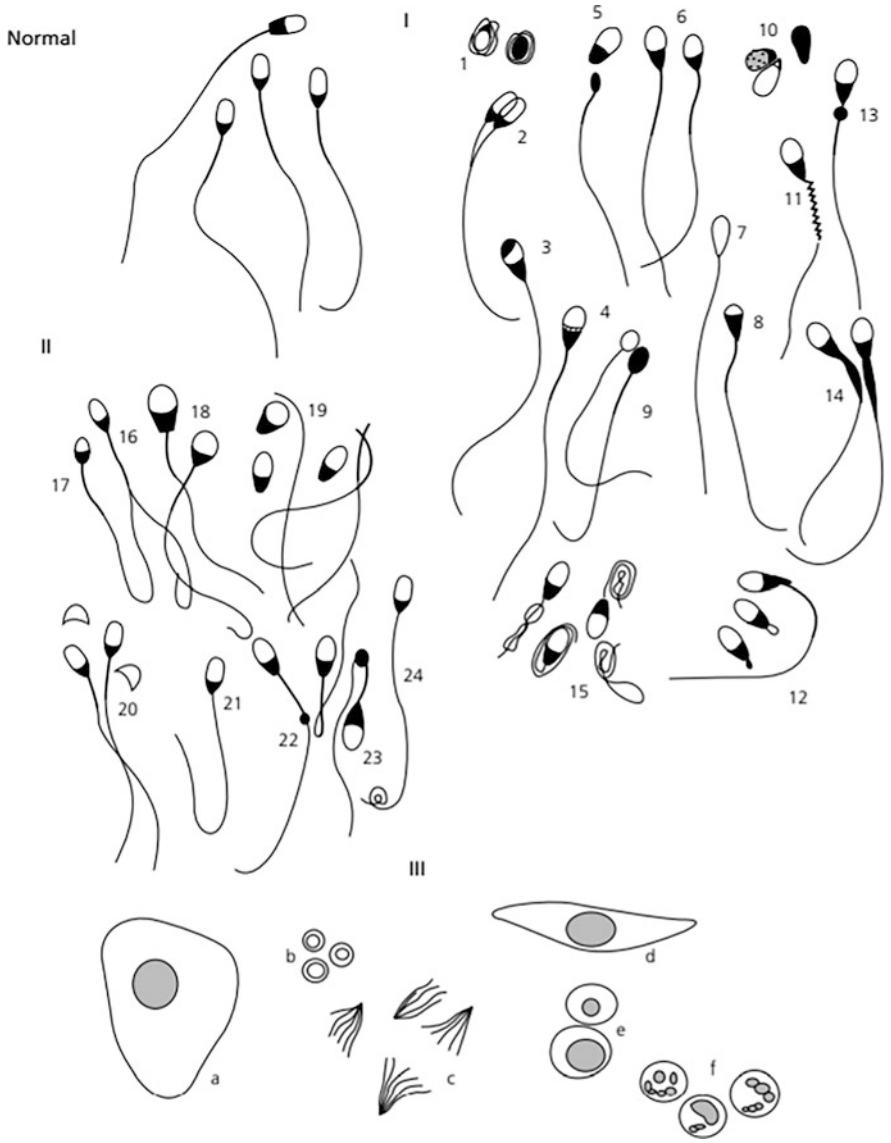

Fig. 17.17 Classification of sperm abnormalities nto major and minor defects according to their effect upon fertility (Blom 1972)

Günzel et al. 1979; Heuer 1980; Vale et al. 1984; Tayel et al. 1988; Rahman et al. 1984; Stoyanova 1991; Vale et al. 1991a, b; Vale 1994).

Thus, buffalo semen is diluted using a suitable extender, with the inclusion of additives, cryoprotectants, and antibiotics. Extended semen is then filled in straw and cryopreserved by various procedures (Vale et al. 2014).

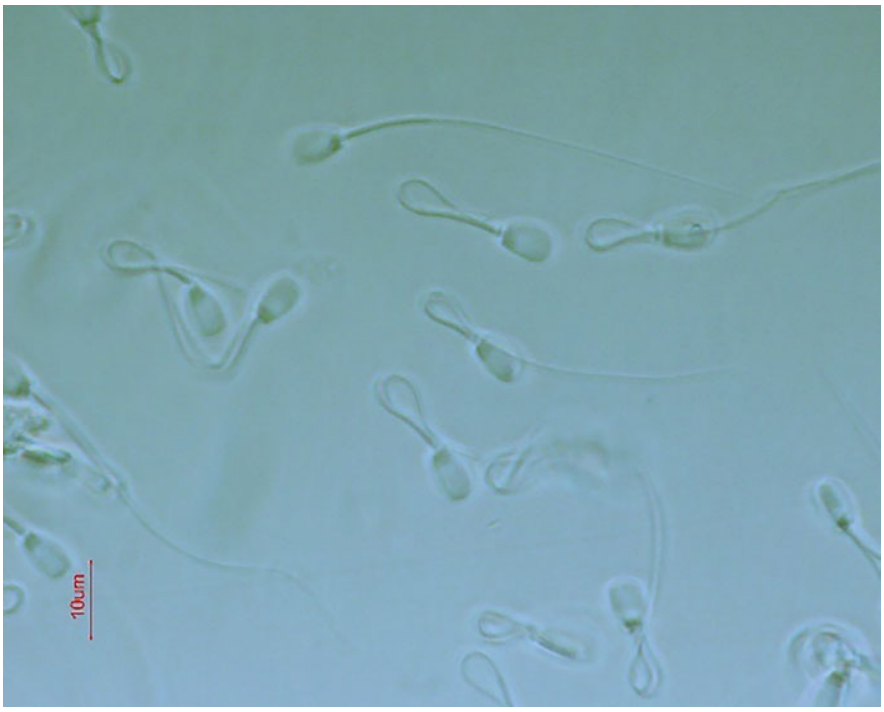

Fig. 17.18 Sperm coiled tails due to testicular degeneration caused by the hot environment, phase-contrast 100×

17.7.1 Semen Extenders

In order to reduce the deleterious effects of cryopreservation, for several years numerous commercial dilutive media have been tested, added additives that help protect the spermatic cells and provide substrates to maintain their viability, even after freezing (Castro 2010). According to Rasul et al. (2000), the dilution of semen with an appropriate buffer is one of the key factors determining the survivability of semen during cryopreservation. An ideal buffer should have pH between 6 and 8, preferably 7; greater water solubility and lesser solubility in any other solvents; lesser salt effects and buffer concentration; the least temperature effect; higher ionic strengths; better cation interactions and chemical stability. According to Dalal et al. (2018), buffalo spermatozoa are more vulnerable to damages during cryopreservation in comparison to cattle spermatozoa (Raizada et al. 1990). The cryo-damages can be minimized by optimizing the cooling and freezing rates and using suitable diluting media (Kumar et al. 1992).

Presently more efforts are being made to develop an appropriate buffering system for cryopreservation of buffalo semen with a composition close to natural medium and assist in maintaining the fertility of cryopreserved semen (Mughal et al. 2017).

In India and Pakistan, various commercial extenders i.e., soybean lecithin-based extender AndroMed® and egg yolk-based Triladyl® (Minitüb, Tiefenbach Germany), have been used for buffalo semen. Triladyl® tended to have high rates of post-thawing motility (Herold et al. 2006). AndroMed® viability has been proven in many studies (Janett et al. 2005; Muino et al. 2007; Leite et al. 2011; Valeanu et al. 2015). In Brazil, Castro and collaborators are studying both AndroMed® and Triladyl® extenders on buffalo semen. Preliminary studies have shown efficacy in the cryopreservation of buffalo semen.

Andrabi (2014) mentioned several types of research that have focused on the utilization of chemically defined buffers for buffalo semen extension that were originally evolved for bovine bull semen, for example, citrate or Tris and/or citric acid or Laiciphos® (IMV, France; containing Laiciphos in unknown buffer) or Biociphos® (IMV, France; containing Biociphos in unknown buffer) or Bioxcell® (IMV, France; containing unknown buffer and animal protein-free formulae of nontoxic cryoprotectant) have been evaluated as buffers for the deep freezing of water buffalo spermatozoa (Chinnaiya and Ganguli 1980a, b; Matharoo and Singh 1980; Ahmad et al. 1986; Dhami and Kodagali 1990; Singh et al. 1990, 1995; Dhami et al. 1994; Akhter et al. 2010).

Vale et al. (1984) reported tris-based buffer as the most appropriate buffer for buffalo semen cryopreservation. Buffers such as Tes and Hepes containing Zwitterion have also been employed in deep freezing of buffalo semen, but with varying success (Oba et al. 1993; Chachur et al. 1997; Rasul et al. 2000). Tris (hydroxymethyl) aminomethane, egg yolk citrate, and egg yolk-lactose are also popular diluents for the liquid storage of buffalo semen (Akhter et al. 2011; Andrabi 2014).

A recent study with Bioxcell® (IMV, France) has shown that it can be a potential substitute to the commonly used Tris-based extender for deep freezing buffalo semen provided that large-scale field fertility results are found to be satisfactory (Akhter et al. 2010). Additionally, Bioxcell® is satisfactory for the liquid storage of cooled buffalo semen (Andrabi 2014). Naz et al. (2018) showed that liposome-based Optixell® (IMV, France, Kurdson Industries, Lahore, Pakistan) extender improved post-thaw semen quality and fertility in buffaloes. From the previous studies, it is evident that buffers, especially Tris-based, may contribute to the most acceptable buffering system to enhance the post-thaw freezability of buffalo spermatozoa. Tris-based buffer is least affected by temperature variations as compared to other buffers and it has a pH nearer to the pKa (acid dissociation constant). In addition, the variation in efficacy of different buffers indicates that buffalo sperm cells are more vulnerable to freezing stress as compared to cattle bull sperm cells, possibly because of biochemical factors that determine the membrane fluidity during cryopreservation (Garcia 2013; Andrabi 2009, 2014).

In an experiment by Vale et al. (1984) with extenders based on yolk citrate or skimmed milk, post-defrost motility was close to 20%. When the lactose was used, post-defrost motility was around 30%, and the best results were obtained with TRIS and TES-based thinners, with post-thawing motility ranging from 50% to 60% for both. In the thermo-resistance test (TTR), the best result was obtained with TES-

TRIS-based extenders, in which motility after 5 h, starting the TTR at 40 °C, was still around 20%. Thus, the recommendation of Vale et al. (1984); Vale et al. 2014) is that TES-TRIS should be the means of choice for cryopreservation of buffalo semen due to the maintenance of motility during the process of cryopreservation and viability of post-defrosting semen. These data are corroborated by those of Barnabe et al. (1994) and Rasul et al. (2000).

As mentioned, in most livestock species including the buffalo, egg yolk is a general constituent of semen extenders which usually acts as a nonpermeable cryoprotectant (Andrabi 2014; Naz et al. 2018). In general, egg yolk is used at the concentration of 20% in a semen extender (Sansone et al. 2000; Andrabi 2014) as higher concentrations may pose deleterious effects when coupled with toxicity (amino acid oxidase activity) of dead spermatozoa, resulting in a lower post-thaw sperm quality (Shannon 1972). It is one of the possible reasons for the detrimental effect of higher amounts of egg yolk on the post-thaw sperm quality (Sansone et al. 2000). The chemical composition of egg yolk used in semen freezing extender may also govern the quality of sperm post-thaw (Andrabi 2014).

Cebran II® is an extender based on ringer lactate, egg yolk, glycerol, milk, antibiotics, fructose, and purified water, proven to be efficient in maintaining the seminal quality of buffaloes, receiving this name for having been developed at the Animal Reproduction Biotechnology Center in Amazon, Brazil. This extender became standard due to its low acquisition cost, as well as the intrinsic qualities linked to the seminal cryopreservation process (Silva et al. 2002).

Studies by Castro (2010) have shown that the use of vitamin C alone or in combination with pentoxifylline can significantly increase progressive sperm motility after performing a thermo-resistance test. Shah et al. (2017) have studied the influence of curcumin as an antioxidant in semen extender and reported an improvement in freezability of water buffalo spermatozoa with the addition of 1.5 mM curcumin in the extender.

For the cryopreservation of buffalo semen, several studies have been implemented to attempt to find the optimum concentration of glycerol and method of glycerolization (Jainudeen and Dass 1982; Kumar et al. 1992; Nastri et al. 1994; Ramakrishnan and Arif 1994; Abbas and Andrabi 2002; Singh et al. 2006). From these previous studies, it is recommended that the inclusion of glycerol at 5–7% either in a single step to the initial extender or in two steps to the milk-based extender is desirable for the deep freezing of buffalo bull spermatozoa (Andrabi 2014).

Ethylene glycol can also be used as a substitute for glycerol in the cryopreservation of buffalo semen (Andrabi 2014). Ethylene glycol possesses greater permeability as compared to glycerol in sperm cells of different species (Gilmore et al. 1995; Phelps et al. 1999). From the preliminary results by replacing glycerol with ethylene glycol it is suggestive that ethylene glycol may be used in cryopreservation of buffalo semen (Valdez et al. 2003; Rohilla et al. 2005). Rasul et al. (2007) tested glycerol and/or DMSO, added either at 4 or 37 °C, as a cryoprotectant for buffalo spermatozoa. The addition of DMSO did not allow satisfactory cryopreservation of buffalo spermatozoa in Tris-citric acid extender. The lethal effect of DMSO is attributed to a toxic rather than an osmotic effect (Rasul et al. 2007).

The fertility rate is the most appropriate parameter to assess the quality of frozen thaw semen. Despite all the efforts, estrus detection, buffalo's hygienic conditions, and insemination time also contribute to poor fertility rates (Vale 1997).

According to Mughal et al. (2017), future research for cryopreserved semen should focus on upgradation of freezing protocols for reducing spermatozoa during deep freezing. To accomplish this goal, diluents composition, upgradation of freezing protocols, and presently used extenders must be emphasized for cryopreservation of buffalo spermatozoa. Also, season influence should be related to cryopreservation of buffalo semen (Castro et al. 2017).

17.8 Technological Processing for Semen Freezing

The basic principle for freezing and thawing the buffalo semen follows a specific protocol with different stages similar to that in bovine species and it is summarized in Fig. 17.19.

Phase I
Collected the semen and diluted with a suitable diluter, followed by packing and sealing in straws;

Phase II
Equilibrium stage at 4–5 °C refrigerator temperature for 4 h, after that the diluted semen be placed horizontally on a freezing tray;

Phase III
Deep freezing, starting with pre-freezing, when the straws are placed horizontally 4 cm above the liquid nitrogen vapor for 20 min. Final freezing when the semen straws are dipped into the liquid nitrogen at −196 °C. It is fascinating to know that the use of a programmable freezer for deep freezing of spermatozoa yielded better quality semen post-thaw (Vale et al. 2014).

17.8.1 The Phase I

Maintaining the highest fertilizing potential of a buffalo semen sample through the freezing/thaw process requires optimizing the conditions for cryopreserving the sperm. In buffalo, many studies over the past 50 years have attempted to optimize the freezing and thawing in this species.

Phase I comprises the start of handling the collected ejaculate and must be carried out after the identification of the ejaculate. The following points are considered in this phase:

– The ejaculate must be evaluated for its physical and morphological characteristics, as described for assessment of buffalo semen in Figs. 17.9,

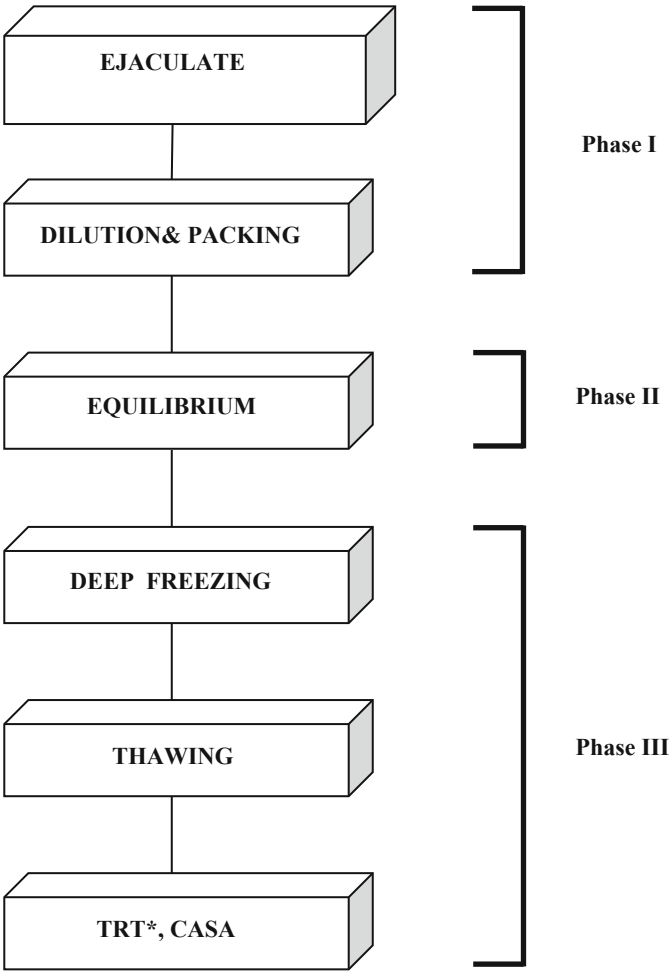

Fig. 17.19 Schematic representation of the routine used in the different stages of technological processing for the freezing of buffalo semen

17.11, 17.13 and 17.17 and Tables 17.5, 17.6, and 17.7. Semen samples with a mass activity and motility >3 should be used for processing. Meanwhile, other parameters of semen should be evaluated. In addition, sperm concentration should be calculated using a hemocytometer or photocolorimeter.

– Decide dilution rate based on sperm concentration and motility, so that the final concentration is maintained at x million live sperm/dose (0.25 ml).

- The dilution must be initially 1:1 or 1:2 depending on the color feature. A very concentrated ejaculate, milk white with a light blue tinge, can be diluted 1:3.
- Both components, semen, and extender must be kept in a water bath (plastic beaker/tray) at 30 °C, until the final dilution; the contents are mixed by gentle rotation and kept at this temperature for 15 min.
- It is advisable to prepare the extender 12 h before the semen evaluation.
- It is suggested that the motility activity should be seen immediately after collection, dilution, and about each 10 min afterward.
- Previously mini-tubes are identified and fixed in a comb and kept under UV rays for 10–15 min.
- Then, diluted semen is transferred to room temperature, at 20 °C for 15 min. During this time mini-tubes are filled and sealed, which is done by a machine, with the straws held in a comb, specially designed for this purpose.
- Then an air space bubble is created inside the straws filled, by a quick movement of the comb downwards and then transferred to the cooling tray and placed to cool horizontally in a refrigerator or cold handling cabinet temperature (4–5 °C) between 4 and 7 h (equilibration time), according to the extender used.

17.8.2 The Phase II

Equilibrium Time

The motility and metabolic activity of sperm are decreased during this time. That is a critical period in the pre-freezing semen processing technology. This time between the addition and initiation of freezing is called the "glycerol equilibration" period. For buffalo semen when TRIS or TES buffer-based extenders are used, a temperature of 4–5 °C for 4 h of equilibration time gave consistently better post-thawing motility (Vale 1994). This operation can be done in a refrigerator or a cooling cabinet.

17.8.3 The Phase III

Semen Freezing and Thawing Evaluation Techniques

Although many extenders were used for semen preservation, the TRIS-buffer, TES-milk with 7% of glycerol, has shown excellent efficacy for semen freezing and fertility (Günzel et al. 1979; Vale et al. 1984). Both extenders can control the ejaculate pH variation, decreasing the superficial tension phenomena among the diluter compounds, and also are a source of nutritive substances that are used in the spermatozoa metabolism.

Deep freezing of buffalo semen requires trained personnel and keen attention, since buffalo spermatozoa are very fragile when compared to the bovine; thus they must be handled carefully. During the freezing procedures, the crucial temperature range is between +4 and −40 °C due to the possibility of crystallization and cold shock occurring (Vale et al. 1991a, b, 2014; Vale 1994).

Different experiments reported that using TRIS buffered extender, with 7% of glycerol, a freezing rate can be used for 20 min between 4 and −140 °C. However, another study observed that a freezing rate of 18 °C per minute between +4 to −40 °C and 8 °C per minute for −40 to −140 °C taking 11 min for the whole process is the most suitable for deep freezing buffalo semen. This rate can be monitored by using a programmable freezer (Mughal et al. 2017; Naz et al. 2018). Furthermore, extended semen packaged in straws (French IMV or German Mini-tubes) using the liquid nitrogen simple vapor-freezing technique, which is very feasible and can be done using a liquid nitrogen container or a simple isotherm box. Deep freezing is carried out in a horizontal position, with the temperature course decreasing, 4 cm above the level of liquid nitrogen vapor (−130 °C) for 20 min and then being transferred into liquid nitrogen at −196 °C. Moreover, there are different methods for processing deep freezing techniques. In some of them, straws were filled and sealed soon after final dilution during the equilibration time in the cooling cabinet and frozen in a vertical position, according to the technical recommendation for each different process.

Recent field works in different countries have shown that both German mini-tubes and French Straws medium type gave comparable conception rates on the use of frozen buffalo semen. At present different semen extenders developed and tested for field use have been recommended, but the most important is the use of successful routine technical procedures described here. After the equilibration time, freezing racks with the straws are transferred to an isotherm box or in a biological freezer and put 4 cm above the level of liquid nitrogen for 20 min. Afterward, the racks with mini-tubes are dipped in liquid nitrogen and maintained at –196 °C in a container. Examine the semen for post-thaw motility in a water bath kept at 37–40 °C temperature for 30 s. Frozen semen samples exhibiting more than 40% of progressive motility are kept for future use (Barnabe et al. 1994; Vale et al. 2014).

17.8.4 Analysis for Thawing Frozen Semen for Fertility Assessment

There are several citations in the literature reporting that the buffalo semen is more fragile and is more vulnerable to damages during freezing/thawing than that of bovine spermatozoa and thus frozen buffalo spermatozoa yield poorer fertility rates in comparison to bovine spermatozoa (Chohan et al. 1992; Dhami et al. 1994).

Although such points are still unclear and not yet fully elucidated, there is some important information on the knowledge of male gametes of domestic animals on the injuries caused by the freezing process and oxidative stress on the morphology of sperm after freezing (Fig. 17.20).

The conventional analysis of semen quality includes the evaluation of sperm concentration and motility (total and progressive). Based on these parameters the number of doses produced from each ejaculate is calculated. Even so, post-thaw viability of sperm is still low, and a greater individual variation exists among breeding bulls. According to Ugur et al. (2019) in bovine semen, these shortcomings are important as they are impeding the advances both in reproductive biotechnology

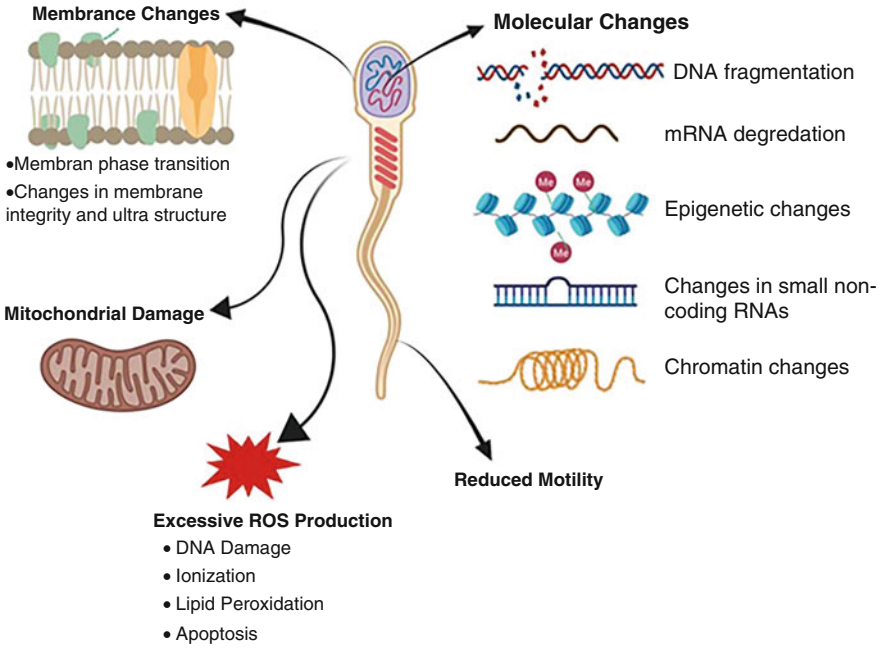

Fig. 17.20 Hazardous effects of the freezing-thawing process on a sperm cell. Morphological and physiological effects of cryopreservation on bovine spermatozoa are summarized. Ugur et al., 2018. *Frontier in Veterinary Science*, doi: https://doi.org/10.3389/fvets.2019.00268, published with author permission

and in the fundamental science of mammalian gametes, since membrane and molecular lesions in the acrosome of the sperm head, DNA fragmentation, and RNA degradation in addition to epigenetic changes, changes in small noncoding RNAs and chromatin changes, and damage in the mitochondrial structure reduce motility and excessive ROS production.

Notwithstanding, it is possible that the same kind of injury also occurs in buffalo semen after post-thaw examination although improved results have been obtained (Vale et al. 2014; Castro 2017). Several attempts have been made to develop different extenders and have been supplemented with additives to lower oxidative stress or cryodamage with varying levels of success. Through the application of advanced techniques in modern molecular and cell biology more explicit insights on sperm morphology and function have been revealed.

The introduction of automated sperm evaluation systems based on computer-assisted semen analysis (CASA) constituted a revolution in the production of doses obtained from an ejaculate to be frozen, representing a major advance in the knowledge of andrology. Computer-assisted semen analysis (CASA) is the potential tool for accurate semen analysis (Amann and Waberski 2014). The CASA system permits the evaluation of a large number of spermatozoa in a short time, thus

delivering a set of quantitative data on the kinematics and morphometry of the sperm head, which allows to optimize the reliability of seminal analyses and predict the semen fertility to be used in AI. However, in buffalo semen, there is some controversy on the subject.

Kumar et al. (2016) conducted a study to find the relationship of sperm motion traits, viability, and membrane integrity with fertility rates. Sperm motion traits in frozen semen from 20 buffalo bulls were evaluated through CASA. The fertility trial was conducted involving 166 buffaloes following standard Ovsynch protocol—Fixed Timed Artificial Insemination (IATF) where it was observed that fertility rate had no significant correlations with sperm motion traits, viability, and membrane integrity. Consequently, the study indicated that fertility rate had no significant correlations with sperm motion traits, viability, and membrane integrity.

On the other hand, Singh et al. (2017) using a computer-assisted sperm analyzer studied some kinematic parameters such as linear velocity (VSL), the curvilinear velocity (VCL), the average path velocity (VAP), the amplitude of lateral head displacement (ALH), the straightness coefficient (STR), the linearity (LIN), and the beat cross frequency (BCF). The proportion of sperm motility was significantly higher ($p < 0.001$) in high fertile bulls as compared to medium and low fertile bulls. Besides, VAP, VCL, BCF, STR, ALH, and LIN were significantly higher ($P < 0.05$) in spermatozoa of high fertile bulls compared to either medium or low fertile bulls. Low fertile bulls had significantly lower ($P < 0.05$) VSL of sperm motion as compared to medium and high fertile bulls. Furthermore, fertility in buffalo bulls was significantly and positively correlated with sperm motility, VSL, VCL, VAP, and ALH.

17.9 Effect of Reactive Oxygen Species (ROS) on Buffalo Semen

The harmful effect of reactive oxygen species (ROS) on sperm was suggested by Macleod (1943), which demonstrated that exposure of human sperm to high concentrations resulted in toxicity, with loss of its motility due to the occurrence of lipid peroxidation (Makker et al. 2009). The effects of this reaction include loss of motility, inhibition of sperm respiration, lesions in sperm DNA, and loss of intracellular enzymes, interfering with the fertilizer capacity of spermatozoa. In fact, spermatic motility is the most sensitive indicator of oxidative stress, a situation in intracellular ATP depletion and insufficient phosphorylation of axoneme protein (White 1993; Aitken and Baker 2002; Sikka 2004). ROS also produces extensive damage to proteins, modifies the cytoskeleton, and causes changes in mechanisms (Sharma and Agarwal 1996). The cytoskeleton provides support for specialized mobile cellular structures, such as eyelashes and flagella, promoting their activation. ROS acts by blocking its function, impairing sperm locomotion (Aitken and Baker 2002).

By relating the excessive production of ROS with the most frequently observed spermatic pathologies, the following changes stand out: abnormal heads, acrosome, intermediate part, and tail defects, fragmentation of DNA, and residual cytoplasmic

drops in the intermediate part (Gomez et al. 1998). Kardivel et al. (2009) observed that progressive spermatic motility decreased by 85.3% in semen in 57.2% after defrosting the semen of buffaloes, and suggested that these results are due to the action of ROS, as the main cause of cell injuries, with a decrease in motility, plasma membrane integrity, and mitochondrial activity.

The oxidative effect generated by ROS can be reduced by the use of antioxidant substances in seminal plasma or added to the diluents used in cryopreservation. In this context, some researchers have added antioxidant substances in buffalo samples (Singh et al. 1995; Nair et al. 2006; Castro 2010). Castro's (2010) studies showed the addition in the buffalo semen extender of vitamin C and pentoxifylline antioxidants, and its association was able to increase the parameters of motility and vigor, and decrease the incidence of defects after the cryopreservation process.

17.10 Adverse Effects of Cryopreservation on Sperm Cells

Cryopreservation of semen is a systematic process that involves an extension, cooling, freezing, storage, and thawing (Mughal et al. 2017). Cryopreservation protocols aim to minimize the deleterious effects on spermatozoa (Kumar et al. 2003). The steps involved in cryopreservation such as cooling, freezing, and defrosting generate physical and chemical damage in the membrane of spermatozoa, which hamper its viability and fecundating ability. A shock from cold in sperm cells is related to oxidative stress induced by the generation of reactive oxygen species, known as ROS. These represent a risk factor because they are highly toxic to spermatozoa. Lipid peroxidation caused by ROS causes structural damage to the acrosome, head, and intermediate part of spermatozoa, as well as initiates the apoptosis process and induces DNA fragmentation. Agarwal et al. (2005) found a significant inverse relationship between ROS levels and in vitro fertilization rate (Agarwal et al. 2005) and it is assumed that the same occurs in in vivo fertilization processes (Castro 2010).

Dilution rate determines the success of semen cryopreservation to a remarkable level. Initially, dilution of semen was made to prevent the damage during cooling, freezing, and thawing, but later on the dilution rate was often changed for various reasons such as specifying the concentration of spermatozoa in a dose of frozen-thawed semen or increasing the number of females that could be served with each ejaculate (Rasul et al. 2000; Kumar et al. 2003; Castro 2010; Andrabi 2014).

After initial dilution, the temperature of semen is brought near to 4 or 5 °C (Andrabi 2014). Cooling aids in adapting spermatozoa to decreased metabolism. The diluted semen cooled slowly to avoid damage due to cold shock. For cryopreservation of buffalo semen, Dhami et al. (1996) determined the relative efficacy of four cooling rates and two equilibration periods at 5 °C. From the results, it is concluded that slower cooling of semen straws from 30 to 5 °C for 2 h is found to be satisfactory as compared to faster cooling (1 h), or a lower initial temperature (10 °C) and 2 h of equilibration at 5 °C, as determined by post-thaw sperm survivability and fertility.

Equilibration is conventionally defined as the period during which sperm cells remain in contact with glycerol before freezing. During this stage, glycerol enters the spermatozoa to maintain a balance between extracellular and intracellular concentration. In the course of equilibration, concentration balance is not only established for glycerol but also for other extender components which are osmotically active. Hence, the equilibration process is associated with the type of extender used (Andrabi 2014). Also, the equilibration process will impact sperm motility (Roy and Ansari 1973).

The adequate cooling rate prevents excessive intracellular dehydration, excessive intracellular solute concentrations, and contraction of the cells (Rasul et al. 2000). During cryopreservation, when the temperature goes down from −5 to −50 °C, the cooling-freezing rate plays an important role. If the rate is more during this temperature range possibility of intracellular ice formation (Kumar et al. 2003).

In the freeze-thaw procedures, the thawing phase is equally important as the freezing phase. In the faster rate of thawing, sperm cells may be exposed for a shorter period to the cryoprotectant glycerol and concentrated solute. The restoration of the intracellular and extracellular equilibrium is more rapid in faster thawing than for slow thawing. Additionally, leaving semen straws at high temperatures for too long a time may lead to fluctuation in pH and eventually result in protein denaturation and cell death (Roy and Ansari 1973; Andrabi 2014).

17.11 Biochemical Characteristics of Semen

According to Andrabi (2009, 2014), certain biochemical constituents affect the capacity of spermatozoa to prevent cryogenic damage. Biochemistry of the seminal plasma is a newly flourishing area of research, specifically related to the biological importance of different biochemical constituents. However, the identification of active biomolecules in the seminal plasma and their mechanism of action is not properly understood in different species. In buffalo, there are few researchers on biochemical concentrations in seminal plasma, and most of them are not recent (Roy et al. 1960; Singh et al. 1969; Singh et al. 1970; Banerjee and Ganguli 1973; Oba et al. 1993; Vale 1997; Princewill et al. 2015; Castro et al. 2017).

According to Oba et al. (1993), the biochemical profile of semen of buffaloes varies throughout the year. In late autumn (May), when temperatures are milder, the buffalo semen has concentrations of fructose and citric acid (526.51 mg/% and 549.22 mg/%, respectively) higher than the annual average, these elements being important for sperm metabolism and the cryopreservation process.

The ionic composition of the semen is extremely important for the processes of cryopreservation of sperm since many enzymatic processes are dependent on ions as cofactors for its activity, as was mentioned previously about the ion calcium, changes in cell volume of sperm that are very large in a short time, during the freezing and thawing processes and also the osmolarity changes during ice formation (Garcia 2013; Castro et al. 2017).

Sansone et al. (2000) reported levels of total proteins, in the seminal plasma of buffaloes, of 2.86 ± 0.14 g/dL. The average total concentration of total proteins in the seminal plasma of buffaloes in India was 2.54 ± 0.51 g/dL, with no influence during the different seasons—summer, from monsoon to winter season (Khawaskar et al. 2012). However, according to experiments by Castro et al. (2017), they found that values of seminal components such as fructose, albumin, calcium, and phosphorus showed interference in normal values, that is, they suffered a decrease in the non-rainy period. This resulted in values with significant statistical differences in the two periods (rainy season and non-rainy season).

17.12 Climatic Effects on Buffalo Reproduction

Though buffaloes are considered as seasonal breeders in some parts of the world, the male of this species does not seem to exhibit seasonality in reproduction, while an important aspect linked to the quality of semen to be submitted to the freezing process is the management and the thermal stress. High temperatures, unbalanced feeding, and poor management lead to the quality of the ejaculate to deteriorate and to lose the quality for freezing (Bhattacharya 1962; Bhattacharya 1968; Shafie 1994; Vale 1994; Vale 1997; Garcia 2013; Castro et al. 2017).

From studies conducted in different parts of the world have demonstrated variations in the freezability of buffalo semen collected during different seasons. Better freezability was achieved with semen collected during shorter days and cooler months compared to hot summer months (Vale et al. 2014).

According to Andrabi (2014), few previous studies have enumerated the variation in the chemical composition of sperm cells and seminal plasma during different climatic conditions (Singh et al. 1969; Singh et al. 1970; Mohan et al. 1979; Sidhu and Guraya 1979, Castro et al. 2017) and also information about the seasonal variation in freezability of buffalo semen. Proper mating ability and good libido of a buffalo bull are prerequisites for a successful artificial insemination program to harvest maximum semen of desirable quality. Wide individual variation exists among breeding buffalo bulls in libido and sexual behavior (Anzar et al. 1993; Andrabi 2014).

In recent studies related to the effect of environmental conditions on the animal, productivity is gaining importance (Vale 2007; Sharma et al. 2014; Bhakat and Mohanty 2015).

Buffaloes are homeotherms and are able to maintain the body temperature within a wide range through some behavioral and physiological mechanisms that assist in thermoregulation. Buffaloes are usually reared across various climatic conditions, which provide them the capacity to adapt to diverse ecosystems. Among different climatic variables, ambient temperature is the one that greatly affects the physical environment surrounding the animal (Castro et al. 2017).

The production of hormones, especially those secreted by the pituitary-gonads and adrenal axis, regulates the expression of sexual behavior. Stressful conditions suppress the secretion of endocrine glands, and hence male animals exposed to

thermal stress exhibit reduced sexual activity owing to less testosterone production and production of stressors secreted by metabolism (Vale 1994; Koonjaenak et al. 2007; Vale 2007; Phogat et al. 2016).

Generally, stress can be defined as any factor or change in the environment that impedes homeostasis, which is a dynamic and complex system of equilibrium governing the proper functioning of living organisms. Stress negatively affects productive performance and is deleterious to reproductive functioning and hinders the animals from exhibiting their total genetic potential (Bhakat and Mohanty 2015; Phogat et al. 2016). Besides, among the various factors that alter the homeostatic balance, the key factors are nutritional deprivation, diseases, improper animal handling, thermal stress, either by cold or heat, and transportation (Marai and Haeeb 2010; Rafidah et al. 2014). The meteorological variables such as ambient temperature between 13 and 18 °C along with relative humidity of 55–65% and wind speed between 5 and 8 km/h with moderate sunshine are considered to be ideal for the proper growth and reproduction in buffaloes (Marai and Haeeb 2010). Accordingly, from the climatic conditions of the countries where the great majority of buffaloes are reared, it can be presumed that the main constraint with the buffalo production system is associated with the thermal stress in tropical regions (Garcia 2013).

Likewise, buffaloes find it difficult to dissipate body heat owing to scarce sweat glands and having a black body color with a high melanin content (Vale 2007, Castro et al. 2017). Besides, the buffalo need much access to water and shade. Hence, proper management interventions to avoid animals from being exposed to intense solar radiation followed by managemental practices to enhance heat loss to the environment have shown beneficial results (Vale 2007, Castro et al. 2017).

Likewise, in male buffaloes, heat stress leads to a decrease in semen quality and reduced sperm viability (Vale 2007; Sansone et al. 2000; Bhakat et al. 2015). Thus, in most parts of Brazil, particularly in the tropical climate of the Amazon region, management interventions to provide a comfortable microenvironment are fundamental for the optimal production of buffaloes (Castro et al. 2017).

Because of buffaloes exhibiting seasonality of reproduction, the changes in quality of semen may occur as a function of the season, with higher reproductive efficiency in short days (hour × light) which represent the winter/autumn seasons (Sansone et al. 2000; Phogat et al. 2016; Castro et al. 2017). The seasonal variation is found to occur even in testosterone, and surprisingly lower blood concentration of testosterone was reported in autumn and winter, which are supposed to be breeding seasons of buffalo rather than in spring and summer (Ohashi et al. 2011). These variations are observed in the regions where there are distinct seasons during the year; it should be considered if there is any seasonal influence on seminal quality in this species (Ohashi et al. 2011). Hence in the tropical humid environment of the Amazon rainforest, where the climate is differentiated as rainy and non-rainy seasons, all around the year the temperature and relative humidity remain very much high leading to discomfort and thermal stress in farm animals, particularly in buffaloes; further studies are essential to enumerate the actual situation prevailing at the local level (Garcia 2013).

Castro et al.'s (2017) studies showed that semen parameters such as motility, vigor, and plasmatic membrane integrity are lower during non-rainy season, and aspects associated with major, minor, and total defects were higher during this period, which characterizes the low quality of sperm. The temperatures of the body and the testicles of the animals in this study during the two seasons did not show any statistical difference ($p < 0.01$), with an average difference between seasons of 2.3 °C. During the non-rainy season, the testicles had temperatures that were 5 °C greater than the body temperature of these animals, which may have caused changes in spermatogenesis due to thermal stress and that could explain the difference in semen quality between seasons. According to Squires (2010), the testicles need a temperature between 2 and 5 °C and a low body temperature to maintain all physiological functions such as hormone production and spermatogenesis, processes that are controlled by the neuroendocrine system and are directly influenced by scrotal-testicular thermal regulation. High ambient temperature is directly linked to a reduction in sperm quality, and spermatogenesis becomes critically compromised at temperatures between 27 and 32 °C, and continuous exposure to temperatures above 30 °C causes a drastic decrease in sperm quality (Skinner and Louw 1966; Castro et al. 2017).

Koonjaenak et al. (2007) in an experiment in Thailand (tropical climate) reported winter and spring seasons as the suitable period for the collection of semen in buffaloes. The semen collected during these seasons exhibited higher stability, membrane integrity, yielded better freezability as well as higher values for the kinematic parameters provided by CASA. The same authors observed significant differences in tail defects (abnormalities) of spermatozoa in various seasons with being high in the thermal stress period.

Koonjaenak et al.'s (2007) experiment in Thailand (tropical climate) showed semen collections were performed between winter and spring when temperatures were mild. For buffaloes, this period was considered more suitable for the collection of semen, destined for freezing, since the ejaculates collected during these months of the year presented higher membrane integrity and stability, as well as better values for the movement (speed) patterns provided by CASA (computer-assisted sperm analysis) to the samples which were thawed, and no effect was observed on the quality of the ejaculates. The same authors did not observe significant differences in seminal characteristics in the different seasonal periods in ejaculates of buffalo bulls, except tail defects (morphology), which increased in the months of higher temperatures. Castro et al. (2017), in a similar experiment, found significant statistical differences for vigor, motility, mass activity, plasma membrane integrity, major defects, minor defects, and total defects. Therefore, the results of these experiments conclude that the environment influences the seminal characteristics in buffaloes.

Photoperiod also influences the seasonality of reproduction in buffaloes along with temperature and relative humidity. The tropical humid environment not only affects the libido but also the quality of fresh and frozen semen, and it has been already reported by a few authors (Vale 2007; Sansone et al. 2000; Castro et al. 2017).

Castro et al. (2017) concluded that the best time for collection and processing of semen for cryopreservation in a humid tropical environment is the rainy season, although it can be done throughout the year by implementing advanced managemental practices to protect the breeding bulls, which include: provision of the shady environment to avoid exposure of animals to direct solar radiation and water baths to hottest parts of the day. As the non-rainy season deteriorates the seminal quality, the managers should look for alternatives to enhance the thermal comfort of the animals if their target is to use them in breeding programs, or for freezing.

17.13 Conclusion

After the period of colostrum feeding, the traditional nutritional strategy for raising dairy breeding male buffalo calves is important for its normal development as a future sire. It is necessary to keep milk consumption; however it is recommended to lower liquid feed consumption, increase solid feed consumption, stimulate early rumen development, and offer solid food of good quality. Attention must be paid to common diseases caused by bacteria, viruses, and parasites, and the use of vaccines against current regional diseases is recommended. Food supply must be controlled and offered in good quantity and quality. Weaning should be done around 6 months when then the best animals should be selected for future breeders. At weaning, the presence of hereditary problems, such as hernias, and other defects of gait and joints morphological defects, should be observed. Attention should be given to problems of diseases of the scrotum and testicles. At 12 months, it is recommended to start to observe the sexual behavior of these young animals which at 15–16 months of age already start sexual activity. Once selected as a semen donor, the bull should be examined clinically, hereditary health, general health, normality of the genital tract, service capacity, and fertilization capacity of semen. Sometimes the conception rate of cryopreserved semen from the good bull is around 30%. It may be due to some alteration in sperm cells during cryopreservation processes. Reduction in percentage motility, decrease in viability, reduction in plasma membrane integrity, and increase in the percentage of apoptotic sperm cells in buffalo due to freeze-thaw procedure are common. Also, buffalo spermatozoa are more susceptible to freezing-thawing processes than cattle. It has been accepted that nearly 50% of buffalo spermatozoa are cryo-damaged during the semen freezing process. By concerning these facts, the semen should only be used for AI if the post-thawing motility is more than and equal to 40%. In addition, the quality of post-thawed spermatozoa is affected by reactive oxygen species (ROS). These substances are also produced physiologically in living cells during respiration as well as by abnormal or dead sperm and phagocytic cells of both the ejaculate and the female reproductive tract. These ROS decrease the sperm quality and render it incapable of fertilizing the oocyte and have deleterious effects on buffalo fertility through AI.

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Advances in Semen Quality Assessments in AI Programs in Buffalo

18

Armando Quintero-Moreno, Hector Nava Trujillo, and Carla Osorio-Meléndez

Abstract

In this document, various aspects related to the evaluation of the seminal quality of buffaloes intended for artificial insemination are reviewed. Aspects about seminal cryopreservation are documented, as well as the evaluation of basic parameters of sperm quality in buffalo using mainly current bibliographic references. In the same way, referential values of the seminal quality parameters are proposed to serve as a guide to the international scientific community.

Keywords

Buffalo · Semen · Quality · Artificial insemination

18.1 Introduction

In tropical and subtropical countries, buffalo species (*Bubalus bubalis*) have been establishing itself as a promising livestock alternative, which although it does not yet compete with cattle farming, complements it. However, it is important to note that despite the gradual and progressive growth in the number of buffaloes as well as the partial technification of some buffalo farms, it is still a species that is not very exploited when compared to cattle, poultry, and pigs, which makes it one of the main challenges for the coming years, which implies establishing the buffalo species as a

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viable and important dual-purpose livestock industry for the progressive livestock community. In addition to this, it is necessary to massively disseminate the genetic material, requiring elite buffaloes to obtain sustained genetic improvement through the application of reproductive biotechnologies such as seminal cryopreservation and artificial insemination (AI), an achievement that is currently being established and strengthened.

Despite the fact that the buffalo population worldwide is over 205 million heads (FAO 2020), there is very little activity linked to seminal cryopreservation and AI (Borghese 2010) compared to cattle, one of the reasons being the deleterious effect that the process of seminal cryopreservation has on buffalo spermatozoa, which is due to the structure of their plasma membrane and biochemical composition, which is an intrinsic factor linked to the species (Vale 2011). However, new protocols have been developed and cryo-protectants have been studied to increase viability rates after cryopreservation (Soltani and Mahdavi 2020). Our experience in cryopreserving of buffalo semen using diluents assembled in our laboratory has been satisfactory (Osorio-Meléndez 2013).

The selection of "good freezer" buffaloes is achieved by choosing those animals potentially suitable for seminal cryopreservation, derived from the results obtained after performing a complete spermiogram and integrated with a previous physical evaluation of the animal. To achieve this objective, high knowledge of physiological indicators is required, mainly related to the genital system (dimensions and morphology) and the scrotal circumference, which are essential for the determination of the reproductive state and the prediction of the potential of seminal production. The scrotal circumference is one of the characteristics to be included in the selection programme of future buffaloes, as it is associated with greater testicular development and better physical and morphological characteristics of the semen (Quirino 2002).

The spermiogram must take into account at least four basic parameters: viability, morphology, number of spermatozoa with progressive motility, and the morphological and functional integrity of the plasma membrane. Once the buffalo with optimal parameters has been selected, the semen is cryopreserved and the post-thaw evaluation is repeated. In buffalo the parameters mentioned and studied in vitro have shown a reliable and repeatable correlation with fertility measured in the field, this being one of the main challenges outlined for the buffalo species (Gillan et al. 2008).

18.2 Seminal Cryopreservation

Sperm cryopreservation is a non-physiological method that involves a high level of adaptation of the biological cells to osmotic and thermal shocks that occur during dilution, refrigeration, freezing, and also during thawing procedures. The procedures associated with cryopreservation attenuate sperm capacitation and may or may not affect sperm motility, but they do reduce the lifespan of the spermatozoon, as well as the fertility of the semen and its ability to interact with the female reproductive system (Medeiros et al. 2002). During cryopreservation process, sperm usually face a number of physiological and structural challenges such as improper osmotic

imbalance, oxidative stresses, and formation of ice crystals, so cryopreservation extenders are supplements with such compounds as cryo-protectants and antioxidants (Mokhtari et al. 2020; Salehi et al. 2020).

The quality of the frozen-thawed semen is one of the factors that limit genetic improvement and the increase of buffalo farming at present, since when AI is implemented with cryopreserved semen the results are poor and of low fertility compared to cattle (Andrabi et al. 2008). Post-thawing motility of cryopreserved buffalo semen is compromised and the success rate using in vitro fertilization with buffalo semen is between 10 and 20% compared to cattle, which is 30–35%. Damage during cryopreservation affects the cell membranes (mainly plasmalemma and mitochondria) having consequences on viability and different metabolic factors of spermatozoa, which can affect fertility (Andrabi et al. 2008).

Cryo-protectants have a role in protecting cells against cryo-injury caused by cryopreservation process. The cryo-protectants are classified into two groups: penetrating cryo-protectants (organic solvents such as DMSO and glycerol) and non-penetrating cryo-protectants (natural and non-toxic compounds such as carbohydrates (trehalose, sucrose) and proteins and polymers (PVA and PVP)) that cannot penetrate into cells (Fuller et al. 2017; Gurruchaga et al. 2018).

Today, the countless diluents used in buffalo semen cryopreservation include egg yolk, lactose, skimmed milk, and Tris-hydroxymethyl aminomethane (TRIS), commonly associated with egg yolk. TRIS can provide the most satisfactory buffer system to improve freezability and consequently can also improve the fertility of buffalo sperm. There are three key components in the cryopreservation of semen in ruminants, and specifically in buffalo semen: egg yolk, skim milk, and glycerol. Relevant research has been carried out to specify the adequate amounts of glycerol, in addition to assessing which species is the best egg yolk or skim milk to use in the seminal freezing process in buffalo. Abbas and Andrabi studied the effects of different glycerol concentrations (2% to 8%; 10% and 12%) in the extender on sperm quality after thawing and reported that frozen spermatozoa with 7% were significantly better than those with other glycerol concentrations, which was tested by evaluating post-thaw motility, survival, and plasma membrane integrity. In addition, a reduction of glycerol below 5% decreases post-thaw motility and/or acrosome integrity of the spermatozoa.

Andrabi et al. (2008) comparatively investigated the use of duck, guinea fowl, Indian chicken, and commercial chicken egg yolks and found duck egg yolks to be the best because they have a higher proportion of phosphatidylethanolamine: phosphatidylcholine unlike the other species, which is why it is said that a better freezability of buffalo sperm was obtained after the following post-thawing parameters were evaluated: motility, survival, plasma membrane integrity, acrosomal status, and evaluation of the head, mid-piece, and tail abnormalities. In reference to the use of milk, the whole buffalo milk-based diluent achieved higher post-thawing motility and viability index than sheep or goat milk (Mohammed et al. 1998).

18.3 Sperm Quality Assessment

18.3.1 General Aspects

To be considered as a reproductively suitable buffalo, it must meet three basic requirements: a) good libido, b) good clinical reproductive status, and c) good sperm quality. Buffalo semen may present problems associated with climatic or seasonal factors, as a consequence of the great sensitivity of the seminiferous epithelium to an increase in environmental temperature. Its sensitivity to heat is due to its difficulty in dissipating body heat, as it has fewer sweat glands and therefore a less efficient thermoregulatory system when compared to zebu cattle. However, special attention should be given to animals subjected to semen collection during the hot season (Vale 2011).

The best time to collect buffalo semen is at the beginning of the day (still dark) or at the end of the evening since this species presents a nocturnal sexual behaviour; and for the collection, the non-lubricated artificial vagina should be used, at a temperature between 44 and 45 °C (Vale 2011). It should also be taken into account that spermatozoa are very sensitive to temperature variations, so it is advisable to control the thermal variations of the material and solutions to be used, keeping it at a constant temperature of around 37 °C. When carrying out the seminal evaluation, it is important to consider the age, breed, nutritional status, body condition, sexual activity, method of collection, time and state of health of the animal. It is worth mentioning that the use of false mounts with penis deviation is recommended to improve the quality of the ejaculate. As a result of the seminal analysis we can qualify the sample as suitable or unsuitable for use in AI (Vale 1994, 1997; Osorio-Meléndez et al. 2011). Among the various tests available, emphasis will be placed on the following:

18.3.2 Basic Parameters for Assessing Sperm Quality

18.3.2.1 Colour

It must be milky white to creamy white, and once placed against artificial light, it has blue stripes. Both the colour and the density of the sample will be directly related to the concentration of spermatozoa (Vale 1994, 1997).

18.3.2.2 Volume

It is measured directly in the graduated collecting tube, varying considerably depending on the breed and age of the buffalo, with a greater volume increase found at age 4–12 year (Pant et al. 2003). Almaguer (2007) states that at the time of puberty the volume is around 1 mL and increases to 3 mL after sexual maturity. Koonjaenak and Rodriguez-Martinez (2007) found a volume of 3 to 4 mL in the Nili-Ravi breed. Osorio-Meléndez (2013) reported in Murrah buffaloes volumes ranging from 2 to 5 mL ($\mu = 3$) in semen samples collected with artificial vagina (implemented 2 collections with 30 min interval). It should be noted that when both

the volume and some physical and morphological characteristics of the semen tend to undergo changes, it may be due to testicular degeneration (Vale 2011).

18.3.2.3 Sperm Concentration

The concentration measures the number of spermatozoa per unit of volume, i.e. per ml of semen, which has been positively correlated with the animal's fertility. In the same way, the presence of a greater number of spermatozoa with normal attributes in an ejaculate increases the possibility of fertilization (Pant et al. 2003; Vale 2011). The sperm concentration is one of the most important tests of seminal analysis, showing a wide range of variation from 600×10^6 to 1200×10^6 sperm per mL. This parameter is very sensitive to seasonal, nutritional, and management disorders, as well as correlated with the genetic aspect (Vale 1994, 1997), which is evidenced by the Murrah breed, in which average values above 1200×10^6 sperm per mL have been described by Osorio-Meléndez (2013). It is also important to consider the age since it has been reported a lower sperm concentration in older buffalo than in younger ones, which could be due to senility, being a determining factor (Javed et al. 2000).

18.3.2.4 Sperm Motility

Sperm motility is the most widely used parameter worldwide in laboratories that process buffalo semen for AI and thus issue a forecast on semen quality. In AI centres located in underdeveloped countries and with great economic limitations, this parameter is used alone or together with the sperm concentration as the only criteria to give a prognosis on the viability of fresh or thawed semen. In fresh semen, progressive mass and individual motility are assessed, while in thawed semen only the second is assessed. Both parameters are described below.

Mass Motility

It indicates the concentration and viability of the sperm cells and is only evaluated on freshly collected semen. A drop of undiluted semen is placed on a slide and then the wave and swirl movements are observed under the microscope at low magnification (10X), looking at various microscopic fields (Vale 2011). It should be evaluated near the edge of the drop, where the depth of the drop is smaller and easier to observe and is given a numerical value on a scale ranging from 0 to 5, although there are other very similar scales (1–5/0–4). In buffaloes destined for AI, only samples with MM equal to or greater than 3 are processed (Osorio-Meléndez 2013).

Progressive Individual Motility (PIM)

Its assessment is based on the observation of the individual movement of the sperm in order to determine the percentage of motile cells in the ejaculate. PIN can be determined by the observation of the spermatozoa by a trained technician, using a simple optical microscope, providing a single value that combines the motility of a group of spermatozoa. Another option is the use of a computerized programme that provides a series of motility descriptors or evaluating the progressive and non-progressive motility, as well as fast, medium, slow, and static spermatozoa.

In the last two decades, the computer-aided sperm analysis (CASA) system for evaluating IM in the semen of many mammals has increased prominence. In fresh semen, Ram et al. (2017) reported PIM values ranging from 72 to 73% and Osorio-Meléndez (2013) described values between 55 and 65% in Murrah buffalos located in South America (Venezuela). Our experience using CASA system on cryopreserved semen was positive, obtaining good results (Osorio-Meléndez 2013). The MI values (progressive + non-progressive) are greater than 40% with MI (linear + progressive) of 30.5% and percentage of rapid 32.6%. These values are similar to those obtained by Shiva et al. (2010), whose reports a progressive MI of 31.67%, however, these figures exceed the reported value by Sohail et al. (2013), where the MI percentage was 20.86%, and Mehmood et al. (2017) described values between 20 and 40%. Most of the publications referred to describe PIM in cryopreserved semen between 20 and 40%, which is lower than the reports described in bull semen (Rubio-Guillen et al. 2007; Quintero-Moreno et al. 2011).

18.3.2.5 Vitality

Sperm vitality is a parameter that allows us to assess the structural integrity of the plasma membrane by evaluating a spermatozoon's capacity to exclude extracellular substances such as dyes or fluorochromes, assuming that only those spermatozoa that exclude staining are those that have an intact membrane and are therefore capable of transporting, capacitating, and fertilizing themselves, as long as their functional integrity is intact (Vale 2011). One of the stains commonly used to assess this parameter in buffalo semen is Eosin-Nigrosin (Osorio-Meléndez 2013; Singh et al. 2013). The appropriate vitality in freshly collected semen should be equal to or higher than 70%; therefore, a buffalo ejaculate with more than 30% dead spermatozoa can hardly be processed and frozen (Vale 2011).

Our experience in American tropic with cryopreserved semen of buffalo, the vitality measured with Eosin-Nigrosin was excellent (70.6%), with similar values to those referenced for fresh semen (Osorio-Meléndez 2013). In similar investigations carried out on another continent have been obtained values of 63.7% (Singh et al. 2013) and 71% (Khan and Ijaz 2008). Based on these works it is proposed that the optimal vitality for frozen-thawed semen could be equal or superior to 50% and for fresh semen it could exceed 70%.

18.3.2.6 Sperm Morphology and Acrosome Integrity

In the semen of other mammals, especially in bulls housed in AI centres, the most commonly used stain to evaluate the morphology of sperm is Eosin-Nigrosin, Diff Quick® or Hemacolor®. These stains are also used in research laboratories at universities with economic limitations; however, Eosin-Nigrosin is associated with Giemsa to assess the integrity of the acrosome (Quintero-Moreno 2003). However, in the research centres and/or andrology laboratories of the universities of developed countries they use more sophisticated stains that generate more reliable results.

It must have a minimum of 70% normal spermatozoa for their ejaculate to be considered satisfactory (Vale 2011). The morphological abnormalities reported for buffaloes located in Venezuela were 32.4 (Osorio-Meléndez 2013), with a slightly

higher value. Saeed et al. (1990) mention that in buffaloes most of the anomalies are found in the head of the spermatozoa (5.78%), while that the anomalies in the intermediate piece are less than 1% and the abnormal tail values oscillate between 3.92 and 5.70. The appearance of cytoplasmic drop is not common and is less than 1%. Endorsing these results, Koonjaenak and Rodriguez-Martinez (2007) using a phase contrast microscope they observed that in buffalo semen, the abnormalities the number of sperms is below 15%, and the most common ones are found on the head, predominantly pear-shaped, followed by goatee acrosomes, proximal cytoplasmic drop, single and folded tails.

The morphometry of the sperm head can be assessed by analysis computerized staining with simple protocols (Hemacolor®, Diff Quick®, or Blue Stain®) capturing images, recording them on video, being digitized, and archiving on magnetic media for subsequent morphometric analysis (Van Der Horst and Maree 2009; Osorio-Meléndez 2013). Length (8.08 μm), width (4.29 μm), area (29.22 μm2), and perimeter (22.25 μm) of the sperm head were described by Osorio-Meléndez (2013). In studies carried out in bulls, the variation in the size of the sperm head was compared between fresh and cryopreserved semen from the same ejaculate (Rubio-Guillen et al. 2007). The cryopreserved heads were significantly smaller and the bulls with a lower proportion of change in head dimensions had better fertility. Due to this fact, the analysis of the morphometric evaluation of the sperm in freshly collected vs frozen-thawed buffalo semen represents a great challenge to demonstrate that this fact is repeated in buffalo.

The integrity of the buffalo sperm acrosome can be assessed with multiple stains proposed by the international scientific community for mammalian sperm, the vast majority of which are very laborious. In our case, we propose the use of BlueStain® (Van Der Horst and Maree 2009), which defines well the sperm head silhouette and acrosome zone, a very useful fact for the evaluation through the CASA system. Eosin-Nigrosin staining is recommended only for assessing sperm vitality; however, our research team has been successfully using it to assess acrosome integrity in buffaloes (Osorio-Meléndez et al. 2012). The reference value for the species is less than 10% of acrosomes reacted for freshly collected semen (Vale 1994, 1997), while for cryopreserved semen it has not yet established a standard value; however, one could extrapolate the referred value in bulls (Catena and Cabodevila 1999). The experience of Osorio-Meléndez (2013) in frozen-thawed semen had values in the order of 12% of acrosomes reacted. Similar values (12–13%) in fresh semen were reported by Ram et al. (2017) by using Giemsa staining. Using Fluorescein isothiocyanate peanut agglutinin (FITC-PNA) staining, Singh et al. (2016) reported values of 76.19% of intact acrosomes for high fertility buffaloes, while in medium and low fertility buffaloes the values found were 65.51 and 61.36, respectively.

18.3.2.7 Assessment of the Functional Integrity of Plasma Membrane by the Endosmosis Test (HOST)

HOST is a simple, practical, and reliable test that can be implemented to determine the functional integrity of the sperm membrane, as well as to predict the reproductive potential of buffalo semen samples to be used for artificial insemination

(Quintero-Moreno et al. 2013). The response to this test for freshly collected semen is in the order of 70% and 50% for cryopreserved semen. In thawed semen, HOST shows a significant correlation with the percentage of quick sperm (0.58; $P < 0.001$) and the percentage of live sperm at thawing (0.49; $P < 0.05$) (Quintero-Moreno et al. 2013). In other geographical areas of the world, findings by researchers (Khan and Ijaz 2008; Ijaz et al. 2009; Sohail et al. 2013) are similar to the investigations carried out in Venezuela. Ram et al. (2017) evaluating recently collected semen showed values between 52% for buffaloes in heat-stressed environments and 56% for colder environments (winter). In our opinion, the values of the endosmosis test obtained in fresh semen in this last work are below the expected optimal values ($\geq$70%). In cryopreserved semen, the HOST values found by Mughal et al. (2018) ranged from 56 to 61%, in contrast, Mehmood et al. (2017) obtained results in the order of 30%. The first results were excellent; however, the results of the second work were suboptimal, although it is important to note that Osorio-Meléndez (2018) obtained results of pregnancy rate (52%) in a breeder that presented values of HOST between 30 and 40%. However, this is an exception, since the HOST values for a pregnancy rate higher than 55% were in the order of 52%.

It is prudent to affirm that this test is a useful and economic tool that allows the selection of the most suitable reproducers for the process of seminal cryopreservation since seminal samples with a good percentage of fast sperm and low percentage of static sperm at thawing are highly associated with a favourable response to HOST (Quintero-Moreno et al. 2013; Osorio-Meléndez 2018).

18.3.2.8 DNA Fragmentation

Sperm DNA fragmentation refers to breaks or lesions in the genetic material of the spermatozoon. The greater the number of lesions, the lower the integrity of the genetic material and the less likely it is that a viable embryo will be produced and therefore a full term pregnancy. These techniques have been used very little to assess buffalo semen and so far there is little research on the subject (Badr et al. 2010; Osorio-Meléndez et al. 2011; Pawar and Kaul 2011). Badr et al. (2010) obtained values ranging from 96 to 98% DNA integrity. Osorio-Meléndez et al. (2011) by using Toluidine Blue stain in fresh buffalo semen obtained lower values (92.5%). Toluidine blue staining is a good alternative to evaluate this parameter, since it is inexpensive and has a good track record in human semen research, where it has been compared with the sperm chromatin dispersion test (SCD), obtaining a good correlation. Pawar and Kaul (2011) using SCD in buffalo semen reported promising results describing a significant association of this technique with viability (0.687, $P < 0.05$) and with DNA integrity quantified by Acridine Orange (AO) staining (0.87, $P < 0.05$). The reviewed investigations present DNA integrity values greater than 90%, highlighting that the majority use AO staining. Ahmad et al. (2017) report 95%; Mehmood et al. (2017) showed values above 90%, and Mughal et al. (2018) obtained 97–98% sperm DNA integrity in cryopreserved semen.

18.3.3 Reference Values for Buffalo Semen with Special Emphasis on AI

Our commitment to continuing this line of research is clear, as it is necessary to generate new results and challenges to achieve the best options in relation to the optimum use of semen, its valuation and its conservation, adopting advanced technologies. On this occasion it is necessary to propose some reference values (Table 18.1) that will serve as a standard for the evaluation of semen quality in buffaloes, especially those destined for semen collection in AI programmes.

18.3.4 Sexed Semen

As in bull sperm, a difference in DNA content was found between X and Y sperm in buffalo; this fact led to the processing of buffalo sexed semen (Lu et al. 2007) with 89% sexual accuracy (Bulletin 2013). The conception rates found in two works ranged between 40 and 50%. Presicce et al. (2005) obtained a conception rate of 42.8% in Italian Mediterranean buffalo following the heat synchronization with Ovsynch protocol and AI with sexed sperm. In a more recent study, a 50% pregnancy rate was achieved by inseminating a dose of sexed semen with four million sperm (Gaviraghi et al. 2013). There is little literature on the use of sexed semen in buffalo AI; however, the immediate future of sexed semen is moving towards the production of embryos, where the efficiency is higher.

18.4 Conclusion

It can be summarized that since the last decade and due to the progressive growth of the farms dedicated to buffalo, the use of reproductive biotechnologies has increased, which include sperm quality control and interest in seminal cryopreservation for commercial purposes to implement AI as a tool for massive genetic

Table 18.1 Proposed reference values for buffalo semen with special emphasis on AI

Parameter	Reference pattern
Colour	White, milky white (with dark blue stripes)
Volume (mL)	2–5
Mass motility (scale: 1–5)	>3
Concentration ($\times 10^6$)	600–1200
Progressive individual motility (%)	≥70 (fresh semen)/≥40 (frozen-thawed semen)
Vitality (%)	≥80 (fresh semen)/≥50 (frozen-thawed semen)
Morphological abnormalities (%)	≤15 (fresh semen)/≤30 (frozen-thawed semen)
Acrosomes damaged (%)	≤10 (fresh semen)/≤30 (frozen-thawed semen)
Endosmosis test (HOST) (%)	≥70 (fresh semen)/≥40 (frozen-thawed semen)
DNA fragmentation	≤10

improvement. These achievements derive in the implementation of an optimal reproductive control of the breeding buffaloes that include aspects of semen evaluation through modern and precise techniques. This commits us to promote research programs that generate new results and challenges. In the short term, it is important to establish AI programmes and establish efficient management protocols of the buffaloes for seminal extraction by means of an artificial vagina. The achievements obtained in the research work carried out by the authors of this paper have allowed proposing some values to be used as a reference in the evaluation of the seminal quality in buffaloes.

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Reproductive Ultrasonography in Buffalo: Basic Concepts and Recent Advances

19

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Abstract

Optimum reproduction is obtained by regular monitoring of reproductive process in livestock species including buffaloes. Therefore, real-time monitoring of reproductive processes through ultrasonography provides better alternative to ascertain the physiological status as well as strategize suitable fertility improvement programs in all livestock species, including buffaloes. Information gained from reproductive ultrasonography helps in proper prognostic, diagnostic, and therapeutic purposes in both males and females without any trauma or invasive procedures. In recent times, other advancements in ultrasonography have improved the efficiency of reproductive ultrasonography manifold both for research and therapeutics in all domestic species.

Keywords

Buffalo · Ultrasound · Follicle · Pregnancy diagnosis

19.1 Introduction

Ovarian and uterine examination is vital for diagnosing the reproductive status and reasons of infertility in large animals. This can be achieved either by observing the reproductive behavior of the animal, status of reproductive hormones, or palpation of ovaries per rectum for the presence of cyclic or pathological structures. Unfortunately, in buffaloes, behavioral observations of estrus signs poorly reflect the accurate information about the ovarian function. Similarly, palpation per rectum

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also does not provide reliable information about the dynamic status of ovarian function as corpus luteum (CL) frequently remains embedded deep in the ovarian stroma in buffaloes. Likewise, measurement of hormones requires sophisticated laboratory procedures/instruments for making a correct diagnosis and in most instance, findings are not available immediately. Therefore, real-time ultrasonography is a better alternative to ascertain reproductive status and for devising suitable strategies to optimize fertility in all domestic livestock species. It is also important to mention that information gained from ultrasound must be correlated clinically with reproductive history of the animal, palpation of genital tract, and other diagnostic procedures rather than in isolation (Warriach et al. 2015).

Fertility of bulls is also a crucial factor in the overall success of the dairy industry. The influence of the bull on herd fertility is often overlooked with the focus being on female infertility. When cow management and infectious disease control are optimum, then the limiting factor in herd fertility is governed by the bull fertility rather than female factors. Infertile bulls (incapable of achieving pregnancies) are rare, but 20% or more breeding bulls may be sub-fertile and fail to perform optimally. Routine breeding soundness evaluation (BSE) is currently based on semen parameters, scrotal circumference, and manual testicular palpation; however, other techniques are often needed to pursue a specific diagnosis. Compared to other more specialized examination procedures (biopsy), ultrasonography is a non-invasive and non-traumatic technique involving no risk to the reproductive potential of the bulls. Considering this, ultrasonography offers the most promising application helpful to establish precisely the type and the location of the pathological conditions (diagnosis) of the different organs of the male reproductive tract and consequently, aid in prompt prognosis. In this context, this chapter throws light on the basic concepts and recent advances in buffalo reproductive ultrasonography techniques.

19.2 Components of Ultrasound

Ultrasounds consist of sound waves of frequencies greater than normal hearing range of the human ear (>20,000 Hz) and operate at frequencies of 1–20 megahertz (1 MHz = one million sound waves per second). Sound waves of these selected frequencies are generated by crystals exhibiting piezo-electric properties. These piezo-electric crystals are housed in a transducer or probe. When an electric current is applied to the piezo-electric crystals, they emit sound waves, which interact with body tissues. In contact with body tissues, these ultrasonic waves travel through it and the tissues have ability to either propagate or reflect the sound waves (echos) depending on their density. The returning echoes are received by same transducer that compresses the same piezocrystals, thereby resulting in electric impulses production, which are subsequently displayed as a display (two dimensional) of dots on a screen. The brightness of image will be directly proportional to the amplitude of the reflected echoes and it provides an image ranging from black through various shades of gray to white. Shape, size, and echotexture (disruption of high and low intensity

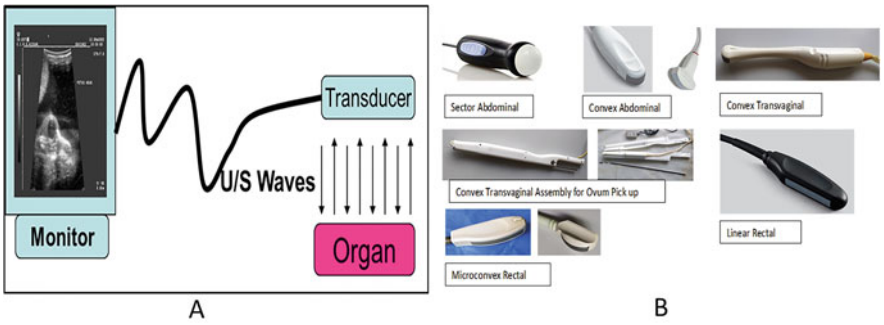

Fig. 19.1 (**a**) Schematic diagram of ultrasound components; (**b**). Different types of transducers

echoes) from the targeted organ are the main parameters that help in the interpretation of normal versus abnormal anatomy (Fig. 19.1a).

19.2.1 Echo Display Modes in Ultrasonography

The returning echoes can be displayed in three modes on ultrasound screen, viz. A-Mode, B-Mode, and M-Mode in diagnostic medical and veterinary field. Among this, real-time B-mode (brightness mode) imaging is most commonly used diagnostic technique for evaluation of reproductive structures in large animals. In real-time B-mode, motion is visualized as it occurs actually. At least 15 images per second is required for real-time observation to give the impression of continuous movement. As the number of frames displayed per second (framing rate) is increased, the smoother is the appearance of any motion. Majority of ultrasound machines typically have 15 to 60 images frames per second. In B-mode ultrasonography, returning echoes are displayed on ultrasound screen as series of gray dots and brightness of the dots is proportional to the intensity (amplitude) of each signal. On ultrasound screen, images appear as cross sectional anatomy of the organ.

19.2.2 Types of Transducer

Transducer/probe is an important part of ultrasound machine as this device converts electrical energy into ultrasound energy and ultrasound energy back into electric energy. Transducers are designed in different shapes, size, and length depending upon organs to be scanned. Sector, convex, and linear array transducers are commonly used in veterinary practice for trans-abdominal and transrectal application. The image produced by the linear array transducer appears rectangular on the screen, whereas in sector or convex transducer it is pie shaped or fan shaped.

Linear array transducer is ideal for imaging superficial structures such as reproductive organs of large animals and tendons as wider field of view is obtained in the

near field. However, this transducer requires a relatively large contact area between skin and transducer. Conversely, in sector and convex transducer, contact area is very narrow that may restrict greater details in superficial area. For examination of reproductive organs, linear as well as convex probe is best. For ovum aspiration, convex transvaginal probe with front firing echos is used. This transducer is housed in an especially designed plastic cover that has a channel for long needle. Other types of transducers include microconvex, curvilinear, etc. (Fig. 19.1b).

19.2.3 Range of Frequencies

For diagnostic ultrasound, sound wave frequencies between 2 and 10 MHz (1 MHz =one million sound waves per second) are commonly used. Higher sound wave frequencies provide greater detail, whereas lower frequency provides greater tissue penetration. Therefore, using lower frequency transducer, a wider area can be viewed, but with lesser details. However, using high frequency transducer, a smaller area can be visualized with more detail. Transducers with lower frequency (<3.5 MHz) are suited for viewing larger structures at a greater distance from the transducer, whereas transducers with higher frequency (>5.0 MHz) can be used for the detailed study of superficial structures close to the transducer (e.g. evaluating the ovaries, uterus, tendons, etc.). Transducer that produces single as well as multiple frequency sound waves are also available.

Based on the echotexture of the organ, the visualized areas can be hyperechoic (high intensity echoes), hypoechoic (low intensity echoes), anechoic (no echoes), and isoechoic (echotexture similar to other adjacent structure/organ). Biological fluids (follicular fluid, cyst, embryonic vesicle, and fetal fluids urinary bladder) do not reflect sound waves and are non-echogenic or anechoic. On the ultrasound screen, fluid appears as black. Conversely, denser tissues such as fetus, fetal skeleton, cervix during diestrus period, cartilage reflect most of sound waves and appear hyperechoic and white on the screen.

19.3 Assessment of Normal Ovarian Structure

19.3.1 Ovaries

Buffalo ovaries are spherical or elongated structures, measuring 1.5–4.0 cm (length), 1.0–1.5 cm (width and height), and weight around 2–4 gm. Identification of ovaries is added by presence of scattered antral follicles (generally 3–4 in each ovary in buffaloes) that appear black in color (Fig. 19.2a).

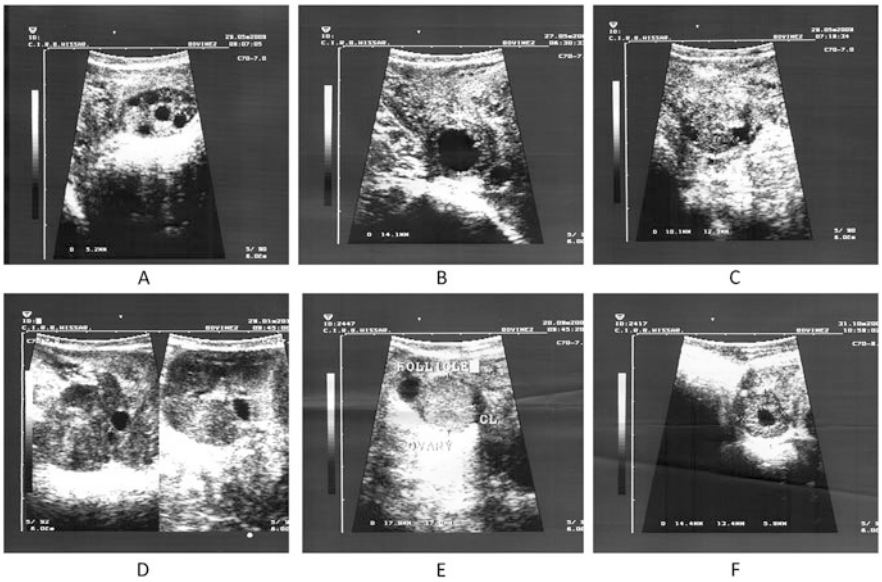

Fig. 19.2 (**a**). A normal size buffalo ovary with several follicles of medium to small size without corpus luteum (CL); (**b**). Buffalo ovary with preovulatory follicle of 14.1 mm; (**c**). Buffalo ovary with recently developed CL on day 2 post-estrus. 2 Small follicles also present; (**d**). Buffalo ovaries with two B-mode images (left image—left ovary; right image—right ovary) day 5 post-estrus having multiple CL; (**e**). Buffalo ovary with a well-developed CL on day 10 post-estrus; (**f**). Buffalo ovary with a cystic CL on day 10 post-estrus. A small cavity is present inside luteal tissue

19.3.2 Ovarian Stroma

Ovarian stroma is more echogenic as compared to other ovarian structures (follicles/CL) and therefore, appear brighter on the viewing screen. Occasionally, ovarian stroma is difficult to appreciate in buffaloes, as luteal tissue almost occupies the entire ovary. Identification of ovarian stroma is aided by the presence of numerous scattered anechogenic follicles, which appear as round and black areas surrounded by thin wall.

19.3.3 Follicle

Follicles as small as 2 to 3 mm diameter can be visualized and quantified by ultrasonography as theses follicles appear round and black area surrounded by a fine wall. Maximum diameter of the preovulatory dominant follicle (DF) at estrus ranges between 12 and 20 mm in buffaloes (Fig. 19.2b). The outline and echogenicity of the follicles can be changed by altering the angle of incidence of the scanning beam. The presence of several follicles or CL can cause compression of the follicles, making them appear irregular in outline. Due to lack of attenuation of

the ultrasound beam, a hyperechogenic border is usually seen at the distal zone of the follicle. This intense echogenic formation beneath a fluid-filled structure is known as enhanced through-transmission. This artifact is especially common beneath images of follicles, embryonic vesicles, and urinary bladder.

19.3.4 Corpus Luteum (CL)

Developing CL is poorly defined with irregular, grayish black structure with echogenic spots within the ovary. But mature CL appears as a well-defined granular and grayish echogenic structure with a clear line of demarcation visible between it and the ovarian stroma (Fig. 19.2c,d). However, regressing CL does not have the line of demarcation due to slight difference in echogenecity between ovarian stroma and luteal tissues (Honparkhe et al. 2004; Barile et al. 2007). CL ranges between 10 and 20 mm diameter in buffaloes and corpus albicans can occasionally be seen as a distinct bright area (Fig. 19.2e). It is especially visible when situated close to a CL or follicle. Two different types of luteal morphology are seen in buffaloes viz. a compact CL and CL with fluid-filled central cavity. Central cavity appears ultrasonically as anechoic (black) to hypoechogenic area surrounded by relatively hyperechogenic luteal tissues. The central cavity is generally present in early CL which get automatically filled in the mature CL (Fig. 19.2f). There is no evidence to support relationship of these two types of CL either with variation in concentration of progesterone or maintenance of pregnancy.

19.4 Detection of Ovarian Pathologies

19.4.1 Ovarian Cysts

Ovarian cysts are fluid-filled structures that persist for 10 days or more and are generally greater than 25 mm in diameter. Cysts appear large (25 to 55 mm), non-echogenic round structures (black) that are single or multiple in one or both ovaries. Cysts may be of either follicular cyst or luteal cyst. Differential diagnosis between a follicular and luteal cyst can be made on the basis of thickness of the cyst wall. The thickness of the wall of a follicular cyst is <3 mm, while it is >3 mm for luteal cyst. Follicular cysts in general are multiple, whereas luteal cysts are generally single. Follicular structures that are in between preovulatory and cystic size require clinical correlation and their persistent in the ovary (Fig. 19.3a-e).

19.4.2 Parovarian Cysts

These cysts appear similar to the follicular cysts, but are located outside the boundary of ovarian stroma. The size of these cysts varies from 3 to more than 40 mm. Small size parovarian cysts may not interfere in the fertility of animal, but

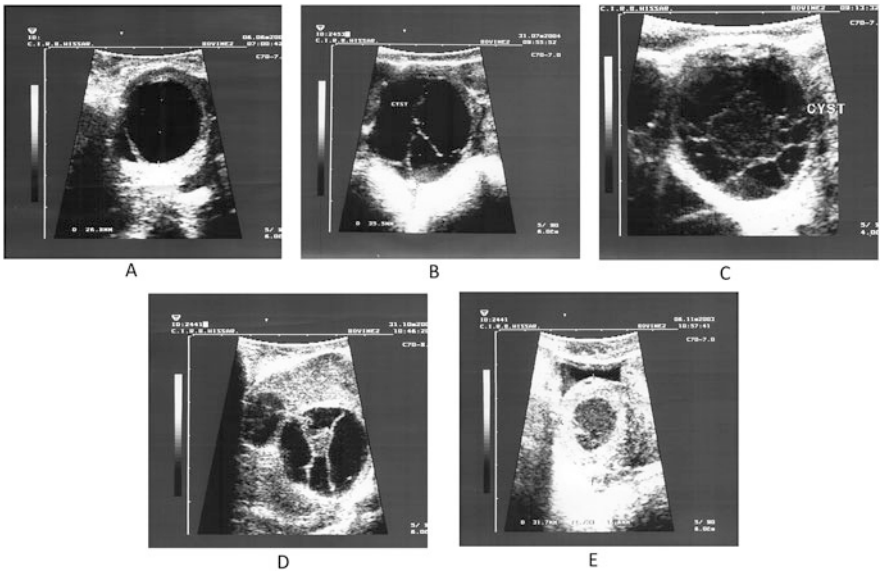

Fig. 19.3 (**a**). Buffalo ovary with a follicular cyst. Size of cyst (26.8 mm); (**b**). Buffalo ovary with a follicular cyst. The inner cavity shows thin septations; (**c**). Buffalo ovary with a luteinized follicular cyst. The inner cavity shows a network of echogenic reflections and thin septations; (**d**). Buffalo ovary with a luteinized follicular cyst, 10 days after GnRH injection. The wall of follicular cyst is luteinized and inner cavity shows thin septations (**e**). Buffalo ovary with a luteal cyst

large size parovarian cyst may physically interfere in the engulfment of ovulated oocyte by fimbriae leading to fertilization failure. Animals with parovarian cysts do not have an altered hormonal profile.

19.4.3 Ovarian Abscess

Pus in the ovarian abscesses usually turns dry and presents hyperechogenic image on ultrasonographic examination.

19.4.4 Follicular Growth Pattern

Ovarian follicular growth, regression, dominance, and ovulation can be monitored with ultrasonography. It is well established that follicular growth occurs in a wave-like pattern and follicular wave involves the synchronous growth of cohort of follicles in both ovaries, from which one dominant follicle (DF) emerges (Fig. 19.4a). Each DF has several phases, viz. growing, static, and regressing phase. There are either one, two, or three waves of follicular growth during buffalo estrous cycle (Baruselli et al. 1997; Taneja et al. 1996; Ali et al. 2003). It has been

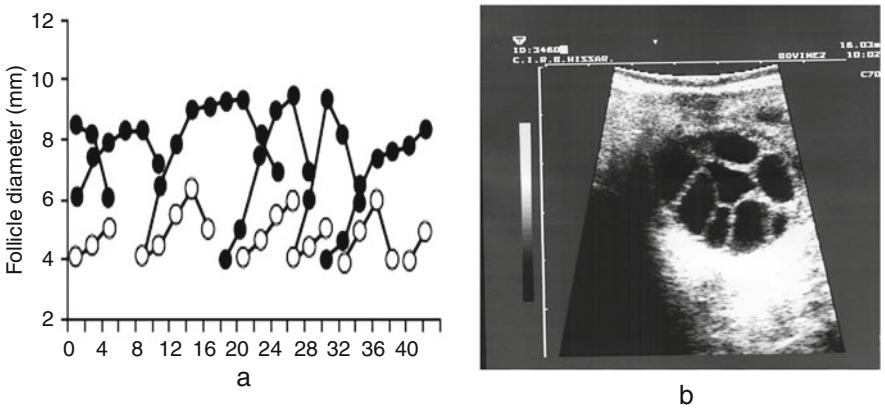

Fig. 19.4 (**a**). Follicular growth pattern in buffalo**.** Data source: Sharma et al. (2011); (**b**). Buffalo ovary with multiple preovulatory follicles on the day of estrus after superovulatory treatment with FSH

documented that the first wave begins on day 1, the second around day 9–11, while the third wave appears on day 17 of the estrous cycle. It has been observed that the DF of the final wave ovulates, whereas the DFs of the preceding waves undergo atresia. The diameter of anovulatory as well as ovulatory DF in buffaloes ranges between 11 and 16 mm. Ultrasonic studies in prepubertal heifers indicate that follicular development occurs in a wave-like pattern as early as 2 weeks, with each wave lasting between 7 and 9 days. As the animal age increases, the DF diameter increased from 8.5 to 12 mm (Evans et al. 1994). During anoestrus condition also, follicular growth occurs in a continuous wave-like pattern without ovulation. It has been documented that the diameter of the largest dominant follicle (DF) may attain a size equivalent to that of the preovulatory follicle (Sharma et al. 2004). The DF undergoes atresia rather than ovulation, possibly due to failure of appropriate preovulatory LH surge. For studying the follicular dynamics, daily examinations are carried out and follicles >3 mm are counted and measured. Ovarian maps during each examination are drawn to record size and relative position of follicles to facilitate sequential evaluation of follicles turn over. Sequential ultrasonic monitoring of individually identifiable follicles is a tool to study the follicular dynamics during the various physiological stages, viz. estrous cycle, pregnancy, postpartum period as well as during superovulation and hormonal treatments (Choudhary et al. 2018; Gaur and Purohit 2019).

19.4.5 Anoestrus and Silent Oestrus

Incidence of anoestrus and silent oestrus condition is high in buffaloes. The diagnosis of ovarian function is usually based on observing the behavioral signs of heat by the owners or rectal palpation of ovaries for the presence of CL by veterinarian.

However, both methods do not provide reliable information as discussed earlier. Transrectal ultrasonography provides accurate information about the status of the CL and cyclicity of the animal. Anoestrus condition can be confirmed by the absence of CL, while small follicles and large follicles >10 mm are invariably present in ovary. Accuracy of identifying cyclic and acyclic condition is >95% using single time ultrasound scanning (Choudhary et al. 2018). Buffaloes that do not manifest estrus signs (Silent estrus) can be diagnosed by presence of CL in one of the ovaries.

19.4.6 Oestrus and Ovulation

Buffalo in estrus can be identified by the presence of good uterine tone by rectal palpation, cervical relaxation, release of cervical discharge on squeezing the vagina, presence of fluid in the uterine lumen, and presence of large follicle (about 11–16 mm in diameter) during ultrasound examination. The ovary may have a regressed CL that may or may not be visualized using ultrasound scanning. Moreover, buffalo immediately after ovulation can be identified by invariably presence of uterine tone and cervical discharge. Immediately after ovulation, the largest ovarian follicle is generally less than 6 mm. Recently developed CL can be detected on the first day after ovulation and developing corpus hemorrhagicum is a poorly delineated, irregular, slightly echogenic grayish black structure within the ovary. During ultrasound examination, the presence of uterine tone, scanty discharge from vagina, and presence of small to medium size (2–6 mm) ovarian follicles and occasionally presence of previous regressing small size CL in one of the ovaries indicate recent ovulation.

19.4.7 Oestrus Induction and Ovulation Synchronization

Ultrasonic scanning of ovaries provides useful information for rational treatment of silent estrus and anestrus conditions in buffaloes. Based on the ultrasonographic observations, buffaloes detected with a mature CL in the ovary can be administered with single or double dose of $PGF_{2\alpha}$, 11 days apart for estrus induction. This is followed by insemination at observed estrus, usually occurring within 2–5 days post-injection. Acyclic buffaloes (i.e. with no CL—true anoestrus: delayed puberty, postpartum anoestrus, summer anoestrus) essentially require progesterone priming to induce fertile ovulatory oestrus. Progesterone implant for 7–9 days along with 400 IU eCG at the time of implant removal is the best treatment to induce fertile estrus in buffaloes. Animals are inseminated at 48 and 60 h after implant removal.

19.5 Ultrasonography in Embryo Transfer (ET) Program

For a successful pregnancy in ET program in buffaloes, embryos must be transferred into the uterine horn, ipsilateral to the side ovary bearing corpus luteum. Accurate identification of early CL by rectal palpation on day 5/6 is extremely difficult especially in buffaloes. Ultrasound is very useful in locating the CL at this stage of cycle. In ET program, ultrasound can also be used to evaluate superovulatory response and devising suitable strategies which could enhance ovulation rate in donors. The response to a given quantity of gonadotrophin is extremely variable both between and within individuals (Armstrong 1993; Sharma et al. 2002). Variability between individuals has been attributed to the number of gonadotrophin sensitive follicles present at the time of initiation of treatment and the number of follicles ovulated subsequent to superovulation (Fig. 19.4b).

19.5.1 Ultrasound-Guided Ovum Pick Up and IVF Technique

In recent years, in vitro embryo production technique using ovaries from slaughter house has attracted the attention of scientists. However, limitation in this technology has been the pedigree status of oocyte collected from abattoir. Curiously, almost 99% population of the follicles present in the ovary undergoes atresia and only less than 1% of it is used in the active reproductive phase of buffaloes. Therefore, use of transvaginal ultrasound-guided oocyte aspiration technique in live animals is a better alternative to trap these unutilized oocytes and produce elite embryos of known genetic makeup using IVM-IVF technology. This technique is less traumatic and less invasive than laparoscopy and it can be repeated over several months without affecting the cyclicity of animal. The technique has been attempted in buffaloes (Kitiyanant et al. 1995) and reported to yield an average of 5–10 transferable embryos per buffalo in 100 days time using IVM-IVF technique (Zicarelli 1997). Transvaginal ablation of largest dominant follicle before any superovulatory treatment is used to enhance the superovulatory response in buffaloes (Singh et al. 2012). Apart from follicular aspiration, ultrasound-guided puncture technique can also be used for aspiration of fetal fluids for sex determination, biochemical analysis, and hormonal estimation, sampling of uterine contents for diagnostic purposes and injection of substances into the ovaries, follicles, corpus luteum, and uterus for research purposes (Aerts et al. 2005; Velazquez et al. 2014).

19.5.2 Early Pregnancy Diagnosis

Early pregnancy diagnosis is very much essential to maintain farms in an economic status. Early identification of pregnant and non-pregnant animals post-breeding helps in rebreeding of non-pregnant animals and improves reproductive efficiency by decreasing calving interval. Pregnancy diagnosis can be made using various methods with varying degrees of accuracy. The accuracy depends upon method of

pregnancy diagnosis, stage of pregnancy, and expertize of the person. Accuracy of the diagnosis is shown as sensitivity, specificity, positive, and negative predictive values (Broaddus and de Vries 2005; Table. 19.1). The various methods of pregnancy diagnosis in cattle and buffalo are non-return to heat after breeding, manual rectal palpation, ultrasound examination, measurement of progesterone hormone and pregnancy associated glycoproteins, and outward changes to appearance. Progesterone hormone is measured in milk or blood between day 21–24 post-insemination to test the functionality of corpus luteum. This test is most accurate in determining a non-pregnant buffalo with greater accuracy (100%) with lower reliability (80–85%).

Rectal palpation and ultrasound examination are most common method for pregnancy diagnosis in large animals (Romano et al. 2006). Rectal palpation has the advantage of being an accurate, fast, and relatively a cheap method. However, findings are subjective and may vary with the experience of the veterinarian. Expert veterinarians can diagnose pregnancy by palpation as early as 45 days after insemination, but others require a time between 60 and 90 days after insemination to increase the accuracy of the examination.

Recently, ultrasonography has emerged a powerful tool for early pregnancy diagnosis in large animal reproduction. The main advantage of the use of ultrasound is diagnosis of pregnancy at an early stage with high accuracy. However, ultrasound machines are relatively expensive. Using ultrasonography, embryo can be visible as early as between days 19 and 24 of gestation (Naikoo et al. 2013). The accuracy varies depending on the stage of pregnancy examined, operator experience, frequency of transducer selected, age and parity of animal, and number of examination. Early diagnosis of pregnancy is based on the detection of a discrete, linear non-echogenic structure at multiple locations within the uterine lumen. However, the diagnosis based solely on detection of fluid within the uterine lumen is unreliable because of the small dimensions of the elongated conceptus approach, the limits of resolution of most available scanners and small amount of free intrauterine fluid are present as early as day 10 and can be indistinguishable from the vesicle (Kastelic et al. 1991; Ingawale et al. 2012). The amniotic vesicle of the embryo can be observed between day 18–20 and fetal heart beat is detectable between days 21 and 22 (Totey et al. 1991; Pawshe et al. 1994; Varughese et al. 2013). Pregnancy diagnosis by ultrasound under field conditions is highly reliable from day 30 onwards. Under field conditions, pregnancy can be diagnosed in buffaloes with certainty on day 26 post-breeding. Disadvantage of early pregnancy diagnosis is that it needs subsequent examination to identify and rebreed buffaloes that experience late embryonic and fetal mortality after diagnosis of pregnancy with ultrasound. Nearly 10 to 16% of cows diagnosed pregnant at 28 days post-AI, experience early embryonic loss by 56 days post-AI. In buffaloes, around 28–30 days post-AI is best time to do ultrasound for pregnancy diagnosis as the fetus and fetal fluid inside uterus will be quite appreciable to diagnose it accurately (Fig. 19.5a-f, Table 19.2).

Table 19.1 Relationship between gestation age and fetal somatic parameters length in buffaloes

Fetal structure	Regression equation	R^2	P value	References
Crown rump length (mm)	$y = 22.71153–1.16234x + 0.02753x^2$	0.95	0.0001	Terzano et al. (2005)
Crown rump length (cm)	$y = 0.0282x^2 + 0.1589x-0.1427$	0.945	0.0001	Ali and Fahmy (2008)
	$y = 0.0036x^2–0.1727 + 2.955$	0.989	0.0001	
Crown rump length(mm)	Gompertz model (gestational age in days = a. $e^{\{-b.e(-c.fs)\}}$ where a, 64.675; b, 01.1915; c, 0.0431; e, exponential; fs fetal size in mm)			Sharma et al. (2012a, b)
Abdominal diameter (mm)	$y = -14.3452 + 0.5631x$	0.94	0.0001	Terzano et al. (2005)
Abdominal diameter (cm)	$y = 0.0323x^{1.736}$	0.865	0.0001	Ali and Fahmy (2008)
Biparietal diameter (mm)	$y = -11.2431 + 0.4455x$	0.91	0.0001	Terzano et al. (2005)
Biparietal diameter (cm)	$y = 0.0179x^2–0.1222x + 1.0688$	0.899	0.0001	Ali and Fahmy (2008)
	$y = 0.0002x^2 + 0.0217x-0.4058$	0.971	0.0001	
Muzzle-occipital length (mm)	$y = -21.4779 + 0.8063x$	0.89	0.0001	Terzano et al. (2005)
Femur length (mm)	$y = -10.3454+ 0.2631x$	0.76	0.0001	
Amniotic vesicle DM (cm)	$y = 0.0004x^2 + 0.0788x-1.8291$	0.853	0.0001	
	$y = -0.1367x^2 + 2.3527x-7.034$	0.978	0.0001	Ali and Fahmy (2008)
Uterine DM (cm)	$y = -0.0427x^2 + 1.1598x-2.6297$	0.964		
Chest depth (cm)	$y = 0.0113x^{2.0302}$	0.866		
Ruminal length (cm)	$y = -0.0322x^2 + 1.3592x-9.1042$	0.673		
Omasal DM (cm)	$y = 0.0146x^{1.7255}$	0.610		
Eyeball DM (cm)	$y = -0.0037x^2 + 0.2599x-1.6977$	0.890		
Placentome DM (cm)	$y = -0.0031x^2 + 0.2712x-0.9265$	0.679		

Data Source: Sharma et al. (2014).

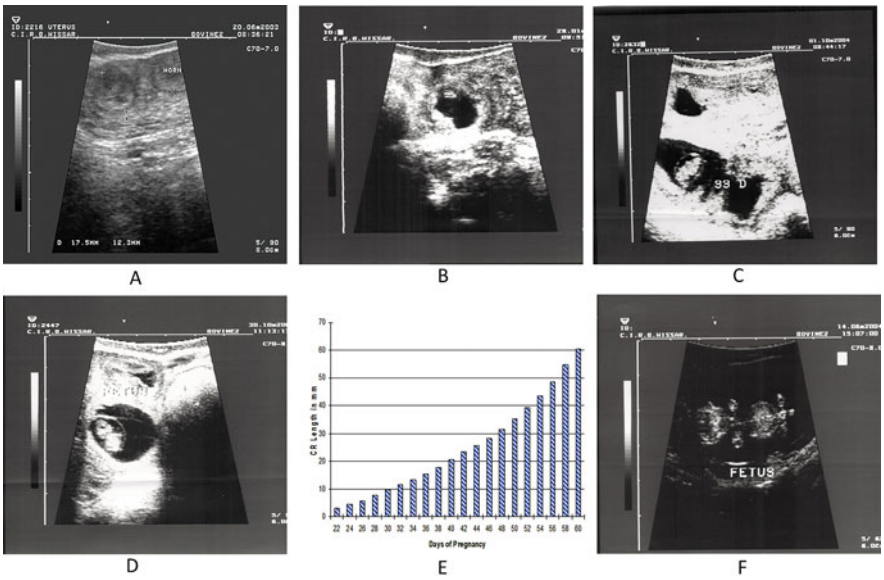

Fig. 19.5 (**a**). Ultrasonogram of a non-pregnant uterus; (**b**). Ultrasonogram at 28 days of pregnancy showing a small fetus; (**c**). Uterine horn at 33 days of pregnancy, fetus is clearly visible as elongated echogenic structure within an amniotic membrane; (**d**). Uterine horn at 39 days of pregnancy, head is clearly visible, developing eye is seen as anechoic spot; (**e**). Crown Rump length of buffalo fetus at different stages of gestation; (**f**). Fetus at 50 days of pregnancy. Head, body, tail, and all four limbs are clearly visible

19.5.3 Uterus and Cervix

Ultrasound helps in visualization of contents within the uterus during various pathological and other physiological conditions in large animals (Fissore et al. 1986). Uterine shape and echotexture varies during the different phases of estrous cycle and estrus phase is evident with good uterine tone with distinguishable endometrial folds visualized using ultrasonography as well as the presence of small accumulations of fluid in uterine lumen. However, during diestrus, fluid is absent and uterine wall is less distinctive in shape and size. Endometrium can be distinguished from more echogenic myometrium. In our personal experience small amount of uterine fluid may also be present in a few prepubertal heifers without being in heat. Therefore, it must be distinguished from early pregnancy and being in heat. Pregnant animals must have a CL and animals in heat have good uterine tone and a large follicle in one of the ovaries. Ultrasonic image of cervix on day 0 is characterized by presence of non-echogenic fluid collections within a hyperechogenic image. However, during diestrus phase non-echogenic area is seen. Uterine pathological conditions, such as endometritis, pyometra, mucometra, and mummified/macerated fetuses are generally characterized by an altered thickness of uterine wall and a distended lumen, filled to varying degrees with partially

Table 19.2 Identification of fetal structure in buffalo during early pregnancy

Fetal structure	Mean gestational age in days at first detection	References
Embryonic vesicle	19.0 ± 2.1	Pawshe et al. (1994)
	24	
	20.55 ± 2.34	
	21	Ingawale et al. (2012)
Embryo proper	19.0 ± 1.69	Pawshe et al. (1994)
	28	
	29.25 ± 0.36	
	25.18 ± 1.91	
	28	Ingawale et al. (2012)
	22	Sharma et al. (2012a)
	26	Naikoo et al. (2013)
Heartbeat	29.6 ± 1.57	Pawshe et al. (1994)
	28	
	26.33 ± 0.52	
	25.27 ± 3.58	
	28	Ingawale et al. (2012)
	27.1 ± 0.3	Sharma et al. (2012b)
	26	Naikoo et al. (2013)
Allantois	30.0 ± 1.14	Pawshe et al. (1994)
	28	Ingawale et al. (2012)
Amnion	33.4 ± 1.64	Pawshe et al. (1994)
	31.64 ± 2.34	
	30.4 ± 0.2	Sharma et al. (2012a)
Optic area	47.78 ± 5.61	
	32.4 ± 0.2	Sharma et al. (2012b)
Fore limbs	34.6 ± 1.34	Pawshe et al. (1994)
	34.8 ± 0.2	Sharma et al. (2012a)
Hind limbs	36.8 ± 2.34	Pawshe et al. (1994)
Optic area	38.2 ± 2.39	Pawshe et al. (1994)
	34.2 ± 0.2	Sharma et al. (2012b)
Spinal cord	35.8 ± 2.52	Pawshe et al. (1994)
Tail bud	36.7 ± 0.2	Sharma et al. (2012a)
Umbilical cord	40.25 ± 2.76	
	27	
	40.9 ± 0.2	Sharma et al. (2012b)
Split hooves	46.0 ± 2.64	Pawshe et al. (1994)
	42.7 ± 0.3	Sharma et al. (2012a)
Fetal movements	49.4 ± 2.31	Pawshe et al. (1994)
	46.25 ± 2.36	
	45.0 ± 0.4	Sharma et al. (2012b)
Genital tubercle	44.5 ± 2.39	
	55	
	49.8 ± 0.4	Sharma et al. (2012a)

(continued)

Table 19.2 (continued)

Fetal structure	Mean gestational age in days at first detection	References
Brain	57.5 ± 1.2	
Ribs and vertebra/vertebral column	59.8 ± 2.39	Pawshe et al. (1994)
	47.33 ± 4.9	
	60	
Liver and gall bladder	60	
	66.63 ± 3.54	
Urinary bladder	57.88 ± 3.08	
Gastric vesicle	60	
Stomach cavities	59.17 ± 4.36	
Omasum	70	Ali and Fahmy (2008)

Data Source: Sharma et al. (2014).

echogenic snowy patches. In fetal maceration, the fetal bones are identifiable as echogenic structures in the uterine lumen suspended in the fetal fluids. In mummified fetus, the uterine fluid is absent and fetal mummy appears as a poorly defined echogenic mass.

19.5.4 Determination of Fetal Age

Determination of gestational age helps in better management of animal around parturition. Per-rectal palpation allows rough assessment of the fetal age. But the stage of gestation in fetus is ascertained by serial measurements of somatic parameters of the fetus, viz. crown rump length (CRL, distance a straight line between the fetal crown and the origin of tail), abdominal diameter (ABD, maximum DM at the region of the umbilical cord), biparietal diameter (DM) of the cranium (BPD, the widest distance between the outer borders of the cranium at an angle of 90° to the falx cerebri), lengths of femur, tibia, and metatarsal bone muzzle-occipital length (MOL), amniotic vesicle DM (AVD, the widest DM), uterine DM (UTD, maximum intrauterine lumen at the level of the conceptus), ruminal length (RUL, the largest intraluminal length), omasal DM (OMD, maximal DM of the omasum), chest depth (CHD, a dorso-ventral distance just caudal to the apex of the heart), eyeball DM (EBD, the largest intra eye distance), and placentome DM (PLD, maximum DM, while it was in cross section). However, crown rump length (CRL distance a straight line between the fetal crown and the origin of tail) is most excellent parameter for calculating ages of embryos less than 60 days and head or trunk diameters are more easily obtained for fetuses above 50 days old. The mean gestational days at first detection of various fetal structures in buffalo fetus are as follows: heartbeat, 27.1 ± 0.3; amnion, 30.4 ± 0.2; optic area, 32.4 ± 0.2; forelimb buds, 34.8 ± 0.2; tail bud, 36.7 ± 0.2; optic lens, 40.8 ± 0.3; umbilical cord,

40.9 ± 0.2; split hooves, 42.7 ± 0.3; fetal movements, 45.0 ± 0.4; and genital tubercle, 49.8 ± 0.4 days (Terzano et al. 2005; Sharma et al. 2012b, 2014).

19.5.5 Determination of Fetal Sex

Fetal sex determination by ultrasonography is one of the recent applications of this imaging technique. Fetal sex determination has the economic advantage for animal owner since a buffalo carrying female fetus is likely to fetch more remuneration. Similarly, it helps in progeny testing program besides helpful in decision making with respect to the suitability of the animals under farm conditions. In bovines, diagnosis of fetal sex is based on the presence of scrotal swelling and mammary glands (Muller and Wittkowski 1986) or the location of genital tubercle which leads to the formation of penis or clitoris (Curan 1992). Identification of sex in bovine is most commonly done by locating the genital tubercle. This genital tubercle develops in between hind legs around day 50 and migrates towards umbilical cord in males and lie beneath the tail in females. Diagnosis based on the presence of scrotum and mammary gland is somewhat difficult as both the structures are present between the two hind limbs and there identification becomes difficult. Furthermore, these two structures are visualized later as compared to genital tubercle. This tubercle is recognized as a prominent bilobular bright structure below the tail in females and in the vicinity of the umbilical cord in males. Fetal sex can be diagnosed between day 55 and 100 post-breeding in buffaloes (Sharma et al. 2011; Fig. 19.6a-c).

To make an accurate diagnosis of sex in buffalo, veterinarian must look the fetus at three positions: 1) nearby umbilicus, where the umbilicus enters the abdomen (possible male genital tubercle); 2) just ventral to the tail (possible female genital tubercle), and 3) if the pregnancy is around 100 days the area between the hind legs (possible scrotum). Fetal sex must be interpreted only after observation of presence and absence of all these structures. There are two limitations that could limit the ability to diagnose the sex of fetus beyond 100 days of pregnancy. First, a large fetus

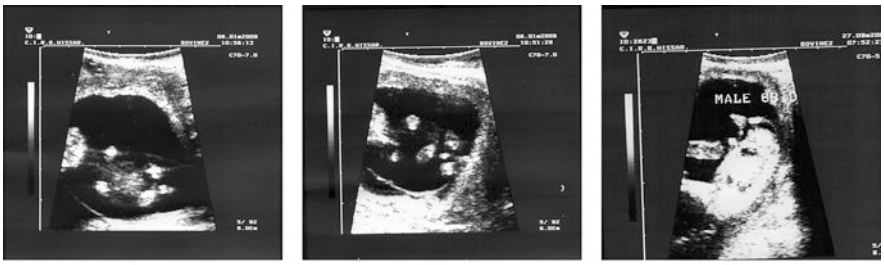

Fig. 19.6 (**a**) A female fetus showing genital tubercle on day 60 post-insemination near tail; (**b**). A male fetus showing echogenic structure (genital tubercle) on day 60 post-insemination just behind umbilicus; (**c**). Fetus showing attachment of umbilicus with body having an echogenic structure (genital tubercle) near its attachment on day 66 post-insemination- A male fetus

may hinder the accurate placement of transducer to get a desired image at important position. Secondly, descending of gravid uterus to abdominal floor makes fetal sexing virtually impossible without retracting the gravid horn in pluriparous animals that are pregnant beyond 100 days.

19.6 Ultrasonography in Male Reproduction

19.6.1 Male Reproductive System

The male reproductive system consists of a pair of testis that produces sperm, ducts which transport the sperm to the penis, and glands that contribute seminal plasma.

19.6.2 External Scanning

Visual appraisal of the external genitalia is performed, while the bull is relaxed. In the posterior approach, the restrained bull is touched to evaluate its attitude. In a continuous and slow movement, the testes, epididymis, and the spermatic cord are located and examined by palpation. While the animal is in standing position, one testis is manually pushed down into the scrotum, while the opposite testis is pushed upward out of the scanning plane, stretching the scrotal wall to allow a good contact between the transducer face and the scrotum (Fig. 19.7a, b).

19.6.3 Internal Scanning

For the examination of internal reproductive organs (bulbourethral glands, pelvic urethra, prostate, ampullae, and vesicular glands), all precautions that apply to transrectal palpation are applicable to transrectal scanning. Aggressive bulls will require stall restraint and sedation (xylazine, 0.01 to 0.02 mg/kg IV). With aggressive bulls, semen collection before the ultrasonographic examination may relax the animal and facilitate the ultrasonographic examination, but the ampullae becomes empty. The transducer face is pressed firmly against the rectal mucosa to allow good transmission, while the index finger directs the transducer and other fingers help to identify and locate the different structures (Kumar et al. 2013).

19.7 Anatomy of the Reproductive System

19.7.1 Testis

Compared to internal organs, ultrasonographic examination of the external genital organs is easier because of better eye-to-hand coordination. The rete testis is the central and most echoic structure of the testicular parenchyma. It is a useful

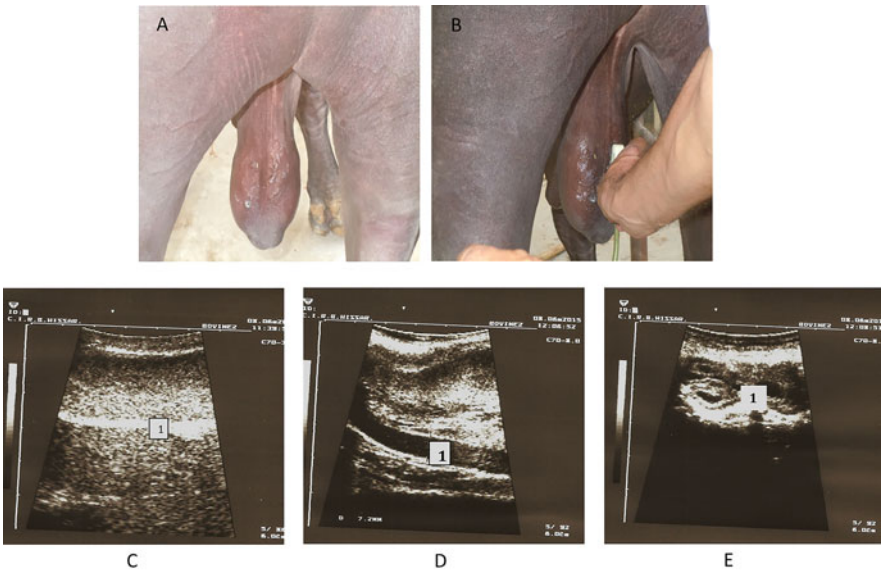

Fig. 19.7 (**a**). Descended scrotum of a buffalo bull; (**b**). Posterior approach to scan the testicles with linear probe; (**c**). Ultrasonographic images of the testes in longitudinal view. 1. Mediastinum; (**d**). Ultrasound image of the pelvic urethra in longitudinal view. 1. Urethral lumen; (**e**). Sonogram of the seminal vesicle. 1: Parenchyma; 2: Lumen of the vesicular gland

landmark to identify the largest diameter and the echogenicity of the testes increases with the age of the bull, representing the most active phase of growth of the seminiferous tubules. Testicular parenchyma has a moderate homogeneous echogenicity as compared to the hyperechoic mediastinum. The testicular tunics are hyperechoic with an apparent anechoic line between the tunica vaginalis parietal and visceral layers. Normally, the testicular parenchyma is moderately echoic and has a fine, homogeneous echotexture with a hyperechoic mediastinum. The mediastinum appears as a central line on longitudinal view and as a dot in transverse view. The testicular border is characterized by a smooth and hyperechoic tunica albuginea. Ultrasonography can be used to predict testicular volume and sperm production as well as any pathological condition of the testis (El-Khawaga et al. 2012; Genedy et al. 2019; Fig. 19.7c).

19.7.2 Epididymis

It is an elongated structure located on the posterolateral side of each testis. It consists of three parts, i.e. head (caput), body (corpus), and tail (cauda). The head of the epididymis is flattened at the upper end of the testis, where vasa efferentia (approximately 12 to 15) merge into a single duct of epididymis. Ultrasonographically, both

head and tail of the epididymis are homogeneous and are less echoic and more heterogeneous in appearance as compared to the testicular parenchyma.

19.7.3 Penis

Penis extends from the ischial arch to near the umbilicus on the ventral abdominal wall. In transverse view, the penis consists of a ventral hyperechoic urethra surrounded by the echoic structures, viz. corpus spongiosum and cavernosum. Corpus cavernosum is in turn surrounded by a dense hyperechoic membrane called the tunica albuginea. Ultrasonography is useful to diagnose several pathological conditions of the penis (hematoma, abscess, urethritis).

19.7.4 Bulbourethral Glands

Bulbourethral glands are two small walnut shaped glands on either side of the urethra (dorsal part) at the level of ischial arch. The bulbourethral glands appear homogenously hyperechoic compared to the other accessory glands. Also, they appear less echoic than the surrounding bulbospongiosus muscle and appears heterogeneous with hyperechoic and less echoic bands (El-Khawaga et al. 2012).

19.7.5 Pelvic Urethra

It is cranial to the bulbourethral glands and runs from the ischial arcade to the bladder neck. Due to the presence of muscular structure around the urethra it assumes cylindrical shape and the ventral portion is thicker than the dorsal one. Pelvic urethra is easy to feel due to its rhythmic muscular contractions when stimulated. The pelvic urethra lumen appears anechoic when visible or filled with urine as compared to the echoic appearance of the surrounding region (Fig. 19.7d).

19.7.6 Prostate

Prostate is a single round to ovoid gland located around and along the urethra, posterior to the vesicular glands ducts. It has two parts, viz. body and pars disseminata. The body of the prostate consists of two lobes which can be palpated transrectally. The pars disseminata is approximately 12 cm long and cannot be identified by transrectal palpation. Ultrasonographically, the prostate body appears to be moderately echoic and homogenous structure present dorsal to the bladder neck with smooth margins (El-Khawaga et al. 2012).

19.7.7 Seminal Vesicles

These are two lobulated and elongated glands situated adjacent to the neck of the urinary bladder and lateral to the ampullae. As they are irregular in shape, determining the accurate dimensions of glands (VGs) is difficult. However, the mean length of the longest axis of the VGs for a 24-month-old bull is approximately 12 cm long and 1.7 cm wide. The size of the VGs increases with age as compared to other accessory glands. By ultrasonography, VGs are shown by isoechoic lobes of the glandular tissue, separated by hypoechoic regions and hyperechoic borders. The collection ducts of the VGs can be viewed as anechoic region running from each gland (El-Khawaga et al. 2012; Fig. 19.7e).

19.7.8 Orchitis

Inflammation of testis is called orchitis, while inflammation of both testis and epididymis is called orchiepididymitis. It is a rare and sporadic disease of domesticated animals except endemic areas of Brucella abortus or tuberculosis and unilateral orchitis is more common than bilateral orchitis. In this condition, the scrotum is painful, hot, and edematous in acute phase. Depending on the condition, the ultrasonographic appearance of orchitis may change. The view of the affected testis is shown with a heterogeneous echotexture. In severe cases, small, round, hypoechoic cystic structures within the testis are observed. The parenchyma has many dense echoic areas scattered through the testis, which may exhibit acoustic shadowing. Some of these affected areas could be foci of mineralization that occur in the chronic phase of orchitis and mostly the mediastinum testis is unidentifiable (Abu-Seida 2012a).

19.7.9 Testicular Degeneration

It denotes the deterioration of a tissue or an organ in which its function is diminished or its structure is impaired. Testicular degeneration is defined as the partial or complete failure of epithelium of seminiferous tubules to proceed with spermatogenesis. It is an acquired disorder and common cause of infertility in male domestic animals. With testicular degeneration, the utrasonographic picture of the testicular parenchyma shows the loss of its tissue architecture with hyperechoic regions over time.

19.7.10 Testicular Neoplasm

Testicular neoplasia or tumor is rare in buffalo bull, but common in dogs. However, they can occur in intact males of any age. Mainly three types of testicular tumor are documented, viz. interstitial cell tumors, seminoma, and Sertoli cell tumors.

Ultrasonographic images of testicular tumors range from circumscribed small nodules to large complex masses. Tumors may be hyperechoic, anechoic, or a mixture of echogenicities and distinguishable from the normal testicular parenchyma.

19.7.11 Inguinal Hernia

Inguinal hernias enlarge the neck of the scrotum and intestinal loops are usually contained within the tunica vaginalis. The echoic content of the intestinal loops can be observed in the inguinal hernia and in real-time scanning using ultrasound, the movement of the contents is apparent and in rare occasions intraluminal gas is also observed.

19.7.12 Hydrocele

Hydrocele is a fluid collection ($>$ 2 mm) within the tunica vaginalis surrounding the testis causing painless scrotal swelling. The excessive fluid may be associated with many conditions, viz. infection, neoplasm of the testis, torsion of the testis, systemic disease (heart or kidney), or idiopathic disease. It is a relatively rare condition in bulls and ultrasonographic scan usually shows a completely anechoic fluid-filled cavity. In an infectious process, adhesion of the tunica albuginea to the scrotal wall can be seen.

19.7.13 Vesiculitis

Inflammation of seminal vesicle is called seminal vesiculitis and it is common in bulls, stallions, and boars. A number of bacteria and viruses are associated with this disease and it has been suggested that the shape of the pelvis of individual animals predisposes them to the development of vesiculitis. In bulls, during the acute phase of the disease, localized peritonitis may occur. Abdominal pain, rear leg lameness, and unwillingness to move may be noted due to localized peritonitis. Later, animals become infertile, despite normal service behavior. Chronic seminal vesiculitis is characterized by firm (fibrotic) and enlarged glands. In young bulls managed in groups, the prevalence of seminal vesiculitis can be as high as 49% and in older bulls ($>$10 years old), vesicular hypertrophy observed is generally normal. Ultrasonographic findings of vesiculitis depict an increase in overall size of the vesicular gland, thickened wall, and increased echogenicity as compared to contralateral unaffected gland.

19.8 Advantages and Clinical Uses of Ultrasound

As a diagnostic aid, ultrasonography is well suited for examination of reproductive organs, particularly in large animals. The greatest advantage of ultrasonography is that it is non-invasive, non-disruptive, relatively simple, and safe to both the subject and the operator. Ultrasonographic findings are accurate, documentable, and rapid as they facilitate an immediate interpretation without causing stress to the animal. Assessment of reproductive status of large animals became easy with ultrasound as ultrasound examination is quick, non-invasive, and non-disruptive and similar to per-rectal examination of the animals. But the foremost criteria is that animals need to be properly restrained in a crate. Ultrasonographic findings are accurate, documentable, and rapid as they facilitate an immediate interpretation during the examination.

It is an imperative diagnostic aid to find out the normal as well as pathological conditions of the reproductive tract. Transrectal ultrasonography is helpful in predicting the day of oestrus and ovulation, studying follicular dynamics during the estrous cycle, pregnancy, postpartum period, superovulation and in relation to hormonal treatments, and differentiating true anoestrus from silent oestrus condition. It has become the most efficient tool to diagnose the early pregnancy as well as fetal viability, identification of twin fetuses and determination of fetal age and gender. In addition, ultrasound-guided interventional techniques can be used for diagnostic or therapeutic purposes. Several pathological conditions of uterus as well as ovary viz. pyometra, mucometra, endometritis, fetal mummification, fetal maceration, cystic ovaries, parovarian cysts. In large animal reproduction ultrasound is mainly used for early pregnancy diagnosis, knowing ovarian cyclicity status of animal, transvaginal follicle aspiration for ovum pick up, fetal age determination, fetal sex determination, fetal viability and assessing the uterus and its content.

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20

Spermatogonial Stem Cells and Testis-Tissue Cryopreservation as a Tool for Conservation of Buffalo Germplasm

Lalitha Devi and Sandeep Goel

Abstract

Buffalo is an economically valuable animal of the south-east Asian counties, and they have made a significant contribution to livestock production. In recent days, due to the rapid decline of valuable breeds of buffalo and their genetic diversity, there is a great need to conserve their germplasm. Development of spermatogonial stem cell technology, testicular stem cell banking, testis-tissue cryopreservation, methods to salvage the cryopreserved-thawed tissues, and mature them in vitro and in vivo to retrieve fertilization-competent gametes are some of the methods which contributed significantly to the conservation of their germplasm. These methods are of specific significance to the juvenile and young buffalo males who die before contributing their genetic potential to the next generation. A combination of these bio-techniques would have substantial and feasible applications in the propagation of their germplasm. However, there is still a need to improve the current methods and introduce new technologies for germplasm conservation and dissemination. This chapter aims to discuss these modern biotechnological methods to provide readers with an insight into how these technologies can find application in buffalo germplasm conservation.

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20.1 Introduction

Asia is the native home for the water buffalo (*Bubalus bubalis*), and India has almost 50% of the total world population. This population includes both river (milk production) and swamp (meat and draught purpose) buffaloes. India is the highest buffalo milk producer globally, with over 20 breeds of river buffaloes (Dutta et al. 2020). Buffalo is considered an important livestock species in India because of its annual milk production of about 135 million tons (Borghese and Mazzi 2006). The buffalos can adapt to harsher environments, utilize poorer quality roughages, and are also more resistant to many tropical illnesses. The strategies like selective breeding and improved management have increased buffalo productivity across the world.

Buffalo shows low reproductive efficiency exemplified by low conception rate, sluggish breeding, anoestrus, seasonality, long calving intervals, and delayed puberty (Perera 2008). Many reproductive strategies have been introduced, with various degrees of success, to improve buffalo production. Furthermore, artificial insemination (AI) is rarely used since oestrus symptoms are mild and oestrus length varies, making oestrus identification challenging. As buffalo is situated mainly in developing countries with inadequate resources, there is limited quality research in basic physiology, health, management, nutrition, and applied reproduction. As a result, there is an urgent need to investigate emerging novel areas of reproductive biology that may be used to improve buffalo's effective genetic improvement.

Interest in buffalo conservation is rapidly growing as several buffalo breeds are experiencing a decline in number and genetic diversity. Ideally, live animals from a given population should be saved. Nevertheless, this method is high-priced, and it is likely to succeed unless the breed can be employed for production (Prentice and Anzar 2010). As a result, there is a great demand for the development of ex situ, in vitro conservation techniques. Some of the emerging technologies are spermatogonial stem cell and testis-tissue cryopreservation and xenografting. In the testes, the most primitive spermatogonia are spermatogonial stem cells (SSCs). SSCs can be defined as undifferentiated germ cells that arise postnatally. These cells are incapable of sustaining spermatogenesis throughout adulthood by balancing self-renewal and differentiating divisions (de Rooij and Russell 2000). Millions of spermatozoa are generated daily in testes following strict quality control to maintain germline integrity; hence SSCs are regarded as a productive stem cell system (Oatley and Brinster 2006). SSCs are the only known adult stem cells known to be self-renewing, and they can differentiate to produce haploid cells, which help in gene transfer to the next generation (de Rooij and Russell 2000). The SSCs can be isolated and cultured in vitro (Nagano et al. 1998; Nagano et al. 2003; Moore et al. 2002; Kubota et al. 2004a; Kanatsu-Shinohara et al. 2004; Kanatsu-Shinohara et al. 2005a; Hamra et al. 2005). Further genetic alterations in these cells can be generated to modify their culture conditions, followed by changing their epigenetic state (Lee and Shinohara 2011).

There is a growing demand for cryopreservation of gametes and embryos to regenerate a particular population in the future (Holt and Pickard 1999). Successful cryopreservation of gonadal (testis and ovary) tissue is critical in preserving fertility

(Jahnukainen et al. 2007). With the development of assisted reproduction technology (ART) and a better understanding of cryobiology, techniques for the prolonged preservation of gametes and embryos have been introduced (Picton et al. 2000). Researchers are more interested in understanding the mechanism or fundamental concepts of cryobiology responsible for low survival rates to develop better cryopreservation techniques. For example, human testicular tissue cryopreservation provides prepubertal boys with cancer, hoping for future fertility preservation before exposure to gonadotoxic treatments (Dumont et al. 2015). Concurrently, in prepubertal girls and women who cannot postpone chemotherapy, ovarian tissue cryopreservation is the sole option available (Donnez and Dolmans 2011). The lack of proper methods for in vivo maturation of premature male and female germ cells led many researchers to focus on developing new methods for isolating and preserving the earliest stage of germ cells. Testis-tissue xenografting in males provides an opportunity for studying spermatogenesis, producing mature gametes testicular maturation in in vivo conditions (Honaramooz et al. 2002b), whereas, in females, ovarian tissue cryopreservation can be used for fertility preservation (Prasath 2008). Long-term oocyte storage would help develop ova banks, allowing to store oocytes in an unfertilized state until suitable sperm is selected (Prentice and Anzar 2010).

Conversely, effective cryopreservation of gonads would preserve the genetic material from animals that die unexpectedly. Several assisted reproductive technologies can be employed later if the tissue is cryopreserved efficiently (Ledda et al. 2001; Checura and Seidel Jr. 2007; Pereira and Marques 2008). The present chapter explored the recent advances in applying male germline stem cell technology, testis-tissue cryopreservation, and xenografting techniques to improve buffaloes' breeding efficiency.

20.2 The Biology of Testes

The testes, also called testicles, are reproductive organs present in a sac of skin called the scrotum in the male. Tunica albuginea, a muscular membrane shell, surrounds the testes, and the testis parenchyma consists of very fine coiled tubes, i.e. seminiferous tubules underneath the albuginea. These tubules consist of three types of cells, germ, Sertoli, and peritubular myoid cells (PTM). Spermatogonia differentiates into spermatocytes followed by spermatids and spermatozoon through spermatogenesis, whereas Sertoli cells are essential for the maturation of germ cells into spermatozoa. Peritubular myoid cells lay down in the basement membrane and surround the seminiferous tubules to offer structural support to the tubules. However, the exact role of PTM was still uncertain. During the prepubertal development in testis, somatic and germ cells proliferate and differentiate systematically to commence the first wave of spermatogenesis. The transfer of genetic information from one generation to another occurs through germ cells. In the case of mammals, during gastrulation, the germline specification starts in the epiblast as primordial germ cells (PGC) (Capel 2000). During embryonic development, the migration of PGC occurs from the base of the allantois via hindgut to the genital ridge (de Rooij

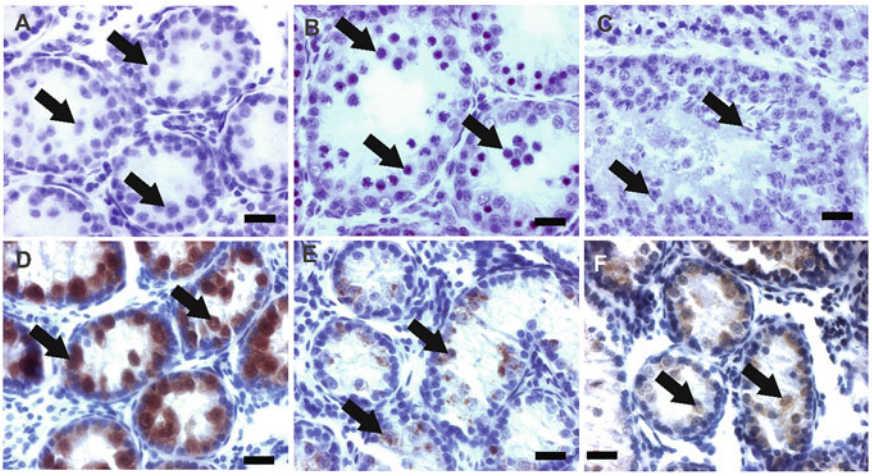

Fig. 20.1 Histological evaluation of buffalo testis at different ages and immunohistological evaluation of immature prepubertal testis for biological markers for spermatogonia. (**a**) Histological appearance of the testis from immature buffalo shows gonocytes (arrows) as the most advanced germ cells. (**b**) Testis from prepubertal buffalo shows pachytene-stage spermatocytes (arrow) as the most advanced germ cells. (**c**) Elongated spermatids (arrows) and spermatozoa are the most advanced germ cells in adult testis. Note that the seminiferous tubule diameter is increasing with the increasing age indicating maturation of testis. (**d**) UCHL1 (ubiquitin C-terminal hydrolase L1, also known as PGP9.5) expression, (**e**) DBA (*Dolichos biflorus* agglutinin) affinity, and (**f**) POU5F1 (POU Class 5 Homeobox 1, also known as OCT3/4) expression is restricted to germ cells in immature buffalo testis (arrows). These markers can be utilized for the isolation and purification of germ cells from immature buffalo testis. Scale bar = 20 μm

and Russell 2000). PGC differentiates into gonocytes in the male genital ridge, which proliferate and differentiate to spermatogonia after birth initiating spermatogenesis in the testis. Like other mammals, germ cell differentiation in buffalo begins with the migration of gonocytes from the seminiferous tubule base to a location on the basement membrane (Dhingra and Goyal 1975). When the gonocytes connect to the basement membrane, they differentiate into spermatogonia and multiply (Guraya and Bilaspuri 1976; Bilaspuri and Guraya 1980). In the seminiferous tubules of 4-weeks-old calves, primitive germ cells such as gonocytes can be found (Fig. 20.1a). Mature Sertoli cells (SC) can be first seen in 36-weeks-old calves (Rana and Bilaspuri 2004). In the early stages of testes development, even though the number of spermatocytes and spermatids are low (Fig. 20.1b, c), after 144 weeks their number quickly increases (Rana and Bilaspuri 2004; Pawar and Wrobel 1991). For example, in 72-weeks-old animals, we can see a higher number of SC. At the age 4–24 weeks, the presence of primitive Sertoli cells dominates in the sex cord, at 36–48 weeks of age spermatogonia (SG), from 60 to 120 weeks, we can see primary spermatocytes, and from 144 to 288 wks spermatids (Dhingra and Goyal 1975; Rana and Bilaspuri 2004). The most advanced germ cell types identified in the different age groups (months) in the testis of Murrha buffalo are gonocytes (0 and 1), SG

(1 and 3), early pachytene (6 and 9), late pachytene (12), secondary spermatocytes (15 and 18), elongating spermatids (21 and 24), elongated spermatids attached to SC (30), elongated spermatids detached from SC (36), and spermatozoa (42 and 48) (Rana and Bilaspuri 2004).

20.3 Spermatogonial Stem Cells (SSCs) and Their Biological Markers

SSCs are the germ cell-based/derived stem cells which are present in the testes. SSCs can produce spermatozoa for a lifetime in an adult male. SSC undergoes self-renewal and differentiation divisions like any actual stem cells, maintaining a stable stem cell population while generating a continuous spermatozoa supply. Following transplantation into a recipient testis, the genetically modified SSCs can develop into spermatozoa, eventually generating transgenic offspring (Nagano et al. 2000; Nagano et al. 2001a; Hamra et al. 2002; Kanatsu-Shinohara et al. 2004, 2005b). Since embryonic stem (ES) cell line is not known to exist in any mammalian species other than rats (Li et al. 2008) and mice (Evans and Kaufman 1981) and more recently in bovine (Bogliotti et al. 2018), SSC could provide an alternative for efficient genetic modification of buffalo using homologues recombination technique. Recently, donor-derived sperm production in recipient bull testis following germ cell transplantation has been demonstrated (Stockwell et al. 2009). As a result, male germ stem cell technology can be used in buffalo breeding, particularly preserving and disseminating the germplasm from prepubertal and immature animals.

The SSCs represent a scarce cell population in the testes of adult animals, (0.01–0.03%) (Helsel et al. 2017; Tegelenbosch and de Rooij 1993). Therefore, isolation and enrichment of SSCs with high viability and purity is essential to their successive culture, manipulation, or identification of these cells following transplantation. The identification and characterization of spermatogonia-specific markers lay the basis for their enrichment. Few high potential biological markers have been introduced to separate SSCs from the other somatic cell population using fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS). Although several markers are reported to mark murine spermatogonia, the known markers in domestic animals for distinguishing spermatogonia are comparatively low in number. In mouse spermatogonia, the expression of Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1), also called PGP9.5, was first reported (Kon et al. 1999). Later, the expression of UCHL1 was observed in pigs (Luo et al. 2006), cattle (Herrid et al. 2007), buffalo (Goel et al. 2010) (Fig. 20.1d), and goats (Heidari et al. 2012). Similarly, undifferentiated mouse spermatogonia express transcription factor proteins like zinc-finger protein promyelocytic leukaemia (PLZF or ZBTB16), which is necessary for SSC maintenance and self-renewal. Furthermore, in pigs, the expression of PLZF was observed in sub-population of gonocytes and SSCs/progenitors, cattle (Reding et al. 2010), goats (Song et al. 2013), equids (Costa et al. 2012) testis. Compared to UCHL1 and PLZF, lectin *Dolichos biflorus* agglutinin (DBA) has no affinity to murine germ cells. Moreover,

among domestic species, the affinity of DBA varies. DBA expression can be seen in spermatogonia (in boars, DBA only binds to gonocytes and primitive spermatogonia). However, loss of binding efficiency to male germ cells with the progression of age occurs (Goel et al. 2007). In the case of prepubertal bovine and buffalo testes, the affinity of DBA is restricted to gonocytes and type-A spermatogonia (Fig. 20.1e), and interestingly the expression is similar to that of UCHL1 (Ertl and Wrobel 1992; Hermann et al. 2007; Goel et al. 2010; Goel et al. 2011; Fujihara et al. 2011). Interestingly, DBA does not bind to germ cells (Borjigin et al. 2010). In addition to the markers mentioned above commonly employed to detect spermatogonia in domestic animals, several undifferentiated spermatogonia markers in murine are also conserved among species.

The expression of DEAD-Box Helicase 4 (DDX4), or simply VASA, was observed in spermatogonia of buffalo (Goel et al. 2010), sheep (Borjigin et al. 2010), and bull (Fujihara et al. 2011) testis. THY1 (also known as CD90) expression was observed in rodents, humans, and non-human primates, and it was used as a surface marker of undifferentiated spermatogonia (Kubota et al. 2003; Ryu et al. 2004; Hermann et al. 2009; He et al. 2010). In cattle and pig testes, THY1 expression is seen in gonocytes and undifferentiated spermatogonia (Reding et al. 2010; Zheng et al. 2014). Among domestic species, THY1 is considered as a conserved surface marker after its expression was reported in goat testes (undifferentiated spermatogonia) (Abbasi et al. 2013; Wu et al. 2013). A cell surface marker, a cluster of differentiation 14 (CD14), is expressed in early spermatogonia and SSCs in pig testis (Park et al. 2019). GDNF family receptor α1 (Gfrα1) has been utilized as a surface marker in mice (Buageaw et al. 2005). It has also been expressed in a sub-population of gonocytes in neonatal porcine testes (Lee et al. 2013). Because of dynamic changes in their expression during germ cell development, transcription factors NANOG and POU5F1 (also known as OCT3/4), which are also related to stem cells' pluripotency, appeared to be conserved but are less reliable markers for undifferentiated spermatogonia from domestic species (Goel et al. 2008; Goel et al. 2010; Goel et al. 2011; Fujihara et al. 2011; Mahla et al. 2012). However, in immature buffalo testis, its expression is restricted to germ cells (Fig. 20.1f). Despite the availability of comprehensive knowledge on spermatogonial characterization, no specific marker for SSCs is currently available. As a result, understanding of biological markers for SSCs/progenitors in domestic species must be expanded.

20.4 SSC Transplantation

Dr. Ralph Brinster developed the spermatogonial transplantation technique in 1994. This technique allowed SSCs to be functionally identified. When transplanted into infertile recipient patient testes, SSCs colonize seminiferous tubules and reinitiate spermatogenesis resulting in the successful production of offspring (Brinster and Avarbock 1994). Later, they also reported that this technique might be used to quantitate SSC numbers when germ cell colonies develop from single SSCs (Zhang et al. 2003; Kanatsu-Shinohara et al. 2006b). Both in vitro and in vivo

retroviral/lentiviral/adenoviral vectors can infect SSCs to alter the rodent genome (Takehashi et al. 2007a; Hamra et al. 2005; Nagano et al. 2001a; Kanatsu-Shinohara et al. 2004). Despite this, the scope of genetic alterations and biochemical studies using SSCs is scanty because of the limited availability of culture methods. Conversely, the discovery of GDNF (glial cell line-derived neurotrophic factor) in the control of SSC self-renewal led to a long-term SSC culture method (Meng et al. 2000; Kanatsu-Shinohara et al. 2003a). Neonatal (pup) testicular cells form grape-like colonies when cultured on mouse embryonic fibroblasts (MEFs) with cytokines, including GDNF cells were designated as germline stem cells (GSCs). Following transplantation, they proliferated logarithmically for more than 2 years, reinitiating spermatogenesis and producing offspring cultures were used to generate transgenic and knockout (KO) mice by in vitro pharmacological selection of genetically modified GSCs (Kanatsu-Shinohara et al. 2005b). With the development of spermatogonial transplantation and GSC culture procedures, genetic manipulation and clonal selection to create children became conceivable.

Transplanted SSC efficiently repopulate seminiferous tubules of recipient sterile male mice, which reinitiate spermatogenesis into the testes. The progeny of these males inherits the donor cells genetic properties. The isolation and expansion of SSC in mice can be done for an indefinite period without losing the stem cell potential and no signs of genetic drift (Shinohara et al. 2000a; Shinohara and Brinster 2000; Shinohara et al. 2000b; Kanatsu-Shinohara et al. 2003a; Nagano et al. 2003; Kubota et al. 2004b; Hamra et al. 2005; Kanatsu-Shinohara et al. 2005a; Oatley and Brinster 2006; Hamra et al. 2008; Oatley and Brinster 2008; Oatley et al. 2010). SSC also serves as one of the alternative approaches for transgenic mice's generation as efficient genetic manipulation can be performed by transfection and transduction (Kanatsu-Shinohara et al. 2004; Hamra et al. 2005; Kanatsu-Shinohara et al. 2006a; Kanatsu-Shinohara and Shinohara 2007; Takehashi et al. 2010; Kanatsu-Shinohara et al. 2011). Mouse SSC can transform into pluripotent cells by providing a suitable culture condition without genetic manipulation (Guan et al. 2006; Izadyar et al. 2008; Golestaneh et al. 2009; Mizrak et al. 2010). These pluripotent cells (GPS), which are germline-derived, acquire an expression profile similar to ES cells and are functionally indistinguishable from ES cells (Silva et al. 2009). Apart from the capacity to create teratomas, the GPS differentiates into three germ layers in vitro and contributes to the development of all tissues in mice derived from chimeric blastocysts (Takehashi et al. 2007b; Izadyar et al. 2008). In any scenario, SSCs provide a very sound basis for stem cell and developmental research, including transgenic technologies and stem cell-based cell therapy in mice.

However, till now, these techniques are restricted to use in small species such as rodents. Nevertheless, in the end, we need the successful application of this technology in large animals for modelling human diseases. The transgenic animal models for preclinical research could be generated using the SSC directly. GSCs would allow researchers to avoid both the ethical problems associated with ES cells and the risk of genetic abnormalities caused by multi-gene insertion techniques to develop induced pluripotent stem (iPS) cells. Other techniques, such as somatic cell nuclear transfer (SCNT) technology and DNA microinjection, have proven difficult and

ineffective in producing transgenic models in big animals. Through multiple lines of canine (Hayes et al. 2008; Wilcox et al. 2009) and other large animals (Kumar De et al. 2011; Vassiliev et al. 2011; Kim et al. 2012; Goel et al. 2009) ES cells have been reported; however, all non-rodent lines show genetic instability and loss of pluripotency over time (Yang et al. 2010a; Gerwe et al. 2011). The establishment of germline transmission and the creation of transgenic large-animal models from ES have primarily proven unsuccessful. Recently, chimeric pigs with diverse genetic constitutions have been created (West et al. 2010; Xu et al. 2019) by implanting iPS into early embryos, but this method has yet to prove successful in other big mammals. Several transgenic dogs (Hong et al. 2009; Hong et al. 2011) and other large-animal models (An et al. 2012; Giraldo et al. 2012; Jung et al. 2013) have been generated using SCNT, but this approach has been highly labour, cost, and animal intensive so far. Several publications have documented the isolation and short-term culture of SSC from large animals (Kim et al. 2006; Rodriguez-Sosa et al. 2006; Goel et al. 2007; Hermann et al. 2007; Aponte et al. 2008) and humans (Wu et al. 2009), as well as the conversion of these cells into pluripotent GSC cells (Golestaneh et al. 2009). Several authors reported about identification, isolation, and short-term culture of buffalo SSC (Goel et al. 2010; Kala et al. 2012; Ahmad et al. 2013; Rafeeqi and Kaul 2013; Yu et al. 2014; Li et al. 2017; Sharma et al. 2019a; Li et al. 2020; Kadam et al. 2013; Sharma et al. 2019b). The stem cell potential of SSCs isolated from prepubertal buffalo testis was determined by transplantation in xenogeneic (Mahla et al. 2012) and homologous (Sharma et al. 2020) recipients. Researchers succeeded in the successful SSC transplantation and subsequent donor sperm production in a wide variety of species like pigs, sheep, bulls, goats, monkeys, and dogs (Izadyar et al. 2003; Kim et al. 2008; Herrid et al. 2011; Jahnukainen et al. 2011; Hermann et al. 2012; Zeng et al. 2012; Honaramooz et al. 2002a). Transplantation of transfected enhanced buffalo spermatogonial stem cells to homologous recipients was recently reported to be effective, although no donor-derived spermatogenesis was seen (Sharma et al. 2020). More notably, SSC transplants in both goats (Honaramooz et al. 2003) and sheep (Herrid et al. 2009) and have led to the birth of donor-derived offspring through normal mating, confirming that this technology can also find application in buffaloes for germplasm conservation and genetic modification (Fig. 20.2).

20.5 Cryopreservation of SSCs

For the cryopreservation of SSCs, there are different opinions on whether to freeze as a testicular tissue, an enriched cell suspension, or testicular cell suspension. Testicular cell suspensions comprise various cell types differing in shape, size, and water content, necessitating distinct freezing conditions and media compositions for the functionality and viability of cells with maximum presence. The type of cryoprotectant, its concentration, and the chilling pace might all impact cell survival. The SSCs must survive the freezing procedure and remain functional in order to be transplanted. As a result, techniques for preserving spermatogonia in liquid nitrogen

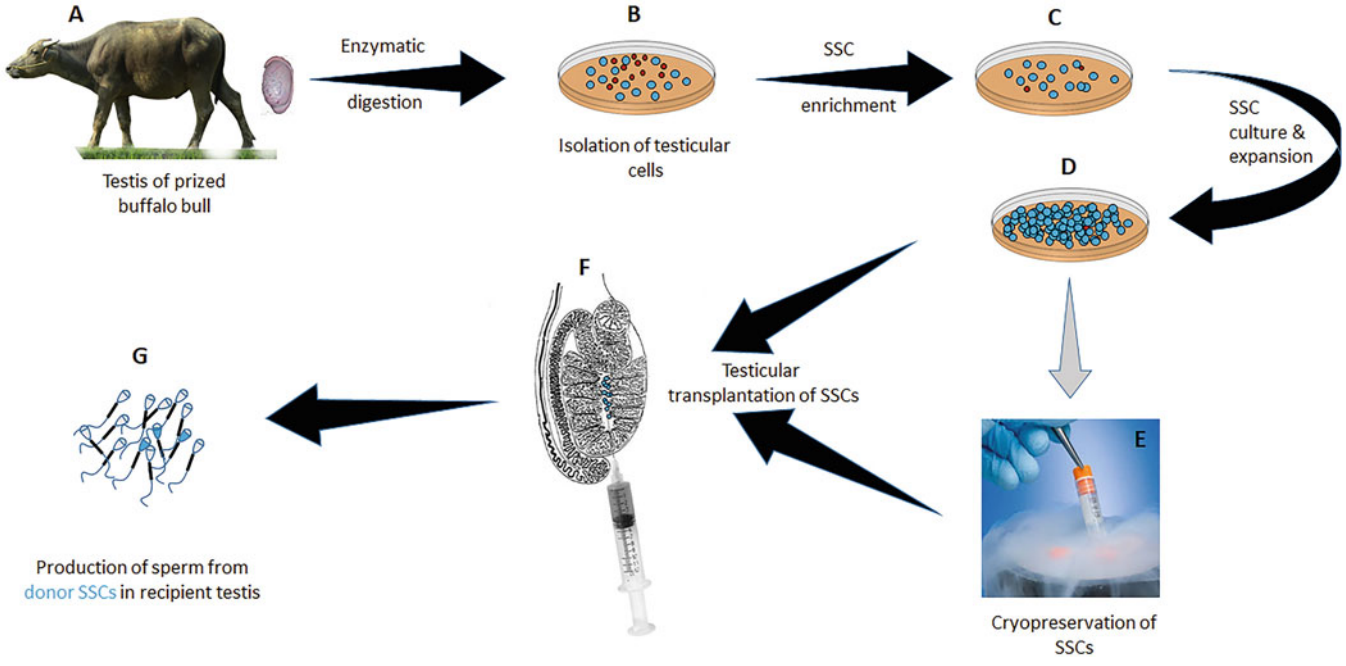

Fig. 20.2 A schematic representation of SSC transplantation in buffalo following enrichment and culture. (**a** and **b**) The testis is collected from the prized buffalo bull, which dies before attaining sexual maturity and is enzymatically digested to get a single-cell suspension of testicular cells. (**c**) SSCs (blue circles) are enriched by different in vitro techniques such as differential plating or density-gradient centrifugation. (**d** and **e**) The isolated SSCs can be expanded in vitro by culturing in a suitable culture medium and then cryopreserved for long-term storage in liquid nitrogen. (**f**) Either freshly cultured or cryopreserved SSCs are used for testicular transplantation. The SSCs are transplanted into the buffalo testis by injecting through the rete testis. SSCs can survive and colonize the seminiferous tubules of the recipient testis and initiated donor-derived spermatogenesis. (**g**) The ejaculated semen contains spermatozoa from the donor SSCs and can be used for insemination to produce offspring from the prized bull.

for an extended period, comparable to those protocols commonly used for somatic cells, have been devised (Avarbock et al. 1996; Izadyar et al. 2002; Frederickx et al. 2004; Sa et al. 2012). Following transplantation, SSCs thawed after 14-year storage in liquid nitrogen retain the ability to regenerate spermatogenesis, and the spermatozoa recovered produced normal offspring by micro insemination (Wu et al. 2012). For individual males who want to provide an immortal life span for their germ cells, cryopreservation of SSC-containing cell populations is the best approach because SSCs can self-renew and expand in number.

Although cryopreservation of semen is used to preserve the germline of certain biologically, scientifically, or economically valuable males, including endangered animal species or livestock breeds, semen cryopreservation is limited by the number of spermatozoa in the sample and the lack of availability of semen cryopreservation protocols for each species. On the contrary, cryopreservation methods for SSCs of several mammalian species are identical and almost identical to the procedures used for somatic cells (Avarbock et al. 1996; Dobrinski et al. 1999, 2000; Nagano et al. 2001b; Nagano et al. 2002; Hermann et al. 2007). Frozen-thawed bovine spermatogonia colonized the mouse testis, although with lower efficiency than the fresh population. We also demonstrated that cryopreserved spermatogonia from an endangered bovid testis could colonize the mouse testis (Goel et al. 2011). To date, cryopreservation of buffalo SSCs remains unreported.

Cryopreserving SSCs is more suited for preserving male germlines and the sole approach applicable for prepubertal males and those species where specialized procedures for cryopreservation of spermatozoa have not been established. Conversely, cryopreserving single-cell suspensions has been proposed as a sensible solution to bypass the increased heat and mass exchange hindrance combated during tissue cryopreservation (Karlsson and Toner 1996). During SSC cryopreservation, the two-step enzymatic digestion procedure required for testicular cell isolation exposes single cells directly to collagenase, and trypsin increases the membrane sensitivity to physicochemical changes (e.g. solution effects, osmolarity, and ice nucleation), thereby resulting in apoptotic cell death (Brook et al. 2001; Karlsson and Toner 1996). Therefore, the cryopreservation of testicular tissue is a better option (Onofre et al. 2020). Testicular tissue freezing preserves the cell–cell contacts but requires more permeable cryoprotectants. Therefore, much focus has been diverted to cryopreservation of testicular tissues than the SSCs.

20.6 Cryopreservation of Testis

20.6.1 Testicular Tissue Cryopreservation

Testicular tissue cryopreservation is a technique that can facilitate the preservation of reproductive potential in different animal species. The testes development stage is vital in cryopreservation; for example, cryopreservation of immature testis varies from cryopreservation of adult testis in tissue texture, and retaining its future developmental potential must be considered. In immature animals, as immediate

transplantation of testis tissue is not always possible, testis cryopreservation provides an opportunity to preserve the germplasm for future use. Therefore, cryopreservation of immature testicular tissue that maintains its potential to produce sperm is desirable. Till now, immature testicular tissues of non-human primates (Jahnukainen et al. 2007; Schlatt et al. 2002), human (Keros et al. 2005; Keros et al. 2007; Baert et al. 2013; Wyns et al. 2008; Unni et al. 2012; Ginsberg et al. 2014; Poels et al. 2014), goat (Honaramooz et al. 2002b), porcine (Yang et al. 2010b; Zeng et al. 2009), rodents (Curaba et al. 2011; Gouk et al. 2011; Milazzo et al. 2010; Milazzo et al. 2008; Travers et al. 2011), sheep (Pukazhenthi et al. 2015) are cryopreserved. Recently our lab reported the successful cryopreservation of immature buffalo (Devi et al. 2014) and Indian mouse-deer (Pothana et al. 2015) testicular tissues.

In an adult animal, sperms can be collected from live animals by electro-ejaculation, but it is impossible with dead animals. In the case of a dead adult animal, when cryopreservation of ejaculated or epididymal sperm is not possible, testis cryopreservation would be an option for germplasm preservation (Honaramooz 2012). Cryopreserved adult testicular tissue can be maintained for a prolonged period and can be used as a source of sperms after thawing (Ehmcke and Schlatt 2008). To date, feline (Mota et al. 2012; Thuwanut and Chatdarong 2012; Buarpung et al. 2013), bovine (Wu et al. 2014), jungle cat, rusa deer, fea's muntjac, and Sumatran serows (Thuwanut et al. 2013) adult testicular tissues are cryopreserved. Our lab also reported the preservation of adult testes of adult non-human primates (mandril, marmoset, and chimpanzee) (Pothana et al. 2016) and cervids (barking deer, sambar deer, and hog deer) (Pothana et al. 2017).

20.6.2 Retrieval of Gametes from Cryopreserved Adult Testis

Cryopreserved testicular tissue from adult animals can be kept indefinitely and utilized as a source of sperm after thawing. So far, no reports of successful sperm extraction from cryopreserved testicular tissues in farm animals or monkey species have been published. The successful retrieval of sperm from cryopreserved-thawed cat testicular tissues was reported (Buarpung et al. 2013). Conversely, human testicular tissues have been cryopreserved following biopsies from non-obstructive azoospermic patients (Dafopoulos et al. 2005a, b), and testicular sperm was retrieved from the cryopreserved biopsies and was further used for fertilization (Zitzmann et al. 2006).

20.6.3 Production of Gametes from Immature Testis after Xenotransplantation

The tight capsule, complicated vascular architecture, and susceptibility to ischemia make the testis look unsuitable for transplantation or grafting at first appearance. Testis grafting has a long history as an experimental endeavour in reproductive biology (Goldstein et al. 1983; Johnson et al. 1996). In the 1950s, testis-tissue

grafting was created as a technique for androgen replacement (Deanesly 1954), and it was later used to understand the roles of Sertoli or Leydig cells and steroidogenesis (Johnson 1995). The application of testis xenografting implies the development of subsequent ART to produce offspring from the dead immature animals.

Fresh testicular tissues from an immature donor of many species have been grafted into immunodeficient mice. The grafted testis has shown successful completion of spermatogenesis in bulls (Oatley et al. 2004; Oatley et al. 2005; Rathi et al. 2005; Schmidt et al. 2006), monkeys (Jahnukainen et al. 2006; Honaramooz et al. 2004), cats (Snedaker et al. 2004), hamsters (Schlatt et al. 2002), dogs (Abrishami et al. 2010; Shirazi et al. 2014), goats (Honaramooz et al. 2002b), pigs (Honaramooz et al. 2002b; Nakai et al. 2010), rabbits (Shinohara et al. 2002), and buffalo (Reddy et al. 2012). When coupled with testis-tissue xenografting, testis cryopreservation can be a potent approach for conserving the germplasm of rare and endangered species (Pukazhenthi et al. 2006). Testicular tissues xenografted ectopically onto mice could survive with induction of spermatogenesis, but the germ cells could not differentiate beyond spermatocytes in monkeys (Jahnukainen et al. 2007; Poels et al. 2012), humans (Wyns et al. 2007; Wyns et al. 2008; Poels et al. 2013) and cats (Mota et al. 2012). However, in pigs (Kaneko et al. 2014; Kaneko et al. 2013; Honaramooz et al. 2002b) mice (Goossens et al. 2008; Shinohara et al. 2002; Goldstein et al. 1983), and rabbits, completion of spermatogenesis with the production of haploid spermatids is reported. Spermatozoa retrieved from cryopreserved immature testicular tissue have produced live offspring in pigs, (Kaneko et al. 2013; Kaneko et al. 2014), mice (Kanatsu-Shinohara et al. 2003b), and rabbits (Shinohara et al. 2002) after intracytoplasmic sperm injection. A similar outcome can be expected in buffalo following xenografting of cryopreserved testicular tissues collected from immature prized buffalo bull. The xenografted testis matures, and spermatogenesis is induced, leading to germ cell differentiation and production of haploid gametes. These gametes can be used for assisted reproduction for the generation of offspring (Fig. 20.3).

20.6.4 Applications in Assisted Reproduction Technology (ART)

ART offers a variety of methods for maintaining fertility in people and financially valuable or endangered animals. Testis, embryo, and oocyte cryopreservation are essential adjuncts to ART such as IVF, intracytoplasmic sperm injection (ICSI), and intrauterine insemination (IUI). Cryopreservation plays a vital role in fertility preservation. It has been decades since we started freezing sperm and embryos successfully and achieved pregnancies after transferring frozen-thawed sperm by IUI and embryos in the IVF program. Cryopreservation has enormous potential and can transform ART significantly. There are several births reported with cryopreserved testis and ovary through ART. At first, the successful pregnancies were reported in 1996, with cryopreserved testicular sperm followed by ICSI in humans (Gil-Salom et al. 1996). Live birth following cryopreservation of immature oocytes, thawing, and IVM, and then fertilization through ICSI took place (Cohen et al. 2005).

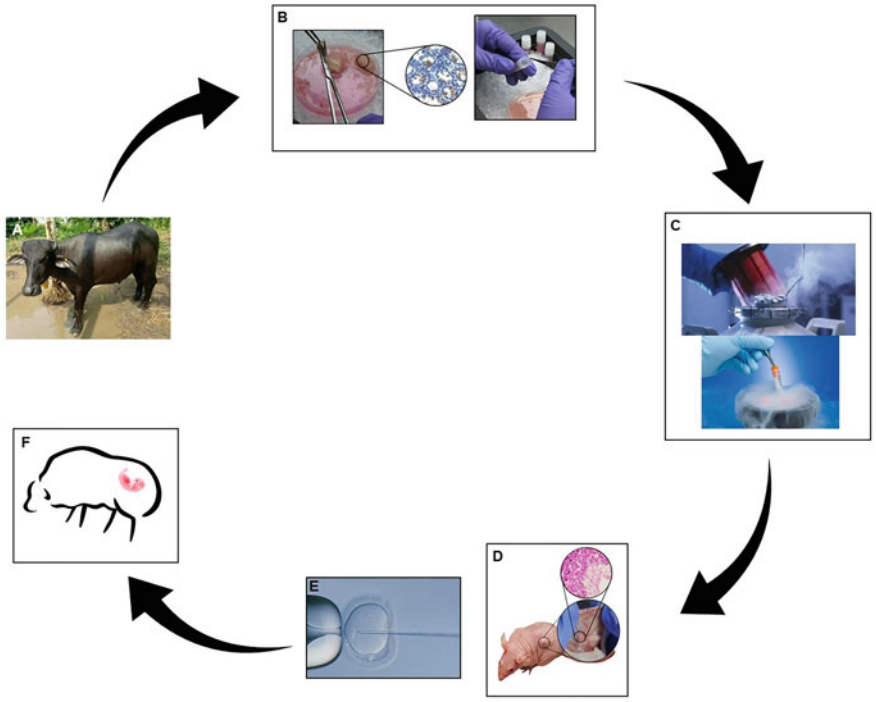

Fig. 20.3 A schematic representation of buffalo testis-tissue cryopreservation and xenografting to preserve the germplasm of prized male buffalo. (**a**) The testis is collected from a juvenile prized buffalo male. (**b**) The collected testis is cut to a desirable size for efficient cryopreservation, and testicular tissues are transferred to cryovial containing suitable cryomedia and subjected to cryopreservation. (**c**) The cryovials containing the cryopreserved testis tissues are then transferred to liquid nitrogen for long-term storage. (**d**) The thawed cryopreserved testicular tissues are xenografted to immunocompromised recipient mice to induce in vivo testicular maturation and germ cell development. (**e**) The haploid gametes retrieved from the xenografted testis are used to generate embryos by intracytoplasmic sperm injection. (**f**) Generated embryos are then transferred to a surrogate mother using the embryo transfer technique. If the transferred embryo is implanted and pregnancy is established, a live offspring can be delivered at the end of pregnancy containing contribution from the germplasm of cryopreserved testicular tissue

Recently, live birth has been reported with cryopreserved ovarian tissue, followed by autotransplantation in females suffering from malignant diseases (Dittrich et al. 2015; Tanbo et al. 2015).

In laboratory animals, cryopreserved mouse and rabbit immature testicular tissues, transplanted into recipient mice produced mature sperm and gave birth to live offspring through IVM and ICSI. In livestock, porcine offsprings were successfully generated utilizing sperm from immature testicular tissues after cryopreservation and xenografting onto nude mice. The piglets born from cryopreserved and xenografted testis maintained normal reproductive development. These findings

further encourage using these techniques to produce offspring from testis of genetically priced large animals that dies unexpectedly.

20.7 Conclusions and Future Prospective

The development of SSC-based reproductive technologies might help maintain the breeding potential of men who die before puberty. When a single individual's genetic contribution may significantly influence long-term survival, these reproductive technologies can benefit. Because SSCs are comparable to ES cells, they can be cultured and provide a platform for genetic modification that terminally differentiated spermatozoa. As a result, the emphasis has been on spermatogonial stem cell transplantation (SSCT) and testis xenografting to maximize the benefits of SSCs. The key criterion for the success of this approach is the evaluation of the viability and quality of cryopreserved gonadal tissue. Additional insights into cryoinjury avoidance aid in the development of enhanced cryopreservation techniques for fertility preservation.

Furthermore, focusing on graft behaviour will enhance follicle survival and create long-term in vitro culture systems. Problems concerning the possibility of male infertility and the identification of genetic abnormalities in embryos before transfer must be investigated. Although the success is meagre currently, strategies are still being developed to successfully preserve unfertilized mature oocytes, yielding a reasonable pregnancy rate. Further refinement of the techniques and modification of cryopreservation strategies will help develop germplasm preservation in several more animals.

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Somatic Cell Nuclear Transfer and its Applications in Buffalo (*Bubalus bubalis*)

21

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Abstract

Buffalo is a multipurpose domesticated animal in South-Eastern Asian countries. Since the domestication, several reproductive bio-techniques have been employed in buffalo to improve the production potential (milk and meat yield). Buffalo cloning (BC), one of the reproductive bio-techniques, allows the faster multiplication of elite germplasm and conservation of endangered breeds. The world's first cloned buffalo was produced in 2007, and later in 2009, the world's prestigious breed (Murrah) has been cloned. Two different BC methods are available to clone a buffalo. Several improvements such as selection of best donor cell types and oocytes, optimization of culture conditions and media, treatment of donor cells or embryos with epigenetic modulations have been done that results in improved blastocyst production rates (30–45%) and live birth rates (8–10%). At present, India is the only country that has reported more than 20 cloned buffaloes, which are alive and growing well. One of the noticeable achievements in BC is the production of seven clones from a single breeding bull. Recent studies reported that clones that had attended maturity have normal growth, life span, reproduction, and production. There has been debate on the use of cloned buffaloes, particularly breeding bulls, for the production of

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next-generation progeny. In this chapter, we discuss the journey, recent developments, and prospects of BC.

Keywords

Buffalo · Cloning · Murrah · Live birth rates · Breeding · Production

21.1 Introduction

Buffalo (*Bubalus bubalis*) is a large and robust domestic animal that provides milk, meat, and work power. For many decades, this species has been playing a significant role in the agricultural economies of several Asian countries. World's buffalo population is 208 million, of which 201 million (96.79%) are present in Asian countries, mainly in India, China, and Pakistan (Minervino et al. 2020). During the last 50 years (1968–2018), the global population of buffalo increased by 98%, whereas the cattle population increased by 40% (Minervino et al. 2020). Globally, the buffalo shares 15.14% of the total milk production, whereas cattle share 80.05% (FAOSTAT 2019). From 2011 to 2018, global buffalo milk production increased by 32%, whereas cattle milk production increased by 10% (Minervino et al. 2020). In Asian countries, buffalo (198 million) contributed 35.59% of the total milk production, whereas cattle (470 million, more than double than buffaloes) account for 59% of the total milk production (FAOSTAT 2019). Among the Asian countries, for example, in India, buffalo shares approximately 50% of the total milk, despite its population is half that of cattle (Minervino et al. 2020). Therefore, buffalo is reared as the main dairy animal, particularly in India and Pakistan.

In addition to milk production, buffalo has also been reared for meat production. The average weight of adult buffalo varies between 450 and 1000 kg, for example, the Jafarabadi buffalo is a heavyweight breed of buffalo (Minervino et al. 2020). As compared to cattle, buffalo have a higher body weight at birth, and then their growth is faster (Naveena and Kiran 2014). Under best husbandry practices, buffalo can achieve more than 1 kg body weight per day (Naveena and Kiran 2014). In 2019, global buffalo meat production was 4.2 million tonnes, of which 3.8 million tonnes (90%) has been contributed by Asian countries. From 2010 to 2019, global buffalo meat production increased by 7.6%, whereas, in Asia, its meat production increased by 11.7% (FAOSTAT 2019). With the humongous contribution, both for milk and meat, buffalo is considered the animal of pride in some countries such as India, Pakistan, and Italy. Despite playing a significant role in the agricultural economies, this species is much less acknowledged, received meager attention, and less scientific care. Also, limited attempts have been made to improve its production potentials through the application of advanced technologies including genomic chips. A major obstacle to implement any strategy for improving the production potential of this valued species is a lack of research studies and paucity of scientific data. Many times, due to the paucity of data, it has been falsely anticipated that scientific studies

Fig. 21.1 Seven clones produced from a single donor animal (left to right) and re-cloned calf (rightmost one)

and information available for cattle can be superlatively applicable to buffalo. Therefore, more research is needed to generate buffalo-specific information and data.

Remarkable improvement in the productivity of livestock is achieved through applications of assisted reproductive technologies (ARTs) such as artificial insemination (AI), embryo transfer (ET), ovum pick-up, and in vitro fertilization (OPU-IVF), and sexed semen. These techniques are aiming to increase the number of progeny of desired males and females and to reduce generation intervals. Animal cloning (AC), one of the ARTs, also allows the production of identical genotype animals of any desirable animal. AC is the only technology that transmits 100% genetics from parents (donor animals) to offspring (cloned copies); therefore, through the applications of AC, it is possible to place high selection intensity for genetic improvement (Faber et al. 2004). In addition to agricultural applications, AC has been efficiently used to produce transgenic/edited animals for the production of therapeutic drugs/proteins in milk, blood, and urine, production of model animals for research studies, including xenotransplantation (transplantation of organs from animals to humans). AC can also protect the sentiment and emotion of animal owners by restoring their lovable animals that died due to some reasons, for example, pet cloning, a very popular bull, and a high milk producer. It is often visualized that AC is a powerful tool to produce vibrant, strong, productive, and uniform animals.

In buffalo, AC is much less explored as compared to other domestic animals such as cattle and pigs (Drost 2007; Singh et al. 2009). The first successful birth of cloned buffalo was reported in 2007 (Shi et al. 2007), which was 10 years down to Dolly's birth (first cloned animal from differentiated somatic cells). At present four countries, namely India, China, Italy, and Thailand are working on buffalo cloning (BC) (Lagutina et al. 2011; Srirattana et al. 2014; Tasripoo et al. 2014; Sun et al. 2015; Selokar et al. 2019). Some of the remarkable achievements of BC are (1) birth of the world's first cloned buffalo (Shi et al. 2007); (2) birth of cloned buffaloes from semen-derived donor cells (frozen as well as fresh semen), the first report in any species (Selokar et al. 2014); (3) birth of a cloned buffalo from urine-derived donor cells (Madheshiya et al. 2015), the first report in any species; (4) production of cloned embryos from dead donor cells (Duah et al. 2016); (5) production of seven cloned copies (Fig. 21.1) from a single animal and successful re-cloning (Yadav et al. 2020); 6) successful cloning of distant animals, the birth of an Assamese cloned

buffalo at a dairy farm, which is 2000 km away from its natural habitat (Selokar et al. 2018a). These reports have witnessed the kinds of advances in BC research that will certainly open new avenues for wider and better applications. In India, our laboratories have been working on BC for more than 10 years. We are currently working to produce multiple copies of the elite stock of breeding bulls so that their semen can be used to compensate for the semen demand for AI and other ARTs, including OPU-IVF. In this chapter, we discuss the development and advances in BC and the roadmap to make use of this technology for buffalo breeding, particularly in India.

21.2 Journey of Buffalo Cloning

AC by somatic cell nuclear transfer (SCNT) is well-established ART in farm animals. On fifth July 1996, the cloned sheep named "Dolly" was born through an SCNT method (Wilmut et al. 1997). Over the years, several domestic, wild, and laboratory animal species have been successfully cloned. In textbooks, a method to produce cloned animals is described as a relatively simple method. In principle, one donor cell, which is usually grown from tissue explants, is transferred into the cytoplasm of an enucleated oocyte. Then, a united oocyte is signaled to start an embryonic division to reach the blastocyst stage of the embryo. The developed blastocyst is then transferred into a synchronized animal to carry the cloned pregnancy, and after the completion of the gestation period, a cloned calf is born which is genetically identical to an animal whose donor cell was used.

In comparison to other farm animals, very few studies have been performed to develop BC methods. In the late 1990s, attempts were made to produce cloned embryos by micromanipulators-based SCNT (mSCNT) in which early embryo cells (blastomeres) and fetal fibroblasts were injected into enucleated oocytes. However, generated embryos did not produce a cloned calf (Singla et al. 1997; Parnpai et al. 1999). In 2007, the first cloned swamp buffalo calf was successfully born (Shi et al. 2007). Shi et al. 2007 used mSCNT to clone the Chinese Swamp buffalo and produced three cloned calves, of which one survived and two died at a very early age. The birth of the world's first cloned buffalo has opened many research directives, including the exploration of SCNT methods to conserve and multiple buffalo breeds. At present, two different methods of SCNT are developed to clone a buffalo (Fig. 21.1). Shi et al. 2007 used mSCNT, which is technically similar to a method that was used to produce the famed sheep "the Dolly." For mSCNT, skilled manpower and sophisticated instruments (micromanipulators, needle grinders, and microforge) are required to perform micromanipulations such as enucleation, transfer of somatic cells into enucleated oocytes. This method of SCNT has been extensively used in many laboratories worldwide to clone domestic, laboratory, and wild animals.

Handmade cloning (HMC) was developed as an alternative to mSCNT (Vajta et al. 2001). In 2009, a significant achievement was made by Indian researchers in the field of buffalo cloning by producing the world's first cloned riverine buffalo

Table 21.1 Cloned buffaloes produced worldwide

Buffalo type	Donor cells type	Sex of donor	Blastocyst rate, %	Recipients for cloned embryos transfer, n	Pregnancy rate, n (%)	Live birth rate, n (%)	Survival status	Reference
Chinese swamp buffalo	Fetal skin fibroblasts	Female	23	16	3 (18.8)	2 (12.5)	One calf survived while the other died after 20 min of birth	Shi et al. (2007)
	Granulosa cells	Female	21	5	1 (20.0)	1 (20.0)	Died after 14 days	
Chinese riverine buffalo	Ear skin fibroblasts	Female	10	15	5 (20.0)	1(6.66)	Survived, normal	Yang et al. (2010)
Thailand swamp buffalo	Adult skin fibroblasts	Male	25	12	6 (50.0)	1(8.33)	Survived, normal	Tasripoo et al. (2014)
Indian Murrah buffalo (riverine)	Fetal skin fibroblasts	Female	24	6	1 (16.66)	1(16.66)	Died after 5 days	Shah et al. (2009)
	Fetal skin fibroblasts	Female	33	9	2 (22.23)	1(11.11)	Died after two years due to heart failure	
	Embryonic stem cells	Female	27	6	2 (33.33)	1(16.66)	Survived and produced progeny after insemination	George et al. (2011)
	Fetal skin fibroblasts	Male	41	4	1 (24.2)	1(24.2)	Died shortly after birth	Saha et al., (2012)
	Newborn skin fibroblasts	Male	40	9	1 (11.1)	1 (11.1)	Survived and produced semen	
	Fetal skin fibroblasts	Male	38	5	1 (20)	1(20)	Died after 4 hours	Panda et al. (2012)
	Fresh semen-derived somatic cells	Male	48	12	1(8.0)	1(8.0)	Survived and producing semen, and progeny through AI	Selokar et al. (2014, 2018a, 2019)

(continued)

Table 21.1 (continued)

Buffalo type	Donor cells type	Sex of donor	Blastocyst rate, %	Recipients for cloned embryos transfer, n	Pregnancy rate, n (%)	Live birth rate, n (%)	Survival status	Reference
	Frozen-thawed semen-derived somatic cells	Male	51	10	1 (10)	1(10)	Died 12 h after birth	
	Adult skin fibroblasts	Male	40	8	2 (25)	1(12.5)	Survived and produced semen, progeny, through AI	
	Adult skin fibroblasts (Assamese buffalo)	Male	25	12	1 (8.5)	1 (8.5)	Survived and producing semen	
	Frozen-thawed semen-derived somatic cells	Male	47	7	1(14)	1(14)	Survived and producing semen	Raja et al. (2019)
	Adult skin fibroblasts[a]	Male	Seven clones from a single breeding bull and a re-cloned calf of a cloned bull				All are surviving well	Yadav et al. (2020)
	Adult skin fibroblasts	Male	45	29	8 (27)	6 (20)	Only three are surviving well	Shyam et al. (2020a, b)
	Urine-derived cells	Female	50	5	1 (20)	1(20)	Surviving	Madheshiya et al. (2015)
	Adult skin fibroblasts	Female	50	4	1 (25)	1(25)	Died after 21 days	Saini et al. (2016)
Overall efficiency, irrespective of conditions			20–50	175	39 (22.22)	24 (13.71)	7 survived and 8 died	

[a]The data of this study is not available; therefore, it has been excluded for calculation of overall efficiency. Over 10 years, several modifications have been made to improve the success rate of buffalo cloning methods. Very recently, we reported the birth of multiple clones from a single donor animal (Yadav et al. 2020; Shyam et al. 2020a). In Table 21.1, we summarize the studies that reported the births of cloned buffaloes. According to our knowledge, more than 20 live buffalo clones have been produced using HMC methods, whereas less than 5 clones were reported using mSCNT methods (Selokar et al. 2018b). The comparison between HMC and mSCNT is presented in Table 21.2.

This table is adopted from Selokar et al. 2018b, and updated with recent data

(Shah et al. 2009). Shah et al. (2009) used HMC to clone Murrah buffalo, the most famous buffalo breed for high milk production. HMC is a micromanipulators-free method of SCNT. To perform the HMC, there is no requirement of expensive micromanipulators and skilled manpower, and several farm animal species such as cow, pig, horse, sheep, goat, and buffalo have been successfully cloned by using HMC methods (Vajta et al. 2005; Du et al. 2007; Zhang et al. 2013; Selokar et al. 2019). Therefore, it has been envisioned that HMC has the potential to replace mSCNT and would be the best choice for those laboratories that have limitations of funds and skilled manpower. Despite several advantages of HMC, there are some issues such as the use of two oocytes per reconstructed embryo increases the requirement of a large number of oocytes and mitochondrial genome likely to be coming from three sources that may lead to heteroplasmy (Fig. 21.2) (Vajta 2007).

21.3 Attempts to Improve the Cloning Success

All protocols of BC have been suffering from a low success rate. Selokar et al. 2018b reviewed that buffalo has approximately 2.5% overall cloning efficiency, which was calculated as a percentage of births following the transfer of cloned embryos. To have successful live births, it is essential to optimize every step of cloning, from oocyte isolation to the transfer of produced embryos into recipient animals to successful calving. Factors affecting the success of BC have been thoroughly discussed by us (see, review by Selokar et al. 2018b). There have been continuous efforts to improve live birth rates. Some of the major steps are (1) appropriate selection of donor cell types (Shi et al. 2007; Shah et al. 2009; Selokar et al. 2014; Madheshiya et al. 2015; Mohapatra et al. 2015a; Jyotsana et al. 2016); (2) selection of quality oocytes (Lu et al. 2011; Mohapatra et al. 2015b; Raja et al. 2019), (3) selection of most appropriate culture surface and media (Shah et al. 2008; Saini et al. 2015), (4) harmony of donor cells and oocyte growth cycle (Saikhun et al. 2004; Shi et al. 2007; Selokar et al. 2012), (5) correction of epigenetic modifications (Panda et al. 2012; Luo et al. 2013; Srirattana et al. 2014; Sun et al. 2015; Saini et al. 2016, 2017; Agrawal et al. 2018), (6) treatment of pathway modulators and miRNAs (Rashmi et al. 2019; Sah et al. 2020). The incorporation of these steps in BC methods has resulted in significant improvements in terms of blastocyst production rates, which is similar to other farm animals; however, the transfer of produced blastocysts into recipients could not achieve more births. Also, few laboratories have been working on BC, resulting in a much fewer number of buffalo clones compared to cattle and pigs.

All SCNT methods are considered inefficient and heavy losses from gestation to adulthood have been reported. It has been reported that clones have abnormalities in the placenta, organs, and muscular growth (Hill 2013). Clones have a heavy birth weight and abnormal postnatal growth, this condition is called large offspring syndrome. It has been anticipated that abnormalities in cloned animals are due to inappropriate genomic reprogramming of differentiated somatic cells by oocyte factors, leading to aberrant epigenetic marks and abnormal expression of

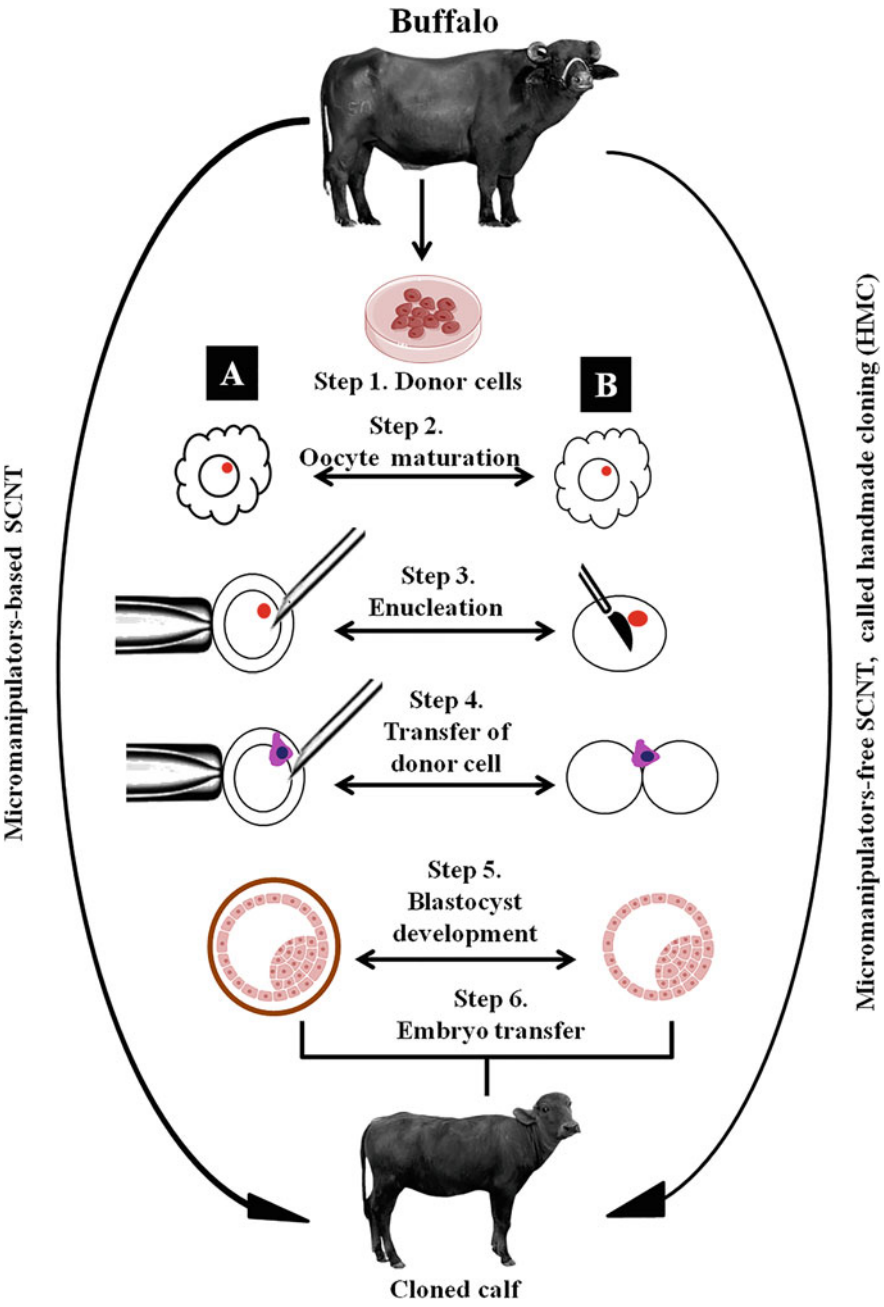

Fig. 21.2 Methods of buffalo cloning: Two methods have been used to clone a buffalo, (A) micromanipulators-based SCNT (mSCNT) and (B) micromanipulators-free SCNT called handmade cloning (HMC). To initiate the cloning, donor cells, preferably skin-derived fibroblasts need to be established in the laboratory (step 1) and be cryopreserved in sufficient numbers for regular use and long-term storage. The immature oocytes, mostly from slaughterhouse ovaries, are

developmental and imprinted genes. In contrast to other farm animals, very few studies have been performed in buffalo to correct the epigenetic abnormalities. In our previous studies, we reported that cloned embryos have abnormal methylation, histone modification, and gene expression as compared to IVF-derived embryos (Saini et al. 2016; Ashok et al. 2018; Sood et al. 2019). For correcting these abnormalities, we treated donor cells or electro-fused embryos or both with a combination of two epigenetic modifiers (50 nM TSA + 7.5 nM 5-aza-dC). We found that irrespective of treatments, the apoptotic index and epigenetic status of four histone markers (H3K9/14ac, H4K5ac, H3K18ac, and H3K27me3) in cloned blastocysts were similar to IVF-derived blastocysts. However, very few attempts have been made to transfer epigenetic modulator-treated embryos to determine their in vivo developmental competence; therefore, further attempts are needed to examine whether improved embryo quality achieves through epigenetic modulators treatment would succeed to live births. Also, extensive research needs to be continued to understand the cause of abnormal epigenetic in cloned embryos.

Recently, Sood et al. 2019 generated global transcriptomics data of cloned and IVF-derived blastocysts. It has been found that transcripts related to embryonic development, including pluripotency and epigenetics, were upregulated in cloned blastocysts as compared to IVF-derived blastocysts. Among the developmental pathways, the WNT signaling pathway was most affected, and many WNT signaling genes were upregulated and its inhibitor genes, particularly Dickkopf-1, were downregulated. Based on this study, later, Shyam et al. 2020b investigated the effects of an inhibitor of the canonical WNT signaling pathway (Dickkopf-1, DKK1) and colony-stimulating factor-2 (CSF2) on the production of cloned

Fig. 21.2 (continued) in vitro cultured for maturation (step 2), in both methods, 20–22 hrs of maturation is performed. Following the maturation, enucleation (removal of genetic material (DNA) from oocytes, step 3) can be performed either through the use of sophisticated micromanipulators under an inverted microscope (mSCNT approach) or a simple cutting blade under a stereomicroscope (HMC approach). The enucleation method of HMC is simple, reproducible, does not require skilled manpower. In HMC, the removal of zona pellucida is indispensable for enucleation, hence also called the zona-free method of SCNT, whereas mSCNT is performed without removal of zona pellucida. Before the transfer of donor cells into enucleated oocytes, it is suggested to synchronize donor cells at the G1 stage of the cell cycle. In mSCNT, one donor cell is transferred into the perivitelline space of an oocyte, and then, electro-fused with the help of an electric current (step 4). Whereas in HMC, one somatic cell is glued with one enucleated oocyte, and then, electro-fused with another oocyte in such a way that the glued donor cell is sandwiched between two oocytes (see right side of step 4). After electrofusion, generated embryos are chemically activated and in vitro cultured for 7 to 8 days. Activation protocol, culture conditions, and media can be similar for both methods. Since HMC embryos are zona-free, it is highly recommended to avoid a group culture. For culturing of HMC zona-free embryos, the Well-of-the-Well (WOW) culture and 4-well culture system have been developed (see previous publications, Vajta et al. (2005) and Shah et al. 2009). The developed blastocysts are non-surgically transferred (step 6) into recipient animals (one or two blastocysts per animal) and after completion of the gestation period (in buffalo, 310 to 320 days), a calf is born which is genetically identical to an animal whose somatic cells are used as nuclear donors

embryos. They found that treatment of these signaling modulators has significantly improved the blastocyst production rates and embryo quality (apoptotic index and ICM/TE ratio), similar to IVF-derived blastocysts. It has also been reported that transfer blastocysts, which were produced from DKK1- and DKK1+CSF2-treated embryos, resulted in high pregnancy rates (25 to 30%), which is the highest reported in the buffalo (Shyam et al. 2020a, b). The results of this study are highly encouraging and suggested treating cloned embryos with DKK1 for achieving more pregnancies. Further attempts are required to understand the exact mechanism of how DKK1 improves the pregnancy rate.

21.4 Growth, Health, and Fertility of Clones

The death of the Dolly, the first cloned sheep, at an early age, has been the main reason for concern and worry about the growth, health, and fertility of produced clones across the species. In buffalo, after the birth of the first cloned calf in 2007, more attention has been diverted to produce more calves. Several studies reported the establishment of cloned pregnancies; however, very few have reported live births (reviewed by Selokar et al. 2018b). In our observation, we notice that cloned pregnancies often continue with a long gestation period as compared to AI-based pregnancies. This could be one of the reasons for fatal deformities and heavy birth weight (clones have 15 to 20% more birth weight compared to AI-based calves). In some cases, induced parturition is needed to deliver a calf. Similar observations have been reported in cattle (Heyman et al. 2002). So far, we have produced 40 cloned buffaloes, of which 20 clones are surviving at different age groups, from 3 months to 11 years old. We observed that if a cloned calf has any abnormality, then it died before 6 months of their age. Died clones may have a wide range of complications such as enlarged organs, enlarged umbilicus, respiratory failure, lameness, and infection in the digestive tract. We anticipated that abnormal gene expression, high apoptosis, placental deformities, and irregular fetal-maternal interchange, and management of recipients could be reasons for heavy embryonic losses and postnatal deaths. Therefore, more studies are required to unravel the cause of abnormalities in clones.

In a recent study, we examined the semen parameters and fertility of three cloned bulls (Saini et al. 2020). This study reported that semen volume, mass motility, post-thaw motility, membrane integrity, estrus mucus migration distance, and computer-assisted semen analysis (CASA) parameters were with the normal ranges that are reported for buffalo. Also, bulls' fertility tested according to IVF and AI was found similar to non-cloned bulls. Semen of one cloned bull has been extensively used to produce progeny, at the time of writing of this chapter, more than 50 progeny have been produced following AI and its conception rate is around 50%, which is considered to be good enough in buffalo species (unpublished data). At present, in India, 5 cloned bulls are producing semen, and 13 clones are expecting to start semen production from December 2021. Similarly, female cloned buffaloes ($n = 3$) have been delivered progeny through conventional breeding (AI was done to conceive

Table 21.2 Comparisons between micromanipulators-based SCNT (mSCNT) and handmade cloning (HMC)

Condition	mSCNT	HMC
Use of micromanipulator	Yes	No
Zona-free	No	Yes
Manual enucleation	No	Yes
Activation and culture methods	Similar	Similar
Problems associated with zona hatching	Yes	No
Problems associated with mitochondrial heteroplasmy	No	Yes
Comparative cell number in produced blastocysts	Less	High
Problems associated with genomic reprogramming	Yes	Yes
Skilled manpower to perform experiments	Yes	No
Involved cost	High	Less

This table is adopted from Saini and Selokar (2018)

them). There has been concern regarding the life expectancy of clones, in buffalo, we observed that clones that attained more than one year of age are survived well and are expected to complete their normal life span. For example, one cloned female buffalo, which was born in 2010, had her first calving at 28 months of age and completed six calvings, and all born calves are normal, healthy, and growing well (unpublished data). In Table 21.3, we show the expected lifespan of cloned animal species, including buffalo. According to the best of our information, the highest age of male and female cloned buffalo is 11 years; both clones are rearing at National Dairy Research Institute, Karnal, India (Fig. 21.3). Since, limited data is available on the life expectancy of buffalo clones and their performance, more efforts are to be ascertained whether clones have the normal lifespan, productivity as their donors and other conventional produced animals.

21.5 Application of Buffalo Cloning, Special Reference to Bull Cloning for India

Research studies suggested that river buffalo is firstly domesticated in north-western India (approximately 6300 years BP) and then migrated to other countries, including Pakistan and Italy (Kumar et al. 2007; Nagarajan et al. 2015). There are two types of buffalo (river type has 50 chromosomes and swamp type has 48 chromosomes). India is ranked first in the global buffalo population and owns nearly 70% of the river buffalo population. River buffalo has been mainly reared for milk production. Indian buffalo contribute nearly 70% of the global buffalo milk production, whereas, in India, they contribute approximately 50% of the total milk production (Minervino et al. 2020). In addition to milk, India has an emerging industry of buffalo meat. According to India's Agricultural and Processed Food Products Export Development Authority (APEDA) report, in 2019, India exported buffalo meat and its product worth INR 22668.47 Crores/3175.09 USD Millions, which accounts for

Table 21.3 Life expectancy of cloned animal species

Species (breed)	Average life span of animal (in years)	The reported life span of cloned animals (in years)
Mouse (C57/BL6)	3	3
Cattle (Jersey)	15	14
Buffalo (Murrah)[a]	12–15	11
Goat (dairy)	15	> 15
Sheep (Finn Dorset)	10	> 9
Dog (afghan hound)	12	>10
Cat	15	11

[a]As per our information, 11 years is the highest age of a cloned buffalo, which is surviving well and expected to complete a normal life span. This aged cloned buffalo is rearing at the National Dairy Research Institute, Karnal, India.
The table is adopted and modified from Burgstaller and Brem 2017

Fig. 21.3 Cloned bull, produced in 2010, is now 11 years old with good health and performance. This indicates buffalo clones that attained maturity have a normal life expectancy

89% of exported animal products (http://apeda.gov.in/apedawebsite/six_head_product/animal.htm, accessed on 8 June 2021). From 2007 to 2019, India's buffalo population increased by 4.36%, whereas the cattle population decreased by 3.3%

(livestock census report 2019, available at the http://dahd.nic.in). The increasing trends of buffalo population indicate that Indian farmers prefer buffalo over cattle. Hence, buffalo is a prime animal for the growth of the Indian milk and meat industry.

Indian researchers have been working to improve the productivity of buffalo through scientific and technological developments in management practices, balanced nutrition, genetic breeding tools, manipulation of reproduction, and the use of biotechnologies. For example, in Haryana, the homeland state of the famed Murrah breed, buffalo produce an average of 9.19 kg of milk per day which is higher than that of the national average (6.34 kg/day). To upgrade the genetic potential of low milk producers and non-descript buffaloes, India an government has proposed increasing the coverage of AI from the current 30% to 70–80% by the end of 2025. Also, non-descript buffalo to be bred with the semen of Murrah bull (Annual report 2019–2020 of Department of Animal Husbandry, Dairying and Fisheries, Government of India, http://dahd.nic.in). Therefore, there is a huge requirement of semen from high genetic merit bulls for breeding (as of 2020, India has 55 million breedable female buffaloes). A recent livestock census-2019 alarmed that the population of males had significantly declined, from 2012 to 2019; males are decreased by 42%. If the male population drop continues, then, there will be storage of genetic pool for selection of breeding bulls. Therefore, there is a need to protect the population of males for maintaining diversity in males. AI and recently OPU-IVF are used to produce bulls. BC, which is well established in the country, can also be used to produce copies of bulls, preferably proven bulls, to compensate the burden of bull production on other techniques. For more than 10 years, India's premier research organization, the Indian Council of Agricultural Research (ICAR) has been utilizing BC technology to produce clones of elite buffaloes. Since the birth of India's first cloned buffalo in 2009, significant advances have been made to improve the success rate of BC. At present, India has 15 cloned bulls, of which five are under semen production, and 4 cloned female buffaloes, of which three had delivered calves. All clones belong to the Murrah breed, except one that belongs to the Assamese buffalo. According to our observations, male clones that attained maturity have produced semen, and their semen parameters are similar to non-cloned bulls. Also, our groups have been reported births of calves following AI using semen of cloned bulls (Selokar et al. 2019; Saini et al. 2020).

Some of the BC applications are: 1) multiplying elite stock: faster dissemination of genetic gain could be achieved through the use of genetics of cloned animals, especially proven bulls. Cloned bulls can produce extra semen to meet national and international demand for AI. It is a wise decision to cryopreserve of somatic cells of elite genetic animals, it might be very useful to restore the genetic of precious animals in case of death due to any means. Thus, we established the first somatic cell bio-bank, particularly fibroblasts from skin biopsies, and the bio-banked cells have been made available to researchers (Selokar et al. 2018c; Dua et al. 2021). This will save money, time, and resources needed to establish somatic cells. 2) Conservation of endangered breeds: BC can be used to conserve traditional breeds that are well adapted to local environments. For example, Chilika buffalo (a heritage animal of Odisha, one of the northeastern states of India) spends the night grazing on weeds

and vegetation in salty waters of Chilika Lake and the morning they usually return to their owners' house. Thus, this heritage buffalo breed is named "the night queen of Chilika." It has been roughly estimated that around 25,000 buffaloes are left, and crossbreeding with other buffalo breeds has been posing an extra threat to extinct this breed. Along with other conservation methods, BC can help to protect the population of this precious breed. Also, attempts should be made to cryopreserve somatic cells of such breeds for future reintroduction of their genetic pool back to the live population. 3) Production of transgenic buffaloes: AC has been used to produce various model animals, including transgenic buffaloes. There are wide ranges of opportunities to genetically modify, including the use of CRISPR, for both agricultural and biomedical applications. There are not many reports available for transgenic buffalo. Therefore, more research efforts along with generous funding are needed toward the generation of transgenic/edited buffalo for various agricultural and biomedical applications.

Despite multiple applications, the major challenges for widespread use of this technology are: 1) many scientific communities are reluctant to accept that product of cloned animals safe for human consumption and there is no need for labeling (despite international regulatory approvals), 2) low success rate, only 8–10% of transferred embryos resulted to live healthily births, productive animals, 3) high cost of animal production, 4) in India, only two research laboratories are working on BC and very few skilled expertise available. Therefore, there is a need to recruit more laboratories to initiate work on BC at a large scale that will create more trained manpower, improve the success rate, and reduce the cost of technology.

Indian researchers are working on a mega project to produce cloned copies of elite breeding bulls. To access the impact of bull cloning, for example, 100 cloned bulls can produce more than 120 million semen doses that can give births to 48 million buffalo calves (2.5 inseminations per calf). In India, the population of low milk producers and non-descript buffalo is humongous, for example, non-descript buffalo contributed 14% of the buffalo milk production. To upgrade the population of non-descript buffaloes, the large-scale use of semen from high genetic merit bulls through AI has been adopted. However, in some states, the coverage of AI is much lesser than that of the national average (30% coverage of AI). For example, Rajasthan (13.7 million) and Madhya Pradesh (10.3 million) have a huge buffalo population, but AI coverage is around 15%. Also, the population of the non-descript population is high. In such states, semen of cloned can promptly be used. While using the semen of cloned bulls, care should be taken to protect diversity. We suggest avoiding the use of cloned bull semen in those locations in which semen of donor bull(s) has been extensively used. Also, breeding through the semen of one cloned bull should not be continued beyond three years in the same locations. These steps can address the inbreeding issue and protect diversity. In Fig. 21.4, we present the impact of the use of cloned bulls for breeding.

For use of cloned bulls for breeding, there are two major requirements, (1) technology must be matured well and repressible, over the 10 years, BC has been regularly used to produce clones. Recently, seven cloned copies of the single bull have been produced, and these cloned copies are healthy and growing well (Yadav

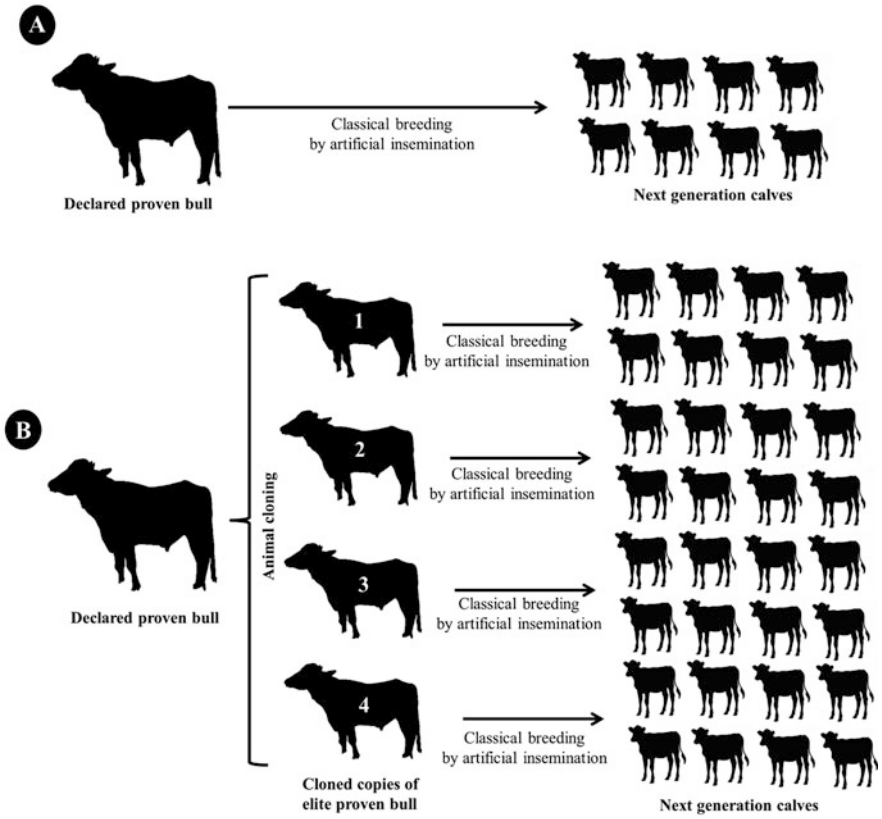

Fig. 21.4 Impact of buffalo cloning (BC) technology. In classical breeding, a lengthy process of progeny testing (requires 8 to 10 years) is performed to select best-proven bulls to produce next-generation calves (A), and limited calves are produced. (B) In BC-based breeding, already proven bulls to be selected for multiplying their genotypes. Identical genotype animals are recommended directly to produce next-generation calves through classical breeding. This way, the genetic of proven bulls is disseminated at a large scale, preferable in those locations in which it was not earlier reached (Image from Selokar et al. 2018)

et al. 2020). We also tested that cloned bulls have similar fertility to non-cloned bulls (Saini et al. 2020), (2) availability of semen from cloned bulls, at present, semen from five cloned bulls have been found normal and more than 30,000 semen doses are available. Also, we could not notice any abnormalities in the progeny of cloned bulls. Since there are no guidelines for use of cloned semen, thus only limited semen doses have been produced from cloned bulls for research studies. In recent years, several sexed semen production laboratories have been established, thus, semen of cloned bulls can also be utilized for sex semen production that will further impact technological advances. It is necessary to develop a roadmap for translation of BC technology in India and cloned bull semen can be included in classical breeding

programs. This will certainly help to reach technology at farmer's farm for enhancing the productivity of their animals. In addition to the production of cloned bulls, support from government agencies is much needed to sustain BC technology in long run. There is a need to make the regulation and guidelines for use of cloned animals for breeding and the use of cloned animal products for human consumptions.

21.6 Perspectives and Prospective

Buffalo cloning is a powerful reproductive tool. At present, both mSCNT and HMC can be effectively used to produce cloned buffaloes. The major applications of BC are (1) faster multiplication of elite germplasm, (2) conservation of endangered buffalo breeds, (3) production of transgenic or genome-edited model animals. Despite the potential applications, the technology has limited use which is mainly due to extremely low live birth rates. Previous studies suggested that cloned embryos have abnormal epigenetic reprogramming that could lead to early abortions, mortality of born clones, and abnormal organ size. Thus, the focus of the current research should be on the understanding of the reprogramming process through next-generation omics tools. It has been reported that clones, which attended maturity, have normal growth, reproduction, and production. In addition to research efforts, to sustain BC in long run, generous support is much needed from government agencies, particularly in India, to translate the technology from the lab to land. The major challenges of translation of BC in those countries where buffalo dominates are: (1) cattle milk and meat are preferred over buffalo due to long traditional practices, (2) high cost of technology, (3) lack of skilled manpower, and (4) absence of coordination and linkage among research scientists, developmental agencies such as animal husbandry departments, and buffalo industries. If these issues address appropriately, then, BC has the potential to hasten the speed of genetic improvement programs, particularly to upgrade non-descript buffaloes, in emerging economies like India and China.

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