Sperm Selection Based on Surface Electrical Charge

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List of Abbreviations

DGC	Density gradient centrifugation
ROS	Reactive oxygen species
GPI	Glycosylphosphatidylinositol
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
AOT	Acridine orange test
PS	Phosphatidylserine
EPS	External phosphatidylserine
HA	Hyaluronic acid
MACS	Magnetic-activated cell sorting

Introduction

For assisted reproduction techniques (ART), different procedures have been developed for separating "normal" viable sperm from seminal plasma, whereas the most commonly employed procedure is density gradient centrifugation (DGC) [1]. In this respect, sperm population with normal morphology, compacted chromatin, and little residual bodies are separated. However, several studies have demonstrated that sperm processed by this procedure does not guarantee genomic integrity of separated sperm [2]. In accordance with this deduction, Avendaño et al. reported that in infertile individuals up to 50 % of sperm with normal morphology may present DNA fragmentation [3].

In vivo, sperm are separated and selected by different screening barriers such as cervical mucus, cumulus and zona pellucida to prevent insemination of defective sperm [4]. Of note, during in vitro fertilization (IVF), zona pellucida remains as the only barrier that may prevent penetration of defective sperm into oocyte, and thereby through this selection, it may increase the chance of early embryo development and pregnancy outcome [5]. However, during intracytoplasmic sperm injection (ICSI), even this barrier is bypassed and the only selection process that is implemented by the embryologist is based on sperm viability and morphology [6]. Considering the fact that selection of sperm based on morphology does not preclude the chance of insemination of defective sperm as suggested by Avendaño et al. Therefore, the role of genomic integrity, with important consequence on early development, maintenance and outcome of pregnancy, as well as future susceptibility of offspring

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to different diseases is ignored in routine sperm selection procedures [3]. To overcome the deficiencies of these procedures, like DGC, advanced strategies for sperm preparation have been proposed or implemented by different researchers.

In advanced strategies, in addition to sperm morphology and viability, sperm are separated and/or selected based on functional characteristics of sperm surface membrane (for more details see review by Said et al. [7] and Nasr-Esfahani et al. [8]). The base of these strategies is that a functional membrane may reflect a normal sperm with intact DNA. It is generally believed, factors such as reactive oxygen species (ROS), influencing integrity of spermolemma also affects the integrity of DNA. Therefore, in this chapter, we introduce two advanced sperm selection procedures based on surface electrical charge and also discuss the importance of these efficient methods in ICSI.

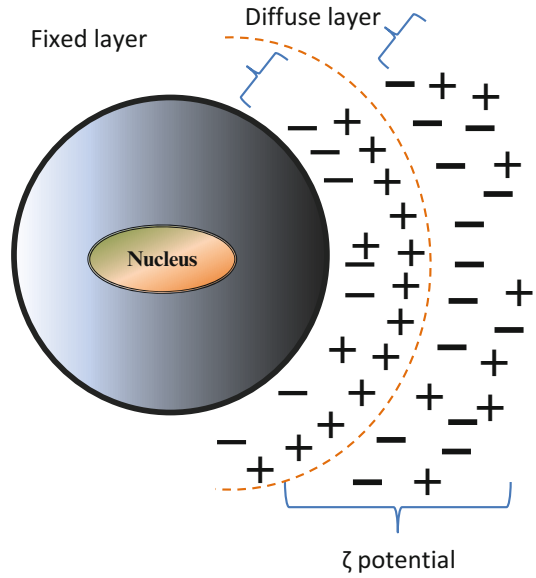

Fig. 4.1 Formation of Zeta potential (ζ potential)

Sperm Plasma Membrane

The sperm plasma membrane plays a dynamic role during sperm–oocyte cross talk and fertilization. Therefore, loss of function and integrity of the sperm plasma membrane is frequently associated with male infertility, notwithstanding normal semen parameters [9–11]. One of the elements playing a central role in this process is glycocalyx. Glycocalyx forms a "sugar coat" composed of complex array of glycans, the oligosaccharides and polysaccharides attached to glycoproteins and glycolipids. In sperm, this coat is rich in sialic acids and is liable for membrane negative charge as is called "Sias." It is intriguing to note that Sias are located in outermost layer of the sugar goat as they cap the majority of glycans at the sperm cell surface [12–14]. These sialoglycoproteins, deposited on sperm surface during spermatogenesis, pass through epididymis by means of epididymosomes and in semen through prostasomes [15]. They account for the electrical charge of the sperm plasma membrane, ranging from −16 to −20 mV, called "Zeta potential" or electrokinetic potential (Fig. 4.1) [16]. Tentative analysis of sialylated proteins responsible for conferring the Zeta potential by MALDI-TOF analysis has nominated four proteins, three of which are aminopeptidase B, fucosyltransferase, and prostatic acid phosphatase [17].

Zeta potential, in addition to preventing intracellular interaction and self-agglutination, it inhibits nonspecific binding with the genital tract epithelium during its transport and storage. It is noticeable that this negative electrical charge in other species such as chimpanzee, porcine, and bovine has been also recognized [18–23].

One of the proteins involved in creating this negative charge in sperm membrane is "CD52". CD52 is defined as a bipolar glycopeptide and a highly sialated glycosylphosphatidylinositol (GPI)-anchored protein on the sperm surface, which is acquired by sperm during epididymal transit and sperm maturation [18, 24]. The presence of high levels of sialic acid residues on the sperm membrane increases its net negative charge, and is taken as a symbol for normal spermatogenesis and sperm maturation within the testis and epididymis [24]. Therefore, transferring GPI-anchored CD52 onto the sperm surface is probably essentials for creating a membrane negative charge. This theory is in keeping with several studies in which they have demonstrated

normal levels of CD52 expression are positively correlated with sperm normal morphology, capacitation, and male fertility [24, 25]. Intriguingly, following capacitation, in addition to loss of Sias including CD52, this molecule shift from a distributed surface pattern toward equatorial region, whereas any disturbance in loss and patterning of Sias is associated with male infertility [24]. This is the reason for reduced Zeta potential following capacitation [24]. Loss of Sias is accounted by their hydrolyzed through means of neuraminidase present on sperm, in the uterus and follicular fluid [12]. Loss of these Sias unmasked the proteins involved in cross talk or signaling between sperm and oocyte during fertilization and thereby allows binding of capacitated sperm with zona pellucida [26].

This "sugar coat" which provides a functional surface electrical charge or the Zeta potential has evoked the researchers in this filed to design two different sperm selection procedures based on this criterion. The procedures are (1) Zeta method and (2) electrophoretic method.

Sperm Selection Based on Zeta Method

The negative electrical charge of the sperm's membrane allows sperm to adhere to surfaces with positive charge (tube, glass slides, and ICSI needle/plate) in a protein-free medium [27]. Based on this property, for the first time, Chan et al. separated sperm based on surface electric charge. These authors showed that the selected population showed higher degree of maturity [16]. Following this report, our research group at Royan Institute and Isfahan Fertility and Infertility Center in Iran used this method for treatment of couple candidate of ICSI [8, 28–35].

Practical Approach to the Zeta Method

Zeta method is carried out according to Chan et al. [16]. Briefly, sperm is mixed with serum free basic sperm processing medium and centrifuged.

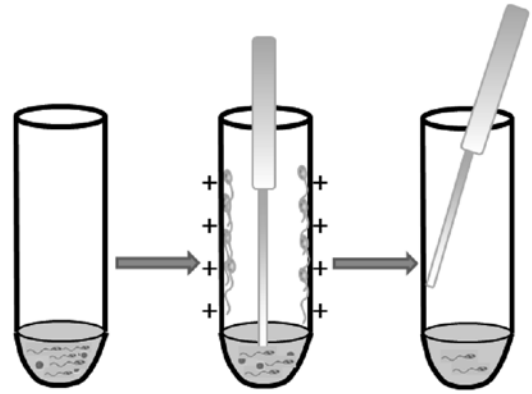

Fig. 4.2 Zeta method: sperm selection based on Zeta or electrokinetic potential

Following centrifugation, the supernatant is discarded, sperm pellet is mixed with serum free medium and sperm concentration is adjusted according to initial sperm count. The adjusted sperm solution is transferred to a new 5 ml Falcon tube which is induced to gain a positive surface charge. To induce a positive charge in lab condition, the tube is put inside a latex glove up to the cap, rotated two or three turns, and rapidly withdrawn from the latex glove. One minute is provided to allow adherence of the charged sperm to the tube wall, and then the medium containing non-adhering sperm (Fig. 4.2) is removed and discarded. Subsequently, tube surface is thoroughly washed with basic sperm processing medium containing serum to detach adhering sperm from tube wall. Subsequently, the sperm is either centrifuged or directly used for ICSI or further assessment [16].

Induction of electrostatic charge on tube surface can be confirmed using an electrostatic voltmeter (Alpha lab, Salt Lake City, USA). Another quick way to confirm the presence of electrostatic charge on tube surface is to check whether the tube will attract very small minute pieces of paper.

It is interesting to note that sperm adheres to glass surface due to their negative charge in albumin or serum-free culture medium. Following Zeta method and washing the tube surface in presence of serum or albumin, serum or albumin binds to anions and cations, so neutralizes the surface charge both on the sperm (Zeta potential)

and the surface of the tube. In accordance with this hypothesis, Chan et al. reported that capacitated motile sperm when exposed to a serum free condition showed lower tendency as compared to when adhered to positive surface charge [16]. Capacitated sperm due to loss of glyocalyx on surface of sperm shows more free movement and partially sticks on the glass surface, while uncapacitated sperm is completely immobilized with occasional twitching [16].

Sperm Quality Following Zeta Method

Following selection of sperm by Zeta method, Chan et al. showed that the quality of sperm selected through this procedure, particularly in terms of morphology, DNA integrity and maturity as compared to routine sperm selection procedure (DGC), was improved. They also reported that percentage of sperm with progressive and hyperactivated motility increases following Zeta method as compared to DGC, while the percentage of total motility remains unmodified. They also postulated that these increments which are associated with increased sperm metabolic activity is likely due to brief exposure to serum free condition or manipulation from the attaching/detaching of sperm to tube surface during this process without inducing premature acrosome reaction [16]. This hypothesis was later proved by Zarei-Kheirabadi et al. [30] in our research group. Further studies in our group, included the comparison of efficiency between DGC and Zeta method for separation of mature sperm in terms of morphology, protamine content and DNA integrity. Percentage of normal sperm morphology and protamine content were significantly increased in both DGC and Zeta procedures compared to neat semen. Unlike percentage of sperm morphology, percentage of sperm protamine content was not significantly different between DGC and Zeta methods [35].

Considering the importance of separation of normal sperm with intact DNA during sperm selection procedure, especially for ICSI, our group assessed percentage of DNA fragmentation by three staining methods; Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), sperm chromatin dispersion SCD and acridine orange test (AOT). The results indicated that percentage of DNA fragmentation was significantly decreased in both DGC and Zeta procedures compared to neat semen. Moreover, percentage of sperm DNA fragmentation rate was significantly decreased in Zeta methods compared to DGC [32]. It is important to note that the efficiency of Zeta and DGC methods relative to semen for DNA fragmentation were 62 % vs. 46 % for TUNEL, 42 % vs. 34 % for SCD, and 41 % vs. 34 % AO methods, respectively [32, 35].

In the above section, we provided evidence that Zeta procedure has a potential to select sperm with intact DNA, and through this procedure, it is possible to certain degree to delete defective or DNA fragmented sperm. However, in nature, the barriers which select "normal" sperm are not in physical contact with sperm nucleus. Therefore, it is the outer cellular characteristics which allow natural barrier to select the "normal" sperm with intact DNA. Therefore, assessment of sperm surface marker may provide evidence how such a sperm is selected in vivo and how these markers may be related to glycocalyx coat, playing the central role in Zeta sperm selection procedure.

Externalization of phosphatidylserine (EPS) from inner to outer layer of plasma membrane is considered as one of early markers of apoptosis in somatic and germ cells [36]. In addition, translocation of phosphatidylserine (PS) can also be considered as physiological event during the process of acquisition of capacitation [37, 38]. Another surface marker which plays a central role in redundancy of defective sperm is ubiquitination during the passage through the epididymis. Highly ubiquitinated sperm are phagocytized by epididymal epithelium [39]. Similar to EPS, it is important to bear in mind that sperm also contain ubiquitinated proteins which are destined to degradation following fertilization. These proteins are masked before capacitation, so they were become exposed to sperm surface during capacitation [40]. Therefore, it is of utmost importance that both EPS and ubiquitination act as a double-edged sword in sperm biology.

Considering important role of these markers, Zarei-Kheirabadt et al. assessed ubiquitination and external phosphatidylserine (EPS) in sperm selected by Zeta, and compared their results with DGC and neat semen. The findings of this study showed that percentage of both externalized PS, and ubiquitin positive sperm were increased in following application of Zeta method compared to DGC and control [30]. Hence, Zeta in addition to selecting sperm with reduced DNA fragmentation and normal protamine content increases the rate of ubiquitination and EPS in this population [30]. This is in agreement with pervious report of Chan et al. in which they postulated that during process of attaching and detaching, the glycocalyx might be altered, and this may induce sperm to undergo a process similar to capacitation. These results are in concordance with pervious report which suggested that increased progressive motility, hyperactivation, and ability to undergo capacitation are associated with higher fertilization rate [16]. To further add to this, Grunewald et al. reported that defective sperm is unable to undergo process of capacitation and acrosome reaction [37].

Recently, several novel sperm separation methods based on functional characteristics of sperm have been introduced [7, 8]. In this context, we compared efficiency of Zeta method with two main sperm separation procedures; HA-binding method and MACS.

Comparison of Zeta Method with Other Functional Sperm Selection Procedures

Zeta Method vs. HA-Binding

One of the sperm surface proteins which is also integral part of "sugar coat" or glycocalyx is a highly sialylated protein called PH-20. This protein has a high affinity for binding to hyaluronic acid (HA) secreted by cumulus cells and is present on Zona pellucida [41]. Therefore, based on this property, sperm has the capacity to bind to HA coated surfaces. Sperm bound to HA shows increased tail cross beat frequency without presenting forward frequency. Sperm selected based on this procedure also shows higher degree of maturity, while displaying normal morphology, low certain kinase activity, absence of cytoplasmic residues, low DNA fragmentation, normal protamine content, and low apoptosis [42].

In regard to this, Razavi et al. compared efficiency of HA-binding and Zeta methods. They reported that percentages of sperm normal morphology and protamine content have improved after HA-binding and Zeta methods compared to neat semen, while percentage of DNA damage has only been improved significantly after Zeta method, not in HA-binding method, compared to control. In addition, these authors reported that percentage of efficiency of the HA method relative to control for normal morphology, DNA integrity, and protamine content were 95 %, 5.9 %, and 19.1 %, while the efficiency of the Zeta method were 67 %, 44.6 % ,and 13.1 %, respectively [29]. One of the reasons for these differences could be the fact that Zeta is accounted for all proteins present in the "sugar coat" or in the glycocalyx while HA procedure is only based on one the component of glycocalyx, the hyaluronic acid. However, HA appears to have higher superiority to recover sperm with normal morphology, and this advantage of Zeta can be overcome by selection of morphology during the process ICSI [29].

DGC-Zeta vs. MACS-DGC

Magnetic-activated cell sorting (MACS) is an efficient method for selecting functional sperm based on membrane surface markers. Therefore, different researchers have used MACS to select non-apoptotic sperm based on phosphatidylserine externalization [7]. Previous studies have shown that sperm selected based on EPS shows improved quality [7, 43]. We showed that combination of DGC followed by MACS (DGC-MACS) improved the sperm quality compared to when DGC and MACS were used independently. Furthermore, we also demonstrated that sperm selection based on EPS before the induction of capacitation during MACS-DGC procedure occurred based on EPS due to early sign apoptosis, while sperm were selected after the process of induction of capacitation by DGC followed by MACS (DGC-MACS), partially capacitated

sperm may also be selected and discarded in the latter procedure [44]. It is assumed that when sperm is separated from semen in DGC procedure, the process of EPS and capacitation are initiated, and this effect is intensified when serum is used. Therefore, we strongly recommended that MACS-DGC rather than DGC-MACS method is more efficient in order to select sperm population with normal morphology, intact DNA, and low apoptosis [44].

Considering that both DGC-Zeta [32] and MACS-DGC [44] methods can improve quality of selected sperm, we compared the efficiency of two procedures in infertile population. It has been demonstrated that although both methods can select sperm with normal morphology, normal acrosome, normal protamine content, and intact DNA compared to neat semen or control, MACS-DGC method was more efficient in separation of sperm with normal acrosome and protamine content. In our study, the DGC-Zeta procedure showed a tendency toward lower DNA fragmentation rate compared to MACS-DGC [31]. However, to verify this point, further experimentation on larger population is required. It is important to remark that some studies expressed concern regarding remnant of micro beads after MACS for ICSI procedure.

Zeta Method and ART Outcome

Considering efficiency of Zeta method in separation of mature sperm population with minor DNA damage, Kheirollahi-Kouhestani et al. assessed effect of this method on ICSI outcome [32]. To initially roll out the confounding effect of female factors, they inseminated sibling oocyte using DGC and DGC-Zeta prepared sperm. They reported that percentage of fertilization (52.4 % vs. 65.4 %, $p=0.03$), percentage of pregnancy (53.57 % vs.33.4 %), and implantation rates rate (26.18 % vs.15.8 %) were increased following DGC-Zeta procedure [32]. Considering this study was performed on a small population, the study was expanded on a larger population which further confirms the outcomes of Kheirollahi-Kouhestani et al. and it was interesting to note in a couple with previous 11 IVF/ICSI failed cycle, it resulted in birth of a healthy child [34].

Advantage and Disadvantage of Zeta Method

The Zeta method is simple, low cost, and fast. It can be carried out on cryopreserved semen samples. The Zeta method has low recovery rate, but can be easily applied to ICSI cases. The procedure cannot be carried out on capacitated processed samples [8, 16, 45].

Sperm Selection Based on Electrophoresis

Similar to Zeta method, Prof. John Aitken's research group also developed a commercialized instrument called Microflow® or SpermSep® (CS-10) to select "normal" sperm. These researchers also separated sperm base on the surface electric charge using electrophoresis technology [17, 46–48].

Practical Approach to Microflow® or SpermSep

Electrophoretic device consists of two outer chambers and two inner chambers (inoculation and collection). The inner and the outer chambers are separated by polyacrylamide membranes with a typically pore sizes of 15 kDa. The inner chambers are further separated from each other by a third membrane with the pore size of 5 μM. The polyacrylamide membranes allow water and solute to flow between the chambers in the micro fluid system, while maintaining the charge on the two platinum plates at the two sides of outer chambers. Therefore, due to micro flow movement in the inner chamber (inoculation chamber), sperm with negative surface charge within the suspension is allowed to move toward the second inner chamber (collection chamber) close to the anode plate where they can be collected. The third membrane between the two inner chambers prevents movement of cells or other elements with negative surface charge and higher than 5 μM size to move toward the collection chamber close to anode plate [17, 49]. Therefore, through this procedure sperm with

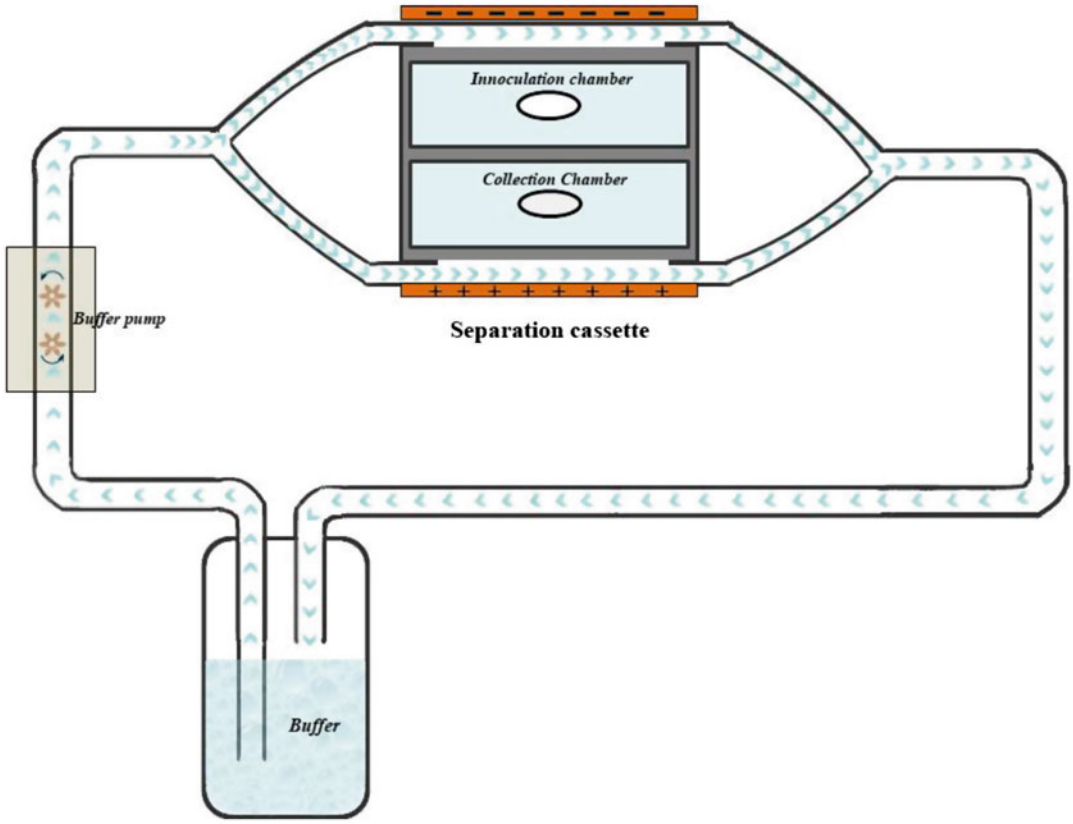

Fig. 4.3 Sperm selection based on electrophoresis

adequate cathode charge moves toward anode plate and due to fluid movement, this sperm can pass to membrane and then the selected sperm can be collected from collection chamber (Fig. 4.3).

Sperm Quality Following Electrophoresis

The research from Aitken group showed that percentages of sperm motility and viability in neat semen were similar to the sperm separated by electrophoresis, and these percentages are maintained in duration different time intervals of electrophoretic treatment. In addition, evaluation of the kinetic characteristics of sperm using CASA indicated that quality of sperm motility has not changed between semen and electrophoretically separated sperm and also during different time intervals of electrophoresis. Comparison of these parameters between sperm separated from DGC, electrophoresis, repeated centrifugation, and neat semen groups have shown that percentage of motility and viability was similar among these groups, except for DGC group in which the percentage of sperm motility were significantly higher than other groups. As a result, percentage of sperm motility has not been improved after the electrophoretic method compared to DGC and/or original ejaculation [46]. Therefore, in the light of this result, these authors have demonstrated that electrophoresis of spermatozoa can be harmful for motility and can lead to disruption of ion fluxes across the sperm plasma membrane [46]. On the other hand, Fleming et al. compared percentage of sperm motility between DGC and electrophoresis methods in infertile men underwent ICSI or IVF. This parameter was similar in DGC and electrophoresis methods in both IVF and ICSI cases. These authors explained that this difference in sperm motility is due to

"differences in donor profile, nature of the gradient used (Percoll versus ISolate) and differences in the susceptibility of spermatozoa to the passage of electric current" [48].

Unlike sperm motility, percentage of sperm with DNA fragmentation was significantly reduced in sperm separated by electrophoresis compared to neat semen sample. This parameter is maintained during different time intervals of electrophoretic treatment. They also showed that percentage of DNA fragmentation significantly increases after exposure to repeated centrifugation compared to DGC and electrophoresis methods. These authors concluded that physical shearing forces associated with repeated centrifugation and cell contamination (leukocyte, senescent spermatozoa, or other cells) are involved factors in production of ROS inducing DNA fragmentation during preparation of sperm. Thereby, they showed that electrophoretic method reduces ROS production and DNA fragmentation, so they contributed these effects, absence of requirement for centrifugation and elimination of ROS, in order to produce cells such as leukocyte [46].

Percentage of sperm with normal morphology was significantly higher in sperm separated by electrophoresis compared to neat semen sample, while this parameter is maintained during different time intervals of electrophoretic treatment. In addition, percentage of morphologically normal spermatozoa was significantly higher in electrophoresis group compared to other groups [46].

These researchers also show that this method is suitable for cryostored semen, snap-frozen sperm suspension and testicular biopsies [47]; furthermore, they showed the efficiency of this procedure to recover sperm is similar to DGC and is around 20 % [46, 47]. This recovery rate also stands for testicular biopsies consisting of complex cellular mixtures [47].

Considering the role of sialic acid in Zeta and electrophoretic method, Ainsworth et al. assessed sialic acid expression in electrophoretically isolated spermatozoa, and higher levels of sialic acid residues were observed in sperm recovered in the vicinity of anode plate compared to DGC-prepared spermatozoa [17].

Electrophoretic Method and ART Outcome

Ainsworth et al. reported the first pregnancy and normal birth using electrophoresis method following ICSI technique in a couple with previous repeated failed fertilization, severe oligozoospermia and high percentage of sperm with DNA fragmentation. They suggested "the electrophoretic sperm isolation procedure could make a significant contribution to good clinical practice in this area" [47].

In the light of these considerations, Fleming and coworkers designed a prospective controlled of electrophoretic method in 28 couples underwent either ICSI or IVF and compared clinical outcome of this method with DGC following IVF and ICSI. They reported that efficiency of two sperm separation methods; electrophoresis and DGC, in terms of percentage of fertilization (62.4 % vs. 63.6 %), cleavage (99.0 % vs. 88.5 %), and high-quality embryos (27.4 % vs. 26.1 %) were similar. But since their trail was not randomized, they did not draw any conclusion regarding their clinical pregnancy outcomes [48].

Advantage and Disadvantage of Electrophoretic Method

The electrophoretic method is fast, but requires commercial instrument which may increase the cost of procedure. It can be carried out on cryopreserved semen samples with recovery of sperm count similar to DGC. But the procedure cannot be carried out on capacitated processed samples. The main advantage of this procedure is absence of centrifugation which can induce ROS and DNA fragmentation [4, 8, 17, 50].

Conclusion

It is well established that even in infertile individuals normal looking sperm might contain fragmented DNA. Therefore, novel sperm selection procedures based on different sperm functional

characteristics have been designed. Among these selection procedures, sperm can be selected based on surface electric charge or the Zeta potential. Sialic acids by coating the spermolemma account for this charge. Population of sperm selected based on this characteristic has been shown to present higher normal morphology, normal protamine content, lower rate of DNA fragmentation, and higher ability to initiate capacitation. Compared to other novel sperm selection procedures, sperm selected based on Zeta potential present lower rate of DNA fragmentation. Such sperm were shown to have higher capacity to support development and lead to pregnancy. Considering that no chemical are used for selection of sperm based on Zeta potential, the data in this chapter support possible potential of both these procedures (Zeta or electrophoretic methods) for future routine clinical applications.

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