Environmental Science

Kavindra Kumar Kesari *Editor*

Perspectives in Environmental Toxicology

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Perspectives in Environmental Toxicology

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Foreword

The present volume is being brought out by Dr. Kavindra K. Kesari which explores in detail multidisciplinary approaches to environmental toxicology, with a focus on the following aspects:

- The effects of man-made electromagnetic fields (RF-EMF) on human health proposed mechanisms and biological effects and measures;
- An overview of nanotoxicity, nanomedicine and cancer research.
- A bio-computational approach to the molecular interaction of environmental carcinogens with DNA.
- The toxicology of environmental pollutants in the air, dust, soil, water and natural toxins in the environment: exposure and health.
- Social insects as environmental indicators of ecotoxicological effects in different ecosystems.

Environmental toxicology deals with the effects of chemicals on human health and the environment. It is an interdisciplinary science integrating biology, microbiology, chemistry, engineering, environmental sciences, ecology and even physical sciences. It pertains to innovative insights into toxic pollutants in the environment and their impacts on natural ecosystems; both freshwater and marine aquatic ecosystems in addition to terrestrial habitats. Evaluation of the environmental effects of chemicals is intricate because it depends on the organisms tested and deals with the toxicity of individual chemicals; their interactive effects, (genotoxicity, mutagenicity and immunotoxicity tests). These chemicals and factors may exhibit synergetic effects on environments which are tricky to quantify or envisage. Subjects covered are anthropogenic and natural pollution as well as feedback mechanisms and multiple stress or response to variable factors including alterations in temperature, pH and radiations.

In the opening Chapters "Neurophysiological and Behavioral Dysfunctions After Electromagnetic Field Exposure: A Dose Response Relationship" and "Induction of LPO and ROS Production in Rat Brain Exposed to Microwaves: Computational Elucidation of Melatonin in Repair System" Sharma et al. and

Kesari et al. discuss the Neurophysiological and behavioral dysfunctions after electromagnetic field exposure: A dose response relationship. In Chapter "Nanoparticles: Applications, Toxicology and Safety Aspects" Anupam Dhasmana discusses the applications and toxicological aspects of nanoparticles and recommendations of safety aspects. In Chapter "Cadmium Toxicity Showing Organ Specific Signature of Responsiveness" Sandeep Agnihotri emphasizes the signature of cadmium toxicity showing organ specific responsiveness.

There is a total of 10 chapters which discuss: biomarkers of ecotoxicological effects in social insects; environmental Toxicology of wastewater, novel treatment techniques and its irrigation reuse potential; antibiotic resistance genes: an emerging environmental pollutant; carcinogenic toxicity of cigarette smoke: a computational enzymatic interaction and DNA repair pathways; determination of *murG* transferase as a potential drug target in *Neisseria meningitides* by spectral graph theory approach; nanotoxicity: current progress and future perspectives; nanoparticles: application, safety and toxicology; nanomedicines in cancer research: an overview and toxicity of synthesized nanomaterial in natural environment.

These discussions highlight experimental and theoretical approaches to the field of environmental toxicology. In a way goad the environmentalists to make the public aware of these maladies. The present volume is a welcome approach with an intent to monitor and analyze levels of pollutants and predict future trends. The excessive use of mobile and its adverse effects call for a wide public awareness. Further topics such as transport of toxic substances, chemical and physical processes, their mitigation to prevent pollution by toxic substances should attract further discussion. The book provides recent topics for individuals interested in the field of toxicology. Overall, the book should be recommended for undergraduate or graduate students as introductory or exploratory text which endeavors the researchers on topical issues.

The present volume is intended for scientists, professionals, and graduate students interested in improving the environment. The book will be extremely useful for biotechnologists, geneticists, molecular biologists, nanotechnologists, nanobiotechnologists, and physiologists. Postgraduate students, honors students, in these disciplines having adequate background in environmental toxicology and spectrum of other researchers interested in biology and agriculture will also find the book a worthwhile reference text. We sincerely hope that the information embodied in the book will enthuse environmentalists and ameliorate upcoming new information.

Dr. Kavindra K. Kesari must be congratulated to undertake this very important topical issue.

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Preface and Acknowledgements

Perspectives in Environmental Toxicology is a comprehensive textbook deciphers the phenomena and highlights the latest developments in environmental toxicology. The fundamental information on the effect of environmental toxicants or pollutants focuses on the multidisciplinary field of science by applying the principles of biology, physics, and chemistry. The *biological* (bacteria, viruses, parasites, fungi, enzymes, biological toxicants) and *chemical* contaminations (metal contamination, pesticides, toxic chemicals, compounds, tobacco smoke, nanoparticles), and *physical* exposures (high power tension wire, electromagnetic field (EMF), electronic gadgets, medical devices) have been discussed in this book. This chemical, physical, and biological contaminants are all around us and pose a problem in the onset of various diseases or physiological disorders to human beings due to man-made substances and compounds. There are a number of different ways to affect the human health by toxic substances such as air, water, soil, and noise. Man-made EMF has been considered as an "electro-pollution" or "electrosmog" in the list of air, water, or soil pollution. The overall objectives of this book are to cover the effects of environmental toxicants in animals, plants and humans. Complementary chapters examine the environmental causes of cancer, classification of carcinogens, metabolism of chemical carcinogens, and DNA damage and repair system. This highlights the latest developments in agriculture solid waste management and ecotoxicological effects. This book has value-added collections of 10 different papers (chapters) and links to multidisciplinary approaches of environmental toxicology with a focus on the following aspects.

Chapter "Neurophysiological and Behavioral Dysfunctions After Electromagnetic Field Exposure: A Dose Response Relationship" represents the introduction of radiofrequency EMF effects on neurophysiology, brain behavior, and dose response relationship. Interestingly, this chapter not only is limited to theoretical or mechanistic view but also explores the experimental examinations. This chapter shows evidence of the effect of EMF on Alzheimer disease and neurodegeneration. Chapter "Induction of LPO and ROS Production in Rat Brain Exposed to Microwaves: Computational Elucidation of Melatonin in Repair System"

investigates the effect of microwave radiations on brain antioxidative levels. The administration of melatonin against microwave radiations and computational elucidation of melatonin in repair system are the main observations. Therefore, Chapters "Neurophysiological and Behavioral Dysfunctions After Electromagnetic Field Exposure: A Dose Response Relationship" and "Induction of LPO and ROS Production in Rat Brain Exposed to Microwaves: Computational Elucidation of Melatonin in Repair System" mainly focus to explore the dose response relationship between EMF exposure and the effect on brain, and also in repair system by introducing melatonin. Chapter "Nanoparticles: Applications, Toxicology and Safety Aspects" discusses good and bad science of nanoparticles. Twenty-first century is known for both technological prosperity and environmental toxicity. This chapter mainly focuses on the applications of nanoparticle in the environmental and biomedical sciences. Also the causative factors come through environmental contaminations and recommended safety aspects have been discussed. Chapter "Cadmium Toxicity Showing Organ Specific Signature of Responsiveness" is in continuation of Chapter "Nanoparticles: Applications, Toxicology and Safety Aspects". The environmental exposures are of several types such as nanoparticle dust, metals or chemical toxicity. Toxic heavy metals like cadmium are found to be more hazardous for the biological system. This chapter mainly explores the toxic effect of cadmium, source of exposure and possible mechanism of health effects. The environmental toxicants that cause severe neurodegenerative diseases are also discussed in Chapter "Toxicity of Protein and DNA-AGEs in Neurodegenerative Diseases (NDDs) with Decisive Approaches to Stop the Deadly Consequences". This chapter's focus is to measure the toxicity of protein and DNA-AGEs in neurodegenerative diseases and to discuss approaches to stop the deadly or severe consequences, which has been associated with toxicity of glycation intermediate (dicarbonyl compounds and ketoamine moieties) and their end products. The measurement and the interaction of enzymes, proteins, and DNA repair pathways suggested to introduce by applying computational approach. Somehow, it is a problem-solving tool of experimental research. Therefore, Chapter "Carcinogenic Toxicity of Cigarette Smoke: A Computational Enzymatic Interaction and DNA Repair Pathways" first time introduces *in silico* approach to find molecular interaction of cigarette smoke carcinogens with enzymes involved in DNA repair pathways. In this continuation, Chapter "Determination of *murG* Transferase as a Potential Drug Target in *Neisseria meningitides* by Spectral Graph Theory Approach" deciphering the fundamentals for the determination of *murG* transferase as a potential drug target in *Neisseria meningitides* by spectral graph theory. Interestingly 3D structure of *murG* transferase has been suggested for further use in *in silico* drug designing by docking methods with suitable inhibitors. This is growing area of research, which has fruitful and novel use in wet laboratory and cancer research. Cancer or associated health concerns are becoming severe nowadays due to an increasing environmental exposure or toxicity. This exposure has a big source of agricultural solid wastes. Chapter "Review processing, Properties and Applications of Agricultural Solid Waste: Effect of an Open Burning in Environmental Toxicology" shows the pros and

cons of solid wastes. The wastes such as rice straw, husk, sugarcane bagasse, leaves or other biomass found good source of bioenergy or biofuel in this chapter, where as the open burning of these agricultural wastes has reported hazardous effect on human health and the environment. This article provides the pathway of mechanism and future recommendations for the use of agriculture wastes as a bioresource for the production of biofuel or bioenergy. Some of the waste has also use in biomedical science like silica nanoparticles, which could be synthesized from rice husk. Concerning medical sciences, Chapter "Antibiotic Resistance Genes: An Emerging Environmental Pollutant" gives an overview on the impact of antibiotics resistance bacteria (ARB) and antibiotic resistance genes (ARGs) as an environmental pollutant into different form of the environment. ARB and ARGs have been extensively detected in wastewater, agricultural soil, animal manure and hospital waste. This type of environmental pollution found more dangerous and causative for human health and need to be exploring by providing the experimental data. Now from Chapter "Neurophysiological and Behavioral Dysfunctions After Electromagnetic Field Exposure: A Dose Response Relationship"–"Antibiotic Resistance Genes: An Emerging Environmental Pollutant" it is very clear that day by day, our environment is getting highly polluted due to man-made sources. The above-reported types of environmental toxicants or pollutants are very dangerous for climate change and ecosystem, insects, soil organisms, and human beings. Chapter "Biomarkers of Ecotoxicological Effects in Social Insects" explores the biomarkers of ecotoxicological effects in social insects. For the ecotoxicity testing, biochemical, morphological, or behavioral parameters of living organisms have been set to biomarkers of exposure, effect or susceptibility or biomarkers of defense and damage. Social insects such as ants, drosophila are well indicators of the lifespan and healthy environment.

This is the first book ever providing comprehensive evidence on multidisciplinary approach of total environmental toxicity for students, research scholars, academicians, scientists, and layman. This book is fundamentally, theoretically, and principally strong to present the mechanisms of interaction of environmental toxicity and human health by flow diagrams. This book analyzes the carcinogenic, mutagenic, genotoxic, and neurotoxic effects of both anthropogenic and natural toxins present in water, soil, air, and our surroundings in the form of electropollution or electrosmog. All Chapters "Neurophysiological and Behavioral Dysfunctions After Electromagnetic Field Exposure: A Dose Response Relationship"–"Toxicity of Protein and DNA-AGEs in Neurodegenerative Diseases (NDDs) with Decisive Approaches to Stop the Deadly Consequences" have followup links from each other, and conclude that reactive oxygen species (ROS) is the responsible factor for all types of induced environmental toxicity and human health. I hope this book will serve as both an excellent review and a valuable reference for formulating suitable measures against environmental toxicology and for promoting the science involved in this area of research.

Finally, I would like to dedicate this book to my mother, late Parwati Devi. She passed away on 31 January 2016, and left her infinite blessings for all my success. I would like to thank my father, Dr. Arjundas Kesari, who has given me much encouragement and support. I would like to thank all authors who have contributed to this book. Last but not least, my special thanks go to series editor, publisher, and entire Springer team for their sincere assistance and support.

Kuopio, Finland Kavindra Kumar Kesari, Ph.D.

Contents

Neurophysiological and Behavioral Dysfunctions After Electromagnetic Field Exposure: A Dose Response Relationship

Archana Sharma, Kavindra Kumar Kesari, H.N. Verma and Rashmi Sisodia

Abstract For decades, there has been an increasing concern about the potential hazards of ionizing and non-ionizing radiations on human health. This chapter provides several evidences related to pathophysiology of electromagnetic field (EMF) and its effects on different tissues and organs with special reference to neurophysiological and behavioral dysfunctions. Developing central nervous system (CNS) is extremely sensitive to EMF due to various factors especially due to presence of the high amount of water content, lipids and low amount of antioxidant enzymes. Therefore, the study is focused on the effects of radio frequency (RF) EMF and extremely low frequency magnetic field (ELF MF) on neurological disorders. The severity of effects always depends on exposure doses like, exposure duration, position of subjects, power density and field intensity, which could be measured in terms of specific absorption rate (SAR). There are several biomarkers, which are very useful to measure the radiation effects in both in vitro and in vivo model. The most intensely studied biomarkers by various researchers in CNS are protein kinase C, micronuclei, mitochondrial pathways, melatonin, calcium ion concentration, antioxidant enzymes like glutathione, superoxide dismutase, catalase etc. EMF may also lead to alterations in neurotransmission and consequently in cognitive and memory functions which are mainly linked to the brain hippocampus. Thus there are various histopathological aspects of hippocampus, which are studied and discussed in this chapter. Additionally, the dose response relationship between EMF and biological effects are discussed in this chapter.

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1 Introduction

Environment surrounding us, contains several type of contaminants, toxicants, pollutants and manmade exposures. These are biological, chemical or physical and could be classified under environmental toxicology. By applying the principles of biology, physics and chemistry, toxicologists can study the toxic behavior of man-made electromagnetic field (EMF) exposure. The hazards of radiofrequency electromagnetic radiation (RF-EMR) pervading the environment have now been increasingly realized and therefore, such radiations have been considered as an "electro-pollution" or "electrosmog" in the list of other environmental pollutants (air, water, soil, and noise pollution) (Behari 2009). Epidemiological evidences indicate that RF-EMF exposures are associated with adverse health effects such as tumor or cancer risk (Ahlbom et al. 2009). Not only the range of RF, but also extremely low frequency magnetic field (ELF MF) have been found to have causative effect on human health. Several epidemiological studies on RF-EMF or ELF MF exposure have investigated the health risks in populations living near cell phone towers, power lines, or who are in electrical occupations. The most common concerns include impaired sperm quality (Akdag et al. 1999; Cleary 1995; Kesari and Behari 2010), liver (Kumari et al. 2012), neurological dysfunctions (Sharma et al. 2014, 2016; Kesari et al. 2014; Kunjilwar and Behari 1993; Meena et al. 2014; Paulraj and Behari 2006) and histopathological changes such as cell injuries (Khayyat and Abou-zaid 2009; Kumari et al. 2012; Verschaeve 2009; Zare et al. 2007). Therefore, RF EMF and ELF MF were classified as a 'possibly carcinogenic to humans' (group 2B) by the International Agency for Research on Cancer (IARC 2002; Baan et al. 2011). Also at higher frequency level, International Commission on Non-Ionizing Radiation Protection reported that the specific absorption rate (*SAR*) *of* mobile phones is legally limited to 2.0 W/kg (ICNIRP 1998). In the USA, Canada and Australia, the maximum SAR level is limited to 1.6 and 2.0 W kg^{-1} in Europe (Dahal 2013), but most have an average SAR of $\sim$1.4 W/kg (Agarwal et al. 2011).

There are more than 2 billion mobile cellular phones or 4 billion people using mobile throughout the world (Stefanics et al. 2007; Roxanne 2009). These handholds mobile phones were normally started with 1G (first generation) and 2G (Second generation) and extended to 3G and 4G (third and fourth generation). With an increasing demand, now 5G (fifth generation) mobile phones are about to launch in market. With an increasing frequencies, the power density and exposure levels are also raised. Not only cell phone but also other electronic appliances like microwave oven has raised serious concern because of their frequent use in houses. The amount of RF EMF radiations absorbed by human tissue depends on the frequency, intensity, polarization and duration of exposure (Agarwal et al. 2011). It also depends on the level of doses, like for how long does a person is getting

exposure? In the case of chemical exposure, what is the amount or concentration level of intake? For the monitoring of radiation exposure, SAR is an important factor to measure the absorbed radiation into the body. The SAR value varies for each type of mobile phone and particular model based on usage conditions (Agarwal and Durairajanayagam 2015) and positions of keeping it with your body. Keeping cell phone near head while talking may lead to more absorption of power in the brain. This may cause an increase of up to 2 °C in the brain temperature on continuously talking for more than 20 min on phone. Microwave radiations have potential to penetrate the cranium, and nearly 40% of these can reach deeper into the brain (Barnett et al. 2007; Kang et al. 2001), where penetration depth is assumed to be 4–5 cm deep into the brain (Dimbylow and Mann 1994; Rothman et al. 1996). An interaction of microwave radiation with tissues arise as a result of mainly three processes; deep penetration into the tissue and their propagation into the living system, then the primary interaction of the waves within tissue, and the possible secondary effects arising from the primary interaction (Rachael 2010). The deep penetration of microwaves within the tissue or living cells is the process that causes the overproduction of free radicals/reactive oxygen species (ROS), will be discussed later in this chapter. Microwave induced oxidative stress may produce ROS which are reported to be the main cause of cellular damage or tissue injury (Dasdag et al. 2008; Kesari and Behari 2010, 2012). Therefore, this chapter provides several important findings related to pathophysiology of microwave radiation and its effects on different tissues and organs. These findings are in agreement with our own previous findings (Kesari et al. 2010a, b, 2012, 2013; Sharma et al. 2014), which indicate that the biological changes could occur due to a microwave exposure induced oxidative stress as also debated by several researchers (De-Iullis et al. 2009; Oktem et al. 2005).

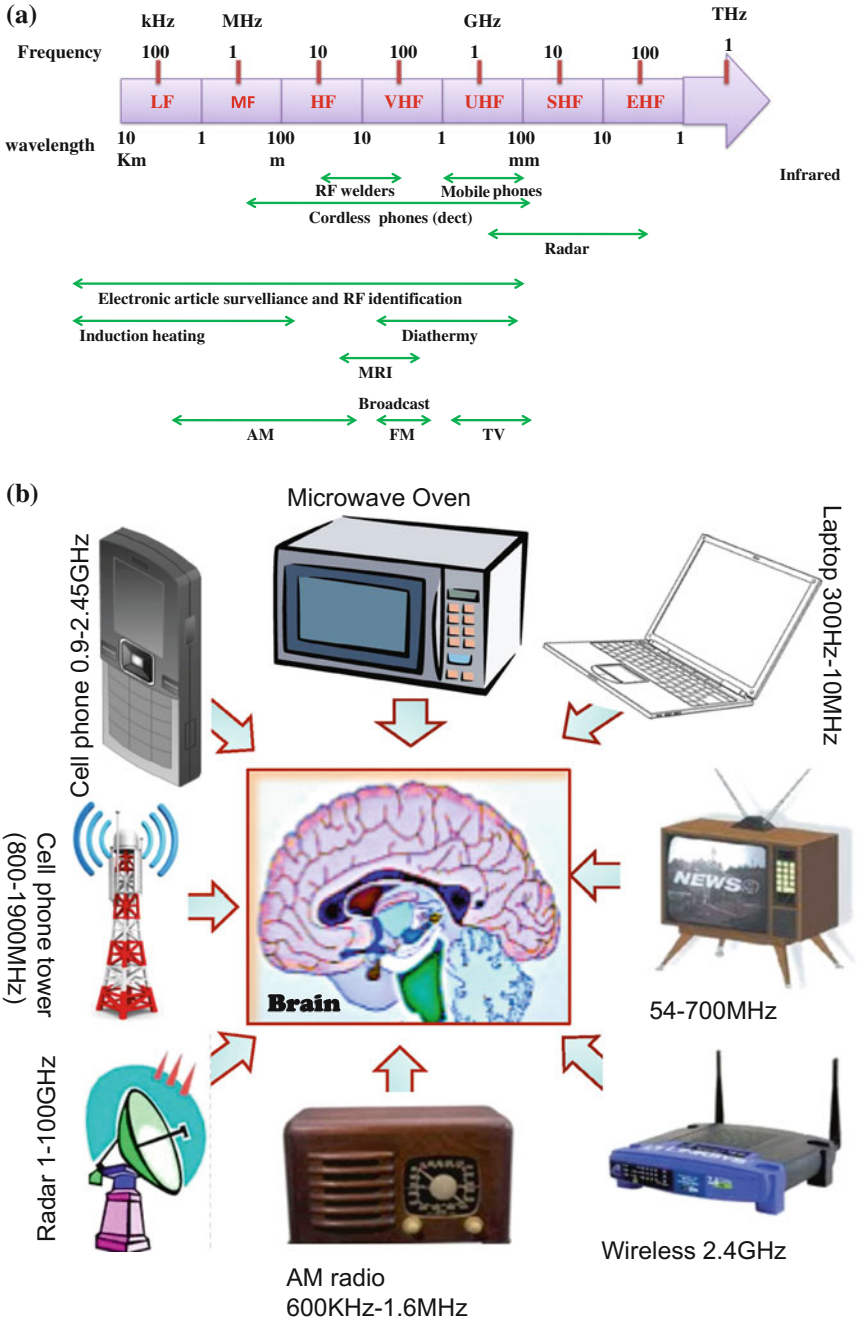
1.1 History and Sources of Electromagnetic Fields

The history of research on the biological effects of microwave radiation effectively begins with the development of radar early in World War II. Prior to this time, the energy levels at which microwaves had been produced were not sufficient to cause widespread concern about harmful effects. Before the invention of radar, artificially produced microwave energy was not a general environmental problem. However, as this field of research began to take shape, it did not do so in a vacuum. Well before the invention of radar, medical researchers had been interested in the controlled effect of RF energy on living things. Once it was discovered that radio waves could be used to heat body tissue, research was undertaken to study how such heating took place and its effect on the whole organism. As a consequence, both continuity and newness characterized this field of research during its early phase of development. Between the early 1940s and 1960, research on the biological effects of microwave radiation slowly shifted from its medical context and the search for

benefits to a military-industrial context and also search for hazards started. Polyashuck (1971) reported for the first time the effect of microwave radiation on the blood-brain barrier in 1971. Since the late 1970s, researches started which revealed that exposure to ELF electric and magnetic fields produces adverse health consequences. The sources of ELF MF comes from wherever electricity is generated or transmitted i.e. power lines, electric wiring etc. However, in many houses and office environments, individuals can experience perpetual exposure to "electromagnetic smog", with occasional peaks of relatively high EMF intensity. This has led to concerns that such radiation can affect health.

The classical example for natural source of non-ionizing radiation (NIR) is the sun and it is emitting ultraviolet radiation continuously. The most common source for NIR is transmission lines (50–60 Hz), computer monitor (60–90 Hz), AM radio transmissions (530–1600 kHz), thunderstorms (30–300 MHz), FM radio transmission (88–108 MHz), television transmissions (50–700 MHz), hand phones (850 MHz–2.4 GHz), wireless data and microwave ovens (2.45 GHz). In the last few decades, many places wireless technology has been introduced for telecommunication, but the long-term health effects of those waves are unpredictable and these emissions may affect human health. The term RF refers to the part of the electromagnetic spectrum that can be readily used for radio communication purposes which lie below the infrared region: specifically, frequencies in the range of 100 kHz to 300 GHz. Frequency bands within this range have been named more formally by the International Telecommunications Union (ITU). Figure 1a, shows these bands together with the ranges of frequencies commonly used for various applications, including those for telecommunications, in medicine, and in industry. Figure 1b, showing the several electromagnetic field exposure sources and effect on whole brain. Human exposure to RF field may arise from their deliberate use- for example, as a part of the global communication networks- or adventitiously, as a part of industrial and other processes utilizing RF energy. The term radio wave is used to denote a RFEMF that is transmitted from a source for communication purposes.

The microwave frequency spectrum ranges from 300 MHz to 300 GHz and RF radiation from 0.5 to 300 MHz. The sources of microwave and RF radiation are air traffic control systems, police and military radar, earth to satellite television broadcast systems, long distance telephone equipment, medical diathermy devices, cancer diagnostic and therapeutic (hyperthermia) equipment, microwave ovens, industrial applications and microwave generators. Among these, mobile phones have been available since the end of the 1980s and have become common in the general population in recent years. In most of the countries, today more than 80% of the population uses mobile phones (Feychting et al. 2005). This worldwide expansion of the use of mobile phones has made EMF exposure ubiquitous in modern society. Additional sources of exposure to RF fields are appearing from new technologies such as domestic meters and airport security scanners. As a consequence, intermediate frequency (IF) has been identified as newest source of exposure. It falls between the low frequency (Low frequency—0.1 Hz–1 kHz) and the RF (10 MHz–300 GHz). The major source of IF are some anti-theft devices


(a)
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Radar
Electronic article surveillance and RF identification
Induction heating
Diathermy
MRI
Broadcast
AM
FM
TV
(b)
Microwave Oven
Laptop 300Hz-10MHz
Cell phone 0.9-2.45GHz
Cell phone tower (800-1900MHz)
Brain
54-700MHz
Radar 1-100GHz
AM radio
600KHz-1.6MHz
Wireless 2.4GHz

◂**Fig. 1** **a** The electromagnetic field spectrum. Abbreviations according to the International Telecommunications Union (ITU) band are given as LF: low frequency, MF: medium frequency, HF: high frequency, VHF: very high frequency, UHF: ultra-high frequency, SHF: super high frequency and EHF: extremely high frequency. **b** Effects of electromagnetic device usage on the CNS or whole brain. Usage of electromagnetic gadgets is associated with alterations in various neurological functions from the central nervous system. Figure shows the various sources of electromagnetic field exposure with their frequency range

operated at the exits of shops, induction hotplates, computers, compact fluorescent lamps, as well as some radio antennas.

1.2 EMF Exposure and Dosimetry

Recently, the National Toxicology Program (NTP) under the National Institutes of Health (NIH) in USA (Wyde et al. 2016) has released animal studies conducted on RF (cell phone) radiation exposure effect and cancer (glioma and malignant Schwannoma in heart). This is the largest ever-animal study reported tumor in the heart. Now the question is, how such a low frequency RF radiation may cause tumor? However, it is not easy to answer the question but possibility to explore by deciphering the role of dosimetry and field measurement within the body can be done. Cell phone emits RF-EMW to nearby relay base stations or antennas. Our bodies act as antennas that absorb the radiation and convert it into alternating eddy currents (DWB 2007). When speaking on the cell phone, the sound wave from speaker goes through a transmitter that converts the sound into a sine wave. The transmitter then sends the signal to the antenna, which then sends it out into space in all directions. The transmitter in cell phone operates on about 0.75–1 W of power, with 2 W at peak usage. This electric sine wave current running through the transmitter circuit also creates an EMF around it. As the electric current moves back and forth, the fields continue to build and collapse, forming EMR. Thus, cell phone radiation is generated in the transmitter, and is emitted through the antenna in the form of a radio wave (Agarwal et al. 2011; TECH 2007). The impact of these RF EMW on the human body is measured via a standardized unit called the SAR value.

The rate of absorption and the distribution of RFR energy in an organism depend on many factors. These include: the dielectric composition (i.e., ability to conduct electricity) of the irradiated tissue, e.g., bones, with a lower water content, absorb less of the energy than muscles; the size of the object relative to the wavelength of the RFR (thus, the frequency); shape, geometry, and orientation of the object; and configuration of the radiation, e.g., how close is the object from the RFR source? These factors make the distribution of energy absorbed in an irradiated organism extremely complex and non-uniform, and also lead to the formation of so called ‘hot spots’ of concentrated energy in the tissue (Lai 2002). For example, an experiment reported by Chou et al. (1985), measuring local energy absorption rates

(SARs) in different areas of the brain in a rat exposed to RFR, has shown that two brain regions less than a millimeter apart can have more than a two-fold difference in SAR.

At lower frequencies (<100 kHz), many biological effects are quantified in terms of current density in tissue and this parameter is most often used as a dosimetric quantity. At higher frequencies, many (but not all) interactions are due to the rate of energy deposition per unit mass. This is why the SAR is used as the dosimetric measure at these frequencies. It is expressed as $W\,kg^{-1}$. The SAR is thus the absorbed power by the absorbing mass. It is always challenging to measure SAR directly inside the human body. Therefore, the most obvious approach towards dosimetric analysis is to experimentally determine the SAR distribution in phantoms simulating animal and human bodies, as well as in real cadavers. Phantoms are well known as tissue equivalent material. It means that, the physical properties existing in human body can fulfill by using phantom material for SAR measurement. Using this makes easy to know the absorbance level in the brain or other delicate organs.

In general, the simple and standard procedure can be applied to calculate SAR values; E-field value is measured with a miniature E-field probe. Indeed, E-field probes/monopole antenna is the most appropriate sensor to measure the SAR, due to their sensitivity and fast response. E-field maybe calculated as-

$$\mathrm{SAR}(\mathrm{W/Kg}) = \sigma E^2 / \rho$$

where sigma (σ) is conductivity of the liquid and rho (ρ) is the density of liquid. The measured E-field values and SAR distribution are 1 and 10 g mass averaged SAR values.

2 Biomarkers of Neurological Dysfunction

Central nervous system (CNS) or brain is a very complicated part of our body and also a carrier for all other organs and metabolisms. Any damage or changes due to environmental exposure in brain may lead to serious health concerns. Biomarkers are often measured and evaluated to examine such changes in various part of human body, especially in brain. The brain is very sensitive and delicate part of human body on which any direct experiments are not possible. Though in vitro and in vivo methods are implemented to measure the neurological dysfunctions. Therefore, several biomarkers like, protein kinases, micronuclei, mitochondrial pathways, DNA damage etc. are very useful to measure the causative factors. An overview of EMF exposure effect on biomarkers, its mechanism and possible diseases are presented in Fig. 2.

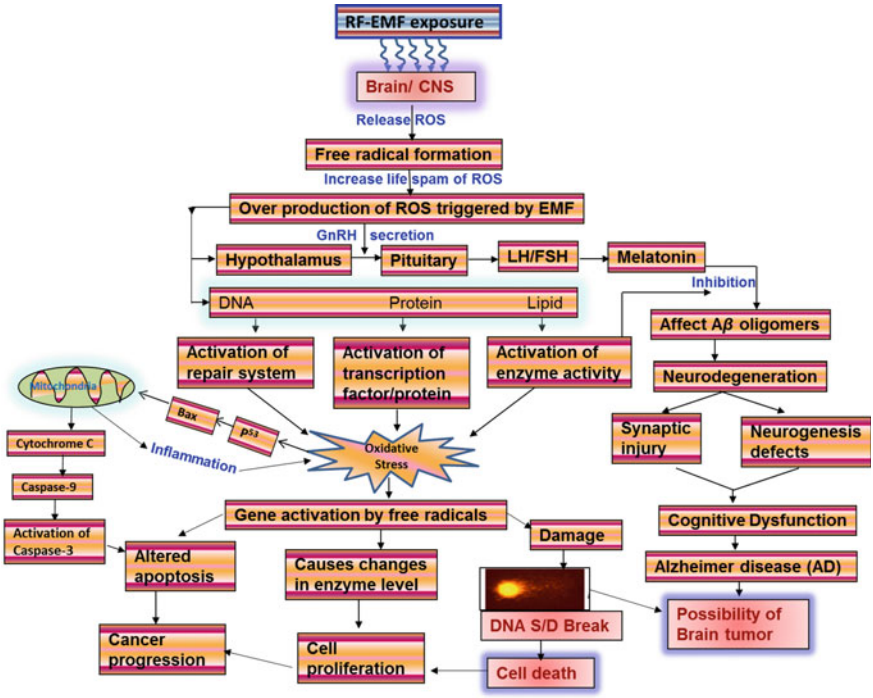

Fig. 2 Summary of the biological effects of RF-EMR exposure on central nervous system. This figure indicates enhanced ROS due to RF-EMR radiation can cause several changes at enzymatic and hormonal level, which may result Alzheimer disease and brain tumor. The activation of transcription and enzyme activity produce oxidative stress due to RF-EMF induced ROS formation. This results apoptosis by release of cytochrome c from mitochondria. The changes due to RF-EMF may enhance the DNA strand break by ROS formation and cause finally cell death

2.1 Protein Kinase C (PKC)

PKC is an isozyme and reported at least twelve in number. It differs in structure, biochemical properties, tissue distribution, subcellular localization, and substrate specificity. The first isoform that were Ca^{++}-activated, phospholipid-dependent protein kinases are ubiquitous enzymes that are highly enriched in the brain (Huang et al. 1986). Ca^{2+}-dependent PKC has been classified as conventional isozymes with α, β and γ. In late 1970s, it was first recognized as proteolytically activated serine/threonine kinase (Takai et al. 1977). PKC plays a major role in brain by regulating both pre and postsynaptic aspects of neurotransmission (Newton 1995; Nishizuka 1992; Stabel and Parker 1991). Any changes in the level of PKC and activation of various isozymes have resulted in brain tumor or neurodegeneration, like Alzheimer disease (Fig. 2). Therefore, researchers reported the structural basis for enhancement of long-term associated memory in single dendritic spines regulated by PKC (Hongpaisan and Alkon 2007). PKC play an important role in

neurological functions, which could be functional in mitochondria. Mitochondria are crucial regulators of energy metabolism and apoptotic pathways that have been closely linked to the pathogenesis of neurodegenerative disorders or malignancies. A malignancy like tumor promoter is well known receptor of PKC (Parker et al. 1984). Figure 2 shows the exposure pathway, that how the EMF interacts with skin and organs and producing free radicals in the cells. Free radicals generation enhance the ROS formation, which may effect several metabolic, enzymatic, transcriptional activity and lead to cell death.

Maximum quantity of PKC is found in the brain hippocampus, which is an integral part of the brain's limbic system. PKC also play an important role in behavior and learning memory—the cellular mechanism believed to underlie learning and memory. Damage to neurons in the hippocampus may therefore lead to impaired learning, memory and behavioral dysfunctions. PKC is known to exist as a family of closely related subspecies, has a heterogenous distribution in brain (with particularly high levels in presynaptic nerve terminals), and together with other kinases, appears to play a crucial role in the regulation of synaptic plasticity and various forms of learning and memory (discussed later in this chapter). Studies from our group have reported the PKC activity (in whole brain of Wistar rat) is reduced significantly ($P = 0.0483$) in EMF exposed group, as compared to sham exposed. Similarly, a significant decrease in the activity of PKC in developing rat brain was recorded more in hippocampus in comparison with whole brain data (Kesari et al. 2011b; Paulraj et al. 1999). PKC activity may play an important role in EMF-induced genotoxicity, and formation of micronuclei may lead to genomic instability.

2.2 Micronuclei: Genomic Instability

Micronuclei (MN) are small, nucleus-like structures present in the cell, especially relevant in the assessment of genotoxic effect. In cell culture studies, the elevated level of micronuclei in neuronal cell (SH-SY5Y) indicates that exposure to ELF MFs may induce genomic instability (GI) (Luukkonen et al. 2014), as also reported by Kesari et al. (2015). Micronuclei are a good biomarker for the detection of GI. Kesari et al. (2015) reported MF induced genomic instability in follow-up study of 15 and 30 days after 24 h of MF exposure. Any late effects due to environmental or chemical exposure may induce GI. Moreover, induced genomic instability (IGI) has also been investigated after exposure to a non-genotoxic agent (Korkalainen et al. 2012). Therefore, genomic instability or genotoxic effect is not only caused due to induced non-ionizing radiation but also non-genotoxic agents and ionizing radiation (well-known inducer of genomic instability) (Baverstock 2000). In the animal study, micronuclei in bone marrow or peripheral blood erythrocytes are widely accepted as a sensitive predictor of the clastogenic potential of chemical and radiation exposure (Criswell et al. 1998). Markers such as micronuclei, which are biomarkers of chromosome malsegregation and/or breakage, have been investigated

in patients affected by one of several neurodegenerative disorders and in groups of subjects at increased risk of neurodegeneration (Kesari et al. 2015, 2016: Trippi et al. 2001; Thomas et al. 2007; Jaworska et al. 2002; Scott et al. 1996; Vral et al. 1996; Migliore et al. 2011). Earlier, Kesari et al. (2011a) showed a significant decrease ($P < 0.002$) in micronuclei of mobile phone exposed group as compared with control group, where a decrease was recorded by comparing the ratio of PCE (polychromatic erythrocyte) and NCE (normochromatic erythrocyte) in animal blood cells. Kumar et al. (2010a, b) also showed the causative effect by lowered percentage of PCE/NCE at two frequency level of 10 and 50 GHz of exposure. The basic phenomenon of micronuclei shows that during RBC formation, erythroblasts expel their nucleus and may also damage the chromosome in the cytoplasm of young erythrocyte (in the form of micronuclei). Due to their relatively small size, the RF-induced MN is likely to change via a clastogenic effect. Therefore, during proliferation, the cells continue to divide and cause chromosomal damage such as breaks and exchanges, which eventually lead to formation of micronuclei. The significant changes in the frequency of micronucleated PCE in the experimental group is an indication of induced chromosomal damage. MN formation occurred with the loss of chromosome fragments due to microwave radiation. Such changes are responsible for the neurodegeneration or neurological diseases in developing brain, which may also cause Alzheimer's disease (Fig. 6).

2.3 The Mitochondrial Pathway: Role in Apoptosis

Mitochondria, which is well known powerhouse of the cell has the main site of oxygen metabolism, where cell consumed approximately 85–90% of the oxygen (Chance et al. 1979; Shigenaga et al. 1994). Oxygen takes part in glucose break down in mitochondria through oxidative phosphorylation and generates energy currency of cells i.e. ATP (Harvey et al. 1999). Mitochondria are vital cell organelles that capture the chemical energy of food to form ATP in the mitochondrial respiratory chain (MRC) (Schapira et al. 2006). Any mutation in mtDNA leads to impaired ATP generation and perturbed oxidative phosphorylation cascade that may further lock the neuronal function (Guido and John 2000). Therefore, a moderate increase in ROS levels can stimulate cell growth, proliferation or apoptosis and also cause cellular injury (e.g., damage to DNA, lipid membranes, and proteins) due to which it may lead to neuronal dementia. Mitochondrial dysfunctions and finally apoptosis have been reported as pathological cause for aging and neurodegenerative diseases in many dementias such as Parkinson's disease (PD), Alzheimer's disease (AD), multiple sclerosis (MS) and amyolotrophic lateral sclerosis (ALS) (Uttara et al. 2009). Not only neuronal dysfunction, but also it is associated with a number of diseases for example including inherited mitochondrial disorders and lifestyle–related metabolic diseases, such as obesity. Obesity increases a risk of many diseases such as type 2 diabetes, cardiovascular diseases, cancers, inflammation, osteoarthritis, breathing disorders and depression, and significantly reduces the life

expectancy, up to 8–10 years in morbidly obese persons. Microwave radiation induced oxidative stress can modify the neuronal proteins and structural components in different neurological disorders leading to neuro-inflammation and loss of cognitive function in these dementias. Exposure to EMF may cause mutational changes in mitochondrial DNA in aged brain leading to oxidative stress and free radical mediated pathological changes in neurons. The cellular response to oxidative stress includes the release of mitochondrial cytochrome c and the induction of apoptosis as presented in Fig. 2.

The central role for mitochondria and cytochrome c in apoptosis was first acknowledged in a cell free system (Newmeyer et al. 1994; Liu et al. 1996). Cytochrome c released from the mitochondrial intermembrane space interacts with dATP and apoptotic protease activating factor (Apaf-1). After conformational changes enabling oligomerization of Apaf-1, the energy demanding aggregate called apoptosome is formed and recruits several procaspase-9 when in proximity becomes activated, leading to an expanding cascade of caspases, controlled digestion and degradation of the cell (Srinivasula et al. 1998; Li et al. 1997). Although the release of cytochrome c is a key event in apoptosis, the permeabilization process of the mitochondria is not fully understood. The findings of numerous Bcl-2 family members in the mitochondria raised the idea that these proteins were channel forming molecules (Muchmore et al. 1996). Although Bax oligomers can form transmembrane channels large enough for cytochrome c in experimental systems the existence of these channels in vivo remains to be conferred (Saito et al. 2000; Antonsson et al. 2000). As an alternative mechanism, Bcl-2 family proteins have been shown to regulate the opening and closing of a pre-existing channel in the outer mitochondrial membrane. This channel is called the permeability transition pore and includes the voltage dependent anion channel, the adenine nucleotide translocator and cyclophilin D (Zoratti et al. 2005). Figure 3 showing mitochondrial pathway of EMF exposure and the formation of apoptosis as discussed above.

2.4 Antioxidant Enzymes

Anti-oxidative enzymes can define the term oxidative stress. In general, antioxidants play an important role in distress of the cells or in other words, to protect the cells by oxidative damage. Oxidative damage can occur occasionally, anytime, anywhere and by any reason. Stress is the main factor for all internal causes to human body. Humans are constantly exposed to free radicals created by EMR from the manmade environment such as electro-pollution or electromagnetic-smog. Natural resources such as radon, cosmic radiation, as well as cellular metabolisms (respiratory burst, enzyme reactions) also add free radicals to the environment. The most common reported cellular free radicals are hydroxyl (OH·), superoxide ($O_2^{-}\cdot$) and nitric monoxide (NO·). Oxidative stress is a condition induced by oxygen and oxygen derived free radicals commonly known as reactive oxygen species

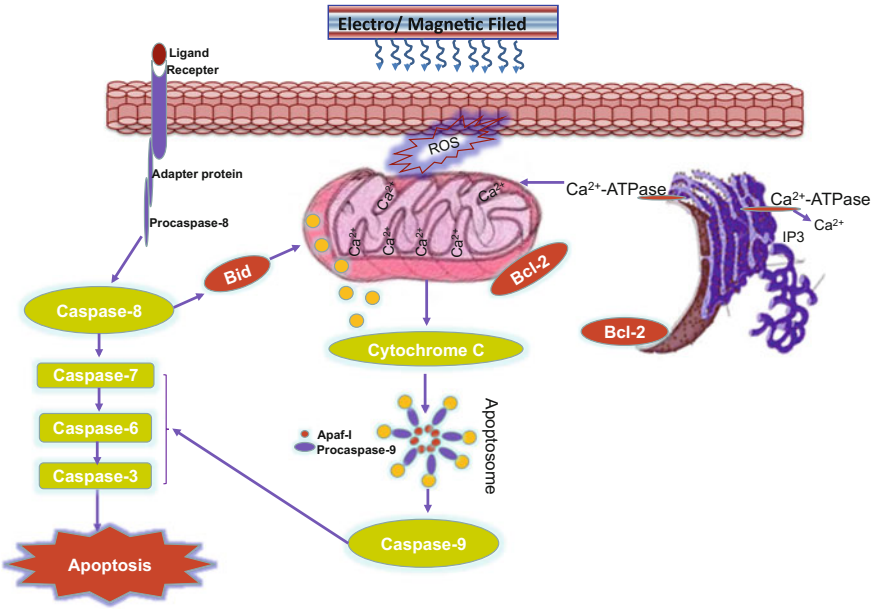

Fig. 3 One possible way in which electromagnetic fields induce changes in the apoptotic process in cells. The EMF, acting especially on Ca^{2+} ions, induces variations in ionic homeostasis. This perturbation of the Ca^{2+}, through its release from the endoplasmic reticulum and uptake by mitochondria initiates the apoptotic cascade. Through Bcl-2 action, this change in Ca^{2+} results in the release of cytochrome c from mitochondria, activation of caspase 9 along with other effector caspases and finally apoptosis or cell death

(ROS) (Schrader and Karnity 1994). ROS are particularly active in the brain and neuronal tissue as the excitatory amino acids and neurotransmitters, whose metabolism is factory of ROS, which are unique to the brain and serve as sources of oxidative stress (Uttara et al. 2009). ROS attack glial cells and neurons, which are post-mitotic cells and therefore, they are particularly sensitive to free radicals, leading to neuronal damage (Gilgun-Sherki et al. 2001).

Cellular antioxidants like superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and malondialdehyde (MDA) are important markers of free radical generation. Adequate level of cellular antioxidants (SOD, CAT, GPx and lipid peroxide (LPO) maintain the free radicals scavenging potential in brain. A dose response relationship based on these enzymes at various power levels is also reported in this chapter later. Oxidative stress is the result of an imbalance between ROS generation and intrinsic ROS scavenging activities. Therefore, melatonin has been found to restrict the effect of EMF induced oxidative damage in the cells (Meena et al. 2014). Several studies from our group investigated significant changes in the level of SOD, GPx, CAT, lipid peroxidation (Chauhan et al. 2016; Sharma et al. 2014, 2016; Kesari et al. 2010a, b, 2011a, b, 2012; Kesari and Behari 2009).

2.5 Melatonin and Calcium Ion Concentration

Recently Meena et al. (2014) have reported the defensive property of melatonin against microwave radiations. All those diseases that involve the death of specific neurons due to changes in calcium ion concentration, melatonin level, protein kinases and oxidative damage may be classified as neurodegenerative diseases. As per neurological aspects studies shows that reduced melatonin can affect the brain and this might be leading to AD as also indicated in Fig. 2. Recently Kumar et al. (2011) also reported decreased level of melatonin after 2.45 GHz exposure of Wistar rats. Pineal melatonin is a vital natural neurohormone. It is a primary signaler of the daily cycle. Hence factors of microwave exposure that alter the melatonin/serotonin cycle can affect the brain and predictably all the vital organs. Melatonin is the most potent known natural antioxidant that scavenges free radicals to protect cells throughout the body, especially brain, heart and immune system.

Calcium ions play important roles in the function of the nervous system, such as the release of neurotransmitters and the actions of some neurotransmitter receptors. Thus, changes in calcium ion concentration could lead to alterations in neural functions. Ca^{2+} ions are essential in the regulation of the resting membrane potential and in the sequence of events in synaptic excitation (Ekert and Tillotson 1978; Seeman, 1972; Shanes 1958) and neurotransmitter release (Katz and Milledi 1967; Llinas and Nicholson 1975). The cell membrane is considered as the primary site for EMF interaction within the cellular systems. RF-EMW may alter intracellular calcium homeostasis by acting on plasma membrane calcium channels (Blackman et al. 1980). Rao et al. (2008) provided evidence supporting the theory that RF-EMW affects the plasma membrane. They studied the effects of RF-EMW on calcium dynamics in stem cell-derived neuronal cells and discovered a significant increase in intracellular calcium spikes in response to non-thermal RF-EMW. The pathway of release of calcium in mitochondria and possible mechanism is presented in Fig. 3.

3 Effects of RF-EMF on Developing CNS

Neurological effects may cause due to changes in the nervous system. The factors that act directly or indirectly in the nervous system causing morphological, chemical, or electrical changes, may lead to neurological disorders. The nervous system is an electrical organ. Thus, it should not be surprising that exposure to EMF could lead to neurological changes. Developing central nervous system (CNS) is especially sensitive to radiation exposure (UNSCEAR 2000; Di Toro et al. 2005). The immature antioxidants defenses and the higher abundance of free iron found in the developing CNS, together with the high proportion of dividing neuroblasts, might be some of the reasons for the high radiosensitivity and susceptibility of developing brain. Involvement of ROS in the pathophysiology of neurodegenerative diseases and brain

injury have been reported by several authors (Ciani et al. 1996; Marzatico et al. 2000; Liu et al. 2003).

Neurons are especially vulnerable to free radical attacks. Insufficient defense with exposure to excess ROS can lead to neuronal dysfunction and neuronal death (Bilici et al. 2001; Khanzode et al. 2003). Figure 2 shows the pathway of free radicals and neuronal dysfunctions by causing DNA damage, apoptosis and cell death. Clinical and laboratory animal studies have shown that environmental conditions during early life can alter brain and behavioral development (Heim et al. 1997; Daniels et al. 2004). Therefore, the issue of mobile phone use by children and adolescents for extended period was first raised and released by 'Stewart Report' constituted by the British independent expert group in 2000 (IEGMP 2000). Expert group reported the use of cell phone near head for longer time period leads to higher exposure in the brain. Theoretical studies on EMF absorption initially indicated a larger absorption in a child's head as compared with the head of an adult (Gandhi et al. 1996; Christ and Kuster 2005). The factors associated with brain and its development either in prenatal or postnatal condition are always important. However, the early foetal period is very active phase of cortical development in the rodent brain (Morgane et al. 1992). There are few reports on the effects of the low dose irradiation at the foetal period on the adult mouse behavior (Hossain and Uma Devi 2001).

3.1 Effects of EMF Exposure on Behavior and Cognition

For a decade, ELF is found to be more effective to cause behavioral changes in animal. ELF or electro-smog (networking of electric and magnetic field) can alter growth, morphology, differentiation, death program and nerve impulse transmission in the cells (Kerr et al. 1972; Pirozzoli et al. 2003; Grassi et al. 2004). Changes in behavior and cognition are important outcomes used to assess the effects of exposure of microwaves in the brain (D'Andrea et al. 2003; Keetley et al. 2006; Papageorgiou et al. 2006). ELF MF exposure causes the behavioral changes, which may lead to Alzheimer disease as discussed later in this article. Lai (1994) has reported the neurochemical and behavioral changes due to EMF exposure in CNS. Behavioral changes especially spatial learning of rodents owes relevance to human health (Anger 1991; Gallagher and Nicolle 1993). Spatial memory is a kind of short-term memory which is responsible for recording surroundings and spatial orientation. Spatial memory formation and consolidation depends on hippocampus, which reflects the influence of external stimulus on organism (Klur et al. 2009). Microwaves exposed rats showed retarded learning, indicating a deficit in spatial and cognitive function. A well-known test, Morris water maze, in which rat learn to locate a submerged platform in a circular pool of opaque water by using cues in the environment is a behavioral paradigm has been widely used to study spatial "reference" memory of rodents. Radial arm maze is used to study working performance of rodents. Radial Arm Maze (Olton and Samuelson 1976) and Morris Water Maze

test (Morris 1984; Morris et al. 1982), have been developed to assess rodent spatial memory and learning.

Liu et al. (2003) reported decline in learning and memory is associated with a significant increase in two parameters of oxidative stress in the brain i.e. levels of lipid peroxidation and protein oxidation. The exposure to EMF may have a facilitator effect on brain functioning, especially in tasks requiring attention and manipulation of information in working memory (Koivisto et al. 2000). Studies also indicate that microwave-induced hyperthermia can impair learning and memory (Moghimi et al. 2009). The hippocampus encodes the spatial relationships between components of scenes or contexts. However, in the absence of this, animals with hippocampal lesions will not be able to form the object-place configurations that are important in episodic memory (Lee and Solivan 2008; Narayanan et al. 2009). A review by Klann and Sweatt (2008) summarizes a contemporary model proposing a role for altered protein synthesis in memory formation and its subsequent stabilization. One defining aspect of the model is that altered protein synthesis serves as a trigger for memory consolidation. Thus, they proposed that specific alterations in the pattern of neuronal protein translation serve as an initial event in long-term memory formation. These specific alterations in protein readout result in the formation of a protein complex that then serves as a nidus for subsequent perpetuating reinforcement by a positive feedback mechanism (Fig. 4). Our earlier study on adult mice (6–8 weeks) exposed to 10 GHz microwaves reported that exposure to microwave radiation causes decrements in the ability of mice to learn the special memory task. This is correlated to the altered protein synthesis or less protein synthesis during this stage of translation, which stabilizes long-term memory (Sharma et al. 2014).

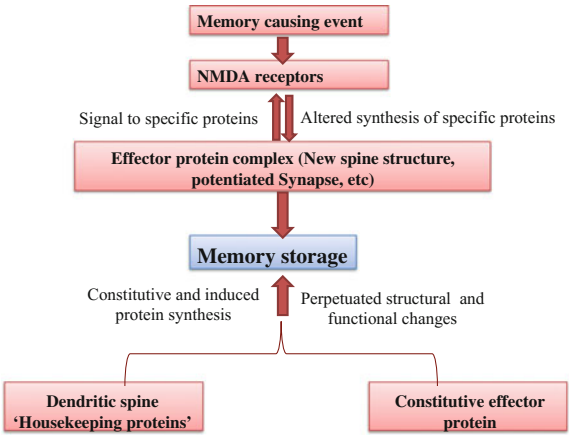

Fig. 4 Role for altered protein synthesis in memory formation and its subsequent stabilization (Klann and Sweatt 2008)

3.2 Effects of EMF on Neurotransmitters

Neurotransmitters are chemicals that carry (transmit) signals from one nerve cell to another. Neurotransmitters are released from one nerve cell and react with molecules called receptors on another nerve cell. The reaction alters the activity of the second nerve cell. Activities in nerve cell could also change the properties of these receptors (mainly by changing the concentration or the affinity of the receptors to neurotransmitters). Manikonda et al. (2007) reported effects of chronic ELF EMF exposure on N-methyl-D-aspartate receptors (NMDA) in the hippocampus of the rat brain. Salunke et al. (2013) reported that ELF EMF-induced anxiety in the rat involved NDMA receptor in the brain. There is a report on the effects of magnetic field serotonin and dopamine receptors in the rat brain (Janac et al. 2009). Changes in a subtype of serotonin receptors 5HT(2A) in the prefrontal cortex were reported. However, Masuda et al. (2011) reported that another types of serotonin receptor 5HT (1B) were not significantly affected after magnetic field exposure in an in vitro experiment. However, the 5HT(2A) receptors, particularly in frontal cortex, are related to psychiatric syndromes of depression in humans. Kitaoka et al. (2013) and Szemerszky et al. (2010) have reported depression-like behavior in the mice and rats, respectively, after chronic exposure to magnetic field. There are two reports on dopamine receptors. Sin et al. (2011) reported an increase in D-1 dopamine receptor and activity in the striatum of the rat after magnetic field exposure. Dopamine in striatum is involved in Parkinson's disease. Wang et al. (2008) reported that ELF MF potentiated morphine-induced decrease in D-2 dopamine receptor. The implication of these data is not readily clear. Both D-1 and D-2 dopamine receptors in the brain are involved in depression and drug addiction. However, study on the cholinergic system by Ravera et al. (2010) reported changes in the enzyme acetylcholinesterase in cell membrane isolated from the cerebellum after magnetic field exposure. Interestingly, these researchers also reported 'frequency window' effects in their experiment. Window effects could be observed at a certain range(s) of EMF frequency or intensity. Study by Fournier et al. (2012) reported 'intensity window' effect of ELF magnetic field on neurodevelopment in rat. The cholinergic systems in the brain play a major role in learning and memory functions.

3.3 Histopathological Alterations in Brain Induced by EMF

The possible effect of RF exposure on nervous system has prompted investigations with animal model mostly focusing on biochemical and morphological alterations. Neuronal damages in cortex, hippocampus, cerebellum, and basal ganglia due to RF exposure have also been reported earlier (Mausset et al. 2001; Salford et al. 2003). Results from our research group also suggested that the reduction in number of pyramidal cells and cerebral cortex of neuronal cells after microwaves exposure might be due to the radiosensitive nature of the cells. Salford et al. (2003) reported

highly significant ($p < 0.002$) evidence for neuronal damage in the cortex, hippocampus, and basal ganglia in the brain of exposed rats to GSM mobile phone of different strengths. The noxious effects of radiation on the cerebellar cortex have been reported by Sisodia and Singh (2009). The hippocampus and olfactory bulb are two structures of CNS continuing neurogenesis after birth. Thus perfect operation of these structures should be affected by neurogenesis (Bruel-Jungerman et al. 2007). Bas et al. (2009) demonstrated that postnatal exposure to 900 MHz EMF reduced the number of pyramidal cells in the cornu ammonis (CA) of the female rat hippocampus. Consequently, Sonmez et al. (2010) determined that a long term EMF exposure may lead to reduced purkinje cells number in female rat cerebellum. Bagher et al. (2008) exposed BALB/c mice to 50 Hz, 0.5 mT EMF for 4 h per day, 6 days per week for 2 months. They concluded that long term exposure to EMF has detrimental effects on the morphological changes of neurons of the frontal cortex and may lead to degenerative phenomenon on pyramidal cells.

Most of the studies done in hippocampus are focused on CA1 region. Figure 5 shows pyramidal neuronal cells in CA1 region of mice hippocampus. Region CA1 receives input from the CA3 subfield, EC layer III and the nucleus reuniens of thalamus (which project only to the terminal apical dendritic tufts in the stratum lacunosum-moleculare). In turn, CA1 projects the subiculum as well as sending information along the aforementioned output paths of the hippocampus. Dorsal CA1 and dorsal CA3 sub regions of the hippocampus have been shown to play an important role in mediating temporal order memory for spatial location information. Histopathological changes in CA1 region were observed in our studies after microwaves exposures in mice brain are in a line with results of Miranda et al. (2006), where they showed that a functional hippocampus is required for the acquisition of spatial tasks in the Morris water maze. Current models of memory consolidation

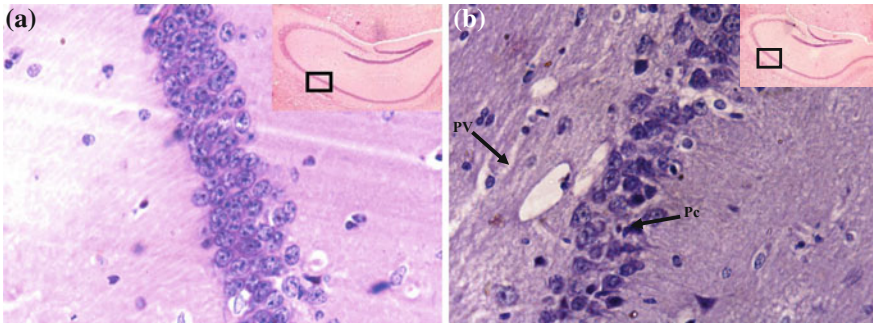

Fig. 5 The effects of 10 GHz microwaves exposure for 30 days (2 h/day) in the diencephalon region of hippocampus. **a** Sham exposed group: the hippocampal neurons and vessels exhibited a regular arrangement, with distinct edges, clear nucleus and nucleolus, and no significant necrosis of pyramidal neurons. **b** Microwaves exposed group: reduced density of pyramidal cells with edema, and neurons exhibited pyknosis (P) and anachromasis with widened perivasicular space (PV). (HE staining, original magnification × 400)

(Dudai 2004; Nader 2003) assume that the storage of long-term memory (LTM) is associated with gene expression, new protein synthesis, and synaptic remodeling. The CA1 region also appears to be involved in retrieval after longer time delays, with rats lessoned in the CA1 region having no difficulty in encoding new information but impaired in retrieval after 24-h of interval (Jerman et al. 2006; Vago and Kesner 2005). Evidences reviewed elsewhere (Rolls and Kesner 2006) indicates that the CA1 region makes a special contribution to the temporal aspects of memory, including associations over a delay period, sequence memory and order memory. The CA1 network is thought to play an important role in retrieval of information to the neocortex, consequently affecting others parts of the brain involved in guiding behavior. Maskey et al. (2010) investigated the effect of RF exposure on rat hippocampus by using both CB (Calbindin) and glial fibrillary acidic protein (GFAP) specific antibodies. The immune-histochemical result shows decrease in CB immuno-reactivity (IR) with the loss of interneurons and pyramidal cells in CA1 region as well as granule cells. Also, an increase in GFAP IR was observed in the hippocampus of E1.6. The change of reactive astrocytosis, which commonly, precedes neuronal death (Petito and Halaby 1993) also supported by Ammari et al. (2008).

4 EMF Links to Alzheimer Disease

Alzheimer's disease (AD) is a most common progressive neurodegenerative disorder of the brain, where Przedborski (2003) reported about the process for the loss of structure and function of neurons. However, if these neuronal changes cannot be compensated may lead to neurodegenerative disease. There are several factors, which are responsible for such disease. One of the important factors is ELF-MF, which is a part of occupational as well as environmental exposure. Several in vivo, in vitro and epidemiological studies have been carried out on manmade as well as natural exposure conditions. The risk in population living near power lines, in electrical occupations and in other groups exposed to ELF-MF have been investigated. The epidemiological studies have provided evidence that exposure to ELF magnetic fields is associated with increased risk of AD (e.g., Håkansson et al. 2003; Feychting et al. 2003; Hug et al. 2006; Huss et al. 2009; García et al. 2008; Röösli et al. 2007). Interestingly these epidemiological associations have been reported at very low magnetic field levels (of the order of 1 μT), much lower than the exposure guidelines (100–500 μT). Therefore, it is important to determine whether the epidemiological findings reflect a true causal relationship with ELF. Most of the epidemiological research on occupational exposure focused on frequency ranging between 3 and 3000 Hz and primarily to ELF-MF (50–60 Hz). Based on these frequencies and certain intensities, there are several studies suggesting that ELF MFs affect the nervous system in humans and animals (e.g., Lyskov et al. 1993a, b, 2001; Fu et al. 2008; Liu et al. 2008; Falone et al. 2008), but considering this the evidence is partly inconsistent and the relevance of the findings to AD is not so well known.

When we talk about biological effect due to MF exposure at the frequency 50/60 Hz, it does not transfer energy to cells in sufficient amounts to directly damage DNA. A possible mechanism of interaction between MF exposure and biological damage is a process where involvement of free radical may have derived from oxygen metabolism is known as ROS. Therefore, a moderate increase in ROS levels can stimulate cell growth and proliferation and also cause cellular injury (e.g., damage to DNA, lipid membranes, and proteins) due to which it may lead to produce neuronal dementia. However, there are several other end points (such as, increased oxidative stress, accumulation of Aβ, mitochondrial dysfunction, DNA damage) which are impetus for apoptosis in AD (Canu and Calissano 2003; Mattson and Magnus 2006). Other potential pathways, which may involve in relationship between ELF-MF and AD include apoptosis and necrosis in brain cells. The pathway for EMF exposure and AD is presented in Fig. 6. Researchers also proposed possible hypothesis that ELF-MF affects the cell membrane structure and permeability to small molecules (Baureus et al. 2003; Grassi et al. 2004; Marino et al. 2003).

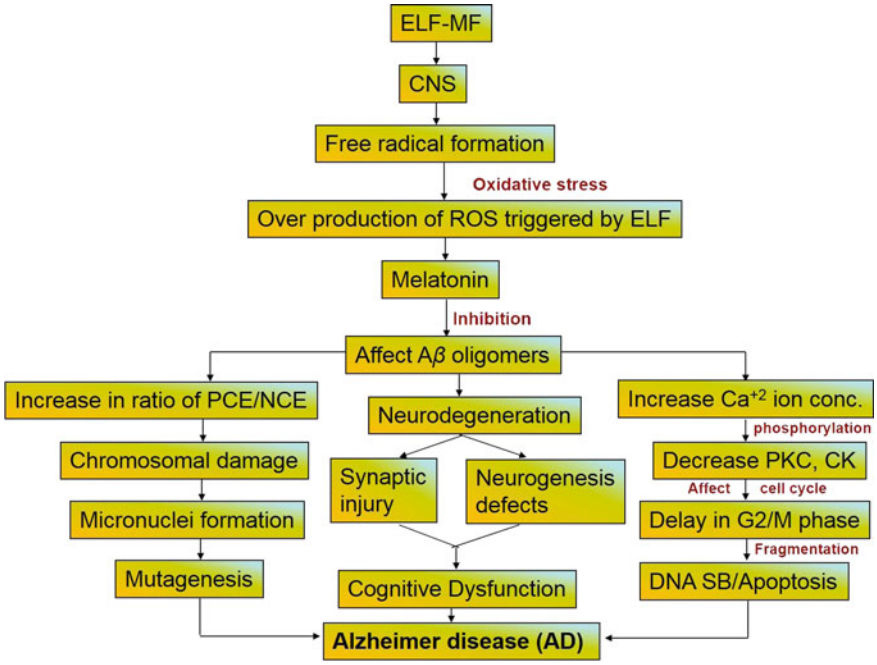

Fig. 6 Interaction mechanism between free radical formation and cell function due to radiofrequency/microwave radiation exposure on CNS. The pathway shows that enhanced ROS due to ELF-MF exposure can cause several changes at enzymatic and hormonal level, which may result Alzheimer disease. ELF induced ROS formation can increase the genotoxic level by increasing micronucleus formation, affect Aβ oligomers and cause neurodegeneration due to which cognitive dysfunctions occur and cause AD. Also another hypothesis shows increased calcium ion concentration and decreased protein kinases (i.e. PKC, histone kinases), delay G2/M phase (DNA synthesis phase) and damage DNA due to which it may transform into AD

Therefore, ELF-MF probably interfere with chemical reactions (O_2, H_2O_2, OH) involves free radical production (Simko and Mattsson 2004). Further, Falone et al. (2007) reported changes in redox and or differentiation status in neuroblastoma cells after short term MF exposure. Indeed, since last few years due to ELF exposure, several data show the redox-related cellular changes (Regoli et al. 2005; Wolf et al. 2005; Zwirska-Korczala et al. 2005). Also study from Katsir and Parola (1998) reported an increase in cell proliferation. Authors concluded that this was due to higher exposure over the frequency (50–100 Hz) and intensity (0.1–0.7 mT) range, where 70% increase in proliferation was recorded with exposure to 100 Hz at 0.7 mT. Though the study presented here suggest the effects due to ELF-MF also depend on exposure parameters like field densities, intensities, modulation and dose response relationship between EMF and biological parameters.

5 Exposure Response Relationship

It is always worthy to discuss about the dose response relationship between EMF exposure and biological effects. The levels of dose for every purpose are very important factor. Recently, Kesari et al. (2016) conducted cell culture studies on two different neuronal cell lines and field intensities (10, 30 and 100 μT). Authors showed strong dose effect at higher field intensity but also suggest that the threshold, if it exists, for biological responses to 50 Hz MFs is of lower intensity at 10 μT or less. From our previous findings at several field intensities (900 MHz, 2.45 and 50 GHz) it is aimed to investigate the dose response relationship between RF-EMF exposure and brain dysfunctions. Overall, the data of this study (Fig. 7a–c) is adopted from our previous findings of Kesari and Behari (2009) and Kesari et al. (2010a, 2011b). Dose response effects on antioxidant enzymes were mostly consistent with a conventional exposure-response relationship: when significant effects were observed, the point estimates of the effect size increased with increasing microwave field strength (900 MHz, 2.45 and 50 GHz).

5.1 Experimental Data Evaluation

For easier judgment of the exposure-response relationships, the data on SOD (Fig. 7a), GPx (Fig. 7b) and CAT (Fig. 7c) were plotted as a function of microwave frequencies. Relative value in RF-EMF-treated group was calculated by dividing the value observed in RF-EMF group by the value observed in control group. SOD, GPx and CAT showed a rising trend of effect size with increasing microwave frequencies in whole brain (Fig. 7a–c). In SOD and GPx, the RF-EMF effect was more in 50 GHz of microwave-exposed group by compared with 900 MHz and 2.45 GHz of exposure group. As described above, this effect was significant at all three frequencies in the

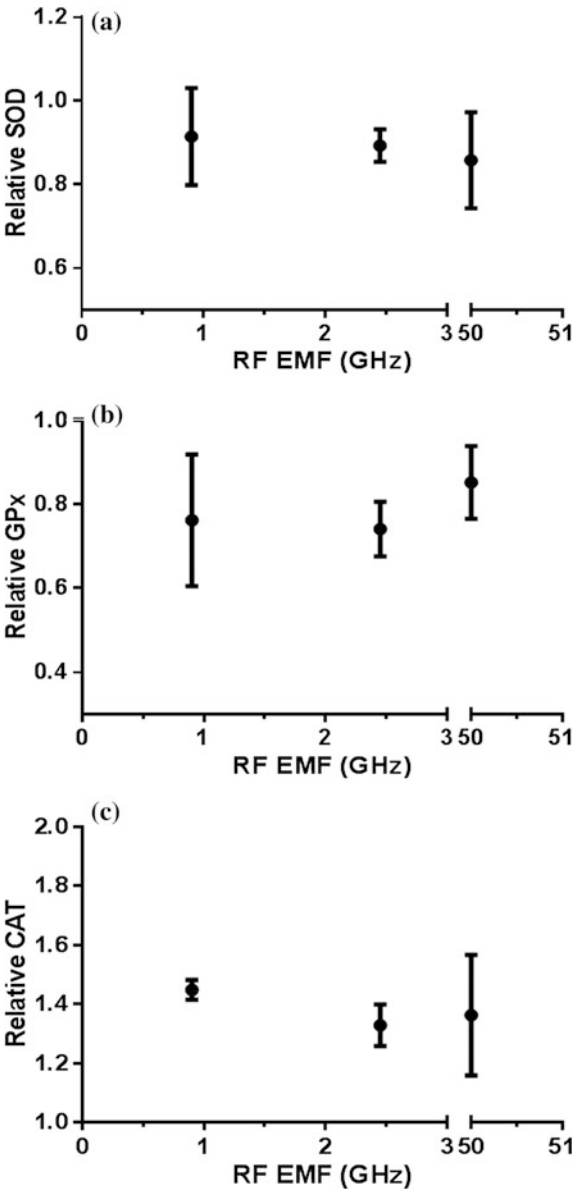

Fig. 7 Exposure-response relationship for antioxidant enzymes, superoxide dismutase (**a**), glutathione peroxide (**b**) and catalase (**c**) in whole brain of male Wistar rat, exposed to 900 MHz (or 0.9 GHz), 2.45 GHz and 50 GHz RF-EMF. The data are given as relative values (value observed in RF-EMF exposed sample divided by the value measured in corresponding non-exposed sample), with 95% confidence intervals

previous studies, where significant ($p < 0.05$) decrease in the level of brain GPx, SOD and increase in the level of catalase activity were investigated in exposed group by comparing with control ones (Kesari and Behari 2009; Kesari et al. 2010a, 2011b). Including these studies, several associated dose response relationship is also discussed in this manuscript to explore the mechanism of field interaction.

Lai and Singh (1995) first reported on dose-dependent changes at DNA level induced by low intensity microwave RFR. A dose-dependent increase in DNA single and double strand breaks in brain cells (exposed at 0.6 and 1.2 W/Kg whole body specific absorption rate) were found after two hours of exposure to 2450 MHz RFR. Several other studies also suggested that microwave induced oxidative stress is able to cause DNA damage in sperm cells (Meena et al. 2014; Kumar et al. 2014) and increased the level of micronuclei at various power densities (Kesari et al. 2011b; Kumar et al. 2010a, b). The results of Ivancsits et al. (2002, 2003a, b) indicate that the interaction of these fields with DNA is quite complicated and apparently depends on many factors, such as the mode of exposure, the type of cells, and the intensity and duration of exposure. Recently, Jankovic et al. (2014) also investigated that the nature and extent of the effect depend on the frequency of microwaves and the total energy absorbed by the microorganisms was dose dependent. Authors reported that low energy, low frequency microwaves enhance the growth of microorganisms, whereas high energy, high frequency microwaves destroy the microorganisms. Therefore, it is obvious to say here that the biological effect of RF-EMF mainly depends on the exposure level, duration of exposure, and the position or organ of body that was exposed to RF Radiation.

6 Conclusion

In light of present debate, we have concluded that neurophysiological and behavioral dysfunctions are affected by EMF exposure. The effects could be measured in terms of one or more of the several biomarkers like protein kinase C, micronuclei, mitochondrial pathways, melatonin, calcium ion concentration, antioxidant enzymes like glutathione, superoxide dismutase, and catalase. Therefore, we hypothesize that any tumor promoting effects of RF-EMF might be due to the effect it has on these biomarkers which may accelerate neuronal cell death and promote neurodegenerative processes (AD) or brain carcinogenesis (Fig. 2). This study also concludes that the dose response relationship is an important factor in an association between RF-EMF and neuronal dysfunction. This leads to a possible conclusion that the effects of RF EMF are cumulative and dose dependent in terms of exposure time and field strength.

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LPO and ROS Production in Rat Brain Exposed to Microwaves: Computational Elucidation of Melatonin in Repair System

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Abstract It is widely accepted that non-ionizing electromagnetic fields are present in the environment and are alarming as a major pollutant or electro-pollutant for health risk. The present study aimed to investigate the protective measures of melatonin against exposure of microwave radiations. Study also explored the mechanistic correlation among microwave radiation, melatonin and biological effects by computational method. For this, 60-day-old male Wistar rats were divided into four groups (n = 4/group): sham exposed (control), Melatonin (Mel) treated (2 mg/kg), 2.45 GHz microwave (MWs) exposed and MWs + Mel treated. Exposure took place in Plexiglas cages for 2 h a day for 35 days where, power density (0.2 mW/cm^2) and specific absorption rate (SAR-0.14 W/kg) were estimated. Results show that melatonin prevent oxidative damage biochemically by significant decrease ($p < 001$) the levels of lipid peroxide (LPO) and

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reactive oxygen species (ROS) in the brain. However, exposure of microwave individually shows significant changes in LPO and ROS level. The effective dose of melatonin was validated by in silico method and which reveals the interaction of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) Eenzmyes of Central Nervous System (CNS) with melatonin. Where, AChE showed better interaction with the binding energy of −9.01 kcal/mol and inhibition constant 3.11 uM by comparing with BuChE. These results concluded that the melatonin has strong antioxidative potential against microwave radiation, which could be achieved by an implementation of computational approach.

Keywords LDH-X · Apoptosis · ROS · Microwave radiation · Cell cycle

1 Introduction

Fast growth in the field of telecommunications has led to markedly increase by a parallel increase in electromagnetic field (EMF) density. Human exposure to radiofrequency (RF) EMF occurs wherever electric and/or magnetic fields are generated, transmitted or used by cell phones or its towers or emitted from various electronic gadgets. There has been an increasing concern about the potential hazards of microwave radiations and possible health outcomes of electric and magnetic fields are being discussed. Although, the primary mechanism of interaction between such fields and living matter (brain or neuronal cells, sperm cells etc.) is unknown. In 2011, the International Agency for Research on Cancer (IARC) classified RF-EMF as "possibly carcinogenic to humans" (Group 2B) (Baan et al. 2011). Carcinogenicity has a significant link between long term RF-EMF exposure and cancer risk (Coureau et al. 2014; Hardell and Carlberg 2013; Hardell et al. 2013a, b, c; Mead 2008).

The effects of EMFs emitted by mobile phones on the central nervous system (CNS) have become a particular focus of concern owing to the fact that mostly mobile phones are kept near head during talking mode and or in close proximity to the brain (Mausset et al. 2001, 2004; Odaci et al. 2008). Therefore, the absorption of SAR is always more in brain and also found more sensitive to microwave exposure. In 1998, the International Commission on Non-Ionizing Radiation Protection (ICNIRP) released guidelines and reported that the specific absorption rate (SAR) of mobile phones could be legally limited to 2.0 W/kg (ICNIRP 1998). Schönborn et al. (1998) investigated that RF-EMFs emitted by cell phones are absorbed in the brain within a range that could influence neuronal activity. Microwave radiations are potentially strong to penetrate the cranium, and nearly 40% of these can reach deeper into the brain (Barnett et al. 2007; Kang et al. 2001), where penetration depth is assumed to be 4–5 cm deep into the brain (Dimbylow and Mann 1994; Rothman et al. 1996). Several studies from our group based on in vitro and in vivo model show the fact that MF (magnetic field) or RF-EMF exposure causes neurological damage (Kesari et al. 2010, 2011, 2013, 2014, 2015, 2016). Consequently, an increased blood-brain barrier permeability and oxidative

damage, which are associated with brain cancer and neurodegenerative diseases, have also been reported by many researchers (Xu et al. 2010; Zhao et al. 2007; Nittby et al. 2009; Awad and Hassan 2008; Leszczynski and Joenvaara 2002).

Therefore, the aim of the present study was to further explore the computational elucidation of melatonin in repair system induced by microwave radiation exposure. In this study, we selected melatonin which is reported as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitor used in the microwave induced cancer treatment. The knowledge of AChE and BuChE structure is essential for understanding its high catalytic efficacy and the molecular basis for the recognition of ACh by other ACh-binding protein (ACh receptors). Therefore, we have selected these enzymes to see the interaction pattern analysis of melatonin. For the validation of present study, the structural interaction by introducing computational approaches explores the binding/inhibition pattern of melatonin with AChE and BuChE enzymes. Considering the current evidences, we have examined two most relevant brain biomarkers like, lipid peroxidation (LPO) and production of reactive oxygen species (ROS). Computational elucidation of melatonin in repair system has also been considered by using docking method.

2 Methodology

2.1 Animal Exposure

Sixty-day-old male Wistar rats (180 ± 10 g) were obtained from an animal facility of Jaipur National University, Jaipur, Rajasthan, India. Animals were divided into four groups: sham exposed, melatonin (Mel) treated, 2.45 GHz microwave (MWs) exposed and MWs + Mel treated (n = 4 in each group). All the experiments were performed during day time and the melatonin was applicated intraperitoneally (2 mg/kg) every morning at 08.00 AM to avoid its effects as neurotransmitter and/or neuromodulator (Drago et al. 2001). Several other researchers used the same dose of melatonin in their studies (Koc et al. 2003; Taysi et al. 2008; Sokolovic et al. 2008). Similarly sham exposed animals were injected with phosphate buffered saline (PBS) as control. All animals were housed in an air-conditioned room, where the temperature was maintained at 25–27 °C, with constant humidity (40–50%) and kept with constant 12-h light and 12-h dark schedule throughout the experiment. The animals were fed standardized normal diet (Tetragon Chemie Private Limited, Bangalore) and provided with water ad libitum. The amount of diet consumed and animal weight were recorded on a daily basis. The protocols for animal experimentation described in the present study were approved by the Institutional Animal Ethical Committee and Committee for the Purpose of Control and Supervision of Experiments on Animals. All subsequent animal experiments were followed as per the “Guidelines for Animal Experimentation” of the University.

2.2 Exposure Chamber

Rats were placed in a Plexiglas cage ventilated with holes of 2 mm diameter on the walls. This was designed so that each animal could be lightly restrained in a fixed position with proper ventilation. Exposure was carried out in an anechoic chamber. All animals were facing horn antenna and the field relative to the long axis of the cages were vertically polarized. The horn antenna gain was 15 dBi calibrated. Anechoic chamber was lined with radar absorbing material (attenuation, 40 db) to minimize the reflection of scattered beam. Rats were exposed to 2.45 GHz radiation source. The temperature in the chamber was maintained around 25 °C throughout the experiment. The animals were exposed to radiation for 2 h per day for 35 days at 0.2 mW/cm^2 power density. The power density was measured within animal cage in the anechoic chamber. The whole body specific absorption rate (SAR) was estimated to be 0.14 W/kg following the method of Durney et al. (1984). For the measurement, power coupled through inner wire was detected by the detector crystal and measured by a power meter (Model-U2000 series USB power sensors, Agilent Technologies), which is a peak sensitive device. The microwave generated power through a series of power generators releasing power through horn antenna as indicated in Fig. 1. Similar exposure setup was used earlier by Chauhan et al. (2016). Each day, the cage was placed in the same position facing the horn antenna. Similar experiment was performed with sham exposed animals without energizing the system.

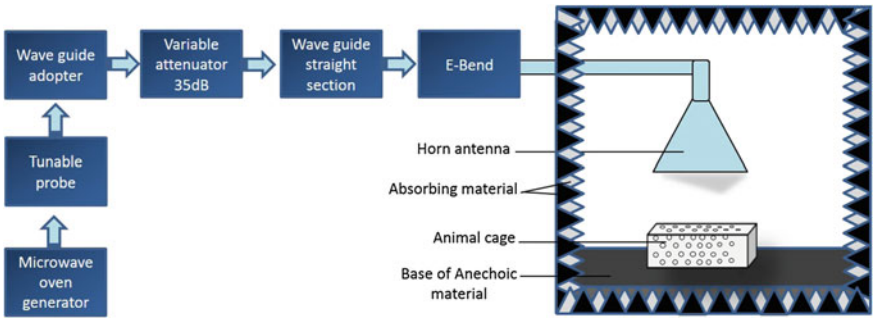

Fig. 1 Schematic diagram of exposure setup with animal cage indicating individual animal's position. Microwave oven was opted here as radiating source (microwave generator) and the generated microwave power further taken into tunable probe and RF coaxial cable transmission line and then power was released through horn antenna. The horn antenna face have wide dimensions, where the cage was placed in the same line of the horn antenna face

2.3 Lipid Peroxidation (LPO) Assay

Lipid peroxidation was measured by the method of Buege and Aust (1978). One gm of the tissue was homogenized in 9 ml of 1.15% KCl. The tissue homogenate (0.8 ml) was mixed with 1.2 ml of trichloroacetic acid (TCA) (15% w/v), thiobarbituric acid (TBA) (0.375% w/v) and hydrochloric acid (HCl) (0.25 N) solutions (Himedia, Mumbai, Maharashtra, India), prepared in a 1:1:1 ratio. The mixture was heated in a boiling water bath for 30 min. Samples were centrifuged at 1000 g for 10 min. After centrifugation, the absorbance was recorded at 532 nm by using Ultra Violet-Vis Double Beam Spectrophotometer (Double Beam Spectrophotometer 2203, Systronics, Ahmedabad, Gujarat, India). A standard curve was prepared by using tetra-methoxy-propane (TMP: purchased from Himedia, Mumbai, Maharashtra, India). After comparison with a standard curve the LPO level was expressed in nmol/gm tissue.

2.4 Reactive Oxygen Species (ROS)

0.1 g of whole brain tissue was homogenized with addition of 1:9 (W/V) phosphate buffered saline (0.1M Na_2HPO_4, 0.1M KH_2PO_4, 1.37M NaCl, 2.7mMKCl, pH 7.4). The homogenates were centrifuged at 14,000 rpm for 10 min at 4 °C (Eppendrof centrifuge 5418 R, Germany) and supernatant was collected for ROS measurement. ROS assay was performed (immediately after centrifugation) by employing DEPPD staining (Hayashi et al. 2007). ROS levels in sample was calculated from the calibration curve of H_2O_2 and expressed as equivalent to levels of hydrogen peroxide (1 unit = 1.0 mg H_2O_2/l). Calibration curve for the standard solution was obtained by calculating slopes from optical density graph.

2.5 Preparation of Drug Enzymes 3D Structures

Ligand file of melatonin was downloaded in *mol* format (Fig. 2) from Chem Spider Chemical Database (Pence and Williams 2010). We have also converted it into *pdb* files. The crystal structure of AChE (PDB ID: 3LII) (Fig. 3) and BuChE (PDB ID: 1P0M) (Fig. 4) was obtained from Protein Data Bank. Published structures were edited to remove HETATM and water molecules, also further the ligands and enzymes were submitted for CHARMm (Brooks et al. 2009) energy minimization protocol using AMBER force field by Chimera tool Pettersen et al. (2004).

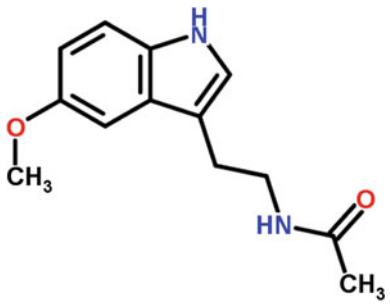

Fig. 2 2D structure of melatonin

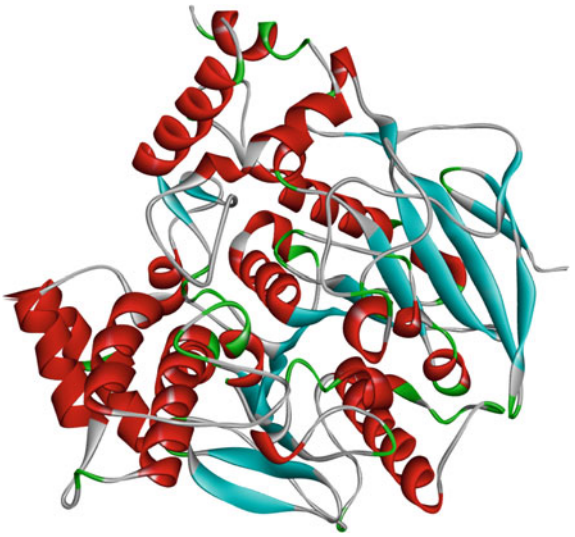

Fig. 3 Human Acetylcholinesterase (*AChE*) PDB ID-3LII

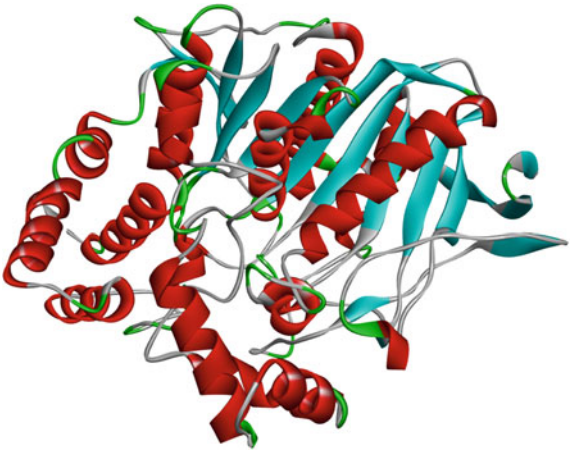

Fig. 4 Human Butyrylcholinesterase (*BuChE*) PDB ID-1P0M

2.6 Molecular Interaction Analysis

The molecular interaction of AChE and BuChE enzymes with melatonin were analysed through computational docking studies.

Autodock 4.2 (Morris et al. 2009) and Cygwin interface was used to dock melatonin on binding site of the enzymes. Molecular docking methods followed by searching the best conformation of enzyme and melatonin complex on the basis of binding energy and inhibition constant. Kollman united charges and salvation parameters were added to the enzymes. Gasteiger charge was added to the melatonin compound. Grid box was set to cover the maximum part of enzymes and melatonin. The co-ordinates were set to 60 × 60 × 60 Å in X, Y and Z axis of a grid point. The default grid points, spacing was 0.375 Å. Lamarckian Genetic Algorithm (LGA) (Goodsell et al. 1996) was used for enzymes-ligands flexible docking calculations. The LGA parameters like population size, energy evaluations, mutation rate, crossover rate and step size were set to 150, 2,500,000, 27,000, 0.02, 0.8 and 0.2 Å, respectively. The LGA runs were set at 50 runs. The generated conformations of enzymes and melatonin were analyzed using Discovery Studio 4.5 molecular visualization software for the interactions and binding energy of the obtained docked complex structure (Ali et al. 2016).

2.7 Statistical Analysis

As a balanced factorial design was used in this study, factorial ANOVA was performed with MW (or sham MW) and Mel. as fixed factors and replicate as random factor. The interactions MW * Mel. (or sham MW * Mel.) were included in the model. The general linear model procedure of SPSS (IBM-SPSS version 24) was used for the analysis.

3 Results

3.1 Lipid Peroxidation

The increased level of LPO was recorded significantly ($p < 0.05$) in the brain tissue of rats exposed to MWs as compared to control and Mel group (Fig. 5). While the melatonin treated group showed significant reduction ($p < 0.05$) of LPO level in brain tissue of animals that were exposed with 2.45 GHz microwave radiation. This suggested MW-related decrease was most obvious in the groups exposed to melatonin (Mel), (Fig. 5), and the Mel * MW interaction was statistically significant.

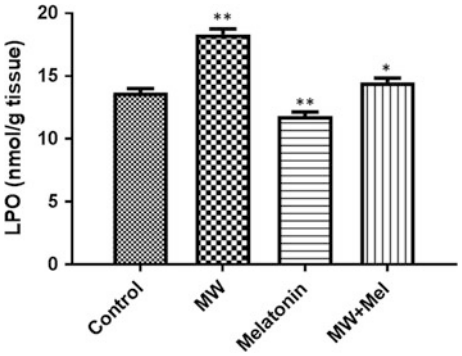

Fig. 5 Lipid peroxidation level in whole brain exposed to 2.45 GHz microwave radiation. The microwave (MW) treatment was combined with melatonin (Mel.). The data shown are mean ± SEM values. Factorial ANOVA was used for statistical testing; statistically significant effects for the two factors (MW and Mel.) and their interactions are reported below: Brain, after 35 days of exposure: MW $p < 0.001$; Mel. $p < 0.001$; and MW * Mel $p = 0.010$. The significances for the MW effects are given in the figure: *$p < 0.05$; **$p < 0.001$

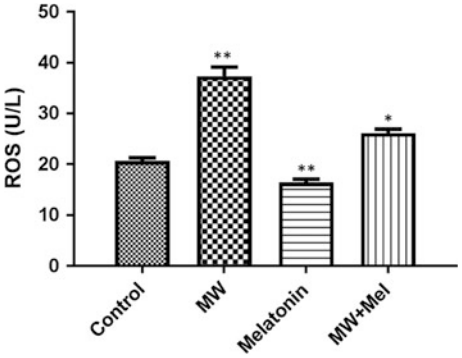

Fig. 6 Reactive oxygen species level in whole brain exposed to 2.45 GHz microwave radiation. The microwave (MW) treatment was combined with melatonin (Mel.). The data shown are mean ± SEM values. Factorial ANOVA was used for statistical testing; statistically significant effects for the two factors (MW and Mel.) and their interactions are reported below: Brain, after 35 days of exposure: MW $p < 0.001$; Mel. $p < 0.001$; and MW*Mel $p = 0.012$. The significances for the MW effects are given in the figure: *$p < 0.05$; **$p < 0.001$

3.2 Reactive Oxygen Species (ROS)

The level of reactive oxygen species was recorded significantly higher ($P < 0.001$) in the brain tissue of rats, exposed to microwave radiation as compared to sham exposed and Mel group (Fig. 6). Whereas, melatonin treatment in irradiated animals were showed significant reduction ($p < 0.05$) in ROS level as compared to irradiated animals. This suggested MW-related decrease was most obvious in the groups exposed to melatonin (Mel), (Fig. 6), and the Mel*MW interaction was statistically significant.

Table 1 Docking results obtained from AChE and BuChE melatonin interaction complex

S. No	Protein-drug complex Name	H bonds	H bonds length	Final intermolecular energy (vdW + Hbond + desolv energy) (kcal/mol)	Inhibition constants (uM)	Residues involved in hydrophobic interaction
1.	AChE-melatonin	A:TYR124:HH—:UNK1:N3	2.23404	−9.01	3.11	Gln71, Tyr72, Val73, Asp74, Trp86, Asn87, Gly120, Gly121, Tyr124, Ser125, Gly126, Tyr133, Glu202, Tyr337, His447, Gly448
		:UNK1:H9—A:GLY120:O	2.061			
		:UNK1:H33—A:TYR72:O	1.95213			
		A:GLY448:CA—:UNK1:O16	3.54374			
		:UNK1:C7—A:SER125:OG	3.14395			
		:UNK1:C4—A:TRP86:O	3.48324			
		:UNK1:C17—A:HIS447:O	3.38297			
2.	BuChE-melatonin	A:HIS438:HE2—:UNK1:N3	2.19484	−7.89	20.46	Trp82, Gly115, Gly116, Gly117, Tyr128, Glu197, Ser198, Ala199, Trp231, Leu286, Ala328, Phe329, Phe398, Met437, His438, Gly439, Tyr440
		:UNK1:H9—A:GLU197:OE1	1.84661			
		:UNK1:H33—A:SER198:OG	1.9853			
		:UNK1:C17—A:HIS438:O	3.08519			

3.3 *In Silico Docking (AChE and BuChE)*

The in silico results obtained by docking analysis are documented in Table 1. The molecular docking results reveal that, drugs exhibited interactions with AChE and BuChE, and obtained binding energies are −9.01 and −7.89 kcal/mol respectively followed by inhibition constant i.e. 3.11 and 20.46 uM respectively (Table 1).

The interaction reveals that Gln71, Tyr72, Val73, Asp74, Trp86, Asn87, Gly120, Gly121,Tyr124, Ser125, Gly126, Tyr133,Glu202, Tyr337, His447 and Gly448 amino acid residues (Fig. 7) are playing crucial role in the hydrophobic interaction with AChE while the amino acid residues Trp82, Gly115, Gly116, Gly117, Tyr128, Glu197, Ser198, Ala199, Trp231, Leu286, Ala328, Phe329, Phe398, Met437, His438, Gly439 and Tyr440 are interacted with BuChE (Fig. 8).

We have also seen seven hydrogen bonds interaction with AChE. On the other hand four hydrogen bonds interaction with BuChE (Table 1).

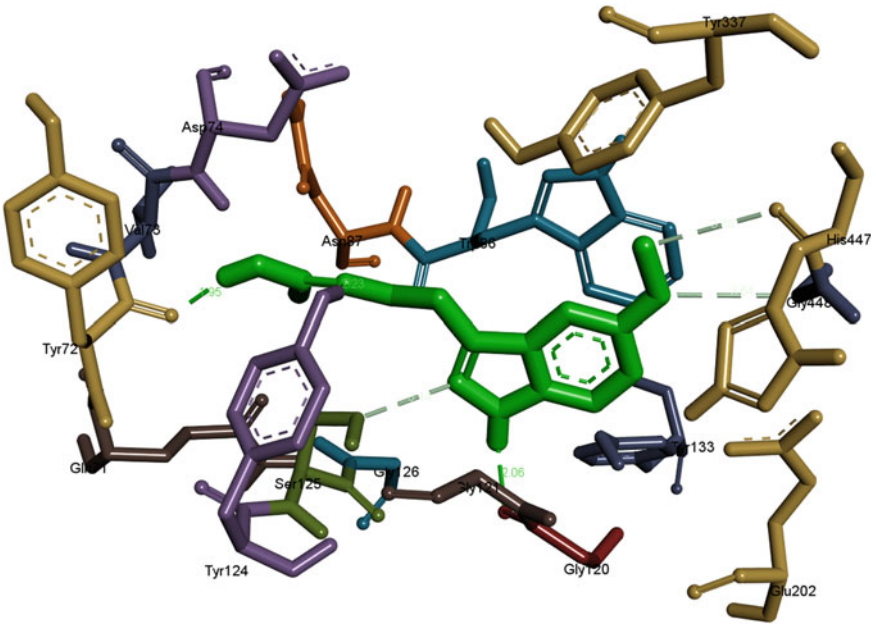

Fig. 7 AChE Amino acids involved in hydrophobic interaction. Hydrogen bonds shown by *green dotted lines*

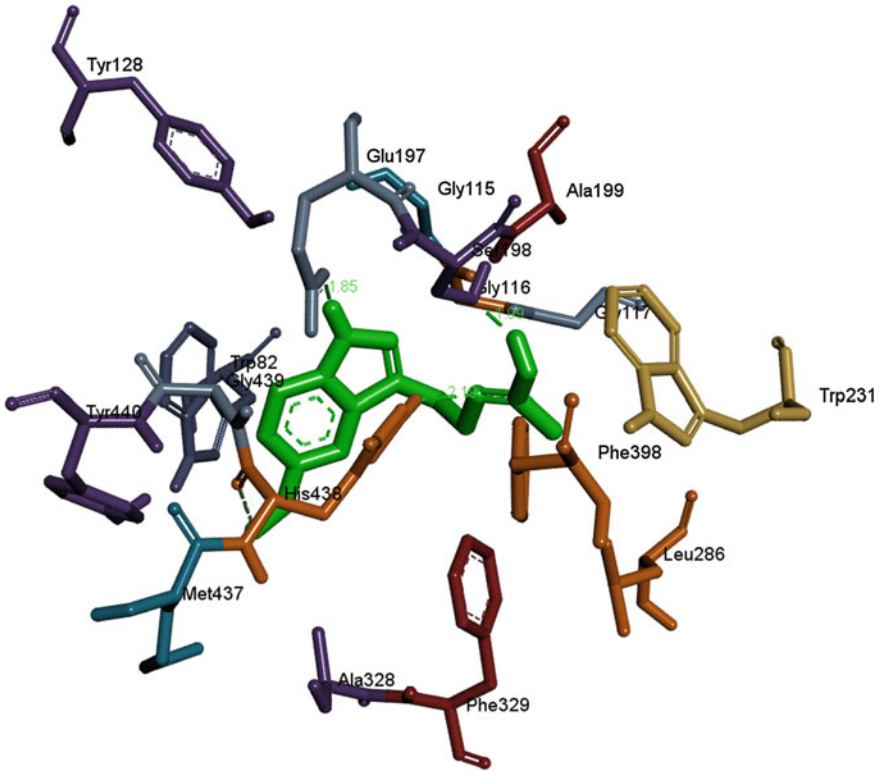

Fig. 8 BuChE amino acids involved in hydrophobic interaction. Hydrogen bonds shown by *green dotted lines*

4 Discussion

In the experiment conducted to confirm the effects of melatonin, expected effects were observed: LPO and ROS levels were decreased. Similarly, decreased ROS was seen in the sperm cells after melatonin treatment (Meena et al. 2014). The study also demonstrated that exposure of microwave alone causes oxidative damage biochemically by increased levels of LPO and ROS. LPO triggers the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, structural damage to DNA, and cell death (Halliwell 1994). The formation of free radicals significantly enhanced the ROS which indicates pathological changes in the brain tissue. There is evidence of free radical generation after RF-microwave exposures (Phillips et al. 2009; DeIullis et al. 2009; Kesari et al. 2012a, b). The increased level of LPO and ROS measured in microwaves exposed group is blocked by melatonin. This has been investigated by showing an interaction between microwave exposed group and melatonin treated group. Interestingly, the results show that melatonin has potential to decrease the increased level of LPO and

ROS after microwave exposure. Significant results from present study justify this statement. Although, melatonin acts as potent antioxidant and therefore is included in protection against oxidative stress (Meena et al. 2014). Moreover, 6-hydroxymelatonin (melatonin metabolite, which is excreted in urine) also shows an antioxidant potential, protecting the DNA molecules from oxidative damage, and reduces the level of hydroxyl radical (Lopez-Burillo et al. 2003; Qi et al. 2000).

The exact mechanism, by which melatonin controls cell death or damages are not entirely known, but however, it has been implied that mitochondria within the cells are targets for melatonin actions. Melatonin plays an effective role in regulating mitochondrial homeostasis (Castroviejo et al. 2011; Srinivasan et al. 2011). Melatonin is a powerful antioxidant that scavenges ·OH radicals as well as other ROS and RNS (Galano 2011). This article summarizes the several mechanisms through which melatonin can exert neuroprotective actions in neurodegenerative disorders. In case of brain disease to understand the mechanism of binding of melatonin to acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), melatonin was selected for in silico docking studies which were carried out using AutoDock 4.2, based on the Lamarckian genetic algorithm principle. AChE is a serine hydrolase whereas, BuChE is known as pseudocholinesterase or nonspecific ChE. These ChEs are highly efficient since they are able to cleave more than 10,000 molecules of ACh per second and produce acetate and choline rapidly (Choi et al. 2007). Its biological role is termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter ACh to acetate and choline. AChE is mainly found at neuromuscular junctions and cholinergic brain synapses. The findings from this study shows a clear indication of brain disorder after exposure of microwaves. The damaging effects of MW radiation on the brain includes brain dysfunction and brain structural damage. The results obtained in this study (increased level of LPO and ROS) after microwave exposures may alter the levels of protective proteins or lipids and this reduction means a greater probability of Alzheimer's disease. AD is characterized by the loss of cholinergic innervations, reduction of choline acetyltransferase and enhanced acetylcholinesterase (AChE) activity (Rutten et al. 2002; Ansari and Khodagholi 2013). The progressive disturbance of cholinergic function is fundamentally close to the short-term memory loss seen in AD. Therefore, it is imperative to see the interaction of melatonin after microwave exposure in the brain as well as the elucidation of mechanism of action underlying the pharmacological and toxicological action of these agents for the purpose of rational drug design (Sussman et al. 1991).

Melatonin has been found to be highly protective against damage to macromolecules resulting from oxygen and nitrogen based reactants (Reiter et al. 2003). Melatonin prevents oxidative damage of biological membranes by preventing harmful lowering of the mitochondrial membrane potential. However, lowering may trigger mitochondrial transition pore opening and apoptosis cascade (Martın et al. 2000; Rodriguez et al. 2004; Winiarska et al. 2006). Friedman et al. (2007) showed that RF-EMW stimulate plasma membrane NADH oxidase in mammalian cells and cause production of ROS. ROS is the affecting factor, where RF-EMW may increase the level and induce formation of large aqueous pores on the cell

membrane. This phenomenon is called electroporation. Present study are in line with several other reports suggest that exposure of microwave radiations may induce oxidative stress by increasing ROS production, which may lead to DNA damage and genotoxic effects (Kumar et al. 2013; Shahin et al. 2013).

5 Conclusion

This study revealed that exposure of 2.45 GHz microwave frequency adversely affects the whole brain and cause oxidative damage. Also, data obtained from in silico analysis shows that the melatonin have a strong capability to interact with AChE and BuChE enzymes of the CNS. The biochemical elucidation of interaction patterns like binding energy, inhibition constants, hydrogen bonds and the residues involved in hydrophobic interactions suggests that melatonin could be used as potent free radical scavenger and antioxidant agent, melatonin for protecting cells and tissues from oxidative damage.

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Nanoparticles: Applications, Toxicology and Safety Aspects

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Abstract Nanotechnology has a global socioeconomic significance. On the brighter side, Nanoparticles (NPs) offer extraordinary technical competencies which allow them to perform enormously novel developments in science and industries. Whereas, on the darker side, just the same novel qualities of nanoparticles can concurrently evoke undesired features, which sometimes lead to adverse and harmful interactions with exposed organisms. Workers involved in manufacturing and handling of NPs in all countries face new hazards from these nanomaterials. The occupational safety and health associations have taken schemes to spot the gaps between awareness and practices. These international agencies formulate the guidelines for handling nano materials and fix their occupational exposure limits. In this chapter authors discussed the source and role of NPs in different areas, NPs induced toxicity, their interaction with different biomolecules, as well as the safety and handling guidelines of NPs in occupational and laboratory areas.

Keywords Nanoparticle · Nanotoxicity · Nanotubes · Cancer nanotechnology

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1 Introduction to Nanoparticles

Nanotechnology is defined as the study of structures having at least one of their dimensions in the nanoscale range (Fig. 1). A nanometer is one billionth of a meter. Nanotechnology is an emerging interdisciplinary field of science, engineering and technology. The structures of the nanometer range (from 1 to 100 nm in size) with novel properties and functions are termed as nanoparticles (NP's). Nanoparticles are small objects that behave as a whole unit. They may be dry, suspended in a gas (nano-aerosol), suspended in a liquid (as a nano-colloid or nano-hydrosol), or embedded in a matrix (as a nano-composite). Nanoparticles exist in several forms such as nanotubes, nanoplates, and nanofibers (CDC/NIOSH 2009).

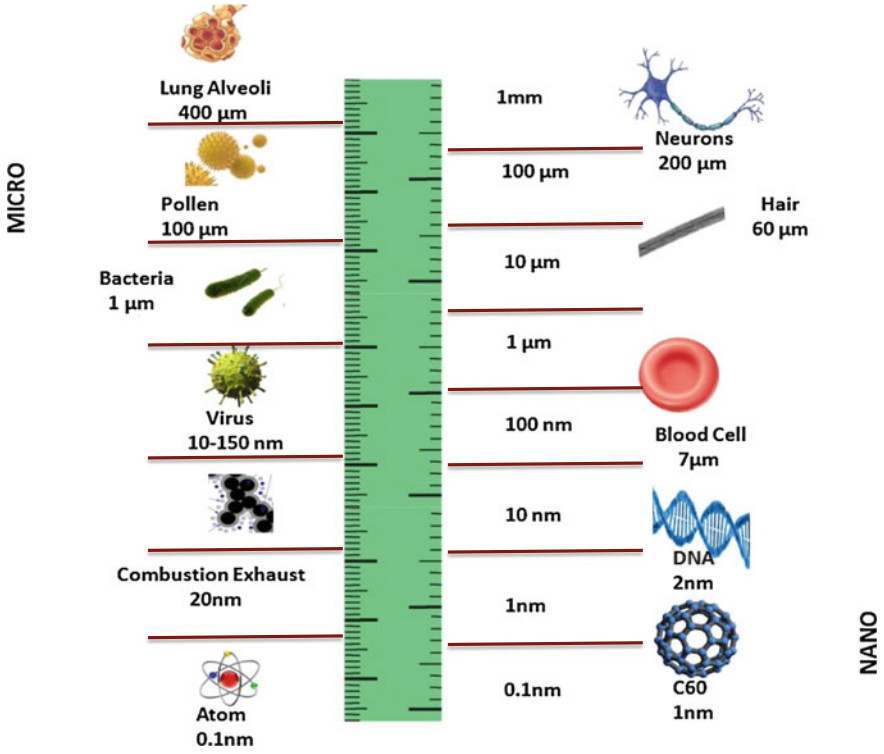

Fig. 1 Scale of science from millimeter (mm) to nanometer (nm)

1.1 Sources of Nanoparticles

Nanoparticles may also be defined as materials with at least one dimension in 1–100 nm range. Nanoparticles may be naturally present in the atmosphere in the form of some viral particles and proteins or as byproducts of photochemical and volcanic activities. Nanoparticles may be manufactured/engineered intentionally or they may unintentionally be produced anthropogenically via the engine exhaust (Fig. 2). They may be used for various purposes such as a catalyst in chemistry, as drug delivery devices in nanobiotechnology, as imaging agents and consumer products. They are also used in engineering and information technology (Aguilera-Granja et al. 1993; Kuzmany et al. 1997; Wilson et al. 1999). The electrical, optical, and chemical properties are very different at 'nano' scale as compared to those exhibited by larger particles; along with very high reactivity as the maximum numbers of atoms of the material are at the surface. Large scale materials have a lower percentage of atoms at the surface.

1.2 Classification of Nanoparticles

On the basis of their existence, nanoparticles may be classified as follows:

(i) **Naturally existing nanoparticles**: They exist in nature, mostly in the form of carbon soot. They may arise from volcanic eruptions, lightening discharge, forest fire, etc. Some biogenic magnetite such as viruses and magneto tactic bacteria also exists in the nanometer range.

(ii) **Manufactured Nanoparticles**: They are produced manually (intentionally/unintentionally). Internal combustion engine exhausts, airplane jets, power

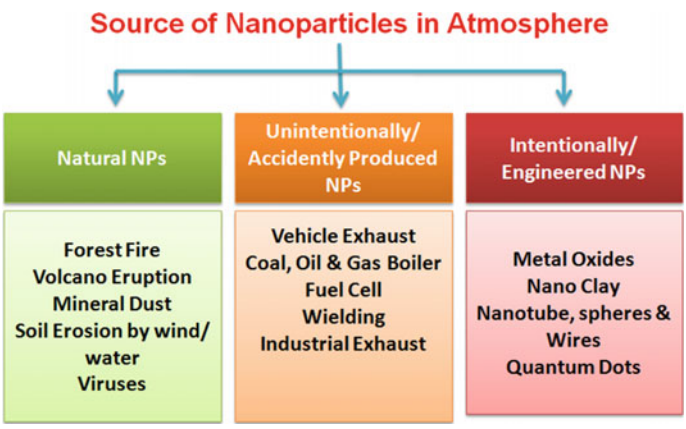

Fig. 2 Major sources of nanoparticles in atmosphere

plants, metal fumes (smelting, welding), polymer fumes etc. produce nanoparticles unintentionally. Whereas nanoparticles with controlled size and shape, designed functionality such as quantum dots/rods, metal oxides, fullerenes, nanotubes, nanowires, nanoshells etc. are produced intentionally for their potential applications in various fields like cosmetics, medical, electronic, optical, pharmacy are termed as intentionally manufactured nanoparticles or engineered nanoparticles (ENP's).

On the basis of dimension nanoparticles can also be classified as:

(i) **Zero Dimension (0-D)**: It represents a special form of spherical nanocrystals from 1 to 10 nm in diameter, which includes nanodots and quantum dots. They have been developed in the form of semiconductors, insulators, metals, magnetic materials or metallic oxides. Quantum dots are used to track DNA molecules in cells, as biosensors for detecting agents of biological warfare and are efficient alternatives to conventional lighting sources.

(ii) **One Dimension (1-D)**: It includes thin films/manufactured surfaces or coatings. They can be used as a corrosion resistant, wear and scratch resistant, dirt repellents, antibacterial and anti-microbial agents, catalytically active and chemically functionalized and transparency modulated surfaces in hydrophobic and self-cleaning.

(iii) **Two Dimensions (2-D)**: It includes nanotubes, nanowires, nanofibers and nanopolymers. ***Carbon Nanotubes***: Carbon nanotubes are a different form of carbon molecule wounded in a hexagonal network of carbon atoms; these hollow cylinders can have diameters as small as 0.7 nm and reach several millimetres in length (Hett 2004). Each end of a carbon nanotube can be opened and closed by a fullerene half-molecule. These nanotubes may have a single layer (like a straw) or several layers (like a poster rolled in a tube) of coaxial cylinders of increasing diameters in a common axis (Iijima 1991).

(iv) **Three Dimension (3-D)**: Three dimensional nanoparticles comprise fullerenes, dendrimers and quantum dots.

  (a) **Fullerenes**: Fullerenes are allotropes of carbon in the form of spherical cages containing 20 to more than 100 carbon atoms. Fullerenes have novel physical properties. They can be subjected to extreme pressures and tend to regain their original shape when the pressure is released. Fullerenes products include drug delivering vehicle and electronic circuit.

  (b) **Dendrimers**: Dendrimers correspond to a new class of controlled-structure polymers with nanometric dimensions. They are considered to be the basic elements for large-scale synthesis of organic and inorganic nanostructures (with dimensions between 1 and 100 nm) and have unique properties. Being compatible with organic structures such as DNA, they can also be fabricated to interact with metallic nanocrystals and nanotubes or to possess an encapsulation capacity (Tomalia 2004). They may have conventional applications, or may be used for drug delivery, environmental and water cleaning.

2 Applications of Nanoparticles

Nanoparticles have become an important topic of research in the recent decades due to their surprising functional properties and find increasing application in the manufacturing industry as well as in biomedical technology. The recently revealed benefits of nanotechnology are due to the novel properties of nanomaterials such as volume-to surface ratio and the capability to organize and control their properties with greater precision and complexity. Nanoparticles are, therefore predicted to play vital role in medical science and technology in the upcoming decades. A number of novel technologies based on nanoparticle–DNA binding and their interactions have been developed and used in molecular diagnosis, gene therapy, sensing, drug delivery and artificial implants (Curtis and Wilkinson 2001; Sachlos et al. 2006; Vaseashta and Dimova-Malinovska 2005; Waren and Nie 1998). These approaches offer an opportunity for the development of efficient and low-cost technologies for disease diagnosis and DNA detection with high sensitivity (Buxton et al. 2003; Yezhelyev et al. 2006). In future, nanoparticle–DNA binding based techniques may have potential implication in medical biotechnology. It is necessary to understand the interactions within cellular membranes or compartmental molecules of the cell. The DNA–nanoparticle interactions through their molecular bindings and biochemical reactions have developed an increasing interest in recent studies. The growth in the course of applications of nanoparticle based technology in medicine and medical biotechnology continues to be a significant factor for research in medical practice, and is expected to trigger more innovations in this field. The industrial use of engineered nanoparticles is expected to increase (such as in the manufacturing of household goods), enabling them to interact with biological molecules and potentially damage the cells in vivo (Fischer and Chan 2007). The functionalization of nanoparticles can provide target specific interaction and thus, can be utilized in varied applications from industrial (Barth et al. 2005), environmental (Gehrke et al. 2015), to biomedical uses (Salata 2004). Generally, nanoparticles exist in three broad categories: organic, inorganic and hybrid particles (Burcham 2010). A polymer-based nanoparticle comes into the category of organic nanoparticles. Inorganic nanomaterials include carbon nanotubes, fullerene particles, metallic nanoparticles, including gold and silver nanoparticles, and many metal oxide species, whereas a combination of organic and inorganic nanomaterials, such as peptide- and DNA-functionalized gold nanoparticles (Lin et al. 2009) and DNA–carbon nanotube arrays are termed as hybrid nanoparticles also known as engineered nanoparticles.

Nanoparticles are, predicted to play an important role in various fields such as medical science and technology in the upcoming decades because of their special electronic, mechanical, and chemical properties (Bernholc et al. 2002). The applications of NP for drug delivery, pharmacy and many industrial and commercial practices are expected to increase considerably (Gu et al. 2007; Gunasekera et al. 2009; Houili et al. 2010; McBain et al. 2008). For example, carbon black is commonly used as a pigment, a reinforcing phase in automotive tire, belts and as a

food color. A number of novel technologies based on nanoparticle–DNA binding and their interactions are invented and used now in molecular diagnosis, gene therapy, sensing (Alivisatos et al. 2005) and imaging (Gunasekera et al. 2009). In future, nanoparticles may help the existing state of medicine in several ways, by providing highly selective and targeted therapeutics i.e. with increased efficacy and minimum side effects of current therapeutics, with increased efficiency of diagnostic and prognostic tools and by impacting the development of drugs. These nanoparticles will overcome the drawbacks of using liposomes, such as low encapsulation, rapid leakage of blood, and poor storage constancy. The nanoparticle-based devices have been developed for the purpose of clinical diagnosis, in the analysis of biological materials and to improve medical technologies. These approaches lead to the development of efficient and low-cost technologies for disease diagnosis and DNA detection with high sensitivity. The nanoparticle–DNA binding based techniques may have potential application in medical biotechnology in the near future.

3 Production and Exposure of Nanoparticles

Nanoparticles are progressively being used in industries, medicines, diagnostics, pharmaceuticals, etc. The unlimited use of nanoparticles leads to their overproduction. It has been estimated that 50,000 kg/year of nano-sized materials are being produced through accidental anthropogenic sources (combustion or nucleation). On the other hand, upon the manufacture of nanoparticles using appropriate techniques, they are released in the air, which may lead to the contamination of soil and food products (Luther 2004). Single-walled and Multi-walled nanotubes had a worldwide production of 2954 kg in 2003 and the Carbon Nanotechnology Research Institute, Japan, proposed the increase in the production of CNT's from $\sim$1000 kg in 2003–120,000 kg per year within the succeeding five years. It was expected that production rates will accelerate exponentially in the next few years (Fig. 3). Considering the tons of engineered nanomaterial planned for production, it is likely that some of these materials will enter the environment during the product's Life Cycle (manufacture, use, and disposal).

The investigation of toxicity of nanoparticles revealed that nanoparticles may adversely affect the biological systems because of their size, strong mobility and reactivity. Upon exposure, some of these nanoparticles may enter the human body and become toxic at the cellular level in the tissues and organs (Jin et al. 2005). These particles may or may not be carcinogenic or allergic, but even inert nanoparticles show harmful effects due to some adsorbed toxic species or formation of toxic products on reactions with body fluids. Their potential toxic effects on the environment and human health are therefore a major concern.

Workers engaged in manufacturing and handlings of nanoparticles are more exposed to the diseases, which poses new challenges for doctors in diagnosis and treatment. A large number of consumer products such as cosmetics, sunscreens, sports clothes, personal care products, food packagings, paints, medical procedures

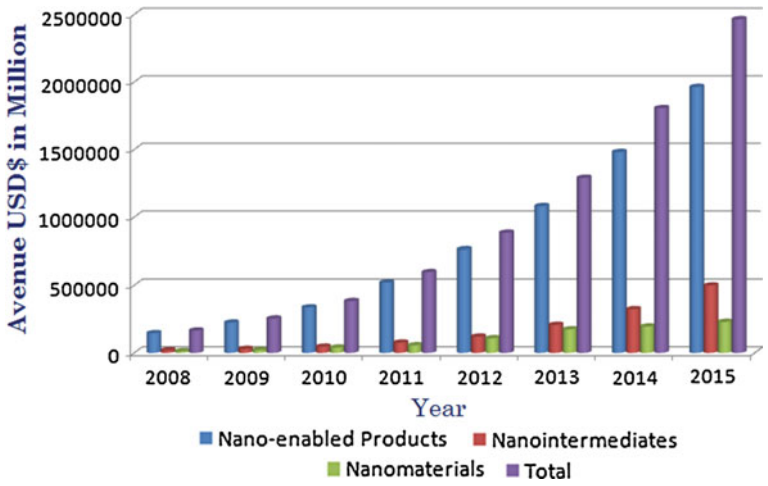

Fig. 3 The world-wide nanomaterial and nano-enabled product markets. *Source* Lux Research, Inc. (www.luxresearchinc.com)

and pharmaceuticals, even tires and auto parts, are already in the market (Christof and Niemeyer 2001). The lack of awareness about the harmful effects of these products, impacts more on consumer health than that of workers. These particles may end up in the environment, settling into the soil, taken up by some plants and accumulated in the bodies, because of their small size (Fig. 4).

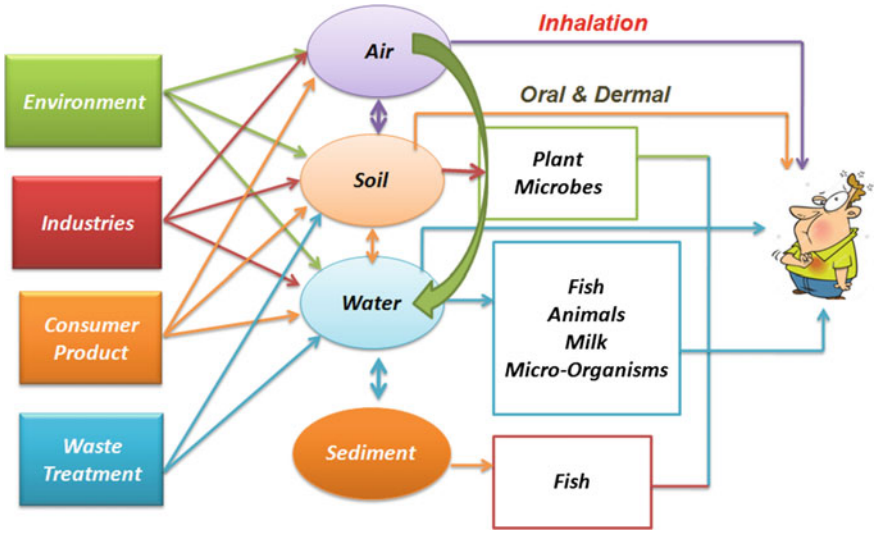

Fig. 4 Exposures routes of nanoparticles from different sources

The interactions of nanoparticles with the body depends on their size, chemical composition, surface structure, solubility, shape and accumulation. Berkeley proposal showed that lower sized (<10 nm) nanoparticles behave more like a gas and easily can pass through skin and lung tissue to penetrate cell membranes. It can easily enter the human body through the food or water we consume, both accidently or intentionally via the nose and lungs just like other aerosols, and also through the skin (Papp et al. 2008). Some of them readily travel throughout the body, deposit in target organs, penetrate cell membranes, lodge in mitochondria, and may trigger injurious responses (Oberdörster et al. 2002) (Fig. 4). This is due to the fact that the sizes of the nanoparticles and biological molecules are comparable, the nanoparticles can easily invade the natural defense system of human body or other species and ultimately enter the cells to affect cellular functions (Alexandra et al. 2010). According to research on the effects of smoking on lung tissue, the foreign particles inhaled into the lungs have the potential to do great damage (Ryu et al. 2001), studies revealed that inhaled nanoparticles, not only cause lung damage, but also can move into the bloodstream; potentially causing cardiac damage. Those inhaled nanoparticles in humans caused damage both to the point of entry, and to the brain also (Fig. 5). The pathway proposed in Fig. 5 is also reported by Terzano et al. (2010). According to a report by the Royal Society, the United Kingdom's National Science Academy, nanotubes are structurally similar to asbestos fibers, which can cause lung fibrosis when inhaled in large amounts over long periods (DelVecchio 2006).

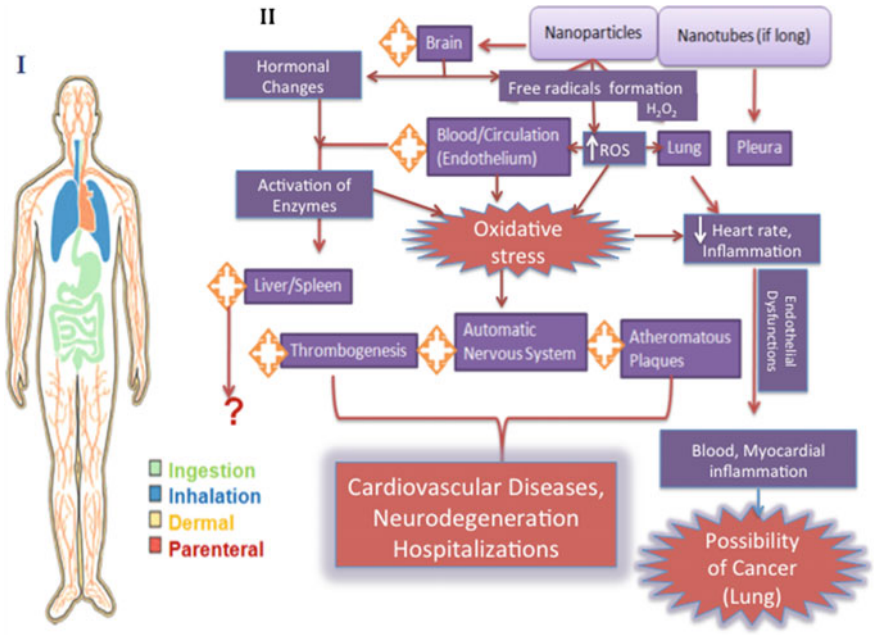

Fig. 5 *I* Exposure of nanoparticles within human body. *II* Potential human effects of nanoparticles and nanotubes

The distinct properties of nanoparticles such as the large surface area, anomalous interface, complicated reactivity, quantum effects, etc. can also lead to changes in physicochemical properties which naturally alter the biological activities in vivo (Fischer and Chan 2007; Warheit et al. 2004; Zhao et al. 2005). It has been already revealed that nanoparticles in medical field may induce cytotoxic effect, in eukaryotic and prokaryotic cells (Brunner et al. 2006; Feris et al. 2010; Hanley et al. 2008). Engineered nanoparticles interact with biological molecules and have potential to damage cells in vivo (Fischer and Chan 2007). All these leads to a great concern about the toxicological effects of engineered nanomaterials and nanoparticles (Brumfiel 2003; Zhao et al. 2008). Although nanoparticles are known to exist in the atmosphere in large concentrations (≈108/cc), the release of manufactured nanoparticles into the atmosphere and the aquatic environment is yet insignificant and unidentified.

Nanoparticles can interact with proteins and enzymes in cells and raise the formation of free radicals, which affects the defense mechanism (Fig. 6). Nanoparticles also induce inflammatory responses and lead to cell death. A number of researches are being undertaken on the biological applications of nanomaterials (drug delivery, cancer treatment, dynamic therapies, etc.), but the amount of data available on the toxicity of nanoparticles is insufficient. More studies are needed to explore the toxic effects of nanoparticles such as alteration in physicochemical properties and

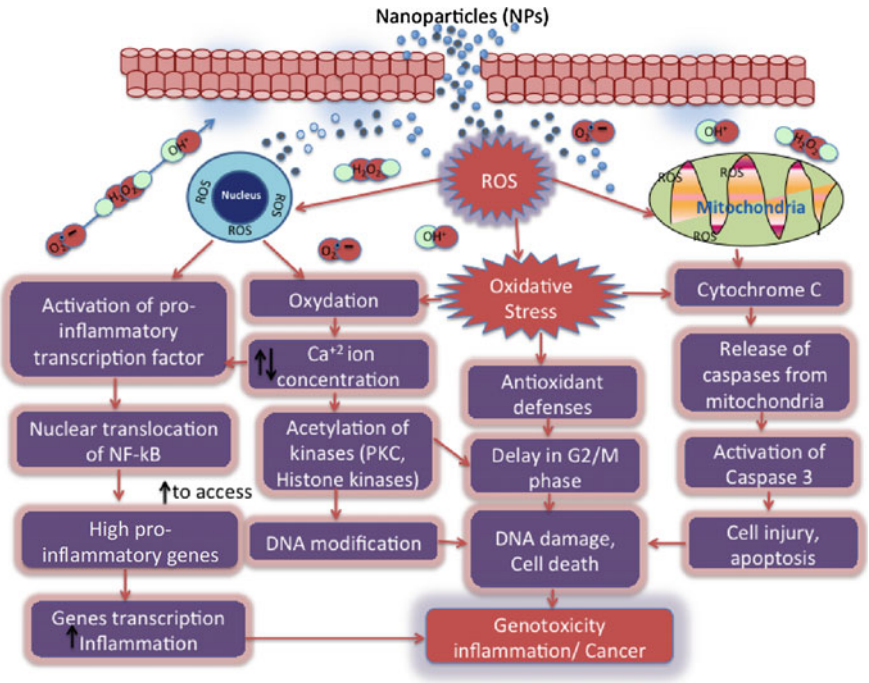

Fig. 6 Pathway shows an interaction of nanoparticles, its toxicity and mechanism of genotoxicity

characteristics of nanomaterials as compared to the bulk material of the same chemical form. Nanoparticle–DNA binding would affect cellular DNA bioactivities, such as replication, transcription, regulation, and repair, even the entire genomes.

4 Carbon Based Nanoparticles and Their Toxicity

Carbon nanomaterials are divided into two categories-fullerenes and carbon nanotubes (CNT). Single-wall carbon nanotubes (SWNTs) and multi-wall nanotubes (MWNTs) are the two main members in the CNT family. SWNTs are formed by rolling graphene into a cylindrical shape, with a diameter of about 0.7–10.0 nm and a length of about 10 nm up to a micrometer scale. Similarly, MWNTs can be considered equal to the spacing of adjacent graphene layers in graphite. With the nested tube structure, MWNTs are concentric ring of SWNTs with different diameters (Iijima 1991). They are allotropes but have individual spatial structures. C60 is the most distinctive fullerene built up by 12 isolated pentagons and 20 hexagons, having a diameter of about 0.7 nm (Osawa 1970). Carbon based nanomaterials have attracted a great deal of attention from scientists in many R&D fields, due to their novel electronic, mechanical, and chemical properties. So, the applications of carbon nanoparticle (CNP) are expectedly increasing in almost every field such as pharmacy, drug delivery, and many industrial and commercial practices. Some of the common applications of CNPs are in the form of a reinforcing phase in automotive tires, belts, or food coloring. However, above mentioned benefits of CNP engineered or released from industrial and human activities may cause significant risk to the environment and, in particular, public health (Medina et al. 2007; Panessa-Warren et al. 2006; Ramón-Azcón et al. 2014). So, CNP's exposure is a major concern because of the possibility of respiratory risks and cardiovascular morbidity and mortality (Peters and Pope III 2002; Pope III 2001).

Inhalation of SWNTs may induce pulmonary multifocal granuloma without any symptom of inflammation or cellular proliferation (Lam et al. 2004; Warheit et al. 2004). A number of studies revealed that ultrafine CNP and carbon nanotubes cause lung injury via inhalation and injection (Donaldson et al. 2004, 2006, Oberdörster et al. 2005; Panessa-Warren, et al. 2006). Some reports have already shown that CNPs affect human cells, resulting in lipid membrane peroxidation, gene down regulation of adhesive proteins, and increased cell death, including necrosis and apoptosis (Bosi et al. 2004; Poulsen et al. 2015). Modern study showed that the microbial evolution of aerobic micro-organism such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) were influenced by the nano-carbon particles from laser pyrolysis. It has been explored earlier that the interactions of CNP with DNA may potentially lead to abnormal effect, mutagenesis or DNA damage (Nel et al. 2006). In vitro studies suggest that DNA molecule may interact with the carbon nanomaterials with unusual responses. It was reported earlier that C60 and its derivatives inhibit the replication of simian immunodeficiency virus (SIV) in vitro

and the activity of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Nacsa et al. 1997). Further these CNPs' have enhanced the amplification efficiency for long PCR reactions (Zhang et al. 2008). Nano-sized carbon and TiO_2 nanoparticles may impair the Alveolar macrophage's (AM) ability of phagocytosis and chemotaxis. Alveolar macrophages (AMs) act as scavengers in the lung by clearing foreign materials, bacteria and viruses, but the poorly digestible particles may be retained in the lung for a long time. To assess the cytotoxicity on AMs exposed to carbon nanomaterials and their effects on phagocytic abilities, the cyotoxicity study suggests that the sequence order of the cytotoxicity of these three types of carbon nanomaterials (on a mass basis) are: SWNTs > MWNT10 > C60 (Jia et al. 2004), where MWNT10 means the diameter of MWNT ranges from 10 to 20 nm. Studies also unveiled that ordinary protein binding site can actively bind to carbon nanoparticle with high affinity and specificity (Noon et al. 2002). Although, carbon–carbon interactions are not specific, but these interactions become more specific due to the nano-structuring of the particle, allowing ordinary protein binding sites to be occupied by carbon nanoparticles. It was investigated by MD simulations, in order to understand possible conformational changes that the carbon nanotube may induce on the structure of protein human serum albumin (Shen et al. 2008) and it was found that α-helical secondary structure of the protein was mostly unchanged whereas the random coils which connect these α-helices were strongly affected. This leads to the alteration in the tertiary structure of protein mostly due to the orientation and conformational changes of the protein structure to fit the arrangement of carbon atoms on the nanotube surface. It can affect the overall structure and function of proteins by hydrophobic interactions and π-stacking between the aromatic residues and hexagonal carbon arrangement on some carbonaceous nanoparticles. The interactions of CNP with DNA have been studied only by simulation (Gao and Kong 2004; Zhao et al. 2005) so, in vivo or in vitro interaction of CNP with DNA and their potential impacts on DNA are yet to be explored.

5 Fullerenes and Its Toxicity

Fullerenes are spherical cage like structures with a diameter of about 1 nm. Fullerenes exist in different molecular weight ranging from C20 to C720, among which most common form, C60, is also known as Buckminster fullerene (C60). Fullerenes may be produced naturally as they are released from combustion processes such as forest fires (Powell and Kanarek 2006). Fullerenes have been found to be useful in almost every field such as chemical and material science applications includes semiconductors and microscopic engineering and polymers (Aguilera-Granja et al. 1993; Kuzmany et al. 1997; Smalley and Yakobson 1998; Spence 1999). Biomedical applications include antioxidant, antiviral, antibiotic and anti-cancerous activities, enzyme inhibition, cell signaling, DNA cleavage as well as imaging and nuclear medicine (Jensen et al. 1996; Tsao et al. 1999; Wilson et al. 1999). Assessing the toxicity of fullerenes is therefore essential for demarcating the risk to the human health.

Previous data clearly show that pristine C60 has no acute or sub-acute toxicity in a large variety of living organism/s but chemical modifications may change the properties of pristine fullerene making them toxic (example polyvinylpyrrolidone, PVP forms a charge transfer complex with fullerenes). Identification of hazards related to fullerene exposure is complex and complicated as a variety of fullerene derivatives exist, a diverse group of moieties can be attached to the fullerene surface, and various preparation processes may be utilized to make fullerenes water soluble. It is also known that C60 is an efficient singlet oxygen sensitizer under light exposure (Krusic et al. 1991). In the presence of O_2, fullerene and some of its derivatives can be extremely toxic through singlet oxygen formation, which can damage important biological molecules such as DNA, lipids and proteins. The first toxicity study of C60 was conducted in the United States (Tucson, Arizona) (Nelson et al. 1993).

Fullerenes do not remain at the site of exposure (lungs and gut) but may cross the cell barriers and get transported through blood. Recognition of potential targets of fullerene toxicity within the body and there in vitro toxicity assessments at particular target sites on the delivery of fullerenes to target organs, such as the liver or kidneys, requires their transfer into blood from their exposure site. This is necessary to access different sites within the body. Few previous studies revealed the absorption of fullerene into blood from their exposure site (Yamago et al. 1995). Fullerene may pass into the blood from the gut (Yamago et al. 1995), may accumulate in kidney, liver (Shipelin et al. 2015) and spleen (Chen et al. 1998). Metabolism of valerian may occur following their accumulation within the liver, but their metabolites are still unknown (Gharbi et al. 2005) and then they might be eliminated through urine (Yamago et al. 1995) and feces (Mori et al. 2006; Yamago et al. 1995). Molecular dynamics studies have shown that the translocation of fullerene across the lipid membrane due to the ability of C_{60} to create a cavity (termed transient micropores) within the membrane helps C_{60} molecules to penetrate into membrane. In contrast, C_{60} $(OH)_{20}$ derivatives barely penetrated the membrane as they are absorbed onto the membrane surface due to its hydrophilic nature (Qiao et al. 2007). $C_{60}(OH)_{20}$ does not interact with the lipid core of the membrane preventing its penetration into the membrane, but shows strong interactions with the membrane surface head groups causing a “pinch” to form in the plasma membrane.

Cyclodextrin-capped fullerenes were able to form stable complexes with bovine serum albumin (BSA), which help to facilitate fullerene transport within the body (Belgorodsky et al. 2006). Computational models were used to reveal the interactions between fullerenes (in a pristine and carboxylated form) and proteins (namely human serum albumin, BSA, human immunodeficiency virus [HIV] protease, and a fullerene-specific antibody) that had all been previously confirmed to interact with fullerene molecules (Benyamini et al. 2006). The binding sites for fullerenes within the proteins were recognized and confirmed to have similarities between the different proteins such as BSA, HIV proteases. On the other hand, the design of the fullerene molecule is a fundamental factor for its interactions with proteins. The fullerene derivatives used were designed in such a way so as to support such

interactions, and the applicability of the response to fullerenes as a whole requires analysis. However, the mechanism of absorption, distribution, metabolism excretion (ADME) profile of fullerenes is yet to be explored and therefore it is necessary to investigate the toxicity of fullerene. The ability of fullerenes to interact with biological molecules such as proteins, DNA and RNA is of major concern as they not only have the ability to alter the normal structure and function of these biological moieties, and enable fullerene transport within the body (as interactions of fullerenes with serum proteins are evident) but also is potentially able to modify the behavior of the particles.

6 Engineered Nanoparticle

Engineered nanoparticles are intentionally produced, however ultrafine particles often referred to as incidental nanoparticles, are normally byproducts of processes like combustion and vaporization. Designed nanoparticles with specific properties or compositions (e.g., shape, size, surface properties, and chemistry) are termed as engineered nanoparticles (Table 1). The potential health risk of exposure to these nanoparticles is associated with the magnitude and duration of exposure, the persistence of the material in the body, the inherent toxicity of the material, and the susceptibility or health status of the person exposed (Fig. 7). More data are needed on the health risks associated with exposure to engineered nanomaterials (Fig. 8). The results of the existing studies on animals and humans, focusing on the exposure and response to ultra-fine or other respirable particles provide a basis for preliminary estimates of the pos-sible adverse health effects from exposure to similar engineered materials on a nano-scale. Experimental studies in rodents and cell cultures have revealed that the toxicity of ultrafine or nanoparticles is greater than that of the same mass of larger particles of similar chemical composition (Oberdörster et al. 1992, 1994a, b; Tran et al. 2000; Brown et al. 2001;

Table 1 Functions and applications of ENP's

Engineered nanoparticles material	Function	Applications
Silver	Biocide	*Wound treatment, prosthesis, odor control*
Titanium dioxide	Photocatalyst: optical	*Cosmetic, sunscreen, pharmaceutical*
Iron oxide	Superparamagnetic	*Electronics, biomedical*
Quantum dots	Semiconductor/fluorescence	*Electronics, biology*
Carbon Nanotubes/fibers/fullerenes	Extraordinary strength, unique electrical properties, efficient thermal conductors	*Health and fitness, electronics, automotive, architecture*
Dendrimers repeatedly branched molecules	Determined by their functional groups	*Drug delivery systems, tissue engineering?*

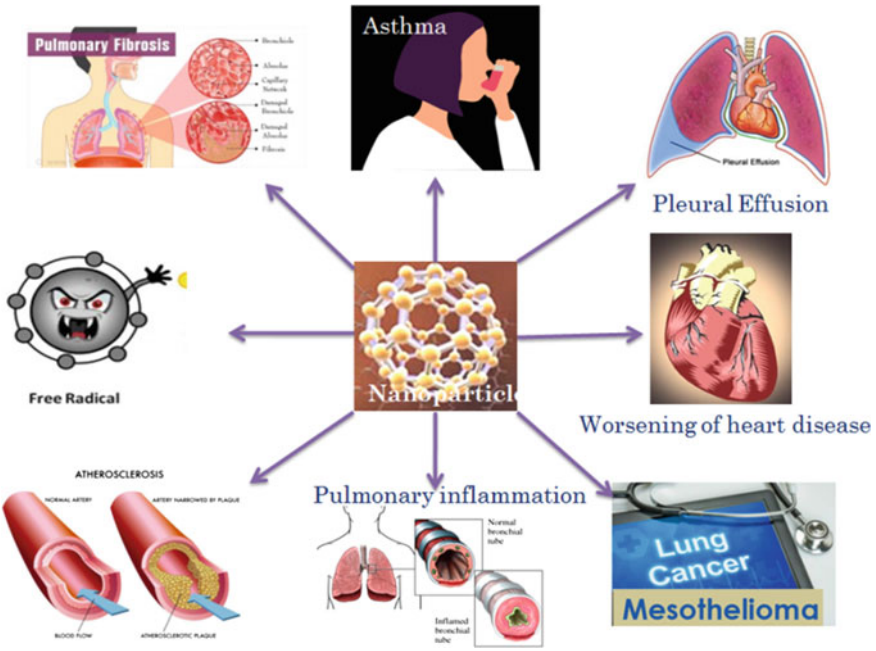

Fig. 7 Factors affecting engineered nanoparticles on human health and severe causes

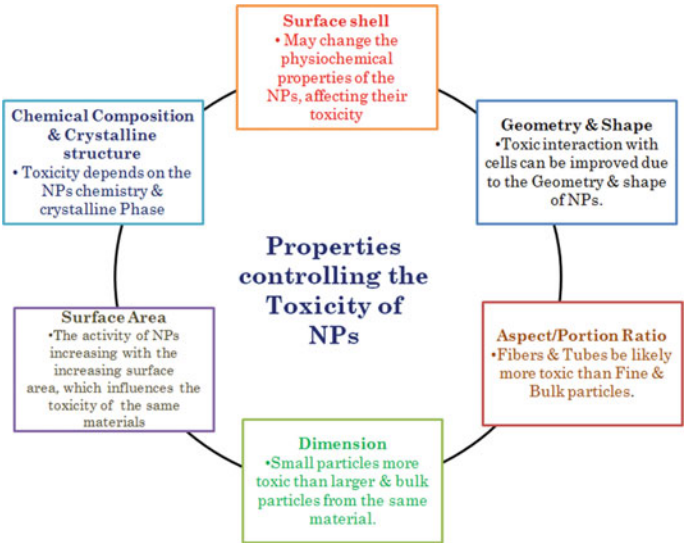

Fig. 8 Nanomaterials properties, characteristics and controlling the toxicity of nanoparticles

Barlow et al. 2005; Duffin et al. 2007). Along with a particle surface area, other particle characteristics may effects, toxicity, include-in surface functionalization or coatings, solubility, shape, and the ability to generate oxidant species and to adsorb biological proteins or bind to receptors (Duffin et al. 2002; Oberdörster et al. 2005; Donaldson et al. 2006).

There are many unknowns as to whether the unique properties of engineered nanomaterials pose health concerns (Table 2). The potential health risk following exposure to a substance is generally associated with the following (CDC/NIOSH 2009):

Table 2 Sources of exposure to nanomaterials through occupational activities (Aiken et al. 2004)

Process synthesis	Particle formation	Exposure source or worker activity	Primary exposure route[a]
Gas phase	In air	Direct leakage from reactor, especially if the reactor is operated at positive pressure	Inhalation
		Product recovery from bag filters in reactors	Inhalation/dermal
		Processing and packaging of dry powder	Inhalation/dermal
		Equipment cleaning/maintenance (including reactor evacuation and spent filters)	
Vapor deposition	On substrate	Product recovery from reactor/dry contamination of workplace	Inhalation
		Processing and packaging of dry powder	Inhalation/dermal
		Equipment cleaning/maintenance (including reactor evacuation)	Dermal (and inhalation during reactor evacuation)
Colloidal	Liquid suspension	If liquid suspension is processed into a powder, potential exposure during spray drying to create a powder, and the processing and packaging of the dry powder Equipment cleaning/maintenance	Inhalation/dermal Dermal
Attrition	Liquid suspension	If liquid suspension is processed into a powder, potential exposure during spray drying to create a powder, and the processing and packaging of the dry powder Equipment cleaning/maintenance	Dermal Dermal

[a]Ingestion would be a secondary route of exposure from all sources/activities from deposition of nanomaterials on food or subsequently swallowed (primary exposure route inhalation) and from hand to mouth contact (primary exposure route dermal)

- Magnitude and duration of the exposure;
- Persistence of the material in the body;
- Inherent toxicity of the material; and
- Susceptibility or health status of the person.

7 Handling of Nanoparticles

Few organizations such as the National Institute of Occupational Safety and Health have started an active program for studying the safe handling of nanomaterials in the workplace. How workers are potentially exposed to nanomaterials and if so what are the characteristics and levels of exposures and workers' health? What work practices, personal protective equipment, and engineering controls are available and how effective are they for controlling exposures to nanomaterials? (Schulte and Salamanca 2007).

During manufacture and handling of these materials, there may be a chance of release and exposure of nanoparticles to workers, which may enter their body through inhalation, dermal contact or ingestion routes (Dreher 2005). Only limited information on the risks of handling of these materials is available, so the workers should implement strict control procedures and engineering safety features to limit exposure when working with nanoparticles and should not allowed to eat or drink in the laboratory. While handling the nanomaterials, they should use laboratory safety measures such as Personnel Protective Equipment (PPE) including gloves, lab coats, safety glasses, face shields, closed-toed shoes etc. avoid the skin contact with nanoparticles or nanoparticles containing solutions (Feder 2004). If necessary to handle nanoparticle powders with exhaust laminar flow hood, workers must wear appropriate respiratory protection. Use of fume exhausts hoods to expel fumes from tube furnaces or chemical reaction vessels is crucial. Laboratory personnel should be trained and made aware of the risk associated with workplace hazards, Material Safety Data Sheets (MSDS), labeling, signage etc. periodically. Disposal of nanoparticles also reflects on the safety of the environment. Disposal should be in accordance with the hazardous chemical waste guidelines.

Research in this field will help us to understand the main causes of toxicity in these materials, so that the safer materials may be engineered which prevent danger to the human life. Industry consortiums, environmental real data on toxicity into the iterative groups and individual corporations need to take strong action to determine the safety of materials and products before they reach into the market. Today engineered nanomaterials also help in handling emergencies. Nanotechnology-based sensors and communication devices can reduce their exposure to risk of injury. Nanosize structures coupled with wireless technology may facilitate development of wearable sensors and systems for real time occupational safety and health management. Nanotechnology-based fuel cells, lab-on-chip analyzers and opto-electronic devices have the potential to be useful in the safe, healthy and efficient design. The development of high performance filter media, respirators, dust-repellant, self-cleaning clothes, fillers for noise absorption materials, fire retardants, protective screens for

prevention of roof falls and curtains for ventilation control in mines, catalysts for emissions reduction and cleanup of pollutants and hazardous substances may support for nano safety.

Proper care should be taken about nanoparticles and nanotechnology safety issues to ensure personal health and safety of the workers who are involved in the nanomanufacturing processes and also of the consumers to eliminate its effect on the environment.

8 Guidelines/Regulatory Measures

At present, there are no federal regulations specifically address the health and safety implications of nanotechnology. There are no national or international consensus standards on measurement techniques for nanomaterials at workplace. However, as with conventional chemicals, research with nanomaterials must be conducted in a manner that is safe and responsible. All chemicals, including nanomaterials, must be transported, stored, used, and disposed in accordance with all federal, state, and local requirements.

The Occupational Safety and Health Administration (OSHA) require employers to maintain a safe and healthful workplace, "free from recognized hazards likely to cause death or serious physical harm" (29 USC 654).

According to OSHA, laboratory personnel must be informed of the risks associated with workplace hazards. This is generally accomplished through training programs, material safety data sheets, and labeling and signage. The Resource Conservation and Recovery Act of 1976 (RCRA) regulate the transportation, treatment, disposal, and cleanup of hazardous waste. Nanomaterials that meet the definition of a "hazardous waste" in RCRA are subject to this rule. Nanomaterials that are defined as "chemical substances" under the Toxic Substances Control Act (TSCA) and which are not on the TSCA Inventory must be reported to U.S. Environmental Protection Agency (EPA). A Premanufacture Notice must be submitted to the EPA by anyone intending to manufacture or import a chemical substance that is not on the TSCA Inventory of Chemical Substances.

The Federal Insecticide, Fungicide, and Rodenticide Act require that the EPA approves all new pesticide products, as well as new uses and changes in the composition of existing pesticide products, before the products may be sold or distributed in commerce. In order to evaluate an application for registration, the EPA requires the applicant to provide a complete characterization of the composition of the product, proposed labeling which describes the intended use of the product, and the results of extensive health and safety testing.

It should be also noted that the U.S. Food and Drug Administration currently regulates a wide range of products including those that utilize nanotechnology or contain nanomaterials (e.g., a drug delivery device).

9 Factors Affecting Exposure

Every attempt should be made to prevent or minimize exposure to nanomaterials. Factors affecting exposure to nanomaterials include the amount of material being used and whether it can be easily dispersed or form airborne sprays or droplets. The degree of containment and duration of use also influence exposure. In case of airborne material, particle or droplet size will determine whether the material can enter the respiratory tract and where it is most likely to deposit. Inhaled particles smaller than 10 μm in diameter have some probability of penetrating and being deposited in the gas-exchange (i.e., alveolar) region of the lungs, but there is at least a 50% probability that particles smaller than 4 μm in diameter will reach the gas-exchange region. At present there is insufficient information to predict all of the situations and workplace scenarios that are likely to lead to exposure to nanomaterials. However, there are some workplace factors that will increase the potential for exposure, including (Approaches to Safe Nanotechnology. Department of Health and Human Services, Center for Disease Control, National Institute for Occupational Safety and Health. 2009—"CDC/NIOSH 2009"):

- Working with nanomaterials in liquid media without adequate protection (e.g. Gloves) increases the risk of skin exposure.
- Working with nanomaterials in liquid media during pouring or mixing operations, or where a high degree of agitation is involved, leads to an increased likelihood of inhalable and respirable droplets being formed.
- Generating nanomaterials in the gas phase in non-enclosed systems increase the chances of aerosol release to the workplace.
- Handling nanopowders leads to the possibility of aerosolization.
- Maintenance on equipment and processes used to produce or fabricate nanomaterials poses a potential exposure risk to workers performing these tasks.
- Cleaning of dust collection systems used to capture nanomaterials poses a potential for both skin and inhalation exposure.

The following engineering controls should be used in conjunction with the aforementioned policy when handling nanomaterials (CDC/NIOSH 2009) and (Nanotechnology and Nanoparticles—Safe Working Practices Information. Virginia Commonwealth University. Office of Environmental Health and Safety. 2007—"VCU 2007"):

- Use of a chemical fume hood is recommended for all tasks with potential of aerosolizing nanomaterials in either liquid or powder form.
- A well-designed local exhaust ventilation system with a local high-efficiency particulate air (HEPA) filter should be used to effectively remove nanomaterials.
- Animals should be appropriately restrained and/or sedated prior to administering injections and other dosing methods.
- If heavy usage of aerosolized nanoparticles is being done, a proper decontamination, or buffer, the area should be utilized to ensure the nanomaterials are not transported outside of the working area.

- Frequent hand washing, especially before eating, smoking, applying cosmetics, or leaving the work area should be employed.
- Laboratories and other spaces where nanomaterials are used or stored must be equipped with an eyewash station that meets the American National Standards Institute (ANSI) and Occupational Safety and Health Administration (OSHA) requirements.

Although traditional permissible exposure limits (PEL) exist for many of the substances that nanomaterials are made from, the PEL for a nanomaterial of these substances is not yet clear. Thus, it is important to incorporate the following administrative controls into all laboratory operations:

- The laboratory safety plan should be modified to include health and safety considerations of nanomaterials used in the laboratory.
- Principal investigators should develop and implement standard operating procedures (SOPs) in the preparation and administration of nanomaterials (with minimal exposure).
- Protocols involving the in vivo use of nanomaterials must be reviewed and approved by the Animal Welfare Committee.
- Laboratory personnel must receive the appropriate training, including specific nanomaterial-related health and safety risks, standard operating procedures, and steps to be taken in the event of an exposure incident, prior to working with nanomaterials.
- Laboratory personnel must be instructed to use extreme caution when performing injections involving nanomaterials since accidental needle stick presents an exposure threat.
- Exposures involving nanomaterials or any other acutely hazardous material must be reported to the office of Safety, Health, Environmental and Risk Management as soon as possible.

10 Work Practices

The incorporation of good work practices can help to minimize exposure to nanomaterials. Examples of good work practices include the following (CDC/NIOSH 2009):

- Projects or applications with the potential for producing nanomaterial aerosols must be conducted within an approved chemical fume hood or ducted biological safety cabinet.
- Needles used for nanomaterial injection must be disposed in an approved sharps container immediately following use. Needles used for nanomaterial injection should never be bent, sheared, or recapped.

- Bench paper utilized during the preparation of nanomaterial stock should be lined with an impervious backing to limit potential for contamination of work surfaces in the event of a minor spill.
- Work areas should be cleaned at the end of each work shift (at a minimum) using either a HEPA-filtered vacuum cleaner or wet wiping methods. Dry sweeping or pressurized air should not be used to clean work areas. Bench tops, chemical fume hood interiors, biological safety cabinet interiors, equipment, and laboratory surfaces with potential for nanomaterial contamination should be routinely cleaned. Cleanup should be conducted in a manner that prevents worker contact with wastes. The disposal of all waste material, should comply with all applicable federal, state, and local regulations.
- The storage and consumption of food or beverages in workplaces must be prevented where nanomaterials are handled, processed, or stored, since exposure may occur via ingestion. Wash hands carefully before eating, drinking, applying cosmetics, smoking, or using the restroom.
- Facilities for showering and changing clothes should be provided to prevent the inadvertent contamination of other areas (including take-home) caused by the transfer of nanomaterials on clothing and skin.

11 Personal Protective Equipment

Typical chemistry laboratory apparel should be worn when working with nanomaterials in accordance with the University's Chemical Hygiene Plan (UTHSC-H 2008). Always wear appropriate clothing (e.g., pants, shirts, shoes) and personal protective equipment, including safety glasses, laboratory coats, and gloves, when working with nanomaterials. Open sandals, shorts, and skirts are prohibited. Laboratory personnel involved in any task with a potential for exposure to nanomaterial must wear the following personal protective equipment.

11.1 Protective Gloves

Glove selection is best determined by a risk assessment and the chemicals used for the procedure. Nitrile or rubber gloves, which cover hands and wrists completely through overlapping sleeve of lab coat when working with nanomaterials, may provide adequate protection. Wearing of two sets of gloves ("double gloving") is advised whenever performing tasks involving nanomaterials and other hazardous substances. Laboratory personnel should thoroughly wash hands with soap and water before and immediately upon removal of gloves.

11.2 Eye Protection

Safety glasses or goggles are considered to be the appropriate level of eye protection for working with nanomaterials. SHERM recommends wearing a full-face shield when conducting tasks posing potential for any generation of aerosol and/or droplets.

11.3 Protective Clothing

Laboratory coats or disposable gowns that provide complete coverage of skin must be worn when working with nanomaterials. Clothing contaminated with nanomaterials should be removed immediately. Do not take contaminated work clothes home—contaminated clothing may require disposal as hazardous waste.

11.4 Respiratory Protection

If engineering controls are not adequate or are not available, and a potential aerosol exposure exists, respiratory protection are required. When working with nanomaterials, one of the following types of respirators must be worn:

- Filtering face piece (N-95 or greater);
- Elastomeric half- or full-face piece with N-100, R-100, or P-100 filters; or
- Powered air-purifying respirator with N-100, R-100, or P-100 filters.

Anyone required to utilize respiratory protection for use with nanoparticle research must contact Chemical Safety at 713-500-5832 to be included in UTHSC-H Respiratory Protection Plan.

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Cadmium Toxicity Showing Organ Specific Signature of Responsiveness

Sandeep Kumar Agnihotri and Ilora Ghosh

Abstract Environmental pollution has a concerned issue now days, increased continuously many fold in current years and has reached the levels, toxic for living things. Toxic heavy metals are the chemicals that more hazards for biological system. Some heavy metals are essential for life, but others like Cadmium (Cd), Hg, As and Pb are non-essential and dangerous for living organism. Cadmium is environmental and industrial pollutant, present in air, drinking water and in food, has several reports to health effect. Cadmium is a toxic heavy metal that accumulates in the body and produce serious illness. Yet being a toxic metal, it's mechanism of toxicity remain unclear but generation of Reactive Oxygen species (ROS) identified as a main role in toxicity. Cadmium induce (ROS) level causes to damage to the organs of body; generate harmful effect and many diseases and also affect the apoptosis inducing factor, caspases and Bax overexpression. Apoptosis disorder are associated with many diseases, such as cancer, autoimmune disorder, and neurodegenerative disease. Chronic cadmium exposure causes several disease and morphological changes in body organs, induce the signalling pathway which caused generation of ROS.

Keywords Cadmium · Reactive oxygen species · Heavy metals · Metal toxicity

1 Introduction

Environment describes the living and nonliving surroundings of an organism or group of organisms. It contains all the elements, factors and condition that have some impact on augmentation and development of organism. Environmental

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conditions are dynamic and often change over time and therefore, organisms must have the ability to adapt to these changes. However, tolerance range is different for all species and exposure to environmental conditions beyond the limit of an organism's tolerance range represents environmental stress which effect on health.

In nature, the Earth is the only planet at the moment, where environment is suitable for life. Lots of living and nonliving factors are associated with each other's, which support life. In nonliving factors nutrients play the major role in metabolism of organism, which are necessary in certain amount must be taken from environment. In this way environment play the important role in development of organism and these nutrients play a crucial role. To achieve optimum living conditions, it is imperative to safeguard environmental health by regulating potentially hazardous material in the environment like heavy metals, lead, mold, and different other types of waste. Out of this segment, the balance of heavy metals in the environment is very crucial since they can be both harmful and essential for life depending on their concentration. Thus, arises the need study heavy metals and their impact on health.

1.1 Environment and Metal Ions

Metals and minerals form keys component in everyday life, their necessities increasing continuously with our home, appliances, tools and cars. We find ourselves becoming increasingly dependent on a vast array of new technologies as computer information system and global communication network all of which need metal. Metals are also integral part of basic infrastructure of our society, transportation system (vehicle, highways, bridges, railroads, and airport), food production, distribution and electrical utilities.

Metals are the natural components of earth crust, they cannot be degraded or destroyed, after mining or industrial use they are released into water, air and in soil in different form and contaminate the environment. In trace amounts they also enter our bodies via food, drinking water and air and maintain the metabolism of human body (Copper, selenium, zinc).

Basically minerals are inorganic substances, present in all body tissues and fluids and their presence is necessary for the maintenance of certain physicochemical processes, they are chemical constituents used by the body in many ways. Even though they yield no energy, they have important roles to play in many activities in the body (Malhotra 1998; Eruvbetine 2003; Hays and Swenson 1985; Ozcan 2003). Minerals may be broadly classified as macro (major) or micro (trace) elements. The third category is the ultra-trace elements. The macro-minerals include calcium, phosphorus, sodium and chloride, while the micro-elements include iron, copper, cobalt, potassium, magnesium, iodine, zinc, manganese, molybdenum, fluoride, chromium, selenium and sulfur (Eruvbetine 2003). The macro-minerals are required in amounts greater than 100 mg/dl and the micro-minerals are required in amounts less than 100 mg/dl (Murray et al. 2000). The ultra-trace elements include boron,

silicon, arsenic and nickel which have been found in animals and are believed to be essential for these animals. Evidence for requirements and essentialness of others like cadmium, lead, tin, lithium and vanadium is weak (Albion Research Notes 1996).

The importance of mineral elements in organism (human, animal) and plant nutrition has been well recognized (Underwood 1971; Darby and Zimmer 1976). Deficiencies or disturbances in the nutrition of an animal cause a variety of diseases and can arise in several ways (Gordon 1977). When a trace element is deficient, a characteristic syndrome is produced which reflects the exact functions of the nutrient in the metabolism of the animal. The trace elements are essential components of enzyme systems. Simple or conditioned deficiencies of mineral elements therefore have intense effects on metabolism and tissue structure. The significance of the mineral elements in humans, animals and plants nutrition cannot be overemphasized. The presence of mineral elements in animal feed is vital for the animal's metabolic processes. Mineral deficiencies or imbalances in soils and forages account partly for low animal production and reproductive problems. Soil acidity and season are factors affecting mineral uptake by plants. Plants use these minerals as structural components in carbohydrates and proteins; organic molecules in metabolism, such as magnesium in chlorophyll and phosphorus in ATP; enzyme activators like potassium, and for maintaining osmotic balance. Calcium is highly implicated in the maintenance of firmness of fruits (Olaiya 2006) and its requirements in fruits are related to cell wall stability and membrane integrity (Belakbir et al. 1998). Mineral elements play important roles in health and disease states of humans and domestic animals, as iron deficiency anaemia and goitre due to iodine deficiency are reported to be problems of public health importance in some communities (Partwardhan 1961; Deosthale and Belavady 1978). Trace elements of significance to people with HIV are zinc and selenium. Selenium is an antioxidant that increases immune function. Zinc, usually taken to stimulate the immune system, has been reported to weaken immune system function and lower calcium levels in HIV positive men (O'Connor 1995).

1.2 Environmental Metal Toxicity and Health

Environmental toxicity due to metals is apparent when they are present above their tolerant limits in high concentrations. Toxic metals comprise a group of minerals that have no known function in the body and, in fact, are harmful. Today mankind is exposed to the highest levels of these metals in recorded history. This is due to their industrial use, the unrestricted burning of coal, natural gas and petroleum, and incineration of waste materials worldwide. Toxic metals have become a major cause of illness, aging and even genetic defects. Heavy metals may have cumulative deleterious effects that can cause chronic degenerative changes (Ibrahim et al. 2006), especially to the nervous system, liver, and kidneys, and, in some cases, they also have teratogenic and carcinogenic effects (IARC 1976). The mechanism of

toxicity of some heavy metals still remains unknown, although enzymatic inhibition, impaired antioxidants metabolism, and oxidative stress may play a role. Heavy metals give many of their adverse health effects through the formation of free radicals, resulting in DNA damage, lipid peroxidation, and depletion of protein sulfhydryls (e.g., glutathione) (Valko et al. 2007). The importance of these metals as environmental health hazards is readily evident on the current Agency for Toxic Substances and Disease Registry Priority List of Hazardous Substances (ATSDR 2005). The effect of metal is based on the toxicity of the substance and the potential for exposure from air, water, or soil contamination. As a result of the extensive use of metals and their compounds in industry and consumer products, these agents have been widely disseminated in the environment. Because metals are not biodegradable, they can persist in the environment for long and produce a variety of adverse effects (Table 1). Maximum levels for heavy metals in food have been set in consideration for possible chemical contaminants.

Table 1 Toxic effect of different heavy metals on health

Heavy metal	Most affected organs	Chronic health effects	References
Arsenic	(i) Central nervous system (ii) Lungs (iii) Digestive tract (iv) Circulatory system (v) Kidneys	(i) Cancers (ii) Peripheral vascular disease, which in its extreme form leads to gangrenous changes (black foot disease, only reported in Taiwan) (iii) Skin lesions (melanosis, keratosis) (iv) Hearing loss (v) Reproductive toxicity (vi) Hematologic disorders (vii) Neurological diseases (viii) Developmental abnormalities and neurobehavioral disorders	(Guha Mazumder 2008)
Lead	(i) Central nervous system (ii) Erythropoiesis (iii) Kidneys (iv) Liver	(i) Cancers (ii) Kidney damage (iii) Neurological diseases (iv) Impaired intellectual ability and behavioral problems in children	(Goyer 1993)
Cadmium	(i) Kidneys (ii) Bone (iii) Liver (iv) Lungs	(i) Cancers (ii) Kidney damage (iii) Bronchiolitis, COPD, emphysema, fibrosis (iv) Skeletal damage, first reported from Japan, the itai-itai (ouch-ouch) disease (a combination of osteomalacia and osteoporosis)	(Nawrot et al. 2010)
Mercury	(i) Central nervous system (ii) Kidneys (iii) Liver (iv) Lungs	(i) Lung damage (ii) Kidney damage (iii) Neurological diseases (iv) Impaired intellectual ability and behavioral problems in children	(Díez 2009)

Toxic metals replace nutrient minerals in enzyme binding sites. When this occurs, the metals inhibit, over stimulate or otherwise alter thousands of enzymes. An affected enzyme may operate at 5% of normal activity. This may contribute too many health conditions. Toxic metals may also replace other substances in other tissue structures. The replacement process weakens these tissues, such as the arteries, joints, bones and muscles.

Divalent cations (Ca^{++}) show hypertrophy and draw attention to areas, which remain unexplored with perspective to cardiovascular disease (Tomera et al. 1994). Ca^{++} and Mg^{++} are of great physiologic importance by their intervention in many enzymatic systems and their function in neural excitability, muscle contraction, blood coagulation, bone formation, hormone secretion, and cell adhesion (Hoenderop and Bindels 2005).

The severity of adverse health effects is related to the chemical form of heavy metals and is also time and dose dependent. As mentioned earlier, heavy metals as environmental pollutants and promoters of oxidative stress are associated with a multitude of disadvantageous impacts on human health. There is a growing concern about the physiological and behavioral effects of environmental heavy metals in human population. Human intoxication has both acute and chronic effects on health and environment (Alissa and Ferns 2011). Albeit the toxicity of heavy metals at high levels of exposure is well known, a major concern of today is the possibility that continual exposure to relatively low levels of heavy metals may entail adverse health effects. Early studies have shown that vascular effects of heavy metals may contribute to a variety of pathologic conditions including diabetes mellitus and hypertension (Prozialeck et al. 2006). Mechanisms of action after heavy metal intoxication are less well studied and are still unclear.

1.3 Metal Ion, Reactive Oxygen Species (ROS) and Disease

Toxic metals (lead, mercury, cadmium and arsenic) are widely found in our environment. Humans are exposed to these metals from numerous sources, including contaminated air, water, soil and food. Recent studies indicate that transition metals act as catalysts in the oxidative reactions of biological macromolecules therefore the toxicities associated with these metals might be due to oxidative tissue damage.

Some of the metals are necessary at low levels for cellular function, such as iron, manganese, cobalt, zinc, copper, selenium, chromium, nickel. These metals are absorbed from food or water through the duodenum and transferred to tissue, where these metals are used as cofactors in enzymatic reactions as they readily transition between oxidized and reduced state. These metals must be regulated and monitored in cellular level because deficiency of these metal causes loss of enzymatic activities while higher concentration causes improper enzymatic reaction which generate ROS, oxidative damage and finally cell death (Nelson 1999).

Some metals serve critical functions in the human body. Iron is a necessary component of haemoglobin in red blood cells. Copper, manganese, zinc, cobalt, chromium, molybdenum, and selenium are all required as enzyme cofactors or prosthetic groups, and human disease results if the diet is deficient in the metals. However, exposure of humans to excessive levels of "physiologic" metals is associated with disease. Other metals, such as lead, cadmium and arsenic, have no known beneficial effects in the human body and have only toxic actions. In relative to toxicity, cadmium is found as a contaminant in the environment and in several ways affect the life with deleterious health effect.

2 Cadmium: A Contaminant in Environment

Cadmium word is form of two words first is Latin word *cadmia* and the second is Greek word *kadmeia* that are ancient names for calamine. Cadmium was discovered in Germany in 1817 by German chemist Friedrich Strohmeyer. Cadmium is a soft, malleable, ductile, bluish-white metal (Table 2) with the biological half-life more than 30 years.

2.1 General Standard

Cadmium is a toxic heavy metal distributed all around the world in earth crust in very small amounts, and found in nature in combination with different chemical elements (Zinc and Sulpher). Naturally 25,000 tons of cadmium is released in a year, nearly half of which goes into rivers through weathering rocks and some released in the air through forest fire and volcanoes. Cadmium metal is also released in the environment by extraction, smelting and refining of other metal as copper, zinc and lead and also from spent nickel cadmium batteries. Cadmium also used in electric batteries, electronic components, nuclear reactors (Friberg et al. 1986; Ros and Slooff 1987) and electroplating of other metals such as steel, iron and copper

Table 2 General properties of cadmium

Name	Cadmium
Symbol	Cd
Atomic Number	48
Atomic Mass	112.411 amu
Melting Point	320.9 °C
Boiling Point	765.0 °C
Number of Protons/Electrons	48
Classification	Transition Metal
Crystal Structure	Hexagonal
Density @ 293 K	8.65 g/cm^3
Color	Silvery

(IARC 1976; Ros and Slooff 1987)

and used in alloys with copper, nickel, gold, silver and aluminium for coating other materials. Cadmium is produced mainly as a by-product of mining, smelting and refining of zinc and, to a lesser degree, as a by-product of lead and copper production. Cadmium used for different purpose in industries like pigments for plastics, ceramics and enamels, stabilizers for plastics, plating on iron and steel; and alloying element of some lead, copper and tin alloys, production of dyes, phosphate fertilizers, photography.

Under Schedule II of the Environment (Protection) Act 1986, general standards for discharge of effluent into different water bodies are notified (Table 3).

Guideline Limit for Agricultural Water and Water for Protection of Aquatic life: CCME (1999) has set the maximum guideline limit of cadmium for agricultural water and for protection of freshwater and marine aquatic life (Table 4).

2.2 *Industry Specific Standards*

The industries produce effluents of varied qualitative quantity and characteristics. In order to reduce the environmental pollution, industry specific standards have been notified by Government of India under schedule I of The Environment (Protection) Act 1986 (Table 5).

Table 3 Discharge (mg/l) Standards for Cadmium content in effluent

Inland surface water	Public Sewer	Marine coastal areas
2.00	1.00	2.00

Table 4 Limit of cadmium in water for protection of aquatic life

Agricultural water (g/l)		Water for protection of aquatic life (g/l)	
Irrigation water	Water for livestock	Fresh water	Marine water
5.1	80	0.017	0.12

Source CCME (1999)

Table 5 Industry specific standards for cadmium

Industry	Cadmium (mg/l)
Small scale industries (located in the Union Territories)	2.00
Dye and dye intermediate industries	2.00
Electroplating industries	2.00
Inorganic chemical industry (wastewater discharge)	0.20
Bullion refining	0.20
Treated effluent quality of CETP	
Discharge into surface waters	1.00
Discharge into coastal waters	2.00

Cadmium is also present in cigarette. It is estimated that single cigarette typically contains 1–2 μg of cadmium. When burned, cadmium is present at a level of 1000–3000 ppb in the smoke. Approximately 40–60% of the cadmium inhaled from cigarette smoke is able to pass through the lungs and into the body. This means that for each pack of cigarettes smoked, a person can absorb an additional 1–3 μg of cadmium over what is taken in from other sources in their daily life. Smokers typically have twice as much cadmium in their bodies as their non-smoking counterparts.

2.3 Food Contaminant

Cadmium is spread in surrounding through waste water and diffuse type of pollution done by fertilizers and local air pollution. Cadmium is taken up by roots of plants to leaves, fruits and seed and also builds up in animal milk and fatty tissue (Kaneta et al. 1986). Hence, animal and human being are exposed to cadmium due to using up of cadmium containing plants or animals, seafood (mollusks and crustaceans) and fish. Mainly crops, which grown in polluted soil or irrigated with polluted water may contain higher concentration of cadmium, as may meat from animals grazing on contaminated pasture (IARC 1976). Some of the plants which are used for daily consumption like potatoes, cereal grains, other vegetables, tobacco, take up cadmium more avidly rather than other heavy metal (Lead and mercury), (Satarug et al. 2003).

As per The Prevention of Food Adulteration Act 1954, Government of India limits the cadmium content 1.50 ppm by weight for all food items. The Ministry of Health and Family Welfare Government of India and the State Health Directorate are responsible for implementing this regulation (Table 6).

2.4 Water Contaminant

World health organization (WHO) primary goal states “all people, whatever their stage of development and their social and economic conditions, have the right to have access to an adequate supply of safe drinking water” (WHO/SDE/WSH/03.04/80).

But due to development, industrialization and various activities of human being affected the quality of water. Direct influence of human involved in spreading the natural or artificial substances which are introduced in nature by human being into the geochemical cycle of the earth, finally these substances reach the groundwater zone and polluted the water. Definition of polluted groundwater states that groundwater that has been affected by man to the extent that it has higher

Table 6 Standard residual limit of cadmium in food products

Country/ Organization	Standard*	Product	Concentration (mg/kg, wet weight)
WHO/FAO	CAC	Fish and fishery	1.00
		Leafy vegetables	0.20
		Other vegetables	0.05
		Stem and rot vegetables	0.10
		Potatoes	0.10
		Wheat grain	0.20
Australia	Max. conc	Root, tuber and leafy vegetables	0.10
	TPHR	Fish and fishery	5.50
	NHMRC	Fish and fishery	2.00
East European Union (EU)	–	Fish and fishery	0.10–1.0
Czech Republic	–	Sea fish	0.20
		Fresh water fish	0.10
		Molluscs	1.00
		Crustaceans and gastropods	0.50
USA	FDA	Fish and fishery	2.00
Japan	–	Fish and fishery	1.00
India	–	Fish and fishery	3.00

Source CPCB (Central Pollution Control Board)
(**CAC* Codex Alimentarius Commission, *FDA* Food and Drug Administration, USA, *NHMRC* National Health Medical Council, *TPHR* Tasmania Public Health Regulation)

concentration of dissolved and suspended element than the maximal permissible concentration fixed by national and international standards for drinking, industrial or agricultural purposes (Matthess 1972). Many industries effluents like mineral fertilizers, municipal, pesticide and livestock farms, poultry farms, agriculture waste, herbicides, refuse contain heavy metals, many of these effluent contain various elements such as cadmium, copper, mercury and lead, which are toxic at low concentration (Khublarian 1989), which are able to penetrate into groundwater, cadmium is one of them major element.

Unpolluted natural water also contain the cadmium, concentration below then 1 μg/l (Friberg et al. 1986), median concentration of dissolved cadmium found <1 μg/l, 110 station around the globe, Rio Rimao in Peru was the place, where maximum value 100 μg/l recorded (WHO/UNEP 1989). The solubility of cadmium in water is affected by its acidity, suspended sediment bound with the cadmium dissolved when there is increase in acidity (Ros and Slooff 1987). In natural waters, cadmium is found mainly in bottom sediments and suspended particles (Friberg et al. 1986).

2.5 Cadmium and Health

First time detection of cadmium observed in Japan, when Itai-Itai bone disease drew the attention of the public and observed the toxicity of heavy metal in environment (Hagino and Yoshioka 1961). After such type of report, a number of epidemiological and experimental studies are being carried out worldwide to characterize the toxicity of cadmium and check the level of cadmium harmful for human health (Table 1).

Consumption of staple foods such as wheat, rice also significantly contributes to human exposure, regardless the route of exposure, Cd is efficiently retained in the organism and remains accumulated throughout life (Friberg 1950), where the percentage of availability is indicated in Fig. 1. After research and data analysis, a relation between cadmium intake and chronic health damage was observed, which would be corresponding to different degree of health injury (Cai et al. 1998; Shang and Cai 1993). The studies also shown that higher value of cadmium in rice caused population higher cadmium exposure and induces series of health effect, such as severe renal injury, higher mortality, shorter survival time, and unfavorable prognosis (Kobayashi et al. 2002; Matsuda et al. 2003; Nishijo et al. 2006), increases of cadmium in environment also increases non-cardiovascular mortality in continuous fashion (Nawrot et al. 2008), cadmium exposure could have immunosuppressive effect, IgG3 was most sensitive inhibitory effect of cadmium, followed by IgG1, IgG2, IgM and IgG2a, IgG could be used as the sensitive indicator of immune suppression of children exposed to Cd (Ritz et al. 1998), type 2 diabetes also have significant dose response relationship with urinary cd concentration (Masayuki et al. 2005). Cadmium also effected the pregnant women in perinatal period e.g. fetal growth retardation, low birth weight, birth deformities and premature (Falcon et al. 2002), serum cadmium level show the significant negative correlation with all biophysical semen characteristics except sperm volume (Epstein et al. 2000) and also show popular mortality (Nishijo et al. 1999). It's also found that some disease in cadmium contaminated areas were higher than non-contaminated areas, such as cancer, respiratory disease, cerebrovascular disease, breast and endometrial cancer (McElroy et al. 2006; Agneta et al. 2008). Cadmium increases the blood pressure (Maria et al. 2008) and mortality observed due to heart failure and cerebral infarction in the cadmium polluted area (Nishijo et al. 2006), cadmium exposure could result in pancreatic dysfunction and the lower urinary cadmium level affected then renal dysfunction (Lei et al. 2007), in lower dose level it risks for chronic kidney disease (CKD), defined by the loss in glomerular filtration rate (GFR) (Ginsberg 2012). It's found that, low level of exposure of cadmium is an important cause of cardiovascular mortality (Tellez-Plaza et al. 2012) hepatocellular carcinoma (HCC) and hepatic adenoma, but long term exposure is associated with pre diabetic and diabetes in dose dependent manner (Satarug 2012). Recently it has been investigated that cadmium affects the brain more adversely respect to the other organs of body in the same dose response (Agnihotri et al. 2015).

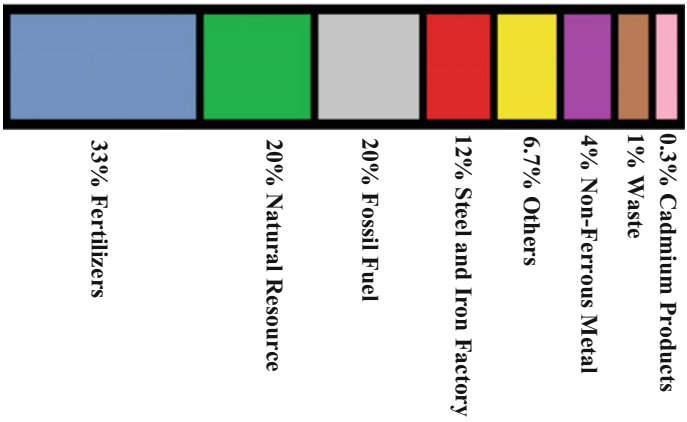

Fig. 1 Relative Contribution (%) of different source of cadmium to human exposure

3 Correlation Between Cadmium and ROS

The critical targets of cadmium binding are the thiol groups of proteins (Sabolic et al. 2002). Thiol proteins are important in cellular anti-oxidant defenses and redox signaling, and it is thought that ROS cause selective thiol oxidation. The relative sensitivities of different cell proteins and critical targets are, however, not well characterized. Most of the cadmium penetrates into cells combined in metallothionein complexes. Cadmium–metallothionein complexes cause endocytosis of brush-border transporters in rat renal proximal tubules (Sabolic et al. 2002). This may lead to a loss of cell membrane function, resulting in absorptive and secretory defects that occur in cadmium-induced nephrotoxicity. Some of the cadmium–metallothionein complexes have been identified in the cytosol of epithelial cells, with secondary evidence that the main target organelles are the mitochondria (Tang and Shaikh 2001). Cadmium penetration of the mitochondria has been identified (Waku 1984; Lee et al. 2005) with the outcome of significant inhibition of mitochondrial function, increased ROS production, and eventual apoptotic cell death (Ossola and Tomaro 1995; Ercal et al. 2001; Lee et al. 2005; Oh and Lim 2006; Cannino et al. 2009) (Fig. 2). The mechanisms behind these changes are the subject of much research, but they still need further definition. Concerning effects on the mitochondrial electron transport chain, heavy metals in mitochondria most likely inhibit the activity of complexes II and III more than that of the other complexes. Since the principal site of ROS production seems to reside in complex III (Wang et al. 2004), its dysfunction may increase ROS production beyond the neutralizing ability of normal anti-oxidants that also reside in mitochondria.

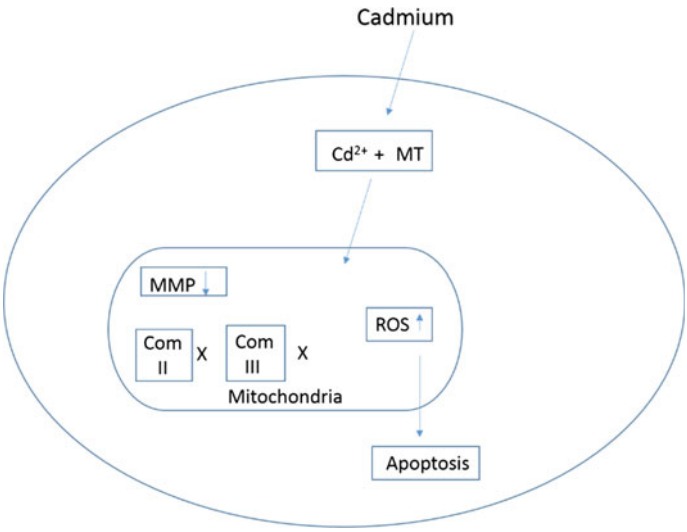

Fig. 2 Cadmium induces the ROS through mitochondria and inhibits the complex II and III of mitochondria

3.1 An Intermediate Pathogenesis: ROS Generation by Metal Toxicity

Reactive oxygen species (ROS) is a phrase used to describe a variety of molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen. Molecular oxygen in the ground state is a bi-radical, containing two unpaired electrons in the outer shell (also known as a triplet state). Since the two single electrons have the same spin, oxygen can only react with one electron at a time and therefore it is not very reactive with the two electrons in a chemical bond. On the other hand, if one of the two unpaired electrons is excited and changes its spin, the resulting species (known as singlet oxygen) becomes a powerful oxidant as the two electrons with opposing spins can quickly react with other pairs of electrons, especially double bonds. The reduction of oxygen by one electron at a time produces relatively stable intermediates. Turrens (2003) has reported that the superoxide anion (O_2^-), the product of a one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions as also indicated in Fig. 3.

'Oxidative stress' describes various deleterious processes resulting from an imbalance between the excessive formations of ROS. While small fluctuations in the steady-state concentration of these oxidants may actually play a role in intracellular signaling (Droge 2002), uncontrolled increases in the steady-state concentrations of these oxidants lead to free radical-mediated chain reactions which target proteins (Stadtman and Levine 2000), lipids (Rubbo et al. 1994), polysaccharides (Kaur and Halliwell 1994) and DNA (Richter et al. 1988; LeDoux et al. 1999).

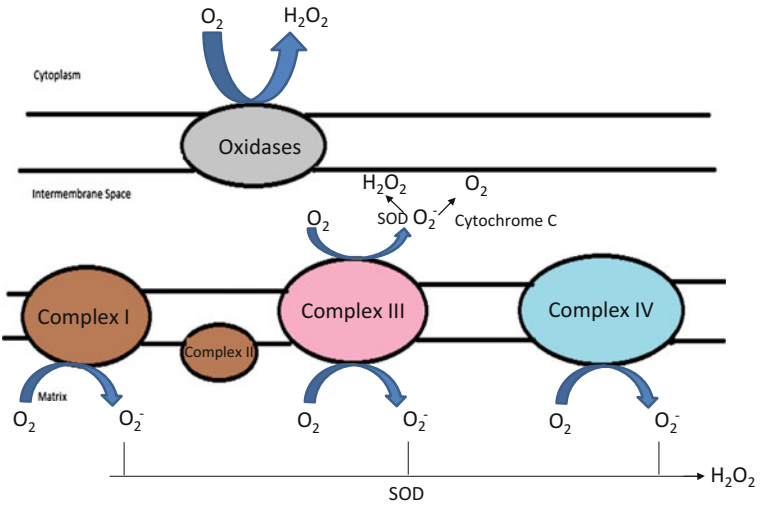

Fig. 3 Showing mechanism for the site of superoxide formation in cytoplasm and intermembrane space

ROS, oxidative stress and environmental insult from a complex relationship with important influence on human health and disease. Exposure to environmental pollutants like UV, chemicals, pollution, heavy metals etc. has detrimental effect on health and is considered crucial contributor to most diseases of major public health significance. Thus, it is important to understand the basic biological processes that are altered or regulated by heavy metal pollutant, and that stimulate the course of the disease process. Environmental stressors are well known to induce oxidative stress and alternations in the cellular redox balance. In addition, oxidative stress has been widely shown to regulate apoptosis.

Oxidative stress is caused by an imbalance between the production of ROS and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. The primary site of ROS generation in a cell is the powerhouse, mitochondria. In other words, ROS is the price by the cell for its energy production by oxidative phosphorylation.

Oxidative phosphorylation is a metabolic pathway that uses energy released by the oxidation of nutrients to produce adenosine triphosphate (ATP). During oxidative phosphorylation, electrons are transferred from electron donor to electron acceptors such as oxygen, in a redox reaction. This step releases energy, which is captured in the form of ATP. In eukaryotes these reactions are carried out by series of protein complexes within mitochondria, whereas in prokaryotes, these proteins are located in the cell's inner membranes. These linked sets of enzymes are called electron transport chains. Although oxidative phosphorylation is a vital part of

metabolism, it produces ROS such as free radicals. ROS is a collective term that describes the chemical species that are formed upon incomplete reduction of oxygen and includes the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO). ROS are thought to mediate the toxicity of oxygen because of their greater chemical reactivity with regard to oxygen (D'Autréaux and Toledano 2007). These free radicals, especially hydroxyl radicals, randomly attack all cell components, including proteins, lipids and nucleic acids, potentially causing extensive cellular damage. Cellular damage consistent with ROS accumulation in the cells includes damage to mitochondria and vacuole membranes.

3.2 *Antioxidant Enzyme and Diseases: Reaction of Toxic Metal*

In our body immunity work against pathogens and defense system of body activated when heavy metal toxicity increases and antioxidants system activated. Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals induced by heavy metal and antioxidant enzymes are endogenous proteins that work in combination to protect cells from ROS damage. ROS are physiologic by-products capable of directly injuring cells, presumably resulting in part from an imbalance of antioxidant enzymes and free radicals.

Aerobic organisms possess antioxidant defense systems that deal with ROS produced as a consequence of aerobic respiration and substrate oxidation. In nature always minute amounts of ROS, including hydroxyl radicals ($^{\bullet}OH$), superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), are constantly generated in aerobic organisms in response to both external and internal stimuli (Hurst et al. 1997; Jornot et al. 1998; Mills et al. 1998). little amount of ROS is crucial in many biochemical processes, including intracellular messaging in the cell differentiation and cell progression or the arrest of growth, apoptosis (Ghosh and Myers 1998), immunity (Yin et al. 1995), and defense against micro-organisms (Bae et al. 1997; Lee et al. 1998). But in high doses or inadequate ROS result in oxidative stress, which may cause severe metabolic malfunctions and damage to biological macromolecules (Wojtaszek 1997; Chopra and Wallace 1998).

3.3 *Role of Mitochondria and ROS in Cellular Pathogenesis of Metal Toxicity*

Environmental stress induced by heavy metal, like cadmium induces the ROS after affecting the mitochondrial dysfunction and produces the less ATP and higher amount of ROS, which causes the imbalance between ROS and antioxidants,

creates the oxidative stress (Gobe and Crane 2010). Metal induced mitochondria loss its membrane potential and release the cytochrome C and activated the caspases leads the cell to apoptosis. In apoptosis cell, mitochondria play an important role in different mechanistic signaling pathway to generate oxidative stress with ROS production.

Mitochondria consume more than 90% of the cells oxygen; a critical by-product of mitochondrial bio-energetic activity is the generation of ROS including superoxide and hydroxyl radicals and hydrogen peroxide (H_2O_2) (Boveris and Chance 1973). In electron transport chain (ETC) inside the mitochondria oxygen directly generate the superoxide anion radical, complex I, II and III of the respiratory chain are the site of ROS generation (McLennan and Degli Esposti 2000). Superoxide radicals can be converted to H_2O_2 by superoxide dismutase (SOD), which react with highly reactive hydroxyl radicals, these metabolic by product are highly toxic and cell damaging oxidant, which neutralized by antioxidant enzyme, some of which found in mitochondria (Mn SOD and glutathione peroxidase) and some in cytosol (Cu-SOD and catalase), Increased ROS generation and impaired antioxidant defenses cause intense effect on cells together with lipid peroxidation targeting membrane phospholipid and proteins (Raha and Robinson 2000), respiratory chain is also effected by ROS generation, and defect increases more ROS level leading to nasty cycle,which diminished the mitochondrial function. Increased ROS generation and oxidative stress have a fundamental role in the regulation of cellular signaling and cytoprotection (Oldenburg et al. 2002), Oxidative species (e.g. H_2O_2) can also function as a signal sent from mitochondria to other cellular sites rapidly and reversibly eliciting an array of intracellular cascades leading to different physiological end points for the cell (e.g. apoptosis, necrosis, cell proliferation) and also affects cell morphology and reorganization of cell architecture.

There is accumulating evidence that mitochondria play an essential role in many forms of apoptosis (Reed 2002) by releasing apoptogenic factors, such as cytochrome c (Kluck et al. 1997; Yang et al. 1997) from the intermembrane space into the cytoplasm, which activates the downstream execution phase of apoptosis with impact of metal toxicity. During apoptosis, the pro-apoptotic Bcl-2 family members are activated; presumably undergo a conformational change (Desagher and Osen-Sand 1999), leading to the exposure of the pro-apoptotic BH3 domain (Zha et al. 1996) and proteolytic cleavage by caspases (Li et al. 1998); and translocate to the mitochondria. Bax translocation to the mitochondria involves homo-oligomerization (Gross et al. 1998). The translocation of Bax, Bid, or Bad to the mitochondria can then induce a dramatic event these organelles release the proteins contained within the intermembrane space, including cytochrome C. Cytochrome C is encoded by a nuclear gene, but when it is imported into the mitochondria, it is coupled with a heme group to become holocytochrome c, and it is only this form that functions to induce caspase activation (Yang et al. 1997). While the anti-apoptotic proteins Bcl-2 and Bcl-xL work to prevent cytochrome C release from mitochondria, and thereby preserve cell survival (Kluck et al. 1997; Yang et al. 1997). This occurs through the formation of an “apoptosome” [consisting of cytochrome C, apoptotic protease activating factor-1(Apaf-1), and

procaspase-9], dependent on either ATP or dATP in the cell. Apaf-1 (Zou et al. 1997, 1999) is a protein contained within the cytosol, and cytochrome C binds and induces it to oligomerize. This, then, recruits an initiator caspase, procaspase-9 (Li et al. 1998). Unlike other caspases, procaspase-9 does not appear to be activated simply by cleavage, but, instead, must be bound to Apaf-1 to be activated (Rodriguez and Lazebnik 1999; Stennicke et al. 1999). The apoptosome can then recruit procaspase-3, which is cleaved and activated by the active caspase-9, and can release it to mediate apoptosis. Once the initiator caspases have been activated, they can proteolytically activate the effector procaspases-3, -6 and -7 which subsequently cleaves a specific set of protein substrates, including procaspase themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed (Hinokio et al. 1999).

Besides amplifying and mediating extrinsic apoptotic pathways, mitochondria also plays a central role in the integration and propagation of death signals originating from inside the cell such as DNA damage, oxidative stress, starvation, as well as those induced by chemotherapeutic drugs (Kaufman et al. 2000; Wang 2004). Most apoptosis inducing conditions involve the disruption of the mitochondrial inner transmembrane potential (DY) as well as the so called permeability transition (PT), a sudden increase of the inner mitochondrial membrane permeability to solutes with a molecular mass below approximately 1.5 kDa. Concomitantly, osmotic mitochondrial swelling has been observed by influx of water into the matrix with eventual rupture of the outer mitochondrial membrane, resulting in the release of proapoptotic proteins from the mitochondrial intermembrane space into the cytoplasm (Bernardi et al. 1999; Loeffler and Kroemer 2000). In addition to the release of mitochondrial factors, the dissipation of Dy and PT also cause a loss of the biochemical homeostasis of the cell: ATP synthesis is stopped, redox molecules such as NADH, NADPH, and glutathione are oxidized, and ROS are increasingly generated (Kroemer 2003). Increased levels of ROS directly cause oxidation of lipids, proteins, and nucleic acids, thereby enhancing the disruption of mitochondrial inner transmembrane potential as part of a positive feedback (Marchetti et al. 1997).

4 Stress Signaling and Apoptosis: Relation to Metal Toxicity

It has been increasingly recognized that mitochondria play an essential role in the early events of apoptosis (programmed cell death). Mitochondrial apoptotic pathway is the release from the intermembrane space into the cytosol of a group of proteins (cytochrome c, Smac/diablo, AIF and endonuclease G) which subsequently triggers a cascade of cytoplasmic changes (Van Gurp et al. 2003). Caspases which are normally inactive, require proteolytic processing for activation that is achieved

by the cytosolic formation of large protein complexes (apoptosome) including released cytochrome c, Apaf-1, and recruited caspases (caspase 9); the assembly and function of the apoptosome are regulated by Smac/diablo, intracellular K^+ levels and inhibitor of apoptosis proteins (IAPs) (Kroemer 2003). Figure 4 shows the key role of proteins in cell signaling pathways, which are translocating to mitochondria. These proteins translocation occurs through outer membrane permeabilization, regulated by the complex interaction of different members of the Bcl-2 family including Bax, Bid, Bcl-2 and Bcl-xL (Kuwana et al. 2002).

Proteins of the Bcl-2 family share one or several Bcl-2 homology (BH) regions and behave as pro or anti-apoptotic proteins. The highly conserved BH domains (BH1-4) are essential for homo- and hetero-complex formation, as well as for their cell-death-inducing capacity. Pro- apoptotic homologues can be subdivided into the two major types: The Bax subfamily and the BH3-only subfamily (e.g. Bad, Bid); both types promote cell-death signaling by targeting mitochondrial membranes though different mechanisms. Pro-apoptotic membrane binding proteins (Bax, Bid, Bad), translocated from the cytosol to mitochondria, potentiate cytochrome c release whereas anti-apoptotic proteins Bcl-2 family and Bcl-xL antagonize this event and preserve outer membrane integrity. This protein release is also associated and potentially regulated by the opening of a membrane mega-channel, the mitochondrial permeability transition (PT) pore, a dynamic multi-protein complex located at contact sites between the mitochondrial inner and outer membranes (Marzo et al. 1998). Recent evidence has established that PT pore opening is a critical early step of apoptosis preceding the caspase cascade. PT pore opening is promoted by elevated Ca^{2+} influx into mitochondria, excessive mitochondrial ROS production (primarily from respiratory complexes I and III), pro-oxidants, fatty acids and nitric oxide; however, the precise mechanism involved in PT pore opening in apoptosis is not known (Kuwana et al. 2002). PT pore opening is

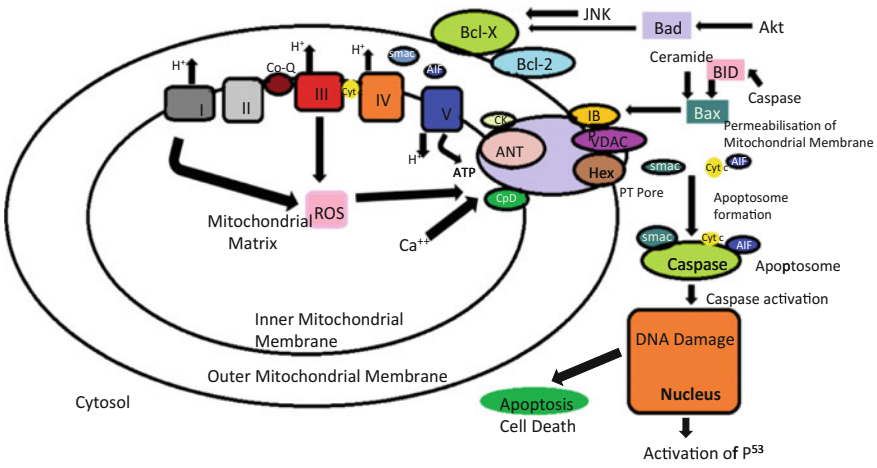

Fig. 4 Induced reactive oxygen species signaling pathway of mitochondria and apoptosis

accompanied by dissipation of the mitochondrial membrane potential and depolarization (Lemasters et al. 1998). Changes in membrane potential can be either a cause or the result of PT pore opening, since extensive proton influx occurs at this site. P38 MAPK stimulated Bax/Bid translocation from cytosol to the mitochondria causes mitochondrial membrane permeabilization (perturbing both inner and outer membranes) (Kuwana et al. 2002) resulting in subsequent cytochrome c release and loss of mitochondrial membrane potential in a variety of cell-types. The anti-apoptotic protein Bcl-2 (a mitochondrial protein) prevents the functional association of Bax with the mitochondria and interferes with the release of apoptogenic peptides (e.g. cytochrome c and AIF) from the mitochondria. Both Bax and Bcl-2 directly interact with VDAC (also called porin), both a component of the PT pore as well as a major contributor to mitochondrial outer membrane permeability. There is a continual dynamic in the balance of the pro-apoptotic proteins (e.g. Bax) and anti-apoptotic Bcl factors in modulating the progression of apoptotic events within the mitochondria indicated in Fig. 5 and reported by Kroemer (2003). In addition to its involvement in apoptotic cell death, opening of the PT pore has been implicated in cell death induced by excitotoxins (glutamate), as well as in necrotic cell death occurring during anoxia and ischemia (Schinder et al. 1996). This supports the emerging view that mitochondrial changes are a pivotal step in the

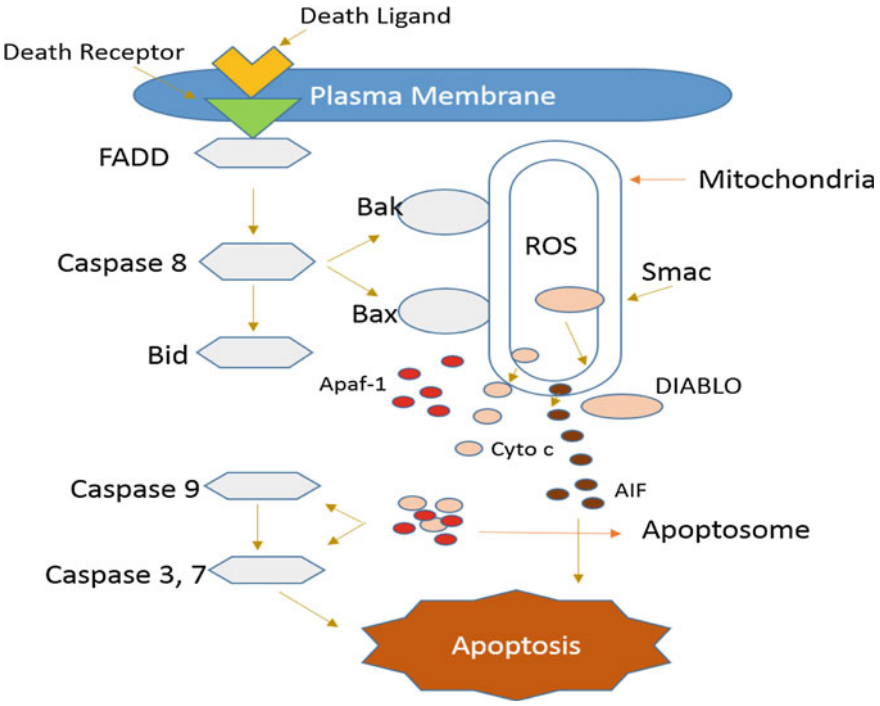

Fig. 5 Mechanistic view of stress signaling of metal toxicity and induction of apoptosis

commitment to both apoptotic and necrotic types of cell death. Decline in respiratory enzyme activities (particularly complex III) and in OXPHOS as well as changes in transmembrane potential contribute to the onset of apoptosis. This has led to the premise that the PT pore represents a site where mitochondria can integrate multiple cell-signaling stimuli and metabolic responses. In addition, opening of the PT pore during apoptosis is temporary allowing mitochondria to maintain ATP levels for fueling the downstream apoptotic responses. The early apoptotic events involve modulation in ATP levels, given the close proximity of the PT pore to respiratory complexes in the mitochondrial inner membrane as well as its involvement in the mitochondrial loss of cytochrome c, a critical molecule in ETC function (Kroemer 2003).

4.1 p53 Mechanistic Role in Stress Signalling

In addition to the pro-apoptotic and anti-apoptotic proteins that are recruited to the mitochondria, the oncogenic p53 protein also translocates to mitochondria subsequently induces the permeabilization of the outer mitochondrial membrane by interacting with the protective Bcl-xL and Bcl-2 proteins, resulting in cytochrome c release (Mihara et al. 2003). Both extrinsic and intrinsic pathways involving the mitochondria are regulated by p53 through transcription-dependent mechanisms or induce apoptosis through transcription-independent mechanisms. p53 is of central importance in apoptosis checkpoints in the defense against malignant transformation because it is inactivated in presumably more than 50% of all human cancers (Pluquet and Hainaut 2001). p53 is a tumor suppressor protein which is activated as a transcription factors in response to e.g. oncogene activation, hypoxia and especially DNA damage, resulting in growth arrest and/or apoptosis by stimulating the expression of various p53 target gene such as p21, Bax, PUMA, Noxa, Apaf-1, Fas, and DR5 or survivin (Vousden and Ryan 2009) or by repressing the expression of antiapoptotic proteins, e.g. Bcl-2, Bcl-xL or survive in (Hoffman et al. 2002). Recent evidence suggests transcription-independent p53 apoptosis pathways in which p53 translocates to the mitochondria, interacts with Bcl-xL, induces PT and the release of cytochrome C (Mihara et al. 2003). In non-stressed, undamaged cells p53 therefore must be kept under stringent control: it is present only at low cellular concentrations, it is retained in the cytosol and prevented to enter the nucleus, and its transactivation domain is inactivated (Chene 2003). Central to p53 regulation is the oncogene Mdm2 which binds to and thereby inhibits p53. Mdm2 is a ubiquitin-ligase which mediates ubiquitination of p53, thereby targeting it for degradation by the proteasome. In this way, p53 levels are kept low in normal cells (Kubbutat et al. 1997). The importance of Mdm2 in the control of p53 is demonstrated by Mdm2 gene knockout mice which die early during development but are rescued from death by additional deletion of the p53 gene (de Oca et al. 1995). In response to cellular stress (such as DNA damage), p53 is phosphorylated at specific serine/threonine residues which prevents the Mdm2-p53 interaction, and

thus p53 is stabilized and activated (Schon et al. 2002). Moreover, p53 is central to oncogene-induced cell death because it is induced by oncogenes such as c-myc, adenovirus E1A, and as as well as by loss of the retinoblastoma tumor suppressor pRb (Henriksson et al. 2001). All those oncogenes activate the transcription factor E2F-1 which not only can promote cell cycle progression and proliferation but at the same time directly triggers expression of the tumor suppressor ARF which leads to stabilization and activation of p53 (Ginsberg 2002). This explains in part why oncogene activation not always leads to uncontrolled cell proliferation but under certain circumstances to the stabilization of p53 and activation of cell death, provided that p53 signaling pathways are intact (Eischen et al. 1999).

Therefore, in many instances, an oncogenic insult only results in increased proliferation and eventually malignant transformation if activators of p53 (such as ARF, Chk2, or ATM), p53 itself (e.g. by Mdm2, the adenovirus E1B, papillomavirus E6, or SV40 large T antigen), or p53 downstream signaling components (p53 target genes) are inactivated. On the other hand, p53-mediated apoptosis pathways can be suppressed by survival signals, such as growth factors binding to their cognate growth factor receptors what eventually results in activation of the Akt kinase. Akt kinase is known to mediate a number of antiapoptotic mechanisms, such as the direct phosphorylation and inactivation of Bad and caspase-9, the activation of NF-kB antiapoptotic signalling via phosphorylation of IkB, but also phosphorylation and activation of Mdm2 as an inhibitor of p53 (Ozes et al. 1999). Besides phosphorylation, ubiquitination and protein-protein interactions, p53 is also regulated by acetylation what affects its transcriptional activity, as well as by sumoylation (Melchior 2003; Appella and Anderson 2001).

4.2 Mitogen-Activated Protein Kinases (MAPKs) Signaling in Metal Induced Stress

Mitogen-activated protein kinases (MAPKs) such as ERK, p38 and JNK (these last two also named stress- activated protein kinases, SAPK) have been widely reported as central players of stress induced apoptosis. More importantly they are very well known for their regulation by oxidative stress and reactive species. MAPKs are all serine/threonine kinases that are directed by a proline residue and are activated by threonine/tyrosine phosphorylation events. They all operate in a cascade fashion with a MAPK kinase (MAPKK) phosphorylating and activating a MAPK kinase (MAPKK) and the MAPKK phosphorylating and activating a MAPK. Inhibition of MAPKs has been demonstrated to protect against apoptosis induced by different condition of stress. However, many examples also exist about the protective effects of MAPK activity against apoptotic cell death. For JNK, its activation kinetics has been proposed to determine its role in induction or inhibition of apoptosis. Sustained activation of JNK has been proposed to have a role in promoting apoptosis. Moreover, the dynamic balance between mitogen (growth factor)–

activated ERK and stress-activated JNK-p38 pathways seems to be important in determining whether a cell survives or undergoes apoptosis. In general, MAPK activation seems to regulate the intrinsic mitochondrial pathway via regulation of the Bcl-2 family members, regulation of p53 and transcriptional regulation of many other genes via transcription factors such as c-Jun, ATF2, p53, and c-Myc.

Environmental stressors are well known to induce oxidative stress and alternations in the cellular balance. In addition, oxidative stress has been widely shown to regulate apoptosis. However, the complexity of redox signaling is evidenced by several reports showing that oxidative stress has been demonstrated to mediate cell proliferation and differentiation, which are considered the opposite of cell death by apoptosis. Earlier studies suggested that long exposure to oxidative stress leads to cell death, whereas low or transient exposure leads to survival/differentiation.

Although oxidative stress has been largely linked to the activation of distinct apoptotic enzymes, the direct mechanisms involved have remained largely elusive. Recently, post-translational oxidative protein modifications (OPMs), through subtle oxidative events that involve targeted amino acids in proteins, have been shown to regulate the activity of a wide variety of proteins such as kinases (ASK, JNK), phosphatases (PTEN, mitogen-activated protein kinase phosphatases), protease (caspase), molecular adaptors and chaperones (heat shock proteins and PDI), and transcription factors (Nrf2, NF-kB, AP-1, p53, HIF-1) involved in apoptosis. Oxidative stress-induced apoptosis has been largely associated to the activation of the intrinsic pathways of apoptosis at the level of the mitochondria. Redox signaling induced by environmental stressors involves both alternations in antioxidant defenses (such as decreases in GSH/GSSG ratio) and accumulation of ROS leading to oxidative stress. These biochemical events mediate a number of processes such as oxidative protein modifications, oxidative DNA damage and alternations in mitochondrial function which in turn trigger the activation of specific signaling cascades.

Environmental stress or toxicity induces apoptosis mainly by the regulation of intrinsic pathways of apoptosis activated by mitochondria, ER-stress and DNA-damage with a high degree of crosstalk between their signaling elements. Activation of SAPKs such as JNK and of transcription-dependent p53 signaling cascades act as important sensors for environmental stress and the induction of apoptotic cell death. In certain circumstances environmental toxicants might trigger other types of cell death pathways such as necrosis and autophagy.

Interestingly, environmental stressors also induce the activation of survival responses including, DNA repair mechanisms, MAPK/PI3 K signaling cascades and up-regulation of antioxidant defenses in an attempt to counteract the deleterious effects of cell death pathways. In fact, in most cases both apoptotic and survival signaling cascades have been observed to be activated in parallel in response to environmental toxicity. Tipping the balance towards either cell death or survival depends in most cases on the intensity, length and type of exposure. Finally, deregulated activation of survival signals as a consequence of mutagenesis is well known to promote cellular transformation aroused by the impairment of apoptotic signaling. Thus, future research in the understanding of both environmentally

induced cytotoxicity/apoptosis and environmentally induced cellular transformation is necessary for a complete understanding of the human health consequences to environmental exposures.

Gaining insight into the mechanisms and alternations by which components of the apoptotic machinery contribute to pathogenic processes, should allow the development of more effective, higher specific and therefore better-tolerable therapeutic approaches, which include the targeted activation of proapoptotic tumor suppressors or alternatively the blockade of anti-apoptotic oncogenes in the case of cancer, whereas for the treatment of premature cell death during e.g. neurodegeneration the inhibition of proapoptotic key components such as the caspases might be promising (Reed 2002).

5 Conclusion and Future Recommendations

Pollution of heavy metal continuously increases with industrialization, uses of fossil fuel and mining, in our environment and now it's effected the human life with decontaminated water, food and air and causes various types of diseases to disrupt the cell behavior inside the body. These heavy metals changed the internal environment of cells and disrupt the signaling pathway and leads to organ specific diseases to affect the mitochondria which induced ROS and apoptosis. In mechanistically, exact molecular mechanism is unknown which plays the important role, but deeper understanding of mechanism is illusive. In future, investigators should emphasis on molecular mechanism of different metals which affect the gene regulation and target the mitochondrial dysfunction, so that ameliorate the detrimental effect of toxic metal in diseases.

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Toxicity of Protein and DNA-AGEs in Neurodegenerative Diseases (NDDs) with Decisive Approaches to Stop the Deadly Consequences

Firoz Akhter, Asma Akhter and Saheem Ahmad

Abstract Protein and DNA-advanced glycation end-products (DNA-AGEs) are toxic by-products of metabolism and are also assimilated by high temperature processed foods. AGEs may be generated rapidly or over long times stimulated by distinct triggering mechanisms, thereby accounting for their roles in multiple settings and disease states. Neurodegenerative diseases (NDDs) are associated with the misfolding and deposition of specific proteins, DNA adduct formation either intra or extra-cellularly in the nervous system. There is also evidence that brain tissue in patients with NDD is exposed to DNA oxidation and glycoxidation during the course of the disease. Although familial mutations play an important role in protein misfolding and aggregation, the majority of cases of NDD are sporadic, suggesting that other factors must contribute to the onset and progression of these disorders. High levels of refined and carbohydrate enriched diets, hyper caloric diets and sedentary lifestyles drive endogenous formation of AGEs via accumulation of highly reactive glycolysis intermediates and activation of the reductase pathway (polyol/aldose) producing high intracellular reducing sugars are the important modifiable environmental factors. Some of these modifications might affect proteins in detrimental ways and lead to their misfolding and accumulation. Reducing sugars play important roles in modifying proteins, forming AGEs in a non-enzymatic process named glycation. Several proteins linked to NDDs, such as amyloid β, tau, prions and transthyretin, were found to be glycated in patients, and this is thought to be associated with increased protein stability through the formation of crosslinks that stabilize protein aggregates causing NDDs like Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), familial amyloid polyneuropathy (FAP), and prion disease (PrD). Moreover, glycation may also be responsible, via the receptor for AGE (RAGE), for an increase in oxidative stress and inflammation through the formation of reactive oxygen species (ROS) and the induction of nuclear factor-κB (NF-κB). Here, we revised the role of protein and

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DNA-AGEs in the major NDDs and highlight the potential value of protein and DNA-AGEs glycation as a biomarker or target for therapeutic intervention. Additionally, the chapter covers several new therapeutic approaches that have been applied to treat these devastating disorders, including the use of various synthetic, natural and gold and silver conjugated nanoparticles (Au, Ag-NPs).

Keywords Agvanced glycation end products (AGE) · Neurodegenerative diseases (NDDs) · Environmental toxicity · DNA glycation (DNA-AGE) · Receptor for AGE (RAGE) · Amyloid β · Tau · Prions · Transthyretin · Nanoparticles (Au, Ag-NPs)

1 Introduction

The incidence of neurodegenerative diseases (NDDs) increases with extended life expectancy observed over the last century has led to the emergence of a rope of age related disorders that pose novel challenges to current societies. Age-related degenerative diseases including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis (ALS) and the prion diseases (PrD) are growing to epidemic proportions, are debilitating and so far deadly disorders that require rigorous research. Increased incidences of mild cognitive impairment due to various environmental factors toxicity in elderly populations are characterized by a slow and progressive loss of neuronal cells, and also intra or extracellular deposition of misfolded and aggregated proteins. Modifiable lifestyle factors cover a critical role in these diseases. The prevalence of rigorous cognitive impairment crucially depends on various factors which are influencing health like energy balance, micronutrient density of food, level of physical activity and exposure to tobacco smoke etc. Recent evidence validates that food production, processing and methods of cooking are life-threatening to health outcomes as well. Induced glycation and reactive oxygen species (ROS) formations are important mechanism by which lifestyle influences. Extracellular and intracellular deposition of amyloid β-peptide (Aβ) (amyloid plaques) and tau protein (neurofibrillary tangles) respectively are the key pathological hallmarks in Alzheimer's disease (AD). Cytoplasmic proteinaceous inclusions, mainly composed of the protein α-synuclein (α-syn), named Lewy bodies (LBs), are the pathognomonic inclusions in Parkinson's disease (PD) (Spillantini et al. 1997). Indeed, the frequency of neurodegenerative disorders, including AD and PD, has increased significantly by about four folds from 9.4 million in 1950 (total population 179.5 million) to 40.3 million in 2010 (total population 831.5 million) (Lekoubou et al. 2014). Production of advanced glycation end products (AGEs) has been regarded as a process of molecular and cellular ageing (Jacobs et al. 2014). There are several mutations are associated with the amyloidogenesis behavior of proteins such as Aβ, α -syn, LBs and transthyretin are very well known while the enormous number of cases of NDDs are intermittent (Haass et al. 1994; Kruger et al. 1998; Polymeropoulos et al. 1997; Zarranz et al. 2004; Saraiva 2001).

Factors such as oxidative stress, protein cross-linking, the sequelae of RAGE signaling and changes of DNA integrity extrapolate this process from the molecular to the biological (cellular and tissue) level (Huttunen et al. 2002), leading to the thrashing of their normal function, to cell dysfunction and death. Various physiological consequences of DNA and protein glycation comprise the development of diabetes mellitus and their secondary complications like retinopathy, nephropathy and neuropathy. Glycation has key clinical relevance, since it may be used as a specific biomarker for several disorders and their secondary complications. AGEs may be used as markers of tissue damage and may predict long-term complications of diabetes mellitus like diabetes-neuropathy and cardiomyopathy (Akhter et al. 2016a, b). Non-invasive techniques like auto-fluorescence reader have been developed to assess the levels of AGE in the skin, which rapidly measures AGE accumulation (Shimasaki et al. 2011). In view of the emerging knowledge about the widespread occurrence of DNA and protein-AGEs in various tissues (Peppa et al. 2003) including the central nervous system (Vlassara et al. 1983) we wants to propose the following hypothesis based on a chapter of recent results and increasing evidence for a putative role of the various environmental factors induced DNA and protein-AGEs in NDDs. We are also proposing the probable approaches to stop the menace of reactions which may open novel avenues for therapeutic intervention.

2 Life Style Dependent Browning Reaction a Deadly Consequence with Sweets

Despite the 100 or so years that have lapsed since French scientist Louis-Camille Maillard (1878–1936) (Maillard 1912), the first scientist to investigate the "browning reaction" in food and in the human body, who reported the story of glycation reaction in the year 1912 after whom the reaction is also known as the Maillard reaction (Maillard 1912). The non-enzymatic reaction starts with the addition of free carbonyl group of a reducing sugar to a nucleophilic free amino group of proteins, lipids and DNA macromolecule to form early, intermediate and advance glycation end-products (AGEs). Exogenous AGEs are acquired from tobacco, brown or high cooked food diet (exogenous sources). Reducing sugars in basic solutions and lipids by β-oxidation generate formyl (an aldehyde) and ketone groups. These aldehydes and ketones have a highly polarized carbonyl (C=O) group, the oxygen atom of which is highly electronegative and may react with nucleophiles in other biomolecules like protein, DNA and lipids. Under hyperglycemic condition (high glucose load), these biomolecules undergo glycation reaction leading to the formation of a complex series of AGEs. This, in turn, results in the altering their structural conformation or deprivation of the functions of the biological macromolecules.

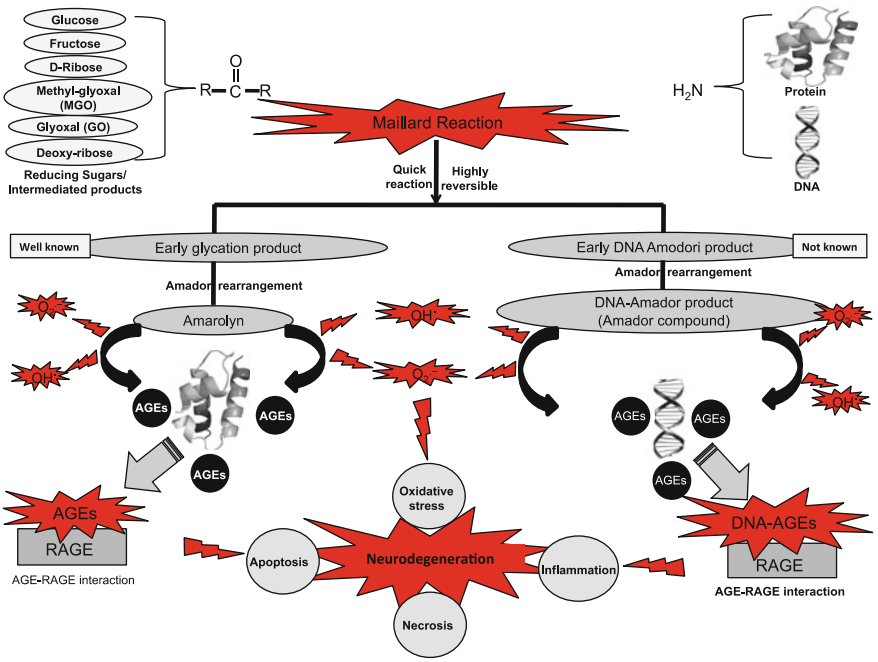

Fig. 1 Schematic representation of proposed pathway for macromolecules reacting with reduced sugars to form protein-AGEs and DNA-AGEs respectively

Figure 1 schematically represents the promising pathway of reaction of biomacromolecules with reducing sugars to form protein advanced glycation end products (protein-AGEs) and DNA advanced glycation end products (DNA-AGEs), which is believed to be involved in the complications associated with several neuronal disorders through various pathways of glycation reaction (Aldini et al. 2013). There are also various pathways direct or indirect depends upon life style causing induced toxic effects of AGEs and ROS, involved to cause neuronal disorders as shown in Fig. 2.

Non-enzymatic glycation of proteins is a post-translational modification conventional process between sugars and proteins, occurring in all living systems. Protein glycation occurs through a complex series of very slow reactions in the body; it is quite dynamic in nature and starts with the formation of unstable Schiff base, which undergoes a series of reactions leading to the formation of heterogeneous AGEs molecules (Akhter et al. 2013a, b). Glucose and glucose-derived glyoxal, MG, glucosone and 3-deoxyglucosone (3-DG) dicarbonyls are the major precursors of AGEs. The levels of these precursors determine the formation of diverse types of AGEs including fructosyl-lysine (FL), carboxymethyl lysine (CML), carboxyethyl lysine (CEL) and pentosidine. Hyperglycemic condition promotes the formation of AGEs in vivo, thus, enhancing the overall accumulation. AGEs cause cell death at various levels, namely alteration of protein structure and

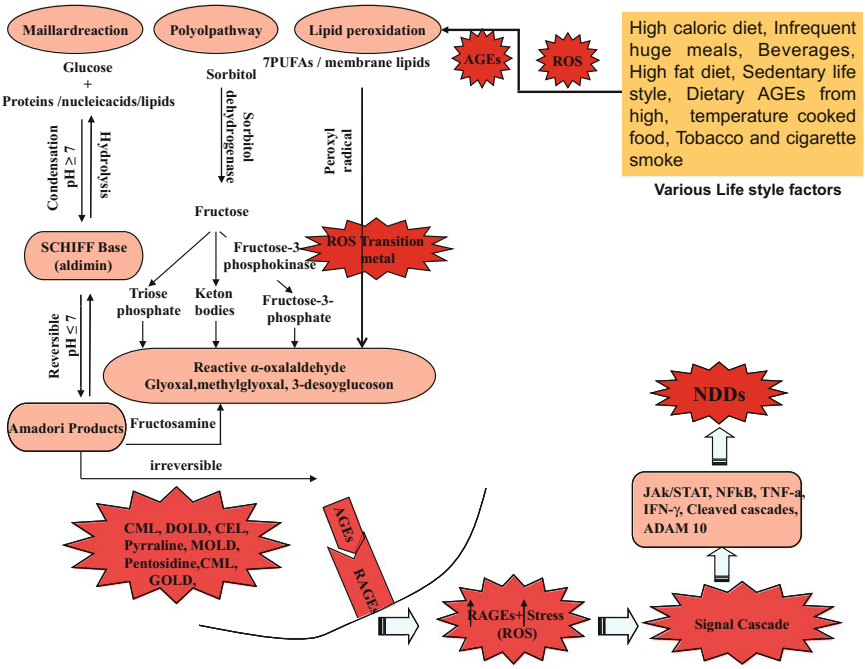

Fig. 2 Schematic representation of direct or indirect modifiable environmental factors dependent various pathways induced toxic of glycation and their cause in neuronal apoptosis

function; protein aggregation, fibril formation and protease resistance (Wei et al. 2012), aberrant signaling through interaction with the RAGE and dysfunction of extracellular matrix. AGEs contribute drastically to the progression of diabetic secondary complications, including retinopathy, neuropathy, nephropathy, cardiomyopathy, accelerated aging and NDDs.

When DNA reacts with a reducing sugar in vitro at a physiological temperature, the formation of DNA-AGEs is observed (Akhter et al. 2015; Shahab et al. 2014). DNA glycation significantly alter the structure of DNA macromolecule, which leads to depurination, strand breaks and mutations such as insertions, deletions and transposition (Ahmad et al. 2011a, b). Therefore, DNA-AGEs could contribute to the loss of genomic integrity, which occurs during aging and may contribute to the age-related complications like NDDs. Few studies on the stability and dynamics of DNA showed that glycation leads to partial unwinding and/or fragmentation of the DNA double helix (Mustafa et al. 2012). The mutagenic potential of the predominant DNA-glycation adduct carboxy ethyl deoxygunaosine (CEdG) was investigated by Wuenschell et al. (2010) and exhibited that CEdG within the template DNA and the corresponding triphosphate possess different syn/anti conformations during replication which influence base pairing preferences. Some reports have shown in the recent past that genotoxicity and immunogenicity are incurred in DNA and proteins by carcinogens and reactive oxygen species (ROS) as well (Ahmad et al. 2014;

Shahab et al. 2012a, b, 2013). Moreover, most recently, it has also been investigated that glycation-induced oxidative stress leads to the modification of DNA macromolecule and results in alteration of its structure (Akhter et al. 2013a, b). The consequence of genotoxic effect is due to the structural perturbations in the glycated DNA macromolecules (Ahmad et al. 2011b). Induced levels of these DNA-AGEs have been implicated in the pathological complications of diabetes and NDDs.

3 Toxic Role of Various Glycated Protein (Protein-AGEs) in NDDs

In the world, millions of elder people suffer from NDDs and diagnosis and treatment of these diseases costs millions of dollars per year. Life style dependent alterations in glycosylated protein have critical role in various human NDDs states, such as AD, PD and Creutzfeldt-Jakob disease (CJD) (Saez-Valero et al. 2003; Silveyra et al. 2006). Although the glycoprotein's structural elucidation is a challenge because of their inherent complexity and heterogeneity in biological systems, advances have been made to identify a few proteins where glycosylation appears to be important in the disease processes of AD and PD (Sihlbom et al. 2004). The role of a few key proteins involved in AD and PD pathogenesis are discussed below.

3.1 Toxic Effect of Protein-AGEs in AD

Among the NDDs, AD is the most common, touching $\sim$5% of people aged 65–75 and nearly 50% of people over the age of 85 (Grossman et al. 2006). The most common reason of NDDs is protein misfolding which leads to protein aggregation and accumulation. The characteristic features of this disease are progressive loss of memory, speech, ability to recognize people and objects followed by confusion, disorientation and disordered thinking. The dysfunction involves degeneration of neurons especially in hippocampus, amygdala, nucleus basalis and entorhinal cortex. Most AD cases are intermittent, whereas approximately 6% show genetic origin (Campion et al. 1999). The great majority of genetic cases are allied to the occurrence of the ε4 allele of the apolipoprotein E (APOEε4) and also to mutations in the amyloid β precursor protein (APP) (Rademakers and Rovelet-Lecrux 2009). It has already been identified that glycation of amyloid-β peptide, the constituent of the senile plaques in AD may contribute to its cross-linking. There are some strong indications that the glycation of amyloid-βeta (Aβ) peptide and tau protein (τ-protein) occurs in the early stages of AD, although it has been hypothesized that peptide free radicals generated by Aβ would cross-link peptides with sugars resulting in protein glycation (Mattson et al. 1995).

The diagnosis of AD can only be confirmed through the presence of characteristic pathological signs such as Aβ plaques and neurofibrillary tangles (NFTs) (Cummings et al. 1998). Some studies have suggested that over-expression of microtubule-associated τ-protein, hyper phosphorylation, and mutations is known to contribute to its aggregation and accumulation of Aβ in mitochondria is linked with the neuronal toxicity observed in this disorder (Andorfer et al. 2005; Sato et al. 2002; Wittmann et al. 2001; Caspersen et al. 2005; Du et al. 2008; Lustbader et al. 2004). However, AGEs are also formed from the reaction of free reactive carbonyl species with free lysine or arginine side chains of proteins (Munch et al. 1999), and are believed to play an important role in NFTs formation as well as in the development of β-amyloid plaques. Increased levels of AGEs in the Aβ plaques and NFTs play an important role in AD (Vitek et al. 1994; Smith et al. 1994). The plaque fractions of AD brains hold higher levels of AGEs than samples from age-matched controls (normal human brain). An immunohistochemically AGEs can be identified in both SPs and NFTs (Sasaki et al. 1998). Furthermore, neurons, microglial cells, and astrocytes in the normal human brain express RAGE (Li et al. 1998), while its expression by cortical neurons increases and becomes more widespread in AD (Yan et al. 1996). Since it was reported that RAGE might be the nerve cell receptor for Aβ protein, the role of RAGE in the pathogenesis of AD has attracted substantial attention (Yan et al. 1997).

Glycation of τ-proteins enhances the formation of paired helical filaments in AD and in vitro reduces its ability to bind microtubules (Ledesma et al. 1994). In addition, AGE modified τ-proteins are responsible for increased fibrillization of the protein. Aβ aggregation follows a nucleation-dependent polymerization mechanism, which is considerably accelerated by AGE-mediated crosslinking (Munch et al. 1999). The Aβ aggregation consists of two characteristic stages i.e. slow, reversible nucleus formation step, involving oligomer formation, followed by a rapid elongation phase. Once the oligomers reach a decisive size, a fast, linear elongation of the aggregates or fibril formation can occur via the addition of Aβ peptides to the eNDDs. The fibril creation step is an irreversible process. Finally, these fibrils grow and form amyloid plaques.

Methylglyoxal (MGO) (AGE precursor), is able to activate p38 MAP kinase, which is able to phosphorylate tau (Liu et al. 2003; Reynolds et al. 2000), a progression believed to occur in neurons in AD (Zhu et al. 2000). MGO glycated Aβ promotes the development of β-sheets, oligomers and protofibrils and also increases the size of the aggregates (Chen et al. 2006). Aβ is also recognized by RAGE, an important player in AD (Li et al. 1998). RAGE is expressed in astrocytes and monocytes across the blood-brain barrier, activating nuclear factor-κB (NF-κB) which consequently increasing reactive oxygen species (ROS) (Sato et al. 2006; Takeuchi et al. 2000; Yan et al. 1998). RAGE-mediated GSK-3 activation, leads to the toxic effect of AGEs, which induces tau protein hyperphosphorylation and consequently impairs synapse signaling and memory in rats (Li et al. 2012).

Surprisingly, levels of protein-AGEs in AD are three-fold higher than healthy subjects (Vitek et al. 1994). A paired helical filament of glycated A68 proteins, a known precursor of NFTs, suggests that it could be an early result (Takeda et al. 2000).

Although protein-AGEs levels increase with age (37.5%), in AD the increase is much greater (72.6%) (Luth et al. 2005). ApoE also seems to be modified under glycation reaction, since in AD the staining patterns of AGEs and ApoE are similar (Dickson et al. 1996). Presence of these protein-AGEs in the cerebrospinal fluid (CSF) of AD patients, signifying that this may be explored as a biomarker for AD.

3.2 Role of Protein-AGEs Toxicity in PD

PD is the second most common progressive neurodegenerative disorder, is caused by a progressive degeneration of the dopaminergic neurons in the substantia nigra of the midbrain, which are involved in the control of voluntary movements (Izumi et al. 2011). The prevalence of PD is around 2% in people over age 60 (Burke 1999). Muscular rigidity, tremor, slow movement, loss of balance and coordination are the most common symptoms of the disease (Forno 1996). Dopaminergic cell loss, excessive accumulation of iron in midbrain neurons, formation of LBs containing α-syn aggregates, presence of extracellular melanin released from degenerating neurons, and increased oxidative stress in substantia nigra region of the brain, known to induce apoptosis, are the main pathological hallmarks of PD.

Several genes like α-syn, Parkin, DJ-1, and Pink1 have already been identified to play an important role in the development of PD (Nuytemans et al. 2010; Martin et al. 2010, 2011) as well as various environmental factors are also lead to the onset of PD, including brief exposure to herbicides, pesticides, heavy metals, increased stress and brain injuries (Nuytemans et al. 2010; Martin et al. 2010, 2011; Hegde et al. 2010; Defebvre 2010). Glycation was first reported to be present in substantia nigra and locus coeruleus of periphery LB (Vitek et al. 1994). It is suggested to be involved in chemical crosslinking making the protein resistant to proteolytic degradation. Glycation of α-syn constitutes a factor that affects the aggregation of the protein into LBs in PD. α-syn has a total of 15 lysine residues which are all candidate sites for glycation, influences the nucleation of protein aggregates (Padmaraju et al. 2011) and induces α-syn oligomerization, thereby stabilizing oligomers. Glycated α-syn may interact with RAGE and activate the release of NF-κB. These signaling proteins trigger the signaling cascades in the brain and damage neuronal cells. By inducing the expression of RAGE proteins, NF-κB also regulates the expression of RAGE. Glycation is also reported to be found in cerebral cortex, amygdala, and substantia nigra of healthy subjects, but levels of glycation are higher in PD patients. The evidence of glycation was first reported in the substantia nigra and locus coeruleus of peripheral LB. The glycated α-syn is one of the important factors that affect the aggregation of the protein into LBs in PD (Guerrero et al. 2013). In comparison to age-matched control induced level of RAGE are expressed in AD patients (Dalfo et al. 2005), suggesting the key role of protein-AGEs in the disease (Fig. 3).

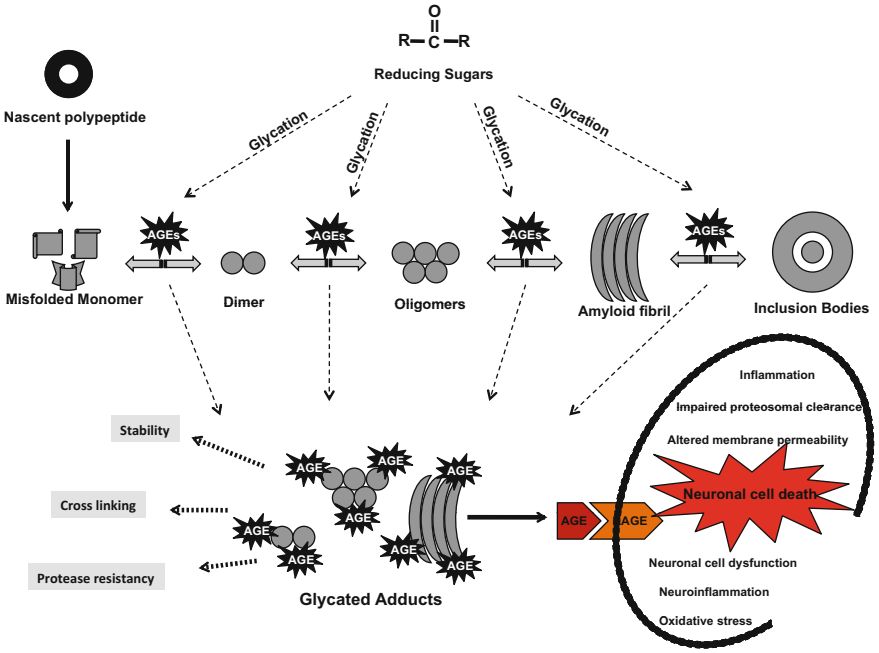

Fig. 3 Presenting probable role of protein glycation in protein aggregation and misfolding. After synthesis, proteins may acquire different misfolded conformations and may form dimers, oligomers and, in later stages, amyloid fibrils, which may be sequestered into inclusion bodies (such as LBs for the case of α-syn in PD). Reducing sugars, increased in hyper-glycaemia conditions, may affect the biology of proteins in early or later stages of the aggregation process (glycating protein monomers, dimers, oligomers and amyloid fibrils). AGEs may lead to cell damage by interacting with RAGE, inducing oxidative stress and inflammatory cell responses causative response in neuronal toxicity

Acute diminish in the levels of cellular glutathione (GSH) in early stage is a fundamental characteristics of PD, results in a lower activity of the glyoxalase system. In catabolic pathway methylglyoxal is the important glycation agent in vivo (Thornalley 1998). The carbonyl content raise oxidative stress will then be responsible for an increase in AGEs concentrations might be responsible to the cell damage. Dopamine auto-oxidation and its deprivation by monoamine oxidase is likely to contribute to an induced level of oxidative stress. Prominently, an in vitro MGO and GO are able to induce oligomerization of α-syn (Lee et al. 2009). Study have been suggested that the membrane-binding ability of glycated α-syn was also decreased (Lee et al. 2009), thus, it is hypothesize that in a glycation-prone environment, more cytotoxic α-syn aggregates or oligomers are present in the cytoplasm, contributing to the development of PD.

3.3 Role of Protein-AGEs Toxicity in Other NDDs

3.3.1 Effects of AGEs in ALS

ALS, also known as Lou Gehrig's disease, is a very common motor neuron disease affecting 4–6/100,000 people, and shares with other disorders of the ageing nervous system a polygenic, multifactorial origin (Choonara et al. 2009). ALS is polygenic and multifactorial in origin, characterized by the degeneration of motor neurons in the brain and spinal cord leading to progressive muscle weakness, atrophy, and spasticity (Véronique et al. 2010). It causes selective loss of upper and lower motor neurons of the brain and spinal cord. The causes of ALS are still unidentified and most of the cases are sporadic and inherited in nature (Gros-Louis et al. 2006). Several inherited cases of the disease are related to copper–zinc superoxide dismutase 1 (Cu, Zn-SOD1) (Gros-Louis et al. 2006; Pasinelli and Brown 2006). This protein catabolizes superoxide radicals, signifying that oxidative stress may participate an imperative role in the disease (Kikuchi et al. 2003). Glycation was first identified in both sporadic and inherited forms of ALS, in the spinal cord and brain of ALS patients. Firstly it was postulated that glycation could be implicated in the time-dependent cross-linking of neurofilament protein, subunits contain multiple Lys–Ser–Pro sequences. Glycation of these lysine residues impairs the self-assembly process; endorse cross-linking in the neurofilament protein, leads to ALS. Studies have revealed that AGE levels were higher in the presence of the Cu, Zn-SOD-1 mutation, while in control human and mouse subjects; AGE immunoreactivities were nearly absent (Shibata et al. 2002). The AGEs presence in astrocytes in the spinal cord demonstrates that carbonyl stress in *SOD1* mutant subjects may stimulate the potentially deleterious effects of AGEs. Remarkably, levels of soluble RAGE (sRAGE), a C-terminal truncated isoform of RAGE, are significantly lower in the serum of ALS patients (Ilzecka 2008). sRAGE, deficient the transmembrane-anchoring domain, found to ameliorate the deleterious effects of RAGE by forming a complex with the ligand (Sakaguchi et al. 2003; Wendt et al. 2003). Therefore, one conspicuous role of sRAGE is to protect humans from ALS, indicates that low sRAGE levels may be a risk factor for ALS.

Arai et al. demonstrated that glycation, modifies Cu, Zn-SOD1, specifically at Lys3, Lys9, Lys39, Lys36m, Lys122 and Lys128 residues (Arai et al. 1987). Higher susceptibility of mutated Cu, Zn-SOD1 to glycation was observed at Lys122 and Lys128 (Arai et al. 1987; Takamiya et al. 2003). Recently, Kaufmann et al. demonstrated that the concentration of *N*-ε-(carboxymethyl) lysine (CML) significantly increases in serum and cerebrospinal fluid (CSF) of ALS patients, possibly representing a novel biomarker for diagnosis (Kaufmann et al. 2004). These results advocate that glycation might be accountable for the observed oxidative stress in ALS.

3.3.2 Effects of AGE Toxicity in FAP

Familial amyloid polyneuropathy (FAP) was first described in 1952 by Andrade, is a systemic amyloid disease, characterized by the extracellular deposition of fibrillar transthyretin (TTR), particularly in the peripheral nervous system (PNS) also affecting the autonomic nervous system. TTR is normally an innocuous protein, present in the plasma and CSF, responsible for the transport of thyroxin hormone (T4) and retinol in the blood (Kanai et al. 1968).

More than 80 TTR point mutations are linked with amyloidotic diseases. The most widely accepted disease model suggests that TTR tetramer instability is associated to point mutations. Nevertheless, this model fails to elucidate that the native TTR also forms amyloids in systemic senile amyloidosis, a geriatric disease and the age onset of disease varies by decades for patients bearing the similar mutation. Indeed, a few mutation carrying individuals are asymptomatic throughout their lives. Protein glycation, play an important role in disease development as methylglyoxal-derived AGE is found in FAP patients (da Costa et al. 2011). The glycated TTR (TTR-AGE) may contribute to cytotoxicity via a mechanism involving oxidative stress, or by interaction TTR-AGE with RAGE (Matsunaga et al. 2002; Sousa et al. 2001; Shorter and Lindquist 2005). Interaction between TTR-AGE fibrils and RAGE results in the translocation of NF-κB to the nucleus, where it induce tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) (97). The activations of TNF-α and IL-1β were abrogated by an anti-RAGE antibody or sRAGE (Sousa et al. 2001). Thus, these studies suggest that glycation plays an important role in FAP. Thus, these data suggest that glycation plays an important role in FAP and blocking RAGE may constitute a good target for therapeutic intervention in FAP.

3.3.3 Effects of AGE Toxicity in Prion Diseases

Prion diseases are a group of NDDs caused by prions, which are “proteinaceous infectious particles” or abnormally folded cellular prion protein (PrPC). These diseases affect a lot of different mammals in addition to humans—for instance, there is scrapie in sheep, mad cow disease in cows, and chronic wasting disease in deer (Knight and Will 2004). The human forms of prion disease are most often the names Creutzfeldt-Jakob disease (CJD), Gertsmann-Straussler-Scheinker syndrome (GSS), variably protease-sensitive prionopathy (VPSPr), kuru and fatal familial insomnia (FFI) (Prusiner 1998). Prion diseases are mostly age-related in nature that can be spontaneous, genetic, or infection-related. Spontaneously occurring prion diseases. All of these diseases are caused by just slightly different versions of the same protein. The ‘protein only’ hypothesis states that these diseases are caused by

the conversion of a normally folded prion protein (PrPC) into an abnormal isoform, which is resistant to degradation by proteinase K (PrPres). However, the precise mechanism is still the subject of extensive discussion (Soto and Castilla 2004). Interestingly recent findings indicate that PrP (C) is involved in signal transduction (Didonna 2013). The presence of AGEs and RAGE in the occipital lobe of CJD patients in higher amounts than in control individuals were confirmed by Sasaki et al. (2002) which revealed a co-localization of PrP-positive granule AGEs and RAGE.

The authors hypothesized that glycation would advance over time and like Aβ, PrP would be degraded through the lysosomal pathway in a RAGE-mediated process. Choi et al. reported that this post-translational modification occurs in the N-terminus of the protein, since upon PK digestion. It cleaves approx 90 amino acid residues from the PrPres N-terminal; glycation was no longer detected (Choi et al. 2004). Although human PrP contains 21 lysine and arginine residues, glycation does not appear to be an arbitrary process. Only 23rd, 24th, 27th lysines and 37th arginine of PrPres can be glycated (Choi et al. 2004). Choi et al. reported glycation in several mouse models infected with 139A, ME7, 22L and 87V scrapie strains, in hamster adapted 263K and 139H scrapie strains, and in both sporadic and genetic CJD. Glycated PrP was detected by using western blot analysis, to form oligomeric species, where immunoreactivity was observed for monomeric and dimeric forms of PrP (Choi et al. 2004). So the role of AGEs in the formation of PrPSc remains unknown, but two hypotheses exist i.e. all AGE modification occurs at the time of PrPSc formation and glycation only occurs after the formation of PrPSc. Hence, glycation plays a key role in the pathogenesis of prion diseases, because glycation would provide enhanced protection for the PrPSc molecules against cellular degradation. Therefore, although the role of glycation in the formation of PrPres cannot be expelled, some discussions still need to be addressed to better understand the role of glycation in prion aggregation and disease progress.

4 Effects of Toxic DNA-AGE in NDDs

DNA bases are vulnerable to glycoxidative stress damage involving hydroxylation, protein carbonylation and nitration (Lovell and Markesbery 2007; Gabbita et al. 1998; Collins et al. 1996). It has been reported that under glycation reaction some free radicals i.e. superoxides and hydroxyl generates (Akhter et al. 2014) that caused oxidative damage to DNA molecules that might be caused the neuronal damaged having direct implication of NDDs. It has been observed in AD that brain ROS induces calcium influx, via glutamate receptors and triggers an excitotoxic response leading to cell death (Mattson and Chan 2003). DNA and RNA oxidation

are marked by increased level of 8-hydroxy-2-deoxyguanosine (8OHdG) and 8-hydroxyguanosine (8OHD) (Nunomura et al. 1999, 2001; Lovell et al. 1999). Furthermore, these markers have been localized in Aβ plaques and NFTs (Mecocci et al. 1994). Increased levels of DNA strand breaks have been found in AD. They were first considered to be part of apoptosis, but it is now widely accepted that oxidative damage is responsible for DNA strand breaks and this is consistent with the increased free carbonyls in the nuclei of neurons and glia in AD. Among all dicarbonyl intermediates formed in the glycation reaction, MG is a potent and most reactive AGE precursor, forming adducts on deoxyguanosine (dG) residues. Furthermore, it has been shown that DNA can be glycated in vitro yielding N2-carboxyethyl-2′-deoxyguanosine (CEdG) as a major product. In vitro, nucleobases and ds-DNA react with sugars in a similar way as proteins (Lee and Cerami 1987; Knerr and Severin 1993; Singh et al. 2001). The exocyclic amino group of 2′-deoxyguanosine is particularly prone to glycation; leading to the formation of N2-carboxyethyl, N2-carboxymethyl, as well as cyclic dicarbonyl adducts (Ochs and Severin 1994). The CEdG is a stable reaction product, formed from a variety of glycating agents, such as glucose, ascorbic acid, glyceraldehyde, dihydroxyacetone (DHA) and MG (Frischmann et al. 2005). Apart from in vitro DNA glycation, under hyperglycemic condition the glycation of DNA (CEdG A and CEdG B) (Fig. 4) occurs in vivo and is considered to be a pathogenic factor for NDDs (Padmaraju et al. 2011) (Fig. 5).

Oxygen free radicals as well as possibly signaling of AGEs to the nucleus through their specific receptor(s) (RAGE) may induce DNA strand scission (Kashimura et al. 1990; Morita and Kashimura 1990) e.g., by stimulation of nuclear factor NF-κB (Lander et al. 1997; Li and Schmidt 1997). DNA replication may become inhibited by intermediate products of the Maillard reaction such as 3-deoxyglucosone, which may lead to impairment of cell proliferation (Shinoda et al. 1990).

Non-enzymatic glycation with production of AGEs has been regarded as a process of molecular (Bunn 1981) and cellular (Li et al. 1995) ageing. Factors such as oxidant stress, molecular cross-linking, the sequelae of RAGE signaling and changes of DNA integrity extrapolate this process from the molecular to the biological (cellular and tissue) level (Huttunen et al. 1999, 2000, 2002). This pertains to ageing (Bjorksten 1977; Monnier et al. 1990) and AD (Heitner and Dickson 1997; Smith et al. 1994).

A few evidences suggests the role of DNA-AGEs in NDDs, yet a comprehensive, potential and prospective study is required to know the deleterious effects of DNA-AGEs in the assessment of neuronal diseases.

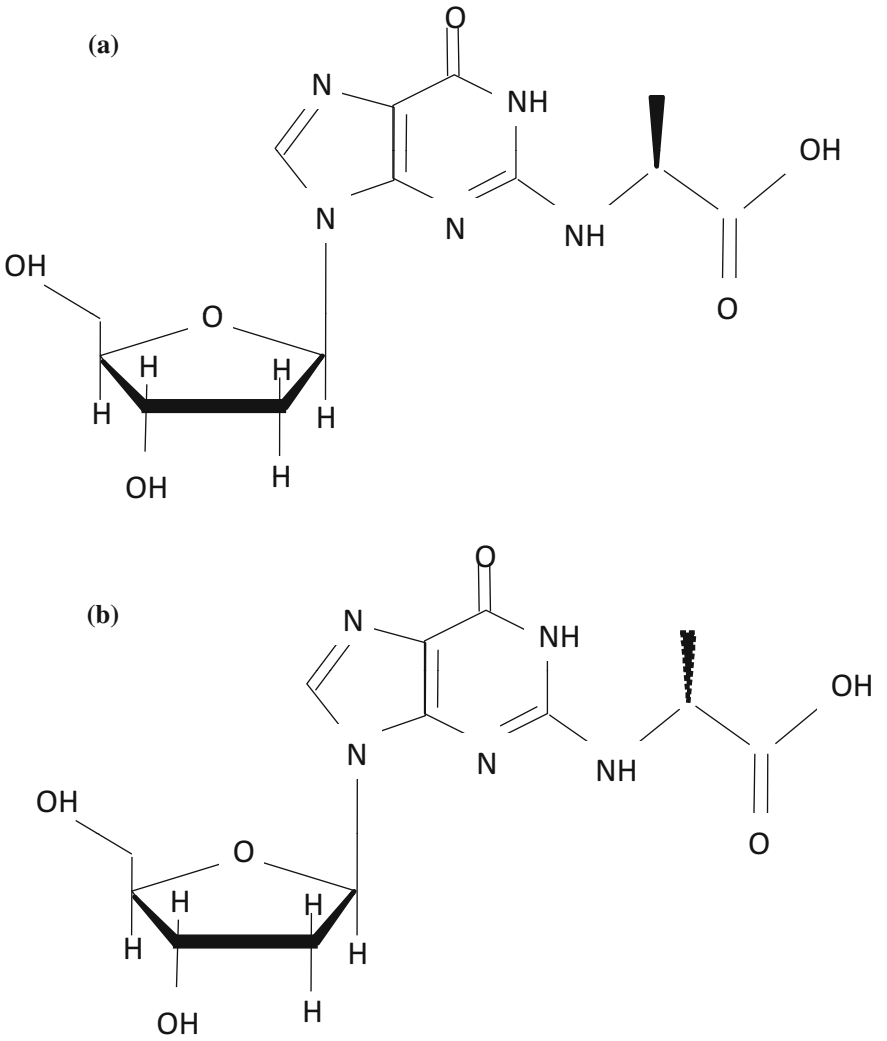

Fig. 4 A structurally representation of glycated DNA adducts i.e. CEdG A (**a**) and CEdG B (**b**) as DNA-AGEs

5 Anti-AGE System to Halt the Menace of Toxicity of Glycation

5.1 Natural and Synthetic Inhibitor

The deterrence of glycation reaction is one of the strategies to reduce DNA-AGEs/protein-AGEs. Both synthetic compounds and natural products have

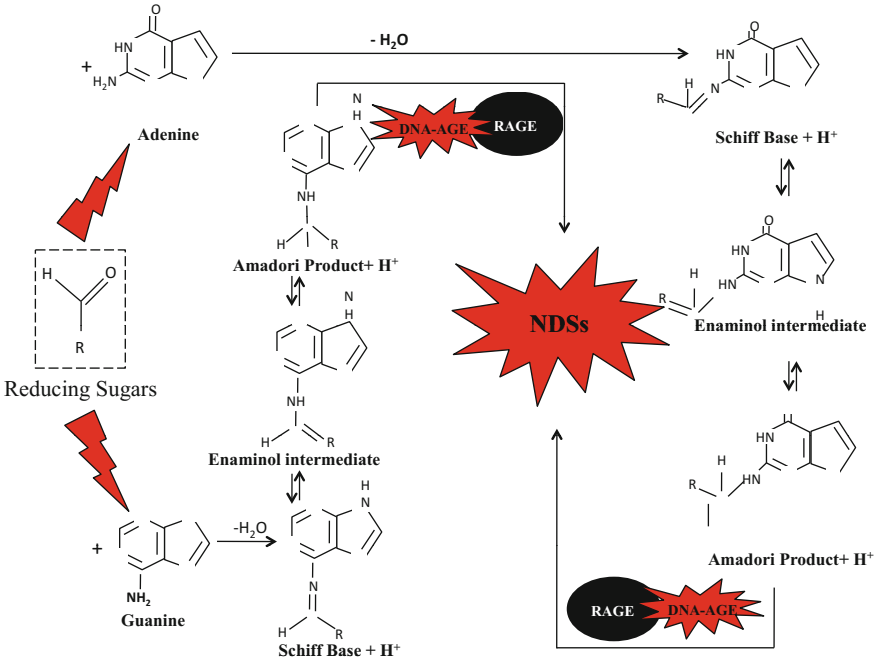

Fig. 5 Probable mechanistic representation of glycation reaction between reducing sugars and adenine/guanine bases, and cause of neuronal toxicity

been evaluated as inhibitors against the formation of AGEs. The formation of ketoamine moieties or intermediate products can be inhibited by reacted biomolecule that react with the reducing sugars, thereby inhibiting the deleterious reaction. Anti AGE systems must be able to grasp with preexistent forms and their formation by scavenging or degrading antiglycation agents. Some protective enzymatic mechanisms are also involved in the degradation of glycation reaction. Glyoxal (GO) and methylglyoxal (MGO) are the most reactive species, able to damage various biomolecules like proteins, DNA and lipoproteins through the Maillard reaction, which can culminate in cell apoptosis (Akhter et al. 2016b; Maeta et al. 2005). Not only glyoxalase system but also some other oxide reductase and dehydrogenases like α-oxoaldehyde dehydrogenase, aldehyde dehydrogenase, aldose reductase, methylglyoxal reductase, and pyruvate dehydrogenase are able to convert MGO into its oxidized or reduced form (Kalapos 1999). Glyoxalase-I (GO-I) is evidently decreased in AD, as already stated by Kuhla et al. (2007), resulting in the accretion of α-dicarbonyls and subsequent deleterious effects. Some specific natural and synthetic inhibitors may also prevent glycation induced cross-linking of proteins and DNA adducts formation. The trapping of the reactive carbonyl intermediates formed in the first stage of Maillard reaction can be carried

out by using guanidine compound such as aminoguanidine (AG). AG was the first AGE inhibitor proposed to scavenge the dicarbonyl compounds such as glyoxal, MG and 3-DG, via its fast reaction with α-dicarbonyl compounds, forms 3-amino-1,2,4-triazine derivatives (Thornalley et al. 2000). Tenilsetam is a known potent AGEs cross-linking inhibitor (Munch et al. 1994), is successfully used for the treatment of patients suffering from AD (Choonara et al. 2009). This is covalently attached to protein-AGEs and blocks the reactive sites for further polymerization reactions. Interestingly, both AG and Tenilsetam having anti neurodegenerative property as were found to protect against the neurotoxic effects of methylglyoxal (Webster et al. 2005). Another category of AGE inhibitors, "Amadorin" (the post-Amadori inhibitors), inhibits the conversion of Amadori intermediates to AGE (Khalifah et al. 1999). Pyridoxamine was the first Amadorin identified that showed a great potential for treatment of diseases by inhibiting AGE formation at different levels. It scavenges carbonyl products of reducing sugars and lipid degradation, sequestering catalytic metal ions, blocking oxidative degradation of glycation intermediates and trapping of ROS. Lalezari-Rahbar (LR) compounds are potent Cu^{2+} chelator, that is more efficiently than PM and which inhibit post-Amadori AGE formation in vitro (Rahbar and Figarola 2003). Benfotiamine; (a lipophilic derivative of vitamin B1), improves the activity of transketolase by reducing the accumulation of hexose and triose phosphates by shunting these intermediates to pentose phosphate pathway (Stracke et al. 2001). Animal model experimental studies have shown that bioactive compounds from therapeutic plant can reduce diabetic complications like neuropathy (Akhter et al. 2013a). The presence of specific enzymes like deglycases or amadoriases is also able to degrade glycated proteins, these include fructosamine 3-kinase enzyme that phosphorylates protein-bound fructosamines and spontaneously breaks down the Amadori rearrangement into inorganic phosphate, along with 3-deoxyglucosone and the amino compound (Akhter et al. 2014; Ahmad et al. 2013). Delpierre et al. observed this process in vivo, leading to deglycation of haemoglobin and protein-bound ribulosamines and psicosamines in erythrocytes (Collard et al. 2004). fructoselysine 6-kinase and Fructose lysine oxidase are also believed to play a role in deglycation of protein (Takahashi et al. 1997; Wiame et al. 2002). Several novel inhibitors like aldehyde, pyridoxal-phosphate, or the acetylating agent aspirin have recently been discovered, react with and thereby cap the free amino groups of proteins and prevent sugar attachment. Thiol-based antioxidants also act as a carbonyl scavenger like glutathione (GSH). Besides this, there are some other dicarbonyl scavengers including L-arginine that trap glyoxal, glycoaldehyde, and glucosones to form substituted triazines. These drugs are potent curative agents for treating neurodegenerative maladies. The ROS and free transition metal ions are known to play an important role in the formation of AGEs. Where, the glycoxidation reactions could be efficiently inhibited by redox metal chelators (Baynes 1991; Price et al. 2001; Nagai et al. 2012), including diethylene triamine penta acetic acid, phytate, and penicillamine. Hence, these redox-metal chelators could be used in the therapy of

NDDs. Nucleation-dependent polymerization of Aβ, the major component of plaques in patients with AD, is significantly accelerated by AGEs in vitro. Drugs like metformin (Ruggiero-Lopez et al. 1999; Beisswenger and Ruggiero-Lopez 2003; Beisswenger et al. 1999) and buformin (Kiho et al. 2005) also showed the potential to protect proteins against in vitro glycation and cross-linking (Bonnefont-Rousselot 2001; Hipkiss 1998). Furthermore, isoferulc acid (IFA) suppressed the formation of β-cross-linked amyloid structures of BSA, is a promising anti-glycation agent, which can be used for the prevention of NDDs. There is one other significant approach to reduce accumulation of AGE formation is to reverse the process by AGE cross-link breakers such as N-phenacyl-4,5-dimethyl-thiazolium chloride (ALT-711/alagebrium) and N-phenacyl thiazolinium bromide. "RAGE blockers" comprise of agents that trap AGE with soluble RAGE (sRAGE), block RAGE and inhibit signal transduction mediated by AGE–RAGE interaction. Administration of recombinant sRAGE in animal models has shown to suppress neuronal dysfunction (Park et al. 1998). Although the synthetic compounds are powerful drugs that inhibit AGE formation or break cross-links, they can also have severe side effects. The importance of anti-AGEs is still divisive, since the resulting degraded compounds are able to react once more with amino groups of DNA, proteins and lipoproteins. However, the inhibition of AGEs and intermediate dicarbonyl compounds formation may correspond to a therapeutic approach also to be explored in various NDDs.

5.2 *Anti-AGE Effect of Bio-conjugated Gold and Silver Nanoparticles (Au, Ag-NPs)*

Anti-AGEs activity of gold nanoparticles (GNPs) was first reported in year 2009, inhibit the consequence of the glycation reaction (Singha et al. 2009). The prevention of glycation of α-crystalline was done by conjugation with GNPs. Formation of protein and using bio-conjugated GNPs prevents DNA-AGEs, even if a strong glycated agent such as fructose is used. In addition, the nano-conjugation approach can provide some important information on the structural distribution of any dynamic chaperone protein and DNA. Because GNPs are biocompatible, their reported antiglycation property may have therapeutic implications.

Furthermore apart from GNPs, silver nanoparticles (Ag-NPs) have also shown to efficiently diminish the menace of the glycation reaction. It has been determined the effects of Ag-NPs on AGEs-induced cell permeability. The AGE-bovine serum albumin (BSA) increased the dextran flux across a PREC monolayer and Ag-NPs blocked the solute flux induced by AGE-BSA. It was also demonstrated that Ag-NPs could inhibit the AGE-BSA-induced permeability via Src kinase pathway. There are reports where researchers have proved that GNPs alone (Seneviratne et al. 2012) and GNPs bio-conjugated with protein (Nowacek et al. 2009) can reduce the rate of non-enzymatic modification of proteins responsible for glycation. The role

of GNPs in the targeted delivery of drugs is one of the most promising aspects. For the bio-conjugation of drug with GNPs a robust and stable protein, albumin was chosen as a capping agent over GNPs. The role of albumin as a drug carrier for last several years has emerged wonderfully because it does not only provide the stability to the drugs but also improves the half-life of drugs/active proteins/peptides. The conjugation was carried out either by simple physical adsorption of the drugs onto GNPs or via the use of alkanethiol linkers (Kratz 2008).

In fact, proteins glycated to different extents showed the formation of nanoparticles of different size and eventually their plasmon intensities were also different. This implied that glycated proteins cause some attenuation of the particle formation that led to only smaller nanoparticle formation. One possible example of such negative control on the particle size formation may arise from proximity of glycation-prone sites on which GNPs preferentially form on the protein surface. This as well as other structural studies with nanoparticle protein conjugates, prompted to explore the possibility of prevention of glycation (Neely et al. 2009). The origin of such resistance against glycation is intriguing. The amino acids containing the free amino group (lysine) are potent sites for glycation in addition to the N-terminal amino acid. Few antiglycating agents have been already reported in the literature, and apparently a nontoxic agent like gold showing antiglycating properties may itself have important clinical significance (Rahim et al. 2014).

In the light of the above explanations, this review is aiming to exploit its preventive effect on glycation by reducing the concentrations of the AG or other drugs showing toxicity at higher concentrations but has antiglycation effect. These drugs might be used for nano-conjugation using GNPs, thus reducing the toxic concentration to minimal. The present concern on bio-conjugation of AG with GNPs is the need of the hour. To enhance activity of the entire above novel drugs at reduced dozes, such as LR-74, LR-90 and others including AG in bio-conjugation with GNPs, might prove to be more accurate and specific novel inhibitor of DNA and protein glycation at reduced doses. Table 1 enlists the several bio-conjugated of GNPs with various proteins and their association with neurodegenerative disorders.

Table 1 Bio-conjugation of GNPs with different proteins and their association with neurological disorders

S. No.	Conjugation of Au with protein	Development of metabolic disorders	Citation
1.	α-Crystallin	Neurodegenerative diseases	Singha et al. (2009)
2.	Serum albumin	Alzheimer's disease	Seneviratne et al. (2012)
3.	Trypsin, bovine serum albumin and transferrin	Neurodegenerative diseases	Rahim et al. (2014)
4.	Aβ peptide	Neurodegenerative diseases	Nowacek et al. (2009)
5.	Anti-tau monoclonal antibody	Neurological disorders	Neely et al. (2009)

6 Concluding Interpretation and Future Prospects

Modifiable environmental factors producing toxicity of oxidized DNA and DNA-AGEs, accumulate in a wide variety of environments and has potential role in the cause of neurological disorders (Wuenschell et al. 2010). These overall studies have conclusively verified that AGEs are complex and heterogeneous in nature. Their mechanism of formation is only partially understood. AGEs play an important role in various NDDs, including AD, PD, ALS, FAP and PrD. In all of these, pathological amyloid glycation induces the formation of α-sheet structure in the amyloid-β- protein, α-synuclein, TTR, SOD1 protein, and prions and causes these NDD. More recently, it has been suggested that oligomeric species of glycated α-synuclein and prion are more toxic than fibrils alone. The query of whether glycation induce protein fibrilization remains unclear and is under intense dispute. Although it seems to induce Aβ fibrilization, α-syn and PrP oligomerization, a direct relationship between glycation and β-sheet formation in vivo is still mislaid. Since glycation is understood as a non-enzymatic process, the modifications are identified to occur at arbitrary. Moreover, only four of the 21 PrPres putative glycation sites are modified. These results suggest that glycation may modify specific targets and be understood as a posttranslational modification that alters protein function, similarly to phosphorylation or acetylation.

A number of AGE-inhibitors have been discovered that inhibit the toxicity of glycation pathway. These inhibitors are either synthetic or natural. One novel approach is bio-conjugated nanoparticles as an antiglycating agent i.e. Au-NPs and Ag-NPs that might be effective at reduced doses for the treatment of NDD. The inhibitors cap the amino groups of DNA and proteins, scavenge free carbonyls or dicarbonyls, block Amadori adducts, break existing cross-links, chelate metal ions, and possess anti-amyloidogenic, anti-oxidative, and anti-inflammatory activities. Thus, they can inhibit glycation reactions. The AGE–RAGE damaging axis is now considered to be a promising drug target. Additionally, there are defense enzymes and protein present in the body, such as glyoxylase systems I and II, fructose-3-kinase, aldose reductase, and carnosine. These enzymes and protein protect the neuronal cell from glycation and carbonyl stress. The formation of toxic oligomeric species could be controlled by blocking conformational changes in monomeric species of these pathological proteins using theses novel inhibitors. More efficient drugs could be designed to be more hydrophobic so that it can easily cross the lipid-bilayer membrane of the brain and prevent efficiently NDD. Using combination therapies, novel drugs could be designed that simultaneously target multiple pathways and may obviously be more efficient than those drugs that modify a single pathway and thereby decrease the risk of side effects.

Eventually, based on anti neurological disorders characteristics of natural products, it is hypothesized that these therapeutic compounds might exhibit antiglycating properties as well with no side effects. AGEs inhibition using nanoparticles as drug delivery system (Kim et al. 2012; Seneviratne et al. 2012); therefore, it would be interesting to see the inhibition of AGEs using various

bio-conjugated inhibitors (IFA, AG and Tenilsetam) with GNPs. In the light of above explanations, in general, this study holds strong future prospects with the development of new, more effective and more specific inhibitors. The inhibitors will be developed based on structure–activity liaison. This study will also help to identify the new targets for AGEs. Since the above explanations confirm that the NDDs such as Alzheimer's and Parkinson's diseases are associated with toxicity of glycation intermediate (dicarbonyl compounds and keto-amine moieties) and their end products (AGEs), which direct or indirect depends on various environmental factors too; therefore, targeting a site and changes in life styles could result in prevention or protection against these drowning and dreaded diseases.

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Carcinogenic Toxicity of Cigarette Smoke: A Computational Enzymatic Interaction and DNA Repair Pathways

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Abstract We have performed the interaction analysis of cigarette smoke carcinogens with the enzymes involved in DNA repair mechanisms. Cigarette smoke's derivatives like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) are well known carcinogens. The binding efficiency of carcinogens with enzymes obtained from docking methods were ranging from +36.96 to −7.47 kcal/mol. Binding efficiency was characterized for the enzymes sharing equivalent or better interaction as compared

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to positive control. Also we have analyzed the interaction pattern of NNK and NNAL with DNA. The present study suggests that NNK and NNAL may alter the DNA repair machinery that could initiate the progression of tumor leading to cancer. Computational method explores the toxicological characteristics of these enzymes and also opening an opportunity for researchers.

Keywords NNK · NNAL · DNA repair enzymes · Docking

1 Introduction

Lung cancer is one of the most common cancer develops in cigarette smoking men and women. It claims the lives of over one million people worldwide each year specially of men and women in North America, Europe, and East Asia. With the increasing number of cancer cases, the research develops several treatment methods of lung cancer including surgery, radiation therapy, chemotherapy, and targeted biological therapies by an increasing rate of 5-year survival (Alberg et al. 2005). Several studies on lung cancer histology indicates the incidence of lung adenocarcinoma, where the lung-specific smoke carcinogens, nitogen oxides and nitrosated compunds are highly released from filter cigarettes during smoking and which has been reported main cause of lung cancer (Zheng et al. 2007; Hoffmann et al. 1996).

Zheng and Takano (2011) reported that the epidemiological and laboratory evidences represents an etiological association with smoking, which contains volatile N-nitrosamines such as NNK.

The tobacco-specific nitrosamines (*S*)-*N*-nitrosonornicotine [(*S*)NNN] and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are carcinogens found in unburned tobacco and cigarette smoke. International Agency for Research on Cancer classified both NNN and NNK as human carcinogens (IARC 2007). NNN induces esophageal and nasal tumors in rodents, and NNK is a potent lung carcinogen in rodents (Anderson et al. 1989). Both NNN and NNK require P450-catalyzed metabolism to exert their carcinogenic effects (Hecht 1998). The metabolic pathway of NNK and NNAL retrieved from KEGG has been presented in Fig. 1.

Each DNA repair mechanism is regulated by a highly specific sets of enzymes. Defects within the system may result in diseases, which have one common feature affects individuals are cancer prone (Bartram 1980). DNA damage and repair, or lack thereof, are thought to be major mechanism underlying cancer development. Most of the carcinogenic chemicals are potentially strong to damage DNA directly and/or indirectly due to inflammation and oxidative stress. DNA damage can cause mutations or loss of expression of DNA repair proteins. However, if these damages not repaired may lead to mutations and finally genomic instability, which are hallmarks of tumor cells. The place of carcinogen adducts at particular site in human DNA has not been found, mostly because of limitations in sensitivity.

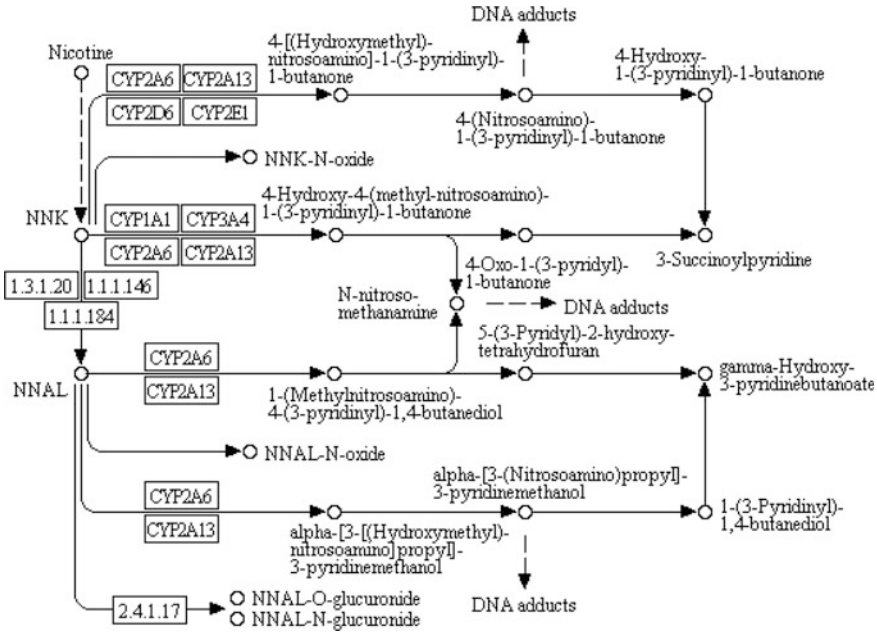

Fig. 1 The metabolic pathways of NNK and NNAL retrieved from KEGG database (http://www.genome.jp/kegg-bin/show_pathway?map00980)

Several studies have been done to identify gene-carcinogen interactions and other mechanistic aspects of the lung cancer process. Moreover, it is important to elucidate those factors, which are responsible for the lung cancer development and also to be found the natural defensive mechanisms (Hecht 1999).

Therefore, we have designed our work to find out molecular interaction of these cigarette smoke carcinogens with enzymes involved in DNA repair pathways by applying molecular docking techniques. The ultimate goal of docking is the prediction of three dimensional structure of the macromolecular complex, which is of interest as it would occur in a living organism. Docking itself only produces plausible candidate structures. These candidates must be ranked using method such as, scoring functions to identify structures that are most likely to occur in the nature. In the present study, we analyzed the molecular interaction of cigarette smoke carcinogens with enzymes involved in DNA repair pathways using AutoDock 4.2 tool (Morris et al. 1996). Docking studies have been executed using AutoDock Tool 4.2 and the results have been interpreted in terms of binding energies.

1.1 Strategies

Using molecular docking techniques, we have performed complete interaction analysis with the help of AutoDock tools. The analyses were divided into two parts as described below:

(i) *Interaction analysis of NNK and NNAL with Enzymes involved in DNA repair pathways*;
(ii) *Interaction analysis of NNK and NNAL with DNA*.

2 Materials

2.1 DNA Repair Enzymes as a Receptor for Analysis

3D structures of DNA repair enzymes have been obtained from Protein Databank (PDB) (www.rcsb.org) and some structures have been developed using homology modeling approach those are not available in the PDB. 3D Structures were validated using Pro-SA online tool (https://prosa.services.came.sbg.ac.at/prosa.php) to check the potential errors within the 3D structures of proteins. Downloaded 3D structures cannot directly use for docking analysis so we have to go for editing and energy minimization process by applying CHARMM force field protocol in Discovery Studio.

3 3D Structures of Different Forms of DNA

The 3D structures of different forms of DNA i.e. A (PDB ID: 1DOU monovalent cations sequester within the A-tract minor groove of [D(CGCGAATTCGCG)]2), B [PDB ID: 3BSE Crystal structure analysis of a 16-base-pair B-DNA) and Z (PDB ID: 390D structural variability and new intermolecular interactions of Z-DNA in crystals of D (PCPGPCPGPCPG)] were also available and downloaded through PDB database.

3.1 Cigarette Smoke Carcinogens NNK and NNAL as Ligand for Analysis

The *.mol* files of cigarette smoke carcinogens NNK (Fig. 2a) and NNAL (Fig. 2b) were obtained from ChemSpider database (http://www.chemspider.com/). They were converted into *.pdb* files using Accelrys Software Inc., Discovery Studio

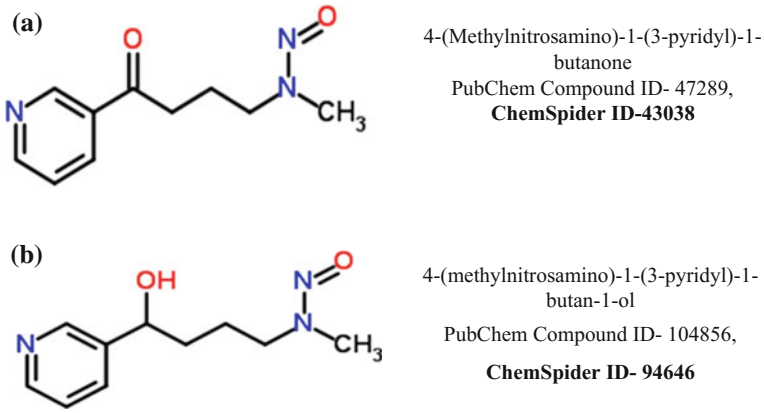

Fig. 2 3Dimentional structure of cigarette smoke carcinogens NNK and NNAL

Modeling Environment, Release 4.0, (San Diego: Accelrys Software Inc. 2013). Discovery Studio makes it easier to examine the properties of large and small molecules. The generated structures couldn't use directly for docking. Therefore, it has been minimized 3D structures of NNK and NNAL using CHARMM force field protocol in Discovery Studio.

3.2 Computational Tools Used

Molecular interaction analyses have been carried out using Auto Dock 4.2 tool. AutoDock 4.2 is faster than earlier versions, and it allows side chains in the macromolecule to be flexible. As before, rigid docking is blindingly fast, and high-quality flexible docking can be done in around a minute. Up to 40,000 rigid dockings can be done in a day.

AutoDock 4.2 has a free-energy scoring function that is based on a linear regression analysis, the AMBER force field, and even larger set of diverse protein-ligand complexes with known inhibition constants. AutoDock uses a computationally (relatively) inexpensive "hybrid" force field that contains terms based on molecular mechanics as well as empirical terms. The prediction of absolute binding energies may be less accurate compared to more computationally expensive, purely force field-based methods. However, the semi-empirical approach is considered well suited for the relative rankings.

The AutoDock semi-empirical force field includes intramolecular terms, a "full" desolvation model, and also considers directionality in hydrogen bonds. The conformational entropy is calculated from the sum of torsional degrees of freedom. Water molecules are not modeled explicitly though, but pair-wise atomic terms are used to estimate the water contribution (dispersion/repulsion, hydrogen bonding,

electrostatics, and desolation), where weights are added for calibration (based on experimental data). The evaluation step in a nutshell: firstly, calculate the energy of ligand and protein in the unbound state. Secondly, calculate the energy of the protein-ligand complex. Then take the difference between 1 and 2.

$$\Delta G = \left(V_{\text{bound}}^{\text{L-L}} - V_{\text{unbound}}^{\text{L-L}}\right) + \left(V_{\text{bound}}^{\text{P-P}} - V_{\text{unbound}}^{\text{P-P}}\right) + \left(V_{\text{bound}}^{\text{P-L}} - V_{\text{unbound}}^{\text{P-L}} + \Delta S_{\text{conf}}\right)$$

Where P refers to the protein, L refers to the ligand, V are the pair-wise evaluations mentioned above, and $\Delta S \sim \text{conf} \sim$ denotes the loss of conformational entropy upon binding (Huey et al. 2007). Graphics has been developed with the help of Accelrys Discovery Studio Visualizer 2.5.5. (http://accelrys.com/products/discovery-studio/visualization-download.php) ICM Browser (http://www.molsoft.com/icm_browser.html) and PyMol (https://www.pymol.org/).

4 Methods

4.1 Ligand Preparation and Optimization for Docking Analysis

PDB structures were generated using the online tool CORINA (http://www.molecular-networks.com). CharMM force field was applied and then subjected to single step minimization using smart minimize algorithm for 1000 steps at RMS gradient of 0.01 and optimized. The computational methodology used in the molecular interaction analysis has been presented in Fig. 3 and Table 1.

4.2 Protein Preparation and Optimization

The crystal structures of DNA repair enzymes and DNA that were taken up in this study were extracted from protein data bank (www.pdb.org). All hetero-atoms of target proteins were removed and further subjected to two-steps energy minimization to remove the bad steric clashes using steepest descent and conjugate gradient methods for 1000 steps at RMS gradient of 0.1 and 0.05 respectively, during the energy minimization process the backbone were fixing the backbone.

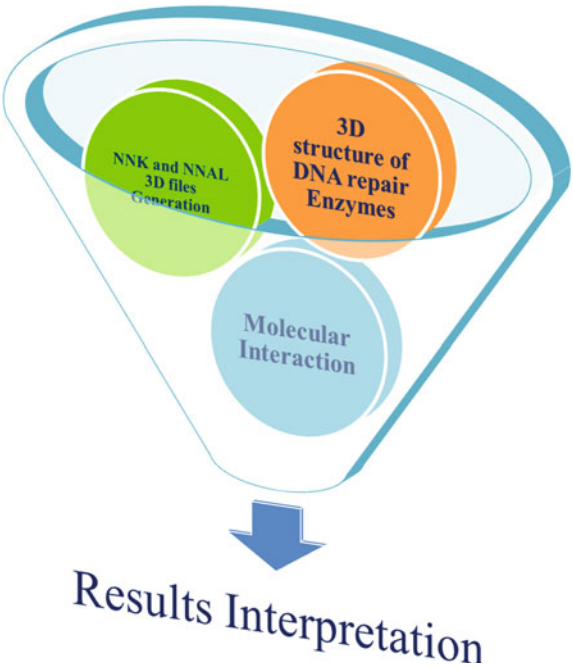

Fig. 3 Schematic diagram of computational methodology used in the molecular interaction analysis

Table 1 Detailed information of selected cigarette smoke compound NNK and NNAL including IUPAC name, molecular formula, molecular weight and their simplified molecular-input line-entry system (SMILES) ID

S. No.	Compound	IUPAC name	Molecular formula	Molecular weight	SMILES
1.	NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone	$C_{10}H_{13}N_3O_2$	207.22912 g/mol	CN(CCCC(=O)C1=CN=CC=C1)N=O
2.	NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butan-1-ol	$C_{10}H_{15}N_3O_2$	209.245000 g/mol	CN(CCCC(C1=CN=CC=C1)O)N=O

4.3 Molecular Docking

Docking experiments were performed using the AutoDock version 4.2 which was set to 50 cycles of run without constraints between the ligands and the specific amino acids of pocket in order to find preferred binding conformations of ligands in receptor. The algorithm exhaustively searches the entire rotational and translational space of ligand with respect to the receptors. The flexibility of ligand is given by

dihedral angle variations. The various solutions evaluated by scoring, which is equivalent to the absolute value of the total energy of ligand in protein environment. The analysis of binding was confirmed by using a scoring function based on free energy of binding. The active site was defined as the collection of protein residues enclosed within 15 Å radius sphere. The annealing parameters for Van der Waals and hydrogen bonding were set to 4.0 and 2.5 Å respectively. Other docking parameters were set to the software's default values. After complete execution of AutoDock 4.2; 50 conformations of ligand in complex with the receptor were obtained, which were finally ranked on the basis of binding energy. The best conformations was visualized in the Discovery Studio Visualizer 2.5 and PyMol (Schrödinger, LLC.).

5 Results and Discussion

5.1 Interaction Analysis of NNK and NNAL with DNA Using AutoDock Tool

In this study, we have analyzed an interaction of 72 enzymes involved in DNA repair mechanisms with cigarette smoke carcinogens NNK and NNAL. According to Hecht et al. (1988) NNK form hemoglobin adducts in Fischer 344 rats, the 1IRD (Crystal Structure of Human Carbonmonoxy-Haemoglobin at 1.25 Å Resolution) was employed as a positive control and 3CI9 (Human heat shock factor-binding protein 1) as a negative control to validate our docking analysis. Furthermore, docking results of these proteins showed that 1IRD docked with NNK, observed binding energy was −6.68 kcal/mol it docked with NNAL, observed binding energy was −6.31 kcal/mol. 3CI9 docked with NNK with observed binding energy of −3.91 kcal/mol it docked with NNAL with binding energy of +2.09 kcal/mol. On the basis of obtained binding energy in comparison with control, we shorted out the enzymes, which were interacted with NNK and NNAL.

The number of biological structures in data bank are rapidly increased, and the molecular docking has become an important approach to evaluate or even to elucidate the interaction between potential ligands and their macromolecular targets. It has been shown that several docking methods described so far can correctly reproduce the binding modes of co-crystallized ligands to their protein targets (self-dockings). There is a large number of study reporting protein-ligand docking. However, limited research has been reported on docking of ligands to nucleic acids, despite DNA being an important molecular target for wide number of antibiotics and antitumor drugs. Disappointingly, most of the scoring functions are parameterized exclusively with protein-ligands sets, and the programs validated only for proteins and their ligands. Nucleic acids differ from proteins due to unique structural features such as high-density charge and helix chiral geometry. Moreover, it is not present a single and well-defined binding site (as occur with most of the

proteins) and, which offers more solvent exposed binding pockets. Consequently, this leads to the question of whether docking programs validated for proteins can also produce reasonable results in ligand-DNA docking. This issue has been recently approached by Holt et al. (2008), where they shown that AutoDock can accurately reproduce the crystal structure of several ligands (minor groove binders and intercalates) bound to DNA, within a resolution of approximately 2 Å. Certainly, this had shed new light on the potential of automated docking programs for virtual screening of DNA binding agents. Indeed, the employment of docking techniques to elucidate unknown DNA binding mechanisms without any conclusive previous experimental data—remains a challenge.

Basically, it was observed that the number of runs has no clear effect upon the predictive skill of docking, although it increases the computational cost in a linear way. In contrast, docking results are improved as the maximum number of energy evaluation increases. However, computational time increases strongly in this case. The maximum number of generations correlates weakly with computational cost, but no prediction improvement was observed when changing from 27,000 to 270,000 generations.

5.2 *Structural Docking Analysis of NNK and NNAL with Enzymes Involved in DNA Repair Pathways*

Total 72 enzymes involved in the DNA repair mechanisms and their interactions with ligands (NNK and NNAL) were analysed during study. NNK showed the binding efficiency with enzymes ranging from +25.41 to −7.47 kcal/mol and NNAL showed the binding efficiency ranging from +36.96 to −6.52 kcal/mol. Simulations were completed and hydrogen bonds were built in docked structures. After analysing the binding energy of distinct formed clusters, top 4 enzymes those shown the higher efficiency to bind with ligands NNK binding efficiency with top 4 enzymes of DNA repair mechanisms ranging from −6.80 to −7.47 kcal/mol and NNAL binding efficiency with top 4 enzymes of DNA repair Mechanisms ranging from −6.17 to −6.52 kcal/mol were selected on the basis of their binding energy obtained from docked conformation files shown in Table 2. Hydrogen bond distances calculated by Discovery Studio Visualizer version 2.5.5.

The study shows that NNK has shown good binding efficiency with proteins 1CKJ (Casein kinases) (−6.80 kcal/mol), 3K05 (−7.04 kcal/mol), 2O8B (centromeric DNA binding; protein binding;) (−7.47 kcal/mol) and 3GQC (Deoxycytidyl transferase (−6.94 kcal/mol). On the other hand it was observed that NNAL shown good binding efficiency with proteins 1CKJ (−6.34 kcal/mol), 1Q2Z (Single stranded DNA-dependent ATP-dependent helicase) (−6.17 kcal/mol), 1T38

Table 2 List of binding energy obtained from molecular interaction between DNA repair enzymes and cigarette smoke carcinogens NNK and NNAL

S. No.	Gene's name	Enzyme's name	PDB ID	Docking analysis with NNK	Docking analysis with NNAL
				Binding energy kcal/mol. Δ G	Binding energy kcal/mol. Δ G
1.	Name = MPG; Synonyms = AAG, ANPG, MID1	DNA-3-methyladenine glycosylase (EC 3.2.2.21) (3-methyladenine DNAglycosidase) (ADPG) (3-alkyladenine DNA glycosylase) (N-methylpurine-DNA glycosirase)	1BNK	−5.05	−5.63
2.	Name = NUDT1; Synonyms = MTH1	7,8-dihydro-8-oxoguanine triphosphatase (EC 3.1.6.-) (8-oxo-dGTPase) (Nucleoside diphosphate-linked moiety X motif 1) (Nudix motif 1)	1IRY	−6.46	−6.18
3.	Name = ABL1; Synonyms = ABL, JTK7	Proto-oncogene tyrosine-protein kinase ABL1 (EC 2.7.1.112) (p150) (c-ABL) (Abelson murine leukemia viral oncogene homolog 1)	2FO0	−5.96	−5.88
4.	Name = APTX	Aprataxin (Forkhead-associated domain histidine triad-like protein) (FHA-HIT)	3KT9	−3.60	−3.94
5.	Name = ATM	Serine-protein kinase ATM (EC 2.7.1.37) (Ataxia telangiectasiamutated) (A-T, mutated)	2WPA	−5.12	−5.60
6.	Name = ATRX; Synonyms = RAD54L, XH2	Transcriptional regulator ATRX (EC 3.6.1.-) (ATP-dependent helicaseATRX) (X-linked helicase II) (X-linked nuclear protein) (XNP) (Znf-HX)	2JM1	−5.63	−6.11
7.	Name = ATR; Synonyms = FRP1	Serine/threonine-protein kinase ATR (EC 2.7.1.37) (Ataxiatelangiectasia and Rad3-related protein) (FRAP-related protein 1)	2KUL	−5.15	−5.16
8.	Name = ATXN3; Synonyms = ATX3, MJD, MJD1, SCA3	Ataxin-3 (EC 3.4.22.-) (Machado-Joseph disease protein 1) (Spinocerebellar ataxia type 3 protein)	2KLZ	−4.41	−4.41

(continued)

Table 2 (continued)

S. No.	Gene's name	Enzyme's name	PDB ID	Docking analysis with NNK	Docking analysis with NNAL
				Binding energy kcal/mol. Δ G	Binding energy kcal/mol. Δ G
9.	Name = BLM; Synonyms = RECQ2, RECQL3	Bloom syndrome protein (EC 3.6.1.-) (RecQ protein-like 3) (DNAhelicase, RecQ-like type 2)	2RRD	−3.41	−3.25
10.	Name = BTG2; Synonyms = PC3	BTG2 protein (NGF-inducible anti-proliferative protein PC3)	3E9V	−4.07	−3.80
11.	Name = CCNH	Cyclin-H (MO15-associated protein) (p37) (p34)	1KXU	−5.37	−5.14
12.	Name = CDK7; Synonyms = MO15	Cell division protein kinase 7 (EC 2.7.1.37) (CDK-activating kinase) (CAK) (TFIIH basal transcription factor complex kinase subunit) (39 kDa protein kinase) (P39 Mo15) (STK1) (CAK1)	1UA2	−4.71	−4.21
13.	Name = CHEK1; Synonyms = CHK1	Serine/threonine-protein kinase Chk1 (EC 2.7.1.37)	1IA8	−4.69	−4.24
14.	Name = DDB1	DNA damage-binding protein 1 (Damage-specific DNA-binding protein 1) (UV-damaged DNA-binding factor) (DDB p127 subunit) (DDBa) (UV-damagedDNA-binding protein 1) (UV-DDB 1) (Xeroderma pigmentosum group E-complementing protein) (XPCe) (XPE-binding factor) (XPE-BF) (X-associated protein 1) (XAP-1)	3I7H	−5.50	−5.26
15.	Name = DDB2	DNA damage-binding protein 2 (Damage-specific DNA-binding protein 2) (DDB p48 subunit) (DDBb) (UV-damaged DNA-binding protein 2) (UV-DDB2)	3EI4	−4.83	−5.09
16.	Name = LIG1	DNA ligase 1 (EC 6.5.1.1) (DNA ligase I) (Polydeoxyribonucleotidesynthase [ATP] 1)	1X9N	−5.69	−5.38

(continued)

Table 2 (continued)

S. No.	Gene's name	Enzyme's name	PDB ID	Docking analysis with NNK	Docking analysis with NNAL
				Binding energy kcal/mol. Δ G	Binding energy kcal/mol. Δ G
17.	Name = LIG3	DNA ligase 3 (EC 6.5.1.1) (DNA ligase III) (Polydeoxyribonucleotidesynthase [ATP] 3)	1IMO	−5.97	−5.82
18.	Name = LIG4	DNA ligase 4 (EC 6.5.1.1) (DNA ligase IV) (Polydeoxyribonucleotidesynthase [ATP] 4)	2E2 W	−4.34	−4.25
19.	Name = POLQ; Synonyms = POLH	DNA polymerase theta (EC 2.7.7.7) (DNA polymerase eta)	2AE9	−4.61	−4.58
20.	Name = EMSY; Synonyms = C11orf30; ORFNames = GL002	Protein EMSY	2FMM	−2.79	−3.89
21.	Name = EXO1; Synonyms = EXOI, HEX1	Exonuclease 1 (EC 3.1.-.-) (hExo1) (Exonuclease I) (hExoI)	EXO1	−6.12	−5.44
22.	Name = FEN1	Flap endonuclease 1 (EC 3.1.-.-) (Maturation factor 1) (MF1)	1UL1	−3.48	−2.88
23.	Name = HMGB1; Synonyms = HMG1	High mobility group protein 1 (HMG-1) (High mobility group proteinB1)	2YRQ	−4.54	−4.05
24.	Name = HMGB2; Synonyms = HMG2	High mobility group protein 2 (HMG-2)	1J3X	−4.21	−4.25
25.	Name = CSNK1D	Casein kinase I isoform delta (EC 2.7.1.-) (CKI-delta) (CKId)	1CKI	−6.80	−6.19
26.	Name = CSNK1E	Casein kinase I isoform epsilon (EC 2.7.1.-) (CKI-epsilon) (CKIe)	3OFM	−3.54	−3.71

(continued)

Table 2 (continued)

S. No.	Gene's name	Enzyme's name	PDB ID	Docking analysis with NNK	Docking analysis with NNAL
				Binding energy kcal/mol. Δ G	Binding energy kcal/mol. Δ G
27.	Name = CIB1; Synonyms = CIB, KIP, PRKDCIP	Calcium and integrin-binding protein 1 (Calmyrin) (DNA-PKcs-interacting protein) (Kinase-interacting protein) (KIP) (CIB) (SNK-interacting protein 2-28) (SIP2-28)	1Y1A	−6.13	−5.61
28.	Name = XRCC6; Synonyms = G22P1	ATP-dependent DNA helicase 2 subunit 1 (EC 3.6.1.-) (ATP-dependent DNAhelicase II 70 kDa subunit) (Lupus Ku autoantigen protein p70) (Ku70) (70 kDa subunit of Ku antigen) (Thyroid-lupus autoantigen) (TLAA) (CTCbox-binding factor 75 kDa subunit) (CTCBF) (CTC75) (DNA-repair proteinXRCC6)	1JEQ	−5.73	−5.64
29.	Name = XRCC5; Synonyms = G22P2	ATP-dependent DNA helicase 2 subunit 2 (EC 3.6.1.-) (ATP-dependent DNAhelicase II 80 kDa subunit) (Lupus Ku autoantigen protein p86) (Ku86) (Ku80) (86 kDa subunit of Ku antigen) (Thyroid-lupus autoantigen) (TLAA) (CTC box-binding factor 85 kDa subunit) (CTCBF) (CTC85) (Nuclear factor IV) (DNA-repair protein XRCC5)	1Q2Z	−6.61	−6.24
30.	Name = MNAT1; Synonyms = CAP35, MAT1, RNF66	CDK-activating kinase assembly factor MAT1 (RING finger protein MAT1) (Menage a trois) (CDK7/cyclin H assembly factor) (p36) (p35) (CyclinG1-interacting protein) (RING finger protein 66)	1G25	−4.37	−4.49
31.	Name = MBD4; Synonyms = MED1	Methyl-CpG-binding domain protein 4 (EC 3.2.2.-) (Methyl-CpG-bindingprotein MBD4) (Methyl-CpG-binding endonuclease 1) (Mismatch-specificDNA N-glycosylase)	3IHO	−5.84	−6.01

(continued)

Table 2 (continued)

S. No.	Gene's name	Enzyme's name	PDB ID	Docking analysis with NNK	Docking analysis with NNAL
				Binding energy kcal/mol. Δ G	Binding energy kcal/mol. Δ G
32.	Name = EEF1E1	Eukaryotic translation elongation factor 1 epsilon-1 (Multisynthetasecomplex auxiliary component p18) (Elongation factor p18)	2UZ8	−4.88	−5.18
33.	Name = MDC1; Synonyms = KIAA0170, NFBD1	Mediator of DNA damage checkpoint protein 1 (Nuclear factor with BRCTdomains 1)	3K05	−7.20	−5.01
34.	Name = MGMT	Methylated-DNA–protein-cysteine methyltransferase (EC 2.1.1.63) (6-O-methylguanine-DNA methyltransferase) (MGMT) (O-6-methylguanine-DNA-alkyltransferase)	1T38	−6.35	−6.62
35.	Name = MLH1; Synonyms = COCA2	DNA mismatch repair protein Mlh1 (MutL protein homolog 1)	3NA3	−4.63	−4.38
36.	Name = MLH3	DNA mismatch repair protein Mlh3 (MutL protein homolog 3)	2O8B	−6.52	−5.47
37.	Name = MSH3; Synonyms = DUC1, DUG	DNA mismatch repair protein Msh3 (Divergent upstream protein) (DUP5) (Mismatch repair protein 1) (MRP1)	MSH3	−6.27	−5.62
38.	Name = MSH4	MutS protein homolog 4	MSH4	−5.74	−5.70
39.	Name = MSH5	MutS protein homolog 5	MSH5	−5.99	−5.92
40.	Name = MSH6; Synonyms = GTBP	DNA mismatch repair protein MSH6 (MutS-alpha 160 kDa subunit) (G/Tmismatch binding protein) (GTBP) (GTMBP) (p160)	2GFU	−5.34	−4.30
41.	Name = NONO; Synonyms = NRB54	Non-POU domain-containing octamer-binding protein (NonO protein) (54 kDa nuclear RNA- and DNA-binding protein) (p54(nrb)) (p54nrb) (55 kDanuclear protein) (NMT55) (DNA-binding p52/p100 complex, 52 kDasubunit)	2CPJ	−4.74	−4.29

(continued)

Table 2 (continued)

S. No.	Gene's name	Enzyme's name	PDB ID	Docking analysis with NNK	Docking analysis with NNAL
				Binding energy kcal/mol. Δ G	Binding energy kcal/mol. Δ G
42.	Name = OGG1; Synonyms = MMH, MUTM, OGH1	N-glycosylase/DNA lyase [Includes: 8-oxoguanine DNA glycosylase (EC 3.2.2.-); DNA-(apurinic or apyrimidinic site) lyase (EC 4.2.99.18) (AP lyase)]	3KTU	−3.93	−3.90
43.	Name = TP53; Synonyms = P53	Cellular tumor antigen p53 (Tumor suppressor p53) (Phosphoprotein p53) (Antigen NY-CO-13)	2WQI	−4.69	−4.59
44.	Name = TP73; Synonyms = P73	Tumor protein p73 (p53-like transcription factor) (p53-relatedprotein)	1COK	−4.31	−4.55
45.	Name = PCNA	Proliferating cell nuclear antigen (PCNA) (Cyclin)	2ZVK	−3.15	−2.71
46.	Name = PML; Synonyms = MYL, RNF71, TRIM19	Probable transcription factor PML (Tripartite motif protein 19) (RINGfinger protein 71)	1BOR	−5.01	−4.92
47.	Name = PMS1; Synonyms = PMSL1	PMS1 protein homolog 1 (DNA mismatch repair protein PMS1)	2CS1	−4.58	−4.66
48.	Name = PMS2; Synonyms = PMSL2	PMS1 protein homolog 2 (DNA mismatch repair protein PMS2)	1EA6	−6.35	−6.10
49.	Name = PNKP	Bifunctional polynucleotide phosphatase/kinase (Polynucleotide kinase-3′-phosphatase) (DNA 5′-kinase/3′-phosphatase) [Includes: Polynucleotide 3′-phosphatase (EC 3.1.3.32) (2′(3′)-polynucleotidase); Polynucleotide 5′-hydroxyl-kinase (EC 2.7.1.78)]	2W3O	−3.27	−3.24

(continued)

Table 2 (continued)

S. No.	Gene's name	Enzyme's name	PDB ID	Docking analysis with NNK	Docking analysis with NNAL
				Binding energy kcal/mol. Δ G	Binding energy kcal/mol. Δ G
50.	Name = POLH; Synonyms = RAD30, RAD30A, XPV	DNA polymerase eta (EC 2.7.7.7) (RAD30 homolog A) (Xerodermapigmentosum variant type protein)	2I5O	−3.86	−3.96
51.	Name = POLI; Synonyms = RD30B	DNA polymerase iota (EC 2.7.7.7) (RAD30 homolog B) (Eta2)	2KHU	−4.76	−4.49
52.	Name = POLK; Synonyms = DINB1	DNA polymerase kappa (EC 2.7.7.7) (DINB protein) (DINP)	3IN5	−6.11	−5.38
53.	Name = POLS; Synonyms = TRF4	DNA polymerase sigma (EC 2.7.7.7) (Topoisomerase-related functionprotein 4-1) (TRF4-1) (LAK-1) (DNA polymerase kappa)	POLS	−5.31	−5.33
54.	Name = REV1L; Synonyms = REV1	DNA repair protein REV1 (EC 2.7.7.-) (Rev1-like terminal deoxycytidyltransferase) (Alpha integrin-binding protein 80) (AIBP80)	3GQC	−6.75	−5.78
55.	Name = RPA1; Synonyms = REPA1, RPA70	Replication protein A 70 kDa DNA-binding subunit (RP-A) (RF-A) (Replication factor-A protein 1) (Single-stranded DNA-binding protein) (p70)	1JMC	−4.92	−5.52
56.	Name = RPA3; Synonyms = REPA3, RPA14	Replication protein A 14 kDa subunit (RP-A) (RF-A) (Replicationfactor-A protein 3) (p14)	2Z6 K	−5.79	−5.68
57.	Name = RFC4	Activator 1 37 kDa subunit (Replication factor C 37 kDa subunit) (A137 kDa subunit) (RF-C 37 kDa subunit) (RFC37)	RFC4	−5.95	−6.10

(continued)

Table 2 (continued)

S. No.	Gene's name	Enzyme's name	PDB ID	Docking analysis with NNK	Docking analysis with NNAL
				Binding energy kcal/mol. Δ G	Binding energy kcal/mol. Δ G
58.	Name = RFC5	Activator 1 36 kDa subunit (Replication factor C 36 kDa subunit) (A136 kDa subunit) (RF-C 36 kDa subunit) (RFC36) (Replication factor Csubunit 5)	1LFS	−6.32	−5.77
59.	Name = SFPQ; Synonyms = PSF	Splicing factor, proline- and glutamine-rich (Polypyrimidine tract-binding protein-associated splicing factor) (PTB-associated splicingfactor) (PSF) (DNA-binding p52/p100 complex, 100 kDa subunit) (100-kDaDNA-pairing protein) (hPOMp100)	SEPQ	−5.64	−5.68
60.	Name = CSPG6; Synonyms = BAM, BMH, SMC3, SMC3L1	Structural maintenance of chromosome 3 (Chondroitin sulfateproteoglycan 6) (Chromosome-associated polypeptide) (hCAP) (Bamacan) (Basement membrane-associated chondroitin proteoglycan)	2WD5	−5.08	−4.73
61.	Name = TNP2	Spermatid nuclear transition protein 2 (STP-1) (TP-2)	TNP2	−5.78	−5.21
62.	Name = TDG	G/T mismatch-specific thymine DNA glycosylase (EC 3.2.2.-)	2RBA	−5.26	−6.62
63.	Name = VCP	Transitional endoplasmic reticulum ATPase (TER ATPase) (15S Mg(2+)-ATPase p97 subunit) (Valosin-containing protein) (VCP)	3HU1	−5.41	−5.56
64.	Name = TYMS; Synonyms = TS; ORFNames = OK/SW-cl.29	Thymidylate synthase (EC 2.1.1.45) (TS) (TSase)	3HB8	−5.46	−5.42
65.	Name = USP1	Ubiquitin carboxyl-terminal hydrolase 1 (EC 3.1.2.15) (Ubiquitinthiolesterase 1) (Ubiquitin-specific processing protease 1) (Deubiquitinating enzyme 1) (hUBP)	3IFW	−4.49	−4.03

(continued)

Table 2 (continued)

S. No.	Gene's name	Enzyme's name	PDB ID	Docking analysis with NNK	Docking analysis with NNAL
				Binding energy kcal/mol. Δ G	Binding energy kcal/mol. Δ G
66.	Name = UNG2	Uracil-DNA glycosylase 2 (EC 3.2.2.-) (UDG 2)	3FCF	−2.82	−2.10
67.	Name = UNG; Synonyms = DGU, UNG15	Uracil-DNA glycosylase (EC 3.2.2.-) (UDG)	2HXM	+7.87	+36.96
68.	Name = XAB2; Synonyms = HCNP, KIAA1177; ORFNames = PP3898	XPA-binding protein 2 (HCNP protein)	2QYQ	+25.41	+29.37
69.	Name = XPA; Synonyms = XPAC	DNA-repair protein complementing XP-A cells (Xeroderma pigmentosumgroup A complementing protein)	2JNW	−4.90	−4.60
70.	Name = XPC; Synonyms = XPCC	DNA-repair protein complementing XP-C cells (Xeroderma pigmentosumgroup C complementing protein) (p125)	2A4 J	−4.89	−4.23
71.	Name = XRN2	5′-3′ exoribonuclease 2 (EC 3.1.11.-) (DHM1-like protein) (DHPprotein)	XRN2	−5.43	−5.73
72.	Name = PARP1	Poly (ADP-ribose) polymerase (PARP)	1UK0	−4.60	−4.39

Table 3 Docking analysis NNK/NNAL with A, B and Z form of DNA [A (PDB ID: 1DOU monovalent cations sequester within the A-tract minor groove of (D(CGCGAATTCGCG)2), B (PDB ID: 3BSE Crystal structure analysis of a 16-base-pair B-DNA) and Z (PDB ID: 390D structural variability and new intermolecular interactions of Z-DNA in crystals of D (PCPGPCPGPCPG)]

S. No.	DNA	NNAL(binding energy in kcal/mol) Δ G	NNK (binding energy in kcal/mol) Δ G
1.	1DOU (A form)	−5.98	−4.20
2.	2DCG_Z (Z form)	−4.63	−4.36
3.	3BSE_B (B form)	−5.19	−3.78
4.	309D_Z (Z form)	−6.57	−6.16

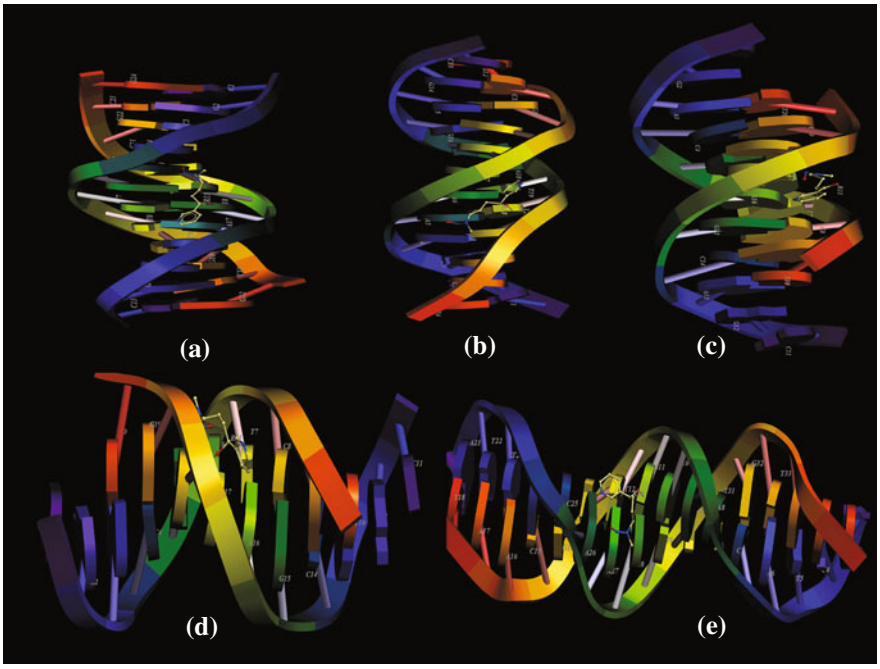

Fig. 4 3Dimentional visualization of an interaction with cigarette smokes carcinogens with different form of DNA, where the nitrogenous base shown in *stick multicolor*. Graphics generated by ICM-Browser visualization software. **a** 1DOU DNA interacted with NNK, **b** 1DOU DNA interacted with NNAL, **c** 390D DNA interacted with NNK, **d** 390D DNA interacted with NNAL, **e** NNAL docked with 3BSE

(Methylated-DNA-protein-cysteine methyltransferase) (−6.52 kcal/mol) and 2RBA (G/T mismatch-specific thymine DNA glycosylase) (−6.41 kcal/mol). As an extension of analysis, we have also analysed possible interaction of NNK and NNAL with different forms of DNA i.e. A, B and Z (Table 3).

5.3 *Results of Interaction of DNA with NNK and NNAL*

The docking results of NNK (Fig. 6a) with all forms of DNA i.e. A (PDB ID: 1DOU monovalent citations sequester within the A-tract minor groove of [D (CGCGAATTCGCG)]2), B (PDB ID: 3BSE Crystal structure analysis of a 16-base-pair B-DNA) and Z [PDB ID: 390D structural variability and new intermolecular interactions of Z-DNA in crystals of D (PCPGPCPGPCPG)] showed binding energy ranging from −4.20 kcal/mol to −6.57 kcal/mol where as NNAL (Fig. 6b) showed binding energy ranging from −3.78 kcal/mol to −6.16 kcal/mol (Figs. 4 and 5). The biochemistry, biology, and carcinogenicity of tobacco-specific

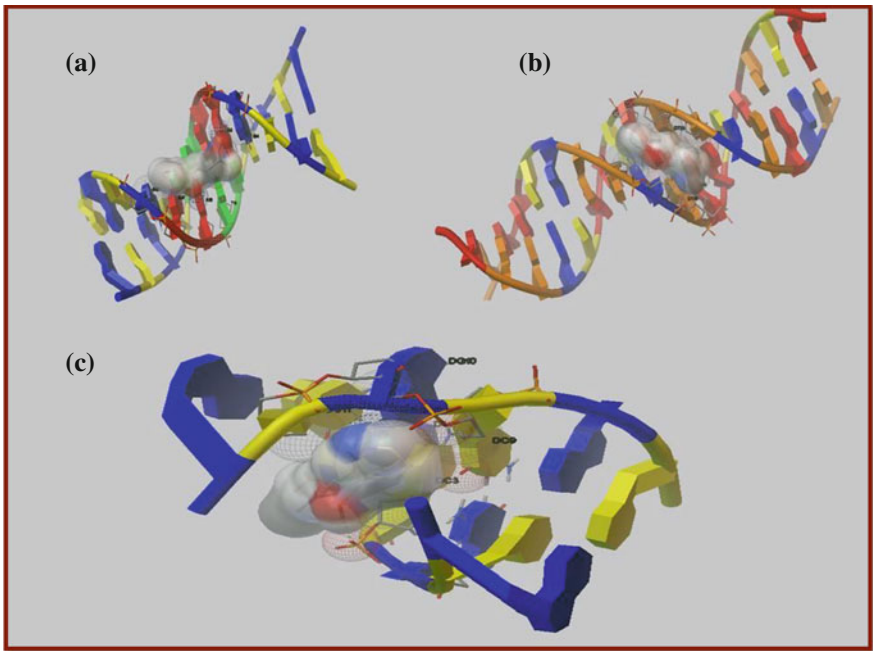

Fig. 5 Visualization of binding site using Autodock 4.2 tool figure **a** NNK docked with 1DOU, **b** NNK docked with 3BSE, **c** NNAL docked with 2DCG (molecular structure of a left-handed double helical DNA fragment at atomic resolution)

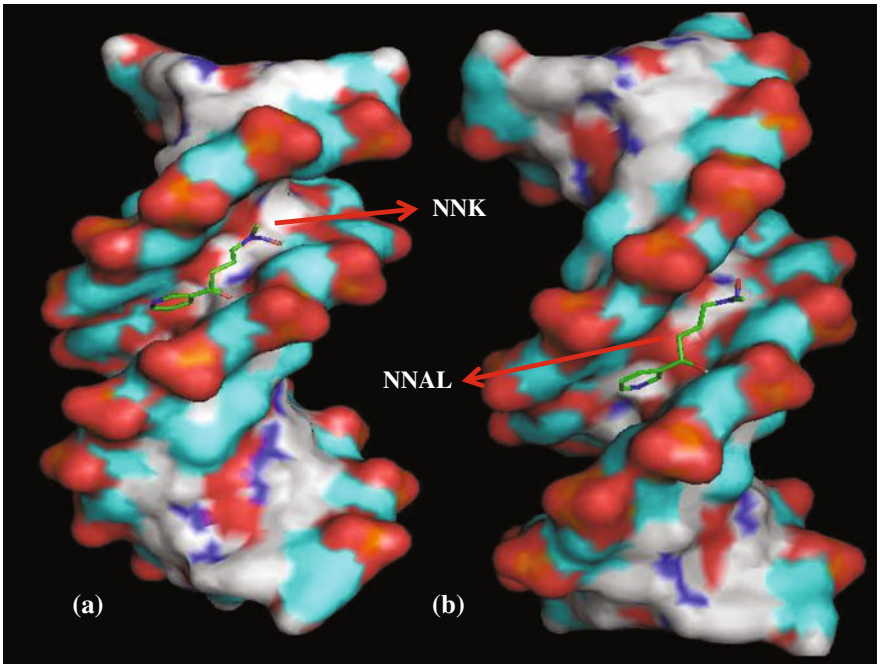

Fig. 6 Visualization of NNK (**a**) (shown in *green stick* pattern) and NNAL (**b**) (shown in *green stick* pattern) as a formed DNA adduct complex. Graphics generated by PyMol 3D visualizer software

nitrosamines have been reported previously (Hecht 2003). Hence, obtained results clearly showed that metabolite of NNK i.e. NNAL interacted with DNA and could able to form DNA adduct and lead to damage DNA double helix.

6 Conclusion

The present study first time introduces *in silico* approach to explores the molecular interaction of cigarette smoke carcinogens with enzymes involved in DNA repair pathways. The computational analysis satisfies that the use of AutoDock Tools effectively characterizing NNK and NNAL as DNA binders. These carcinogens will in due course inhibit the DNA replication and may possibly induce cell cycle arrest and cause cancer. The computational analysis in association with laboratory research have better possibility for future extension in medical sciences, specially for the cancer treatment.

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Determination of *murG* Transferase as a Potential Drug Target in *Neisseria meningitides* by Spectral Graph Theory Approach

Pooja Tripathi and Vijay Tripathi

Abstract The *Neisseria meningitides* has overcome the several front line drugs, which inhibit penicillin binding protein synthesis and develop resistance or tolerance to these drugs. To overcome this situation, here we have attempted to reconstruct the metabolic network of peptidoglycan biosynthesis pathway of *Neisseria meningitides*, to obtain the potential drug target other than the penicillin binding proteins, as the biological networks like transcriptional, gene regulatory, metabolic or protein-protein interaction networks of organisms are widely studied, giving an insight into metabolism and regulation. The metabolic network was constructed based on the KEGG database, followed by graph spectral analysis of the network to identify hubs as well as sub-clustering of the reactions. Analysis of the eigen values and spectrum of the normalized laplacian matrix of the reaction pathway indicate the enzyme, *murG* transferase, catalyzing N-acetylglucosamine (GlcNAc) may considered as a potential drug target. As a case study, we have built a homology model of identified drug target *murG* transferase and various information have been generated through molecular dynamics, which will be useful in wetlab structure determination. The three-dimensional (3D) structure is essential for functional annotation and rational drug design. Accurate models are suitable for a

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wide range of applications, such as prediction of protein binding sites, prediction of the effect of protein mutations, and structure-guided virtual screening. The generated model can be further explored for *insilico* docking studies with suitable inhibitors.

Keywords *Neisseria meningitides* · Peptidoglycan biosynthesis pathway · Graph spectral analysis · *murg* transferase

1 Introduction

The complete genome sequence of several pathogenic bacteria and human genome project has revolutionized the field of drug-discovery against threatening human pathogens (Miesel et al. 2003). Novel drug targets are required to design new drugs against antibiotic sensitive pathogens. In general, a drug target should provide adequate selectivity yielding a drug which is specific or highly selective against the pathogen with respect to the human host. The target should be essential for growth and viability of the pathogen at least under the condition of infection (Sakharkar et al. 2004). The search for potential drug targets has increasingly relied on genomic approaches. The entire approach is built on the assumption that the potential target must play an essential role in the pathogen's survival and constitute a critical component in its metabolic pathway. *N. meningitidis*, also simply known as meningococcus, is a gram-negative bacterium best known for its role in septicemia and meningitis. It only infects humans; there is no animal reservoir. The bacterium can traverse the epithelium and reach the blood stream causing septicemia. From the blood meningococcus is able to cross the blood brain barrier and infect the meninges, causing meningitis potentially leading to shock and death. The total cases of meningitis are caused by five major serogroups: A, B, C, Y and W135. In the present work we have analyzed the peptidoglycan metabolic pathway of *Neisseria meningitides* serogroup B strain MC58 for the determination of alternate drug target against the bacterium. The study shows that murG transferase is the alternative potential drug target using the spectral graph theory approach. The murG is potential drug target after the peptidoglycans in *Escherichia coli* has been confirmed by the recent study (Trunkfield et al. 2010), and it can be further use for the development of new antibacterial agents. Metabolic networks have also been constructed for a number of genomes such as *E. coli* (Edwards and Palsson 2000) and *Staphylococcus aureus* (Becker and Palsson 2005). The spectral graph theory approach in the metabolic network analysis of *M. tuberculosis* and *M. leprae* is applied (Verkhedkar et al. 2007) as a comparative study.

2 Materials and Methods

2.1 Spectral Graph Theory

2.1.1 Node-Degree and the Adjacency Matrix

For an undirected graph G, the degree of a node n in vertex set $V(G)$, is the total number of edges incident at n. The adjacency matrix, $A(G)$ is given by $A_{ij} = 1$ if $v_i v_j$ belongs to edge set $E(G)$, $A_{ij} = 0$ if $v_i v_j$ doesn't belong to $E(G)$. Thus, the adjacency matrix of an undirected graph is symmetric while this need not be the case for a directed graph (Patra and Vishveshwara 2000; Vishweshwara et al. 2002; Ma and Zeng 2002, 2003).

2.1.2 Diagonal Matrix

A diagonal matrix is a square matrix in which the entries outside the main diagonal are all zero. The diagonal entries themselves may or may not be zero (Bollobas and Riordan 2002). Thus, the matrix $D = (d_{ij})$ with n columns and n rows is diagonal if: $D_{ij} = 0$, if $i \neq j$, for all $i, j = \{1–n\}$.

2.1.3 Laplacian Matrix

The Laplacian matrix, or Kirchhoff matrix, of a graph G, where $G = (V, E)$ is an undirected, unweighted graph without graph loops (i, i) or multiple edges from one node to another, V is the vertex set, $n = |V|$, and E is the edge set, is an $n \times n$ symmetric matrix with one row and column for each node defined by $L = D - A$, where $D = diag(d_1,\ldots, d_n)$ is the degree matrix, which is the diagonal matrix formed from the vertex degrees and A is the adjacency matrix.

2.1.4 Eigen Values

Given a linear transformation A, a non-zero vector x is defined to be an *eigen vector* of the transformation if it satisfies the eigen value equation $Ax = \lambda x$ for some scalar λ. In this situation, the scalar λ is called an *eigen value* of A corresponding to the eigen vector x (Quemard et al. 1995; Ravasz et al. 2002; Papin et al. 2004; Hu et al. 2005).

2.2 Spectral Graph Analysis

Spectral graph analysis was performed to find and analyze spectra of nodes in the graph. To obtain Eigen value spectra of the graph, the adjacency matrix is converted

to a Laplacian matrix L, by the equation: $L = D - A$, where, D being the degree matrix of the graph, is the diagonal matrix in which the ith element of the diagonal is equal to the number of connections that the ith node makes in the graph digitalization of the Laplacian matrix yielding the spectra of the graph comprising the Eigen values and corresponding Eigen vectors. The highest eigen value of a node reveals that the node is highly connected in the network and the disruption of that node in the network can lead to the disruption of network flow.

2.3 Softwares Used

VisANT was used for analyzing networks of the pathways. Given user-defined sets of interactions or groupings between genes or proteins, VisANT (http://visant.bu.edu/) provides supporting function and annotation data for different genomes from the Gene Ontology and KEGG databases (Golub and Van der Vorst 2000). MATLAB was used for calculating Eigen values.

2.4 Database Used

KEGG (*Kyoto Encyclopedia of Genes and Genomes*): We have used the KEGG database to reconstruct the reaction networks of *Neisseria meningitides* serogroup B strain MC58. A list of metabolic pathways and their constituent biochemical reactions were downloaded as flat files. These files contain information about reactants, products, and reversibility and steady state stoichiometry of biochemical reactions (Kremer et al. 2002).

2.5 Metabolic Network Reconstruction

The metabolic network MAP **nme00550** of the strain was reconstructed. Each metabolite of the network is a node, and reactions are the edges. Enzymes that catalyze these reactions are the potential drug targets. All edges have an equal weight. In order to make the network amenable to network analysis, it is represented in the form of adjacency matrix A or reaction-interaction matrix (RIM), which is an $n \times n$ matrix; n being the number of nodes (biochemical reactions) in the graph. The elements of A_{ij} of the RIM have values:

$$\begin{aligned} A_{ij} &= 1 \text{ if } V_iV_j \text{ belong to the set of edges} \\ &= 0 \text{ if } V_jV_i \text{ do not belong to the set of edges,} \end{aligned}$$

where V is the vertex i.e. the nodes in the metabolic network graph.

To construct the RIM, the set of reactions in the flat file representing the metabolome was first represented as stoichiometric matrix $S(m \times n)$, with every metabolite being represented by a row and every reaction by a column (Altschul et al. 1997; Berman et al. 2000; Marrakchi et al. 2002).

2.6 Molecular Modeling of Drug Target Protein MurG Transferase

Three dimensional structure of drug target murG transferase of *Neisseria meningitides* serogroup B strain MC58 is not available in PDB database, so we have predicted the 3D structure of murG transferase by using homology modeling method. Protein sequence of murG transferase was obtained from the NCBI database in FASTA format (Accession No. NMB0422). Protein-BLAST algorithm (Henikoff and Henikoff 1992) against Protein Data Bank (Thompson et al. 1994) was carried out for the sequence homology search, in order to identify homologous sequences with known 3-D structure. Blast-p (protein query–protein database) program was run with BLOSUM62 as a scoring matrix (Laskowski et al. 2005), word size 3, gap penalty of **11** and gap extension penalty of **1**. High resolution crystal structure of homologous protein as a template was considered for homology modeling. The Blast-p alignments were further refined by using Clustal, *W* 2.0.10 program (Heinig and Frishman 2004) with default parameters. The protein sequence and 3D structure of template protein were extracted from the PDB database. 3D structure of murG transferase of *Neisseria meningitides* serogroup B strain MC58 predicted by homology modeling method based softwares MODELLER 9v8 (Sali and Blundell 1993) and SWISS-MODEL server (Arnold et al. 2006). Homology modeling of this protein was performed in the following steps: template selection from Protein Data Bank (PDB), sequence-template alignment, model building, model refinement and validation (Marti-Renom et al. 2000).

2.7 Protein Structure Validation

MODELLER generated several preliminary models which were ranked based on their DOPE scores. Some models having low DOPE score were selected and stereo-chemical property of each models was assessed by PROCHECK (Laskowski et al. 1993). PROCHEK server was used for the validation of modeled murG transferase protein structure. PROCHECK analysis of the model was done to check whether the residues are falling in the most favored region in the Ramachandran's Plot or not. The model with the least number of residues in the disallowed region

was selected for the further studies. Quality of models was evaluated with respect to energy and stereochemical geometry. ProSA-Web server (Wiederstein and Sippl 2007) to evaluate energy and Verify 3D (Lüthy et al. 1992) to evaluate the local compatibility of the model related to good protein structure.

2.8 Molecular Dynamics Study

3D structure of murG transferase was refined through energy minimization and molecular dynamics. Molecular dynamics (MD) stimulations were carried out using 43A1 force field of Gromacs96 implemented in the GROMACS 4.0.1 package (Hess et al. 2008). It was performed using GROMACS 4.0.1 package by applying steepest descent gradient algorithm (Wiberg 1965) on LINUX environment. All-atom were solvated with SPC water model in a truncated octahedron box (1.0 nm) and the system net charged was neutralized with the addition of Na^+ and Cl^- ions, randomly placed in the simulation box. The system is then followed by energy minimization for 2000 steps by steepest descent gradient algorithm. The leap-frog algorithm was used for integrating Newton's equations in MD simulation. After that the equilibrated system were subjected to molecular dynamics simulation for 1000 ps at 300 K. The protein stability was evaluated by calculating the root mean square deviation (RMSD) value between the structures generated from the simulation. Pymol (DeLano 2002) software was used for the visualization and analysis of generated protein structure. The energy minimized protein model was used for the further studies.

3 Results and Discussion

Meningitis continues to be a major health challenge in case of infants, warranting the need for newer strategies for therapeutic intervention and newer approaches to discover them. Some issues that need to be addressed specifically are to increase efficiency rates of bacterial clearance, so as to minimize both treatment time and persistence. One way of achieving that could be by significant disruption of meningococcal metabolism. This, however, would have to be done efficiently using minimal points of attack for any practical application in drug discovery.

The metabolic network for the chosen pathway was reconstructed as described earlier in the previous section and analyzed using VisANT software. VisANT (http://visant.bu.edu/) creates a network of the given pathway where each metabolite was treated as node and reaction as an edge. The adjacency and the diagonal matrix for the network were constructed as described earlier using Fig. 1. It was formed in a 38 × 38 matrix where each cell (i, j) represents a link between the ith and jth metabolite. If $(i, j) = 1$; it means that upon enzyme action, the ith metabolite leads to the production of the jth metabolite. Followed by the adjacency

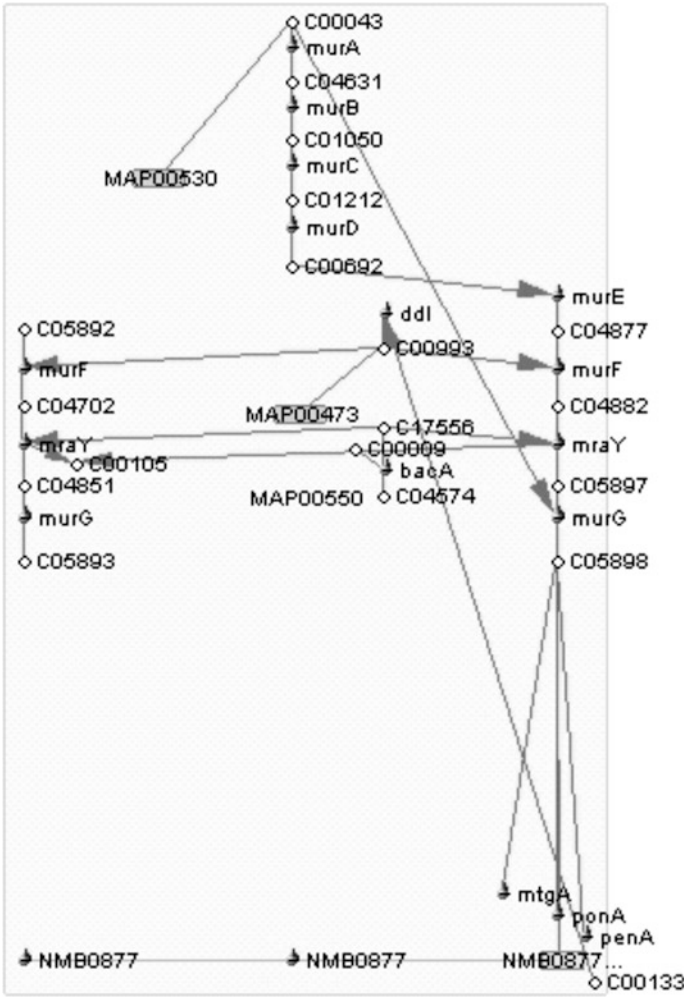

Fig. 1 Peptidoglycan biosynthesis pathway of *Neisseria meningitides* serogroup B strain MC58 as generated by VisANT

matrix and diagonal matrix reconstruction, Laplacian matrix was found using: $L = D - A$. L was then normalized in MATLAB using: $L' = [1/sqrt\ (D)]\ *\ L\ *\ [1/sqrt\ (D)]$. The maximum eigen value calculated by the MATLAB is the spectral radius which is equal to maximum eigen value 0.25 for the node 18 which is the node for N-acetylglucosamine (GlcNAc), and the enzyme involved in this reaction is murG transferase (Fig. 2). In very simple words the node with the highest connectivity in any network when disconnected affects the network flow to a large extent, whereas on the other hand the node with less connectivity affects little or doesn't at all affects the network flow in the network because the product can reach to final

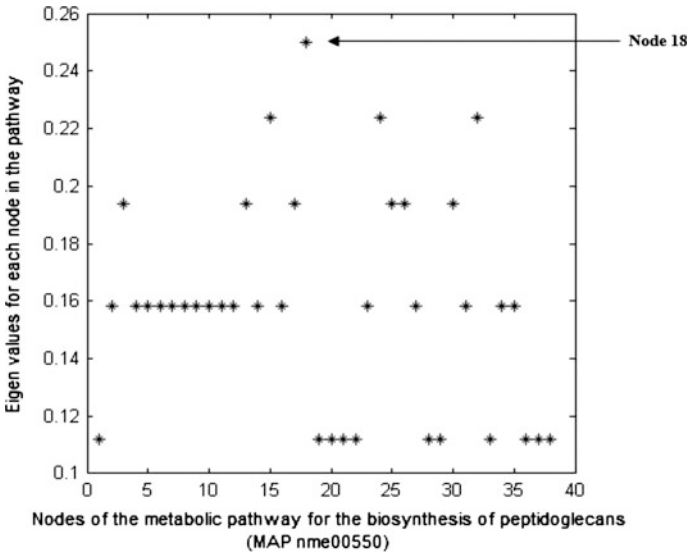

Fig. 2 Eigen values on a scatter plot. We can clearly see that node 18 i.e. the enzyme murG transferase has the highest Eigen value and defines the spectral radius

destination by some other alternate paths present in the connected network (path). Since it is difficult to look through a graph and tell which is the highly or next highly connected node in the large networks, thus we have mathematically computed this connectivity by using the eigen values and eigen vectors concept. Thus, the enzyme murG transferase involved in this reaction can be explored as a potential drug target against meningococcal disease.

3.1 Homology Modeling of murG Transferase

murG transferase of *Neisseria meningitides* serogroup B strain MC58 (Accession No. NMB0422) is 355 amino acids long and shows structural similarity with the crystal structure of murG protein of *E. coli* (PDB ID: 1F0K.A). murG protein of *E. coli* was selected as a template on the basis of lowest e-value (0.00e-1) and maximum identity (45.5%) (Data shown in Table 1). MODELLER 9v8 was used to generate the homology model of murG transferase of *Neisseria meningitides* serogroup B strain MC58 according to the crystal structure of 1F0K.A. Total five models were generated and their discrete optimize potential energy (DOPE) was calculated using "model-single.top" script (Table 2). The model no. 3 (murG.B99990003.pdb) having maximum score was considering as a best model shown in Fig. 3. Pymol software was used to visualize the model and find out the maximum

Table 1 Target and template protein information

Target	Template		
	PDB ID	Protein	Sequence identity
murG transferase	1F0 K	murG	92
(*Neisseria meningitides* MC58)		(*E. coli*)	
	1F2A	Beta-Amylase	90
		(*Sweet Potato*)	
	3KHN	Putative Motb Like Protein	91
		(*Desulfovibrio Vulgaris*)	

Table 2 Comparative study of DOPE score of five models predicted through MODELLER and overall quality factor determination through ERRAT

S.N	Model predicted through MODELLER	DOPE score kJ mol^{-1}	Overall quality factor ERRAT
1.	murG.99990001	−12225.373	78.71
2.	murG.99990002	−12225.373	78.71
3.	murG.99990003	−20151.761	93.562
4.	murG.99990004	−20016.876	91.953
5.	murG.99990005	−20128.563	92.392

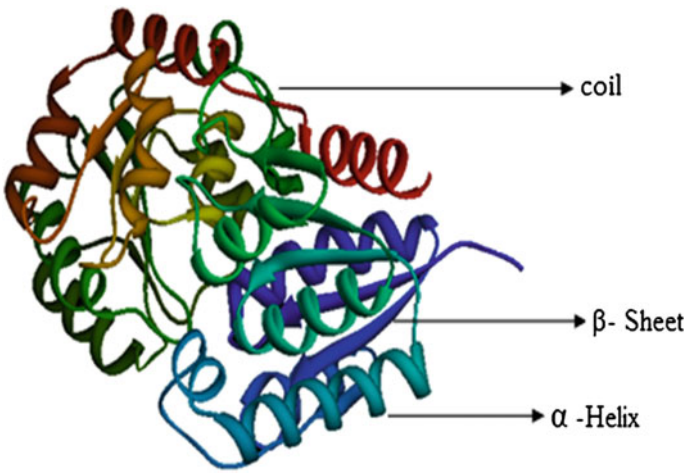

Fig. 3 3D structure of murG transferase of *Neisseria meningitides*

numbers of helixes, turns and sheets in the protein. The 3D model of murG transferase of *Neisseria meningitides* serogroup B strain MC58 was submitted to Protein Model Data Base (PMDB) database with identifier no. PM0077428.

3.2 Protein Structure Analysis

The final model was validated using different tools: PROCHECK, Verify3D and ERRAT programs were used for the validation of predicted model. PROCHECK analysis of the modeled protein showed that 94.17% of the residues were found in allowed regions of Ramachandran plot (Fig. 4). Among the 355 residues 270 residues found in most favored region, 25 in additional allowed region, 3 in generously allowed region and 1 residue in disallowed region. The statistical score of the Ramachandran plot shows that 90.3% are in the most favored region, 8.4% in additional allowed region, 1.0% in generously allowed region and 0.3% in disallowed region. The above results indicate that the protein model is reliable (Table 3). Verify 3D score profile access the quality of the model. Figure 5 shows the Verify 3D profile of the modeled protein, residues have an averaged 3D-1D score greater than zero should be considered reliable. The Verify3D determines the compatibility

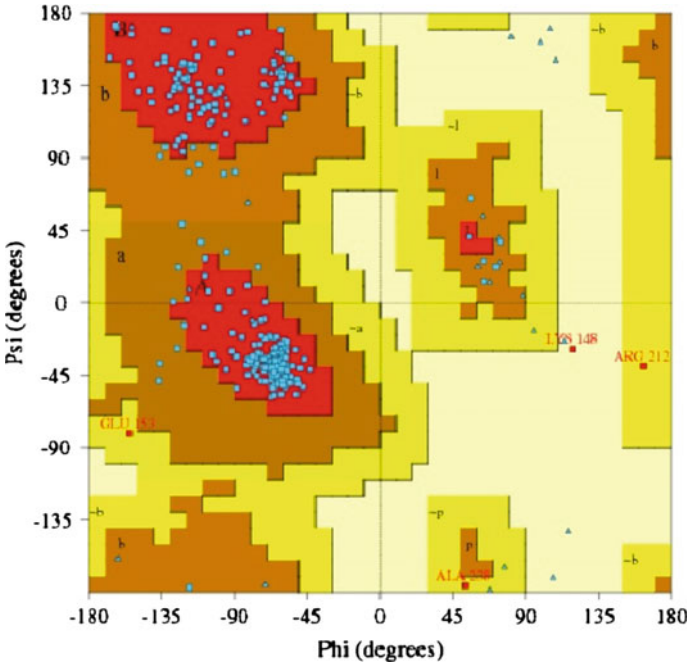

Fig. 4 Ramachandran's map of murG transferase of *Neisseria meningitides*

Table 3 Ramachandran plot calculation for 3D model of murG transferase

Ramachandran plot statistics	Modeled protein	Template
% Amino acid in most favored regions	90.3	95.6
% Amino acid in additional allowed regions	8.4	4.4
% Amino acid in generously allowed regions	1.0	0.0
% Amino acid in disallowed regions	0.3	0.0
RMS Z-Score		
Bond angle	0.898	0.703
Bond length	0.617	0.565

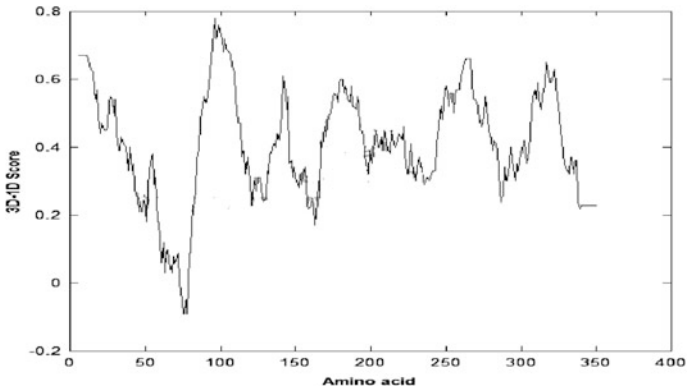

Fig. 5 Verify 3Dimentional score profile

of an atomic model (3D) with its own amino acid sequence (1D) by assigning a structural class based on its location and environment (alpha, beta, loop, polar, nonpolar etc.) and comparing the results to valid structures. The computability score for all the residues in the modeled protein are above zero.

3.3 Molecular Dynamics Analysis

Molecular dynamics simulation was applied for improving and verifying the stability of predicted 3D structure of murG transferase in equilibration with solvent molecules. During molecular simulation, the obtained MD trajectory were monitored and found to be stable. The potential energy of the modeled protein was decreased in the energy minimization procedure and RMSD value of modeled protein structure was increased only. The changes in structural conformation have been monitored in terms of RMSD and RMSF. Figure 6a shows the RMSD value of the murG as time dependent function of MD simulation. It shows that protein–

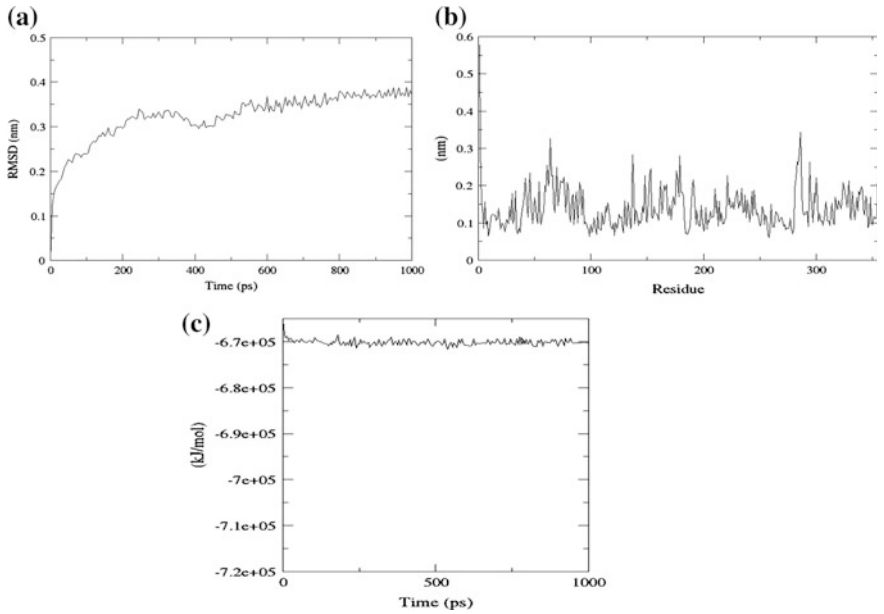

Fig. 6 **a** RMSD of protein and backbone during 1000 ps; **b** RMS fluctuation of protein residue wise during 1000 ps; **c** Total energy of protein during 1000 ps simulation

protein RMSD and backbone–backbone RMSD has become almost stationary at about 1.0 and 2.5 respectively in 1000 ps simulation time. Figure 6b shows 0.1 (approx.) as the average RMSF and Fig. 6c shows the total energy equivalent to −6.7e + 05 kJ/mol (approx.) of protein. Protein stability analysis has been performed through RMSD and energy data. murG transferase protein of *Neisseria meningitides* is reliable and more stable. On the basis of above analysis, the homology model of murG transferase can be further use for the docking studies.

4 Conclusion

Reconstruction of the metabolic network for peptidoglycan biosynthesis, and on further carrying out the spectral analysis for the same, the enzyme murG transferase is identified as a potential drug target against *Neisseria meningitides* and has been demonstrated in Fig. 2. We can clearly see that node 18 i.e. the metabolite N-acetylglucosamine (GlcNAc) has the highest Eigen value and defines the spectral radius. Such essential metabolites can be good targets for drug designing, and they can serve as strategic point to combat meningitis. Locating the protein which affects the maximum number of proteins in a given network will help for finding the intervention strategies against meningitis. murG transferase is also identified as an

alternate drug target in case of *E. coli*, this has been proved experimentally (Mengin-Lecreulx et al. 1991). Thus our finding, by applying spectral graph theory, is in accordance with the wet lab experiments. Homology modeling has been done for identified drug target murG transferase which is shown in Fig. 3 and stero-chemical checking has been done through Ramachandran plot (Fig. 4). RMSD, RMSF, total energy, has been calculated (Fig. 6a–c) and *insilico* 3D modeling data has been generated for experimental support. The 3D structure of murG transferase will be helpful in the *insilico* drug designing with suitable inhibitors.

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Review Processing, Properties and Applications of Agricultural Solid Waste: Effect of an Open Burning in Environmental Toxicology

Kavindra Kumar Kesari and Qazi Mohammad Sajid Jamal

Abstract India is the one among the major country in agricultural production and second largest producer after China and Brazil in rice and sugarcane production respectively. The cultivation of rice and plantation of sugarcane results in various types of residues: rice straw, rice husk and for sugarcane: bagasse, press mud or filter cake and molasses. During the harvesting of rice crop, the top portion grains are harvested and transported to the mill, while the stem (straw), rice husk, leaf and sugarcane residues are left on the field. It could be an appropriate to reuse and utilize a portion of crop residue for liquid fuels or combusted/gasified to produce electricity and heat. This chapter provides an overview of solid waste residues for energy production and also their toxic effects in the environment an open burning process. This chapter reviews the process for the production of ethanol, charcoal, paper and building materials (concrete, cement, bricks) from biomass. Additionally, a significant role of rice husk in silica production and then synthesized silicon nanoparticle use in biomedical research (i.e. drug delivery, cancer treatment etc.) has been introduced. Interestingly, the role of bio-computational approach of silica nanoparticle for drug designing also discussed. Comprehensive review processing of vermicomposting is discussed as a potential tool to bio-convert rice or sugarcane residues into enabling recycling of organic matter or organic fertilizer. After harvesting of rice or sugarcane, these residues remain on agriculture land and processed further for open burning by farmers. This open burning has hazardous effects on human health and the environment, is also discussed in this article. This article provides the possible pathway of mechanism and future recommendations for the use of agriculture waste as a bio-resource.

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1 Introduction

For many decades, agriculture has been associated with the production of essential food crops such as; rice, wheat, nuts. With these food crops, the existing solid waste is also of interest. These solid wastes are available in the form of residual stalks, straw (rice, wheat), leaves, roots, husk (rice, wheat), nut or seed shells, waste wood and animal husbandry waste (cow dung). Crop residues are value added organic byproducts generated from harvesting and processing of agricultural crops. After harvesting of rice, their straw, leaves and husk are majorly using for bioenergy or biofuel production. On the other hand, during the harvesting of sugarcane crop, the stem portion is harvested and transported to the mill, while the leaf residues are left on the field. Moreover, after extraction of juice from sugarcane stem, the remaining baggage, molasses and press mud are also one of the major sources for bioenergy. India is the second largest producer of sugarcane and rice with an estimated production of around 341 million tons (FAOSTAT 2015) and 159.2 Mt respectively in 2013. Therefore, the term biomass energy refers to those crops, residues, and other biological materials that can be used as a substitute for fossil fuels in the production of energy and other products. Agricultural wastes can be in the form of liquids, slurries, or solids. Agricultural solid wastes (rice and sugarcane) are the focus of this paper by focusing on both beneficial and environmental toxicological aspects.

Due to lack of knowledge regarding the significance of crop residues, they are often burned in the field (Samra et al. 2003). However, discharged to the environment, agricultural wastes can be both beneficial and detrimental to living matter. Therefore, the better options for the utilization of biomass (*rice*: straw, husk, bran, leaves; *sugarcane*: bagasse, leaves, fly ash, press mud, molasses) are to reproduce bioenergy products instead of burning or leaving them on agricultural land after harvesting. The beneficial aspects of biomass are to produce biofuel, ethanol, paper, charcoal, coal, bricks, cement, silicon and many more ecofriendly products. On the other hand, detrimental things are associated with human health and the environmental pollution. They can affect surface and ground waters, soils and crops, as well as animals and humans. Sugarcane open burning increases soil temperature, decreases soil water content and bulk density and, consequently, leads to soil compaction, higher surface water runoff, and soil erosion (Dourado-Neto et al. 1999; Oliveira et al. 2000; Tominaga et al. 2002). Moreover, the quantity of the crop residues burned and the fire intensity strongly influence the amount of carbon and nutrients released during fire (Sharma and Mishra 2001). One of the recognized threats to the rice-wheat cropping system sustainability is the loss of soil organic matter as a result of rice-wheat residue burning in the fields. More dangerously, burning of biomass either of sugar or rice may cause serious health issues. It pollutes the air and emission of particulate residues represents the environmental

problem, which can trigger respiratory diseases such as asthma (Cancado et al. 2006; Dawson and Boopathy 2007). In addition to affecting the human health, it may substantially contribute to the formation of Atmospheric Brown Cloud that affects the local air quality, atmospheric visibility and Earth climate (UNEP and C4 2002). This paper presents an important findings associated with an application of rice and sugarcane solid wastes in the production of bioenergy, agriculture, biofuel, animal feed and most considerable aspects of human health due to open burning of these solid waste materials.

2 Solid Waste Production in Rice and Sugarcane Industry and Their Applications

The cultivation of rice results in two types of residues: rice straw and rice husk. The importance of Rice Husk and Rice Straw is an attractive, alternative and ecofriendly source of energy. It is a renewable natural resource, which makes it as an alternative energy source to replace the depleting fossil fuel (Shyam 2002; Kopetz 2007; Gadde et al. 2009). The pathway and the application of bio-resources are presented in Fig. 1. The whole-sole application of rice straw, husk and bran are discussed here in detail. Rice straw is a good source to produce ethanol via microbial fermentation; charcoal via fly ash; animal feed; electrical power and heat via combustion and gasification; manufacturing composite and paper products, manufacturing useful building materials like, cement via silicon, bricks, plywood etc. Because, the nutrient content of the rice straw is high with 25% nitrogen (N) and phosphorus (P), 50% sulfur (S) and 75% potassium (K), though use in controlling soil erosion. Moreover, the components of rice husk are cellulose (25–35%), hemicelluloses (18–21%), silica (15–17%), lignin (26–31%), solubles (2–5%) and moisture content 7.5% (Gerardi et al. 1998; Leiva et al. 2007; Mansary and Ghaly 1998; Stefani et al. 2005), which are very useful components in energy production. With these very high nutritional values, it is a big loss if the agricultural residue is not being exploited and utilized to the optimum. Zafar (2015) provided the statistical value of rice straw and husk as presented in Fig. 2.

On the other hand, the four main byproducts of the sugarcane industry are cane tops, bagasse, filter muds and molasses. Sugarcane is a rich source of carbohydrates; it used as a food (sucrose, fructose, syrups, and jiggery) for human; fiber (cellulitic materials) feed for animal (green leaves, top portion); fuel/ethanol (bagasse, filter cake) and chemicals (alcohol, bagasse, filter cake) in various forms and used as a fertilizer in crop production across the globe (Dotaniya et al. 2016). This solid waste contains lignocellulosic material and mainly composed of cellulose, hemicellulose, lignin, nitrogenous compounds and ash. Therefore, it is a value added source of bio-fertilizer by converting through bio-compositing or vermicomposting. Vermicomposting is a very natural and economical process of organic fertilizer production.

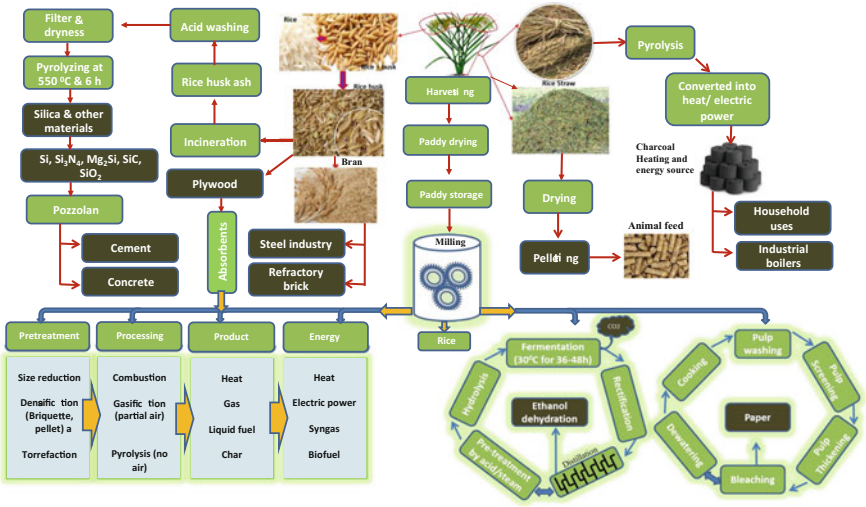

Fig. 1 An overview of strategic applications of different part of rice plant after harvesting (rice husk, bran and rice straw) for the production of building materials (cement, concrete, bricks) ethanol, charcoal, animal feed, toilet paper and silicon. This figure highlights the processing of different parts of rice in bio-energy production. This pathway somehow applies for sugarcane plant of its different parts (bagasse, leaves, filter cake)

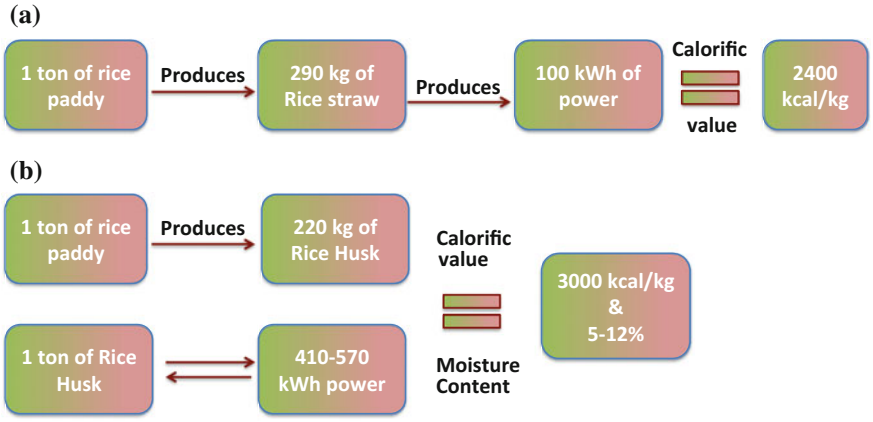

Fig. 2 Conversion of biomass (rice straw and husk) into energy production **a** statistical evaluation, production and value of rice straw and **b** rice Husk

2.1 Production of Ethanol

The solid wastes are a good bio-resource for energy production. Lignocellulosic biomasses like wheat straw, sugarcane bagasse and rice straw are the world

renewable resource in the biosphere (Gruno et al. 2004). The residual wastes rice straw is used for ethanol production. Ethanol is produced from rice straw by following steps: Lignocellulosic biomass could be washed, dried and milled into powder form and thereafter, processed for fermentation by using microorganism (i.e. *Sacchromyces cervisae*). Then, it was pretreated by soaking (3% H_2O_2 + 2% NaOH), steaming (130 °C for 60 min) and processed further for filtration and washing. After washing, it could be process for hydrolysis (first 1.25% H_2SO_4, 2 h and then 72% H_2SO_4, 4 h) thereafter the measurement of lignin. Then the main steps for further treatment and measurement of ethanol production are- enzymatic hydrolysis by using enzyme solution, fermentation by adding yeast extract and measurement of ethanol yield. However, residues from sugar waste production of ethanol contain 60% fermentable sugars with fermentation of molasses (sugarcane). Molasses is first diluted with water (1:5 ratio/vol.). It is fortified with ammonium sulphate (supply of nitrogen to yeasts) and then acidified with a small quantity of sulphuric acid. The resulting solution is then transferred to a large tank and yeast is added to it at 30 °C and left to ferment for 2–3 days. After fermentation, it is finally converted to 92% pure alcohol, by fractional distillation, which is commonly referred to as rectified spirit or commercial alcohol. The overall process also presented in Fig. 1.

2.2 Production of Charcoal

Charcoal from rice straw, sugarcane leaves and bagasse is another possible source of heating and cogeneration of energy. In most of the developing countries it is used as domestic fuel for cooking and heating. Charcoal production is done through a method called pyrolysis of biomass. The process of charcoal production can be defined into following steps: dry leaves, straws or bagasse are collected and packed into a cylindrical, metallic container (drum or cans) for pyrolysis (Fig. 1). Pyrolysis is defined as the irreversible chemical change brought about by heating the biomass in the absence of oxygen. Once the biomass gets fully carbonized, water is sprinkled over the charcoal kiln and resultant powder briquetted. This carbonized char powder is mixed with a binding material such as starch and boiled with water. Thereafter, the mixture is poured into heating machine to form desirable shapes of charcoal. High quality charcoal can also be produced from coconut shell and rice husk for local domestic and industrial use.

2.3 Production of Building Materials

The application of solid waste for building material is another creation of man-made construction material in the world. Rice husks, together with sugarcane bagasse, are one of the two highest-volume agricultural process residues. Rice husk

ash (RHA), fly ash, coconut ash, bagasse etc. are the major source of cement or concrete production as also indicated in Fig. 1. Safiuddin et al. (2010) have demonstrated the utilization of solid wastes for building materials. Interestingly, another agriculture solid waste, 'coconut fibers' were used in reinforced concrete beam along with rice husk and sugarcane waste fibers (Sivaraja and Kandasamy 2009). The production and consumption of concrete is about 25 billion tons, where the consumption of cement increased to 2.9 billion tons worldwide in a year and which is predicted to increase to 4 billion tons by 2020 (Chana 2011). The commercial way for the production of cement has several health hazards and environmental toxicity issues. Therefore, the use of agriculture solid waste like rice husk ash, sugarcane bagasse ash and fly ash for the production of building material has gained increasing interest because of various ecological, economical, technical, and diversified product quality reasons (Oner et al. 2003). The production of building material like cement and concrete rice husk ash is obtained by burning rice husk in a controlled manner without causing environmental pollution. The controlled burning of rice husks between 500 and 800 °C produces non-crystalline amorphous RHA (Mehta and Monteiro 1993; Malhotra 1993). Fly ash and rice husk ash are pozzolanic materials. Sustainable concrete manufactured from pozzolanic materials is one of the solutions recommended by several researchers in the past few decades (Mehta 1999; Nehdi 2001; Malhotra 2006). A pozzolanic material is essentially a siliceous (containing SiO_2) and aluminous (containing Al_2O_3) material, which possesses no cementitious properties. Rice husk ash is a very good source of amorphous silica (Mehta 1992). Silicon has promising applications in energy production. Rice husk ash has high strength to produce concrete (Ismail and Waliuddin 1996) and supplementary cementing material by using this concreate (Zhang and Malhotra 1996). Not only concrete or supplementary cement, but also rice husk ash can be used to make bricks and blocks by several researchers (Nasly and Yassin 2009; Rahman 1987, 1988). These bricks, blocks, concrete and cement are ecofriendly and protect from high temperature of sunlight. Lertsatitthanakorn et al. (2009) investigated that these blocks made of rice husk ash along with sand-cement reduce the solar heat.

On the other hand, many researchers examined the suitability of sugarcane bagasse ash in cement and concrete production (Chusilp et al. 2009; Fairbairn et al. 2010; Frías et al. 2011). Depending on the incinerating conditions, the resulting sugar cane bagasse ash (SCBA) may contain high levels of SiO_2 and Al_2O_3, enabling its use as a supplementary cementitious material (SCM) in blended cement systems. Cordeiro et al. (2009b) reported that the use of SCBA in high-performance concrete as a partial replacement of Portland cement improved the rheological properties. Besides the utilization of sugar industry wastes in cement and concrete production, few reports are also available where sugar industry wastes were used in bricks production, ceramic materials, glass-ceramic materials (Faria et al. 2012; Madurwar et al. 2014; Souza et al. 2011; Teixeira et al. 2014).

2.4 Production of Toilet Paper, Office Paper

Paper can be made from any fibrous plant but the best materials are rice straw and sugarcane bagasse as presented in Fig. 1. Rice straw or bagasse is not only useful in ethanol production but also used in making of paper such as toilet and normal paper (Kumaraguru et al. 2014; Rainey 2009; FPRDI 1991). Paper production from fibers or bagasse undergoes three major processes: preparation of the bagasse, pulping and papermaking. The procedure for the papermaking is very similar for each fibrous agriculture solid wastes. Poopak and Reza (2012) reported that bagasse contains 65–68% fibers, 25–30% pith, 2% sugar and 1–2% minerals, which are well suited for the making of tissue, corrugating medium, newsprint and writing paper.

3 Silica from Rice Husk Ash: Applications

Rice husk is an excellent source of high-grade silica (Ghosh et al. 1999b; Conradt et al. 1992; Real et al. 1996). The first report of the presence of silica in rice husk has been highlighted in 1938 (Martin 1938). The information of silica ash comes through burning of rice husk in air and which varies from gray to black depending upon inorganic impurities and unburnt carbon amount (Rao et al. 2001). Pure silica can be obtained from rice husk ash by following procedures: Fluidized bed (Huang et al. 2001), Chemical pre and post treatment using acid and base solution (Yalcin and Sevinc 2001), Pressurized hot water treatment processes (Mochidzuki et al. 2001), Carbonization and combustion (Liou 2004a), Non isothermal decomposition in oxidizing atmosphere (Liou 2004b). The silica in the ash undergoes structural transformation depending on the various conditions like time, temperature, chemical composition, and minerals content of combustion. At 400–1200 °C amorphous ash is formed and at temperature greater than the crystalline ash is formed (James and Rao 1986). This kind of silica has been shown to be a good material for the synthesis of very pure silicon, silicon nitride, silicon carbide, magnesium silicide, and other applications (Real et al. 1996; Chandrasekhar et al. 2003). Amorphous silica powder is basic raw material used in industries associated with rubber, ceramics, electronics, catalysis, pharmaceutics, and dental materials (Sun and Gong 2001). There is a growing demand for fine amorphous silica in the production of high performance cement and concrete, use in bridges, marine environments, nuclear power plants etc. Silica aerogels prepared from rice husk ash finds application in super thermal insulators, catalyst supports and dielectric materials. It can be an economically viable raw material for the production of silicates and silica (Chandrasekhar et al. 2003).

3.1 Rice Husk Ash Based Silicon Nano-particles

The use of nanotechnology for medical applications has undergone rapid development in various disciplines like, nanocarrier for delivering nanomedicine and nanoparticles for therapeutic applications (Peer et al. 2007; Jain and Stylianopoulos 2010; Petros and DeSimone 2010; Oanh et al. 2010). Silica nanoparticles (SNPs) have drawn widespread attention due to their applications in many emerging areas. Slowing et al. (2007) reported that mesoporous silica nanoparticles (MSNs) with a pore size ranging from 2 to 50 nm are excellent candidates for drug delivery and biomedical applications by comparing with other porous silica nanocarriers. Silica nanoparticles are developed for a host of biomedical and biotechnological applications such as cancer therapy, DNA transfection, drug delivery and enzyme immobilization (Hirsch et al. 2003; Slowing et al. 2008; Kumar et al. 2004; Barik et al. 2008). Not only in medical sciences but also in energy production, waste management, drug development, wastewater treatment, agriculture and environment management. Zapata et al. (2013) studied the rheological and hardened properties of super plasticized cement mortar with SNPs. Highly dispersed SNPs enhance the thermal, mechanical, optical, abrasion resistance and surface hardness properties of composite coatings (Hoshikawa et al. 2010; Slowing et al. 2007). The application of nanoparticles sounds more interesting, after synthesizing from agriculture waste materials like rice husk. The synthesis of amorphous silica nanoparticle is one of the best examples of solid waste management. Figure 3 represents the application of silica nanoparticle in biomedical sciences. In the research area of nanotechnologies, the silica-based NPs have an advantage and dominant role because of their particle size from 5 to 100 nm. Also it has been fundamentally characterized by unique optical properties, high specific surface area, low density, capacity for encapsulation and adsorption and biocompatibility and low toxicity (Halas 2011). Adam et al. (2011) synthesized spherical nanosilica from agricultural biomass as rice husk via the sol–gel method. A small size, nanostructured silicon materials have promising applications in the area of advance technologies, such as nanoelectronics (Cui and Lieber 2001), energy harvesting (Tian et al. 2007; Hochbaum et al. 2008; Boukai et al. 2008), photonics (Pavesi et al. 2000), biotechnology (Lin et al. 1997; Park et al. 2009; Tian et al. 2010), and energy storage (Chan et al. 2008; Magasinski et al. 2010; Kovalenko et al. 2011; Wu et al. 2012).

Jung et al. (2013) demonstrates that rice husks, a major by-product in rice harvest, can be used to produce silicon with an ideal porous nanostructure for use in high-capacity lithium-ion battery (LIB) anodes. Silicon anodes for lithium-ion batteries can store 10 times more capacity than graphite anodes, the material most commonly used in today's batteries. Nano-Si has attracted considerable attention to several other researchers as a promising anode material in next generation Li-ion batteries for electric vehicles and portable electronics (Hatchard and Dahn 2004; Obrovac and Krause 2007).

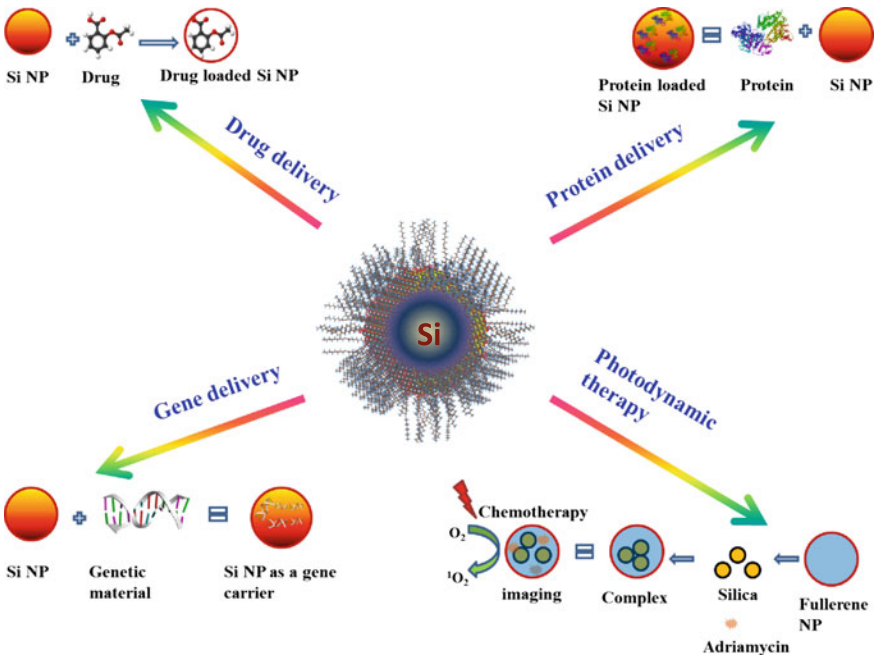

Fig. 3 A graphical representation of the application of silica nanoparticles (SiNP) in biomedical research. Majorly it is useful in drug delivery as drug delivery carriers and protein. The genetic materials (e.g. DNA, miRNA and siRNA) could be delivering into the cellular system through gene delivery methods using SiNP and photodynamic therapy

3.2 Bio-computational Modeling and Nanoparticle Drug Designing

The bio-computational modeling techniques have been widely used in drug discovery fields for rational drug design and compound screening. It has great application to measure the amount of dose to be delivering in diseased or infected human body (or specific region). Now these techniques are used to model or mimic the behavior of molecules, and help us to study formulation at the molecular level. Therefore, computational pharmaceutics enable us to understand the mechanism of drug delivery, and to develop new drug delivery systems. Proteins, enzymes or any biological macromolecule interacts with nano-particles and binding pattern depends on the size, shape, and chemistry of the surface of nanomaterials (Sun et al. 2014).

Recently, titanium dioxide nanoparticles have been used in scavenging the high molecular weight polycyclic aromatic hydrocarbons (PAHs) from the contaminated soils (Karnchanasest and Santisukkasaem 2007). The scavenging capability of nanoparticles for PAH and other toxicants could be accredited to their higher affinity towards xenobiotics due to surface chemistry, large surface area and other intrinsic properties of nanoparticles (Dhasmana et al. 2014). Some studies also have

shown that titanate nanotube has the capacity to scavenge the PAHs from water sample from the environment (Bochra et al. 2011).

The implementation of bio-computational approach in drug designing can explore the interaction of nanoparticles including binding pattern with several biological macromolecules. In silico analysis is able to generate 3D interaction graphics for the better understanding of drug discovery and targeting. It is now very successfully using in wet lab experiments. Moreover, it is also possible to find out scavenging property of silica through computational approach.

3.2.1 Computational Tools for the Designing of Nanoparticles

Synthesis of nanoparticles is an expensive and time taking process. At present, commercial, non-commercial, online and offline software's are available to computationally design, analyze the nanoparticles into three dimensional (3D) structures and perform molecular interaction between nanoparticles and bio macromolecules. Moreover, nanotube modeler (http://www.jcrystal.com/products/wincnt/), CoNTub (http://www.ugr.es/~gmdm/contub.htm) and TubeGen (http://turin.nss.udel.edu/research/tubegenonline.html) are the best example for the generating xyz-coordinates for Nanotubes and Nanocones.

4 Vermicompositing of Biomass: Conversion of Organic Waste into Organic Fertilizer and Its Applications

Rice and sugarcane industries are one of the largest growing industries in India. In rice and sugarcane production, India is the second largest producer after China and Brazil respectively. Therefore, it is obvious that production of agricultural wastes will be produced in large amount. Because, during the harvesting of rice crop, the top portion grains are harvested and transported to the mill, while the stem (straw), leaf residues and rice husk are left on the field. On the other hand, a large amount of solid waste is generated during the manufacture of sugar that contains high levels of suspended solids, organic matter, press mud and bagasse (Muthusamy et al. 2012). Therefore, the proper disposal of these agriculture solid wastes by recycling can supply plant nutrients and improve soil physical conditions and environment quality (Mishra et al. 1989; Bhardwaj 1995). Vermicomposting can be used as a potential tool to bio-convert rice residues into composting or vermicomposting to enabling recycling of organic matter (Said et al. 2010). Rice residues are agricultural wastes; they are rich in silica plant material and are potential sources of plant nutrients (Mandal et al. 2004; Kadam et al. 2000). During vermicomposting, it is also important to maintain nutritional value to supply nutrients to earthworm for the bio-degradation process. Therefore, vermicomposting promotes reuse and bio-transformation of organic waste into organic fertilizer, which helps to contribute

significantly towards sustainable agricultural practices (Yan et al. 2012). Vermicomposting is a bio-oxidative process entails the combined action of earthworms and microbes in presence of cow dung to accelerate the stabilization process of organic materials (Sim and Wu 2010; Bhat et al. 2015). In this combined action of earthworms and microbes, earthworms ingest, break and digest waste and converts into finer, humified, microbially active material (Khwairakpam and Bhargava 2009). The biodegradation of bio solids and organic residues is performed in an aerobic environment. These earthworms participate in recycling the organic waste residues and significantly increase the amount of N, P and K, Ca, Mg and also useful microorganisms (bacteria, fungi, actinomycetes and protozoa) hormones, enzymes, vitamins and certain micronutrients needed for plant growth (Lee 1985; Bansal and Kapoor 2000; Jambhekar 1992). All these nutrients can be effectively recycled for the improvement of soil fertility index and crop yield (Hussain and Anjum 1999).

Vermicompost is a peat-like material with a high porosity, aeration, drainage, water holding capacity and rich in microbial activity and plant nutrient substances that may act as suitable plant growth media for sustainable agriculture (Suthar 2012). Composting or vermicomposting is not possible directly from agriculture waste, because earthworms could not survive in pure rice residues or wastes. Therefore, a mixture of cowdung with organic waste (green rice straw and leafs) is necessary for composting. Cowdung is a good source of microorganisms, which increases the nitrogen content of the vermicompost. Therefore, it is imperative to implement the C/N ratio to promote new earthworm's juveniles through vermibed. The agriculture solid waste (rice straw or sugarcane bagasse) was prepared based on the initial C/N ratio between 20 and 55. As Mupondi et al. (2010) reported that the wastes with a C/N ratio of 30 are the most suitable substrates in vermicomposting processes. The earthwarm survival depends on the percentage of composition (cowdung and agriculture waste) used inside the bricks container. For example, in sugarcane waste (bagasse, filter cake, molasses or all three together) it should be 60% sugar waste and 40% of cowdung (Umar and Sharif 2013). Several steps of composting and vermicomposting are presented in Fig. 4 and also discussed briefly by Nagavallemma et al. (2006). Organic waste transforming rapidly into vermicompost because earthworms intestine contains a wide range of microorganisms, enzymes and hormones which may able to decompose half-digested material in a short time period (about 4–8 weeks) (Ghosh et al. 1999a; Nagavallemma et al. 2006). Therefore, we conclude that compositing or vermicomposting is a well-known system for rapid stabilization (Adani et al. 1995) and also environmentally friendly and economical alternative method for treating solid organic waste (Saranraj and Stella 2012).

Vermicomposting is majorly using as fertilizer for plant growth. It is a good source of nutrient for plant growth. Fundamentally, if the plant or crop is healthy to eat then it can maintain healthy environment. The use of vermicomposting is a way to help cut down on methane gases (greenhouse gases). It is eco-friendly and produces one of the most nutrient enriched fertilizers around for plant growth promoting activity. With nutrient enriched fertilizers, vermicomposting improved crop growth and yield. Therefore, it will help to make reduction in soil C:N ratio

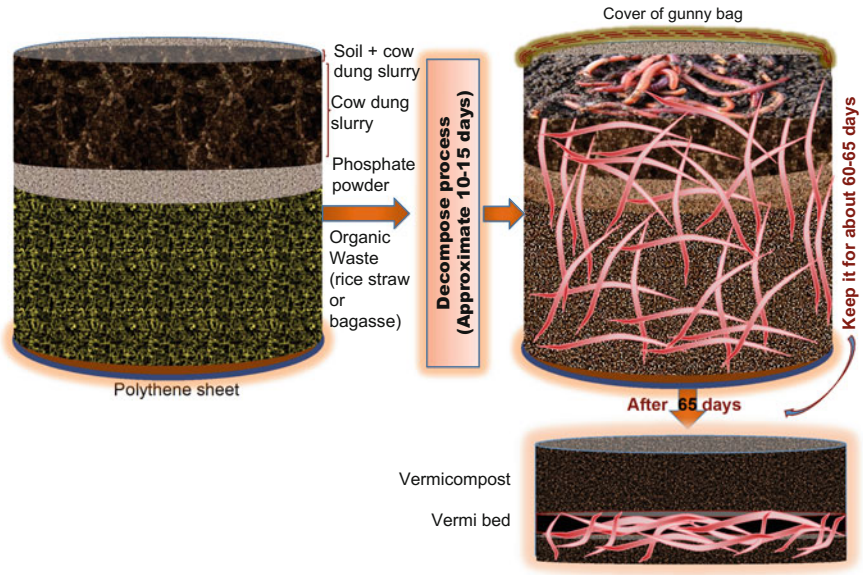

Fig. 4 Process of different steps for composting or vermicomposting of rice residues, bagasse or solid organic waste

and play a major role in nitrogen cycle. Also it may improve soil physical, chemical and biological properties. More importantly, it is cheap and low maintenance to produce.

5 Open Burning of Solid Waste (Biomass) and Health Concerns

Open burning of disposed solid wastes, such as rice residues, sugarcane leaves, are leading to severe air, land, and water pollution as also found detrimental effects on human health (Sim and Wu 2010; Jorapur and Rajvanshi 1997). Burning biomass (rice straw, sugarcane leaves, stems) smoke could deteriorate air quality and create an atmospheric brown cloud (UNEP and C4 2002; UNEP 2008). This may lead to high personal exposure (Wu et al. 2006) and result in adverse health effects (Torigoe et al. 2000). During open burning of rice straw, the emissions of fine particles are a major cause of concern due to their harmful effects on human health (Pope et al. 2009) as well as the earth's climate change (Bond et al. 2004). Such climatic changes due to biomass burning is also a critical source of greenhouse gases such as methane (CH_4) and carbon dioxide (CO_2) (Andreae and Merlet 2001), which contribute to global warming (Sun et al. 2016). Biomass burning is a matter of incomplete combustion in nature and therefore, due to open burning of rice

straw, large amount of pollutants is emitted in various form of toxic gases (carbon monoxide (CO), volatile organic compound (VOC), and carcinogenic polycyclic aromatic hydrocarbons or PAHs) and fine/inhalable particles (Tipayarom 2004; Manandhar 2003; Kim et al. 2005). The fine inhalable particulate matter contains mutagenic/carcinogenic airborne contaminants such as PAHs (Andrade et al. 2010a; Umbuzeiro et al. 2008; Bosso et al. 2006; Godoi et al. 2004; Azevedo et al. 2002; Santos et al. 2002). In this consequences, Silva et al. (2010) also reported potential cancer risk due to the influence of sugarcane burning and emission of PAH exposure. Batelle (1975) and Friedman and Edward (1977) reported that burning a ton of leaves would produce 117 lb of CO and 41 lb of particulate matter (easily absorbed in the lungs if >10 μm). Lung cancer is the principal cause of cancer related mortality and which has been associated to PAHs exposure (Farmer et al. 2003; Lewtas 2007; Silva et al. 2006). Figure 5, shows the overall burning effects due to release of toxic gasses in the environment and how the human beings are getting exposure through inhaling, direct contact or water contamination. Several other researchers have also reported biomass smoke has potentially toxic effect on human health and the environment (Oanh et al. 2002; Smith 1987; WHO 1999). The open burning biomass (wood, leaves, trees and grasses, agricultural waste such as rice, wheat straw, sugarcane) produces 40% of carbon dioxide (CO_2), 32% of carbon monoxide (CO), 20% of particulate matter, and 50% of PAHs, which may releases in the environmental atmosphere (Kambis and Levine 1996),

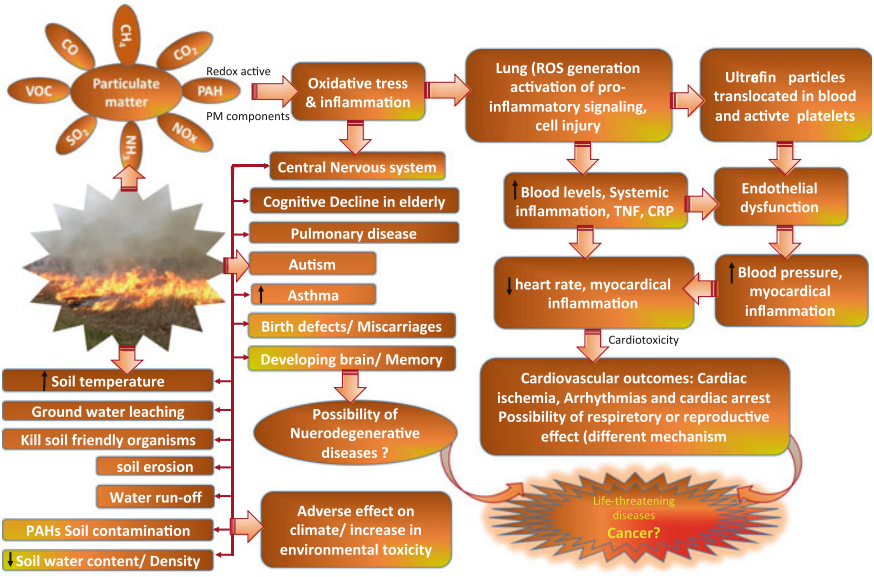

Fig. 5 Biomass open burring and the emission of toxic gases. This pathway represents the effect of toxic gases exposure to human and severs heath effects like cardiovascular disease, neurodegenerative and cancer

where exposure to PAH may cause cardiovascular, respiratory and reproductive diseases in human and animal (Brito et al. 2010; Lewtas 2007; Cançado et al. 2006) including cancer risk (Silva et al. 2010).

Open burning of biomass not only affects the human health, but also it increases soil temperature, decreases soil water content and bulk density and, consequently, leads to soil compaction, higher surface water runoff, and soil erosion (Dourado-Neto et al. 1999; Oliveira et al. 2000; Tominaga et al. 2002). Consequently, Netto et al. (2004) detected high concentrations of PAHs contamination in soils located near sugarcane burning areas. Soils contaminated with these compounds, which are often carcinogenic, represent a risk for human health when leaches to water bodies. Also, human get exposure directly through the soil contamination by inhaling process. Therefore, based on the carcinogenic potential of inhaled particulate PAHs the recommendation of World Health Organization for maximum permissible concentration is 1 ng m^{-3} benzo[a]pyrene (BaP) in the atmosphere (Ravindra et al. 2008). The pathway of an open burning exposure to human and environmental toxicity represented in Fig. 5. Given pathway provides the mechanistic view on serious health concerns such as cardiovascular diseases and cancer and also effect on climate change.

6 Conclusion and Future Recommendations

It is now worldwide accepted that lignocellulosic biomass (rice straw and sugarcane bagasse) has tremendous potential for provided economic, energy saving, ecology benefits and improvements in properties of material. Production of ethanol, charcoal, building materials (concrete, cement, bricks), paper from rice straw, rice husk, sugarcane bagasse are the major renewable or recyclable residues. Also, production of silicon nanoparticle from rice husk has potential to contribute majorly in biomedical research especially in drug delivery system. A computational approach presented in this article purposely to solve the dose related response and the molecular interaction mechanism by docking method. Another an important aspect is production of organic fertilizer by vermicomposting method. Sugarcane bagasse and rice straw are major source of organic fertilizer, which has been discussed in this paper. The review processing, properties and applications of agricultural solid wastes shows better options for the utilization of rice and sugarcane biomass instead of burning or leaving them on agricultural land after harvesting. As it has been discussed here that the open burning or leaving them on field may cause sever eco-toxicity and heath problems like, cardiovascular disease, neurodegeneration, infertility and possibly cancer.

Cultivation of rice needs major amount of water for irrigation. In present scenario, the agricultural sector is known to be a largest user of water, where approximately 70% of water uses on average (Winpenny et al. 2010). However, the cultivation of rice alone demands maximum percent of water for irrigation.

Therefore, the future recommendation from this study is to use treated wastewater for rice irrigation. But contrary, there are several reports shows that untreated or partially treated wastewater is very toxic or hazardous for human health especially for farmers and consumers of wastewater irrigated crops. Therefore, it is suggestive and imperative to implement a well treated wastewater by advance treatment methods before taking it in use for irrigation. This method will solve the major crisis of fresh water especially in developing countries. Because, developing countries are the major producer of both rice and wastewater. Though, it is fruitful to establish a wastewater treatment plant in rice producing countries or sates. Bhatnagar et al. (2016) have proposed a model based on solid wastewater treatment and further irrigation prospects.

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Antibiotic Resistance Genes: An Emerging Environmental Pollutant

Vijay Tripathi and Pooja Tripathi

Abstract Antibiotics are the most effective group of antimicrobial drugs used for humans and veterinary therapy, but it is a rising trouble for the modern healthcare. However, the maximum use of antibiotics may be introducing the emergence and development of antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs), which increase the dissemination of resistance bacteria and genes in clinical and nonclinical environments. Horizontal gene transfer (HGT) and mobile genetic elements like plasmids, transposons and integrons are facilitating the environmental dissemination of the ARGs. The main source of antibiotics spreading is the uncontrolled uses of antibiotics, are not only used for the human therapy but it is widely used for the agricultural and livestock farming purposes. ARBs and ARGs have been extensively detected in wastewater, agricultural soil, animal manure and hospital waste, so that they must be consider as environment pollutant as well and that can contaminate the natural environment. This chapter gives an overview on the impact of antibiotics and antibiotics resistance genes as an environmental pollutant in different environment.

Keywords Antibiotics · Antibiotics resistance bacteria · Antibiotics resistance genes · Horizontal gene transfer · Environment pollutant

Abbreviations

ARB	Antibiotic resistance bacteria
ARG	Antibiotic resistance gene
HGT	Horizontal gene transfer

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WWTP	Wastewater treatment plant
MRSA	Methicillin-resistant *Staphylococcus aureus*
LGT	Lateral gene transfer
MGE	Mobile genetic element
GC	Gene cassettes
IS	Insertion sequence
CI	Chromosomal integrons
MI	Mobile integrons

1 Introduction

Antibiotics introduced in medical science as a medicine to treat and reduced the infectious disease causality and it was a biggest medical revolution of the 20th century. Penicillin was isolated by Fleming (1929) and it was the first commonly used antibiotics. In World War II, it was significantly used and reduced the death cases caused by the bacterial infections (Quinn 2013). Most of the antibiotics were discovered and introduced in the medical market form mid to last of 20th century and these molecules are still in use.

A large number of studies have demonstrated that the widespread use of antibiotics to treat microbial infections in humans, animals and less commonly in crop plants. Many antibiotics are also used for to promote growth and increase the feed efficiency in animal. After use, the proliferation of antibiotics residues, ARBs and ARGs introduced in the environment. ARGs have been detected in surface water (Reinthaler et al. 2003), groundwater (Chee-Sanford et al. 2001), sediments (Pei et al. 2006; Storteboom et al. 2010a) and wetlands (Cummings et al. 2010). The use of antibiotics is increasing day by day and the development and dissemination of antibiotics resistance genes among both the clinical and environment bacteria will be a consider a universal threat to human, animal and environmental health in the 21st century.

The antibiotic resistance can contaminate the environment because environmental bacteria develop resistance against the antibiotics which are used in the treatment of human and veterinary (Allen et al. 2010; Baquero et al. 2008; Martinez 2008; Riesenfeld et al. 2004; Zhang et al. 2009). In recent time, lots of interest has been expressed to the role of environmental bacteria because Many ARGs carried by pathogenic bacteria are originated in environmental bacteria (Martinez 2008), and ARGs have been found everywhere in a large range of environments (Zhang et al. 2009). Human antibiotic contamination may play a vital role in the distribution of clinical antibiotic resistance (Martinez 2009b; Wright 2010).

The antibiotics use in Livestock operations are very much responsible to increases the ARBs in animal workers (Bertrand et al. 2006; Boerlin et al. 2001), meat processors (Borgen et al. 2000). Now day's animal manure is widely used in

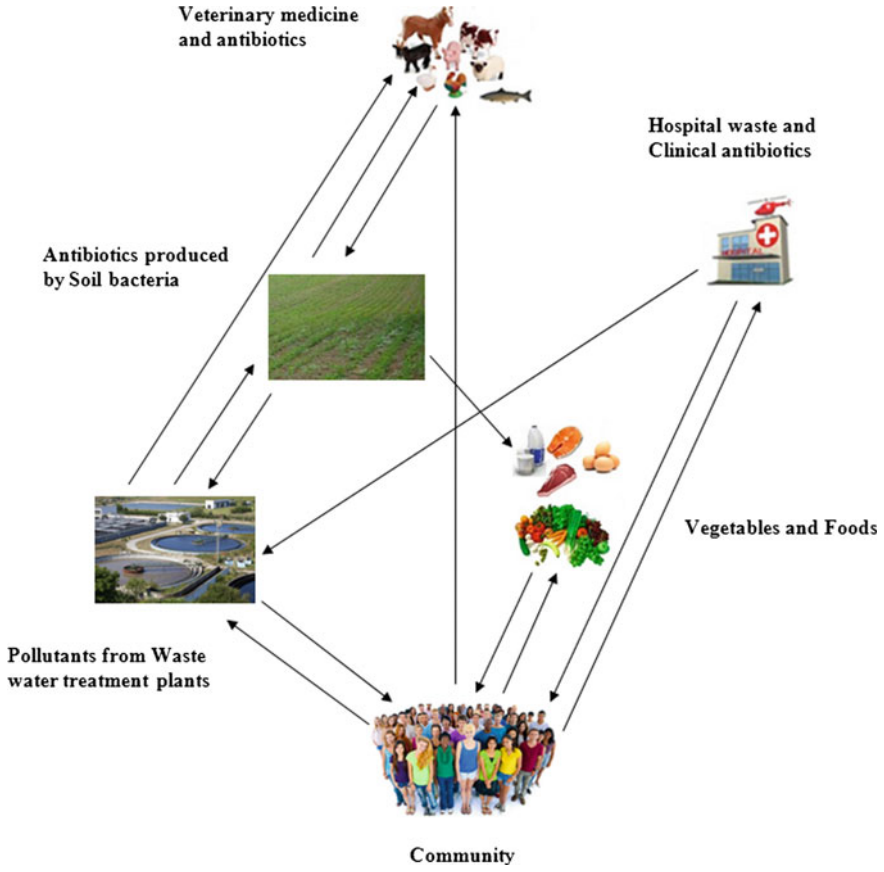

Fig. 1 Dissemination of antibiotic resistance bacteria and genes among different environmental conditions

agricultural fields and these antibiotic-treated animals are main source of antibiotics and antibiotic-resistant bacteria, and animal manure frequently increase the levels of antibiotics bacteria and genes in the soil. Dissemination of Antibiotic resistance bacteria and genes among different environmental conditions are presented in Fig. 1.

In the present chapter, we explore the dissemination of ARBs and ARGs in different environment like Wastewater Treatment Plants (WWTPs), Agro-ecosystems and Veterinary farming. Additionally, this chapter focuses on occurrence of ARBs and ARGs, because antibiotic resistance remains one of the major threats to public health.

2 Transmission of Antibiotic Resistance

Horizontal gene transfer (HGT) is mainly responsible for the transfer of genetic material between the bacteria (Davies 1997) and this process is also responsible for the transmission of antibiotics resistance among the pathogenic bacteria in world-wide (Levy and Marshall 2004). The main mechanisms of resistance gene transfer in a bacterium are plasmid transfer, transfer by viral delivery, and transfer of free DNA. The three mechanisms are responsible for facilitating the HGT are: Conjugation, transformation and transduction.

Transformation: Bacteria take up DNA from their environment.

Conjugation: Bacteria directly transfer genes to another cell.

Transduction: Bacteriophages (bacterial viruses) move genes from one cell to another.

The conjugation is a horizontal gene transfer process from donor cell bearing one or more conjugative plasmid to a plasmid free recipient cell through the construction of Conjugation Bridge (Lederberg and Tatum 1946). Conjugation could be seen to depend on the presence in the donor cell of a so-called fertility factor F, a relatively small autonomous DNA molecule. Bacteria can acquire antibiotic resistance genes from other bacteria in several ways and Conjugation is simple process which including genes encoding resistance to antibiotics (found on plasmids and transposons) from one bacterium to another.

Transformation involves the natural capability of uptake, integration, and functional expression of naked DNA such as plasmid or genome fragments from the environment and has the potential to transmit DNA between very distantly related organisms. Natural transformation is basically active uptake of free DNA by bacterial cell (Chen and Dubnau 2004; Dubnau 1999; Lorenz and Wackernagel 1994) also including human pathogens, such as *Acinetobacter* spp., *Haemophilus* spp. and *Neisseria* spp. Now it is very much clear that bacteria could exchange ARGs by using transformation and it has been responsible for the evolution of β-lactam resistant strains of *H. influenzae* and *N. gonorrhoeae*, as well as penicillin-resistant *S. Pneumonia* (Dowson et al. 1994; Saunders et al. 1986).

Transduction involves transfer of DNA from one bacterium into another mediated by bacteriophages (Fig. 2). There are two types of transduction method: (1) Generalized transduction (2) Specialized transduction. Through specialized or generalized transduction, bacteriophages can transfer genes that are advantageous to their microbial hosts and endorsing their own survival and distribution (Modi et al. 2013; Lacey 1973). Transduction is also responsible for the transfer of some ARGs among clinical strains of *Staphylococcus aureus*, (Lacey 1973, 1984) and occurs among bacteria in natural water systems (Miller 1998; Miller and Ripp 1998).

The bacteriophage mediated mobilization or transfer of ARGs has been recognized in various bacterial species like the transfer of tetracycline and gentamicin resistance between *enterococci* (Fard et al. 2011), β-lactamase genes *Escherichia coli* (Billard-Pomares et al. 2014) and *Salmonella* (Schmieger and Schicklmaier 1999); or the transfer of antibiotic resistance plasmids in MRSA (Varga et al. 2012)

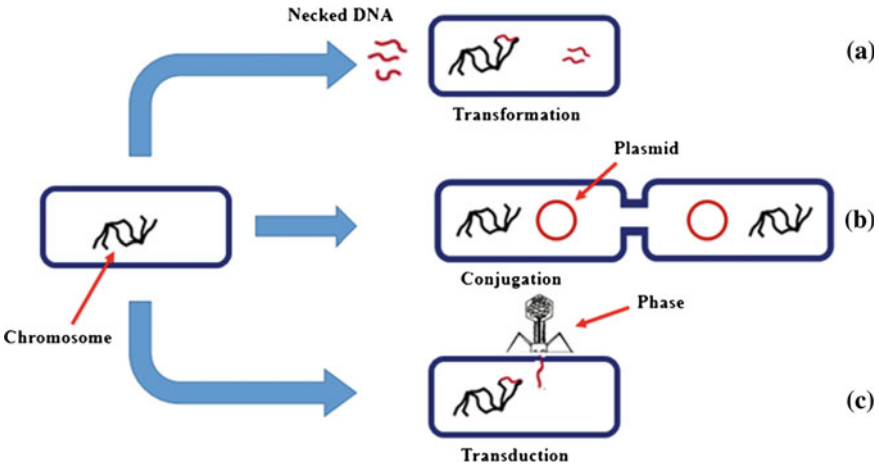

Fig. 2 Primary mechanism of Horizontal gene transfer in bacteria **a** Transformation: Necked DNA is taken up by the cell from surrounding environment **b** Conjugation: Transfer of conjugative plasmid through direct cell to cell link established by donor cell **c** Transduction: Bacteriophages move genetic materials from one cell to another by infection

but few studies have been reported that bacteriophage considered transfer genes between different bacterial communities (Fard et al. 2011; Jensen et al. 1998) and environment and play a major role in the dissemination of ARGs in microbial ecosystems.

3 Mobile Genetic Elements

Movement of foreign DNA within the genome or between bacterial cells is known as horizontal or lateral gene transfer (LGT), and the mobilization of DNA into the host bacterium is referred to as mobile genetic elements (MGEs) (Frost et al. 2005). Mobile genetic elements are a DNA segments which encode the enzymes and proteins that help in the movement of DNA. MGEs play a major role in bacterial evolution, infectious diseases, antibiotic resistance, bacterial symbioses, and biotransformation of xenobiotics and contribute very much to adaptation to new and changing ecological niches (Wiedenbeck and Cohan 2011). The three main processes transformation, conjugation and transduction are involved in the transfer of MGEs from one bacterium to another (Fig. 2). Intracellular mobility (DNA transfer within the genome) facilitated by transposons, gene cassettes, and integrons and Plasmids, bacteriophages, and conjugative resistance transposons can facilitate in the intercellular mobility (DNA transfer between the bacterial genome) (Bennet 2008).

Integrons are genetic assembly platforms that capable in the integration, assembly and expression of gene cassettes (Davies and Davies 2010; Su et al. 2006; Mazel 2006; Kovalevskaya 2002). Integrons are natural gene capture systems that are located on MGEs like transposons and plasmid and play a major role in the dissemination of resistance genes (Hall and Collis 1995; Rowe-Magnus and Mazel 2002; Rowe-Magnus et al. 2002). The functional integron platform consists of a gene (intI) that codes for an integrase catalyzes the incorporation of gene cassettes (GCs) by sit specific recombination and a proximal primary recombination sequence called an attI site. The integrase mediates recombination between the attI site and a secondary target called an attC site. Integrons are divided into two major groups: chromosomal integrons (CIs), and mobile integrons (MIs). CI is also known as Super integrons, located in the chromosome of hundreds of bacterial species and they can carry up to 200 cassettes that mainly encode proteins with unknown function but MIs are located on mobile genetic elements and contain a limited number of GCs, usually encoding antibiotic resistance determinants and promote their dissemination among particular Gram-negative bacterial pathogen. Recently Class-1 integrons introduced as potential targets for source tracking of ARGs from anthropogenic environments because they are generally extensively more abundant in anthropogenic sources than in pristine environments. The majority of integrons which share a pool of gene cassettes are encoded resistance to antibiotics and the mobile resistance integrons are highly abundant in environment because most of the antibiotics are used in clinical and agricultural practice. Resistance genes and integrons are present in floc and sewage sludge (Drudge et al. 2012; Zhang et al. 2011), and considerable quantities are released in reclaimed water (Wang et al. 2014) or directly into rivers (Graham et al. 2011; Koczura et al. 2012). Resistance genes and integrons are also disseminated in effluent from hospitals and in wastewater from tanneries (Stalder et al. 2013). Further, the use of animal wastes as manure introduces resistance genes and integrons into agricultural soils (Binh et al. 2009; Byrne-Bailey et al. 2009, 2011; Cheng et al. 2013). Antibiotic resistance genes and integrons are now viewed as significant environmental contaminants and as markers for tracing sources of pollution (Pruden et al. 2006; Storteboom et al. 2010a, b).

The bacterial plasmids move many bacterial genes from one bacterial cell to another and the process called horizontal gene transfer. Specifically, conjugative plasmids that is able to promote their own transfer and the transfer of other plasmids from one bacterial cell to another. Plasmids carry a considerable variety of genes, including those that present antibiotic resistance and resistance to a number of toxic heavy metals, such as mercury, cadmium and silver. A resistance plasmid can carry one or more antibiotic resistance genes. Many resistance plasmids are conjugative, that is they encode the functions necessary to promote cell-to-cell DNA transfer, particularly their own transfer. WWTPs have been recognized as a reservoir for ARB and ARGs, including plasmids encoding resistance to antibiotics (Tennstedt et al. 2005; Schluter et al. 2008; Moura et al. 2010; Allen et al. 2010). Antibiotic resistance plasmids can harbour genes that confer resistance to most if not all clinically significant antibiotic classes such as macrolides, tetracyclines,

cephalosporins, fluoroquinolines, aminoglycosides and β-lactams (Bennett 2008; Martinez 2009a; Szczepanowski et al. 2009).

Transposons are also belonging to the mobile elements called transposable elements that includes small cryptic elements called insertion sequences (IS elements), transposons and transposing bacteriophages that facilitate the movement of DNA fragments from one location to another on bacterial chromosome and plasmid (McArthur 2006). Tansposons are well structured modular systems that have different classes and are widely distributed among bacterial populations conferring various antibiotic resistance phenotypes. There are many transposons that are strongly related to antibiotic resistance such as Tn5 and Tn10 which encode resistance to kanamycine and neomycine, and tetracycline, respectively (Berg and Berg 1983; Merlin et al. 2011) but the Tn21 family of transposons represents one of the largest and first recognized groups involved in the accumulation and dissemination of ARGs. The Tn21 confers resistance to streptomycin, spectinomycin, and sulfonamides.

4 Reservoirs of Antibiotic Resistance Genes

4.1 Wastewater Treatment Plants

Most of the studies prove that antibiotics resistance bacteria are present in the wastewater treatment plants and it is the main source of both ARBs and ARGs (Munir et al. 2011; Rizzo et al. 2013). Figure 3, shows the route of dissemination of ARGs and ARBs from WWTP into different environmental conditions. The waste water treatment plants are the reservoir and suppliers of antibiotic resistance genes and also hotspot for the horizontal gene transfer because WWTPs receive sewage and bacteria from various sources or different natural environments (Rizzo et al. 2013). Antibiotics can enter WWTPs through human excretion, farm animals and direct disposal of medical and industrial wastes and it is a serious threat to spread of resistance, may be pathogenic bacteria resistance pathogen and antibiotic resistance bacteria (Martinez 2009a). Basically untreated water, treated water and sludge are the main reservoir of the ARGs. Although most of the studies have reported that the quantities of ARGs in treated wastewater effluents are significantly higher than the surface water (Lachmayr et al. 2009; Rodriguez-Mozaz et al. 2015; Czekalski et al. 2014). During the treatment process in WWTPs some antibiotics are removed (Batt et al. 2006), but not all antibiotics are completely removed (Giger et al. 2003). For example, β-lactam and vancomycin genes have been found in natural environment in very low quantity but resistant bacteria and genes encoding resistance against certain β-lactam and vancomycine have been reported in WWTPs. Resistant and multi-resistant bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter* spp. and *Enterobacteriaceae* are present in many processes of WWTPs (Kümmerer 2009). One of the study (Tsai et al. 1998) shows that the

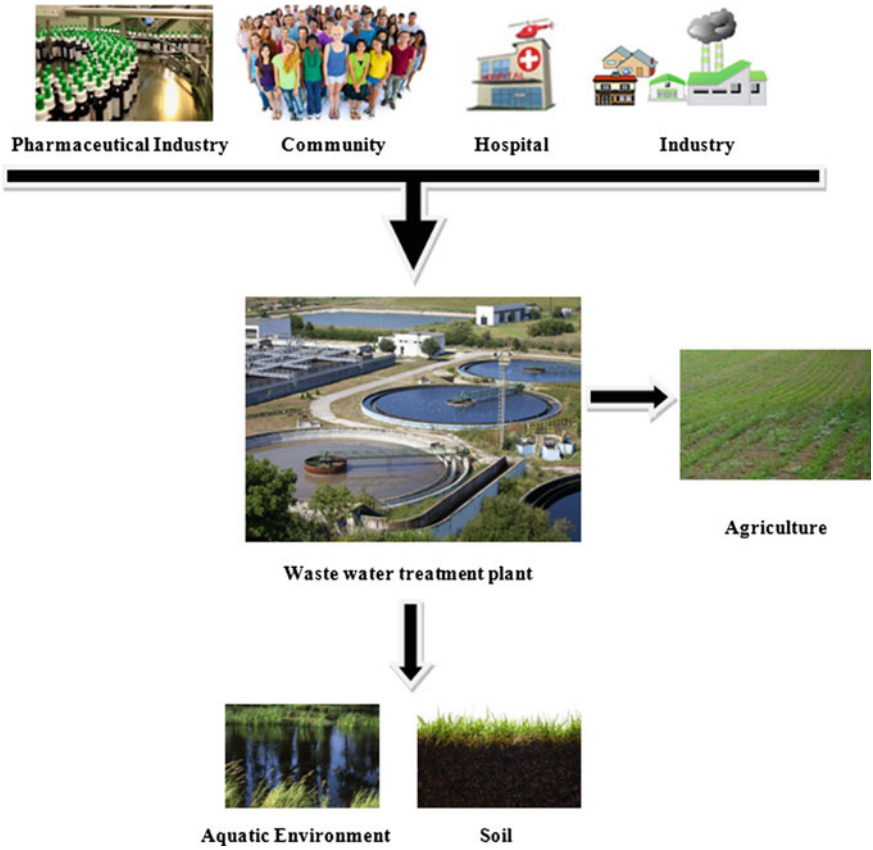

Fig. 3 The route of dissemination of ARGs and ARBs from WWTP into different environments

Salmonella species are very frequently appeared in activated sludge from hospital waste treatment facilities and it is a potential source of infectious organisms. Some recent studies have inspected the tetracycline resistance in activated sludge WWTPs (Guillaume et al. 2000; Auerbach et al. 2007; Kim et al. 2006; Al-Ahmad et al. 2009). The study (Guillaume et al. 2000) has occurred different tetracycline resistance determinants and one more study (da Costa et al. 2006) has investigated the presence of various multi-resistant enterococci in urban sewage and sludge in WWTPs in Portugal and showed that treatment procedure of these bacteria in wastewater treatment plants did not prevent dissemination to the environment. Another study showed that the blaTEM, qnrS, ermB, sul-I and tetW and vanA resistant genes have been detected in both the hospital effluents and in treated and without treated effluent of urban wastewater treatment plants (Narciso-da-Rocha et al. 2014; Rodriguez-Mozaz et al. 2015). The mobile genetic elements can promote the horizontal gene transfer and induce the occurrence of ARGs in different environmental compartments (Schwartz et al. 2003). The aforesaid studies are

showing the importance to find out the efficient solutions for minimising antibiotic resistance spread. In this situation, disinfection process can be a best way to control the diffusion of antibiotic resistance into the environment, mostly in reuse of treated wastewater for agricultural purpose (Fatta-Kassinos et al. 2011; Ferro et al. 2015). Till now few works have been done regarding the effect of disinfection processes mainly chlorination and UV radiation, of ARGs removal (Guo et al. 2013; McKinney and Pruden 2012; Munir et al. 2011; Yuan et al. 2015; Zhuang et al. 2015).

4.2 Agricultural Soil

Soil is a large reservoir of microbial diversity and the majority of antimicrobial compounds used today in human and veterinary health care have been isolated from soil microorganisms.

Most of the antibiotics used in agriculture are given as growth-promoting and prophylactic agents, rather than to treat infection. In all over the world antibiotics are used between 60 and 80% of the total antibiotics production in animal husbandry (Silbergeld et al. 2008). These antibiotics are used as growth promoting agents and are frequently detected in animal manure. The animal manure is a main reservoir and source by which ARBs and ARGs first enter the environment. When soils are treated with animal manure, the antibiotic resistance bacteria carrying ARGs are introduced in the soil because manure enhanced the horizontal gene transfer process in which ARGs are transferred to soil bacteria (Jechalke et al. 2014). Anthropogenic activities can transfer ARB and ARGs from natural environments to soils is generally associated with horizontally transferred elements like class-1 integrons, while selective pressure that can also increase the abundance of native ARGs. The study has (Forsberg et al. 2012) strongly suggested that mobile genetic elements such as plasmids and integrons carry ARGs between soil and human microbiomes. Moreover, the physical properties of soil can also play an important role on the activity and stability of both antibiotics and ARGs in soil matrices.

One study quantifies four broad-spectrum β-lactam AR genes (blaTEM, blaSHV, blaOXA and blaCTX-M) and class-1 integron genes (int1) in soils from manured (M) versus inorganic fertilised (IF) fields and the ARGs levels were significantly higher in manure versus inorganic fertilizer in soils. This was supported by another study that investigated the integron gene cassette diversity in manure-amended field and compare with without manure treated filed, detected class-1 integrons harbouring the aminoglycoside resistance gene aadA in manure-amended soils but not in without manure treated soil. The aadA genes were also abundant in the manure, confirming that aadA gene localized on class-1 integrons are introduced via pig manure into agricultural soil (Binh et al. 2009). The aforesaid studies indicate that application of antibiotic-containing manures expands AR levels in amended soils.

Now days treated wastewater irrigation is progressively more popular in arid and semi-arid areas of the world, applied in agricultural sector but WWTPs encompass a huge collection of environmental and clinically relevant ARB and ARGs, which can be detected in influent, activated sludge and discharged effluent, potentially enhancing AR reservoirs in the downstream environments like river and lake. The wastewater biosolids contains high concentration of ARBs and ARGs are regularly applied to agricultural soil as fertilizers. The study (Negreanu et al. 2012) evaluated the long-term effects of treated wastewater irrigation on AR in agricultural soils, using standard culture-based isolation methods and culture-independent molecular analyses and the study indicated that ARB levels in the treated wastewater itself were significantly higher than those measured in the freshwater.

Using quantitative real-time PCR, (Knapp et al. 2010) showed a significant increase in the relative abundance of selected ARGs in archived soils from Holland from the 1940s when antibiotics were first used, to the present time. Over the past 15 years a huge number of antibiotics from a broad range of classes, including macrolides, lincosamides, sulfonamides, chloramphenicol, florfenicol, fluoroquinolones, tetracyclines have been detected in agroecosystems (Campagnolo et al. 2002; Hamscher et al. 2002; Kolpin et al. 2002; Halling-Sorensen et al. 2003; Aga et al. 2005; Batt et al. 2006; McKinney et al. 2010; Watanabe et al. 2010; Bartelt-Hunt et al. 2011; Pruden et al. 2012; Zhang et al. 2013). Although we have enough amounts of data generated on the occurrence of veterinary-use antibiotics in agroecosystems but our understanding of their overall distribution is still quite limited.

4.3 Livestock's Farming

The main interest regarding the use of antibiotics in animals are for various reasons, including disease treatment, prevention, control, and growth promotion/feed efficiency. The transmission of ARGs between farm animals, the wider environment and humans are resented in Fig. 4. Antibiotics are extensively used as a growth promoter in livestock farming and play a major role in the emerging public health crisis of antibiotic resistance (Preston 1987; Nagaraja and Chengappa 1998; Gaskins et al. 2002). ARBs related to livestock's can be transmitted to humans through the environment (Graham et al. 2009) and food products (Price et al. 2005) and to agricultural workers by direct contact (Smith et al. 2013). Although most of the antibiotics are used in agricultural purposes but relatively little interest has been paid to how antibiotic use in farm animals contributes to the overall problem of antibiotic resistance. In European countries, antibiotics are widely prohibited as growth promoters in animal husbandry (Singer et al. 2003; Kümmerer 2009; Cabello 2006) but in developing countries the situation is totally opposite because the demand of animal proteins are very high and the consumption of antimicrobials are very unregulated.

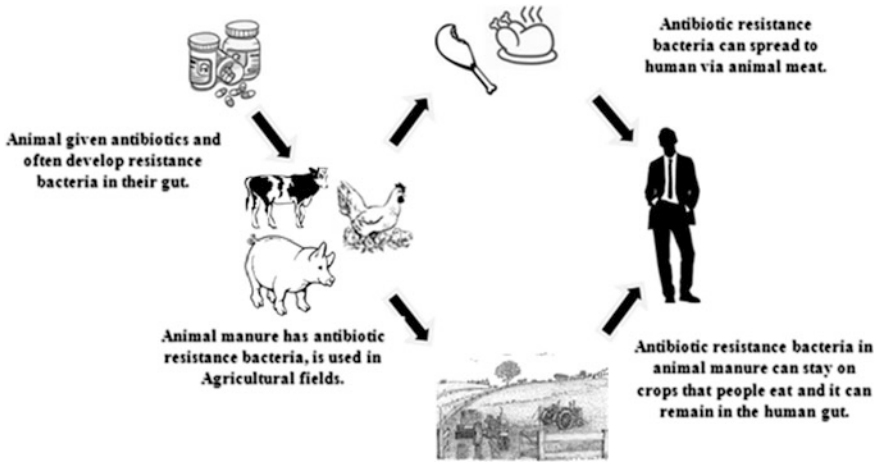

Fig. 4 Diagrammatic representation of transmission of ARGs between farm animals, the wider environment and humans

The workers who worked in Farm and slaughterhouse and those in close contact with them are directly at risk to infect with resistant bacteria through close contact with infected animals. Most of the studies have been proven that the transmission of antibiotic bacteria from animal to farm worker. The study (Levy et al. 1976) first reported the direct transfer of resistance bacteria form animal to farm worker, who got the same tetracycline resistant *E. coli* strain in the gut flora of chicken caretakers as in the chickens receiving tetracyclinelaced feed.

In boiler production gentamycin is commonly used antibiotic but it is not approved in United States as a growth promoter although it remains the most widely used antibiotics in boiler production (Luangtongkum et al. 2006). Some of the antibiotics resistant genes found in food bacteria have also been identified in humans, providing indirect evidence for transfer of antibiotic resistance genes by food handling or consumption. Most of the food-borne pathogens such as *Escherichia coli*, *Salmonella, Enterococcus* and *methicillin-resistant Staphylococcus aureus* (MRSA) are showing the homologous relationships between bacterial resistance genes in humans and farm animals and there is ample of evidence these pathogens can spread resistance genes between animal and people. The rise of antibiotic-resistant bacteria among farm animals and consumer meat and fish products has been well documented (Kümmerer 2009; Lee 2003; Zhao et al. 2003). Some of the antibiotic resistance genes identified in food bacteria have also been identified in humans, providing indirect evidence for transfer by food handling and/or consumption. Expert opinion suggests that global consumption of antimicrobials in animals is twice that of humans (Aarestrup 2012).

5 Impact of Antibiotic Resistance Genes on Human and Environmental Health

Antimicrobial resistance is a worldwide problem in human and livestock farming. The major risk factor involves to increase this situation is broadly use of antimicrobial compound that play a main role in the dissemination of resistant bacteria and resistance genes in animal and humans (Van de Bogaard and Stobberingh 2000). Most of the antibiotics are used for the prevention and curing of infections in human and animals as well as for promoting faster growth of livestock (Dolliver and Gupta 2008) and partially metabolized products are then discharged in sewage treatment plants or directly in water along the excreta. The intensive use of antibiotics in hospitals and agricultural cause the dissemination in aquatic and soil environments and with the help of selective pressure these compounds may affect the treatment of human disease (Thiele-Bruhn 2003; Segura et al. 2009).

The European Union banned the feeding of human related antibiotics to livestock for growth promotion but in after few years the ban was expanded to all antibiotics and related drugs. Similarly, many countries have restricted the use of antibiotics in animals those are still use in therapy of human infections but many developing countries are still using these drugs in very high amount. For example, the class of β-lactam antibiotics is widely used groups of antimicrobial in human and animals but the emergence and dissemination of ESBLs has caused a major concern in several countries and the utilization of quinolones for aquaculture has been banned in several countries, because those antibiotics can remain active in sediments for long periods and spreading the resistance against the quinolones. These infections have a great impact on public health due to an increased incidence of treatment failure and severity of disease.

The household waste and hospital effluents have been shown to be rich in resistance genes and affect the diversity of resistance bacteria because these wastes are discharge in wastewater (Czekalski et al. 2012; Thevenon et al. 2012; Vignesh et al. 2012; Tacão et al. 2012; Schwartz et al. 2003; Santoro et al. 2012). These contaminations in aquatic environments contribute to the spread of human pathogens along with the dissemination of antibiotic-resistant bacteria. The overexposure to antibiotics is leading to increasing levels of resistance in the human.

Antibiotic-resistant bacteria is not only found in pathogenic bacteria but also in the wildlife (environmental organisms of terrestrial and aquatic habitat) and these are very important to human health because of the increasing importance of some zoonotic species of the genera *Salmonella, Campylobacter*, *Listeria*, *Staphylococcus*, *Enterococcus and Escherichia*, which are known to exhibit high levels of acquired antibiotic resistance (de Jong et al. 2013; Garcia-Migura et al. 2014; Brooks et al. 2014).

Transfer of resistance genes from environmental bacteria to human pathogen is basically the main risk for the public health and *Escherichia coli* have been recognized as a major player in the dissemination of antibiotic resistance (Henriques et al. 2006; Zhao and Dang 2012). Freshwater environments are well-known

systems for the distribution and evolution of antibiotic resistance (Baquero et al. 2008; Figueira et al. 2011; Tacão et al. 2014). Water bodies are sites of genetic exchange through horizontal gene transfer where environmental bacteria interact with microbes originated from humans and other animal sources. Aquatic ecosystems may become a threat to human health when they are affected by pollutants carrying resistant bacteria (Baquero et al. 2008).

6 Conclusion

The occurrence and dissemination of antibiotics resistance is a growing problem in different environments like clinical, veterinary, husbandry and agricultural that needs to be paid much more attention regarding to the major factor responsible for the origin and spread of such pollutants. The antibiotic resistance genes are now widely spread through the biosphere and affect the global microbiome because antibiotic contamination promotes the mobilization of resistance genes between the environments. Now it's time to pay more attention to these pollutants which are spread through different sources and active in the environment.

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Biomarkers of Ecotoxicological Effects in Social Insects

Oksana Skaldina and Jouni Sorvari

Abstract Complementing ecotoxicity testing, a biomarker approach is widely used in ecological risk assessment programs. Biomarkers provide information about early warning biological responses to one or several chemical pollutants and can be revealed in an organism or its products. Biochemical, morphological or behavioral parameters of living organisms can be set to biomarkers of exposure, effect or susceptibility or biomarkers of defense and damage. This concept is more developed within aquatic than terrestrial ecotoxicology and social hymenopterans insect (ants, bees, bumblebees, wasps and termites), which are already actively used as bioindicator species, can be furtherly studied for revealing novel sets of biomarkers. They can provide sufficient information about ecosystem health because social insects usually occupy high trophic levels and are important predators, pollinators, scavengers and ecological engineers. Social insect colonies stay in a certain place (except army ants), which makes them excellent model group for biomarker studies on ecotoxicological effects in nature. Despite their high ecological significance, wide spread distribution and sampling convenience, social insects are not intensively studied within biomonitoring programs and still not widely used as sentinel species. Revealing direct biological responses of social insects to toxic substances at the different levels of biological organization, systematization of scientific data and creating of simplified recommendations for practical biomonitoring purposes may facilitate progress in current terrestrial ecotoxicology.

Keywords Bioindicators · Biological responses · Terrestrial ecotoxicology · Sentinel species · Social hymenopterans

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1 Introduction

Over the last past century numerous classes of contaminants have been elaborated and released into environment. The most harmful of them are persistent organic pollutants (POPs) and inorganic compounds such as heavy metals and nonmetallic toxic substances, pharmaceuticals and personal care products. Aerial pollutants, such as aerosols and dust particles, are widely spread from tropics to the Arctic, and emissions still remain at high level (Gong and Barrie 2005; Chen et al. 2008) as well as POPs and polycyclic aromatic hydrocarbons (PAHs) are widely distributed, producing global adverse effects (Bartrons et al. 2016). However, at the same time advanced techniques and new approaches for monitoring environmental changes and preserving nature have been discovered. One of them is a biomarker approach, which was intensively developed over last 20 years and now significantly complements ecotoxicity testing (Romeo and Giamberini 2013). Biological responses toward toxic substances can be revealed at different levels of biological organization. However, at the lowest: molecular, cellular and tissular, which are the most sensitive, early warning signals can be discovered long before visible effects at the population level (Amiard-Triquet et al. 2013). Because of high sensitivity these early warning responses are especially useful for ecological risk assessment and preventing the harmful effects of pollution for the whole ecosystem. As soon as a xenobiotic is released into environment, it starts to affect it gradually from the level of an organism to the level of the whole ecosystem. That is why bioindicator organisms, or sentinel species, are useful for revealing the presence and toxicological effects of one or several contaminants (Berhet 2013) and biomarkers of these species may facilitate progress of ecotoxicological studies.

To be a sentinel species an organism should meet several requirements and the most desirable characteristics are: (1) sedentarity in the studied site; (2) ease of collection and identification; (3) sufficient population size, so the impact of specimens collection can be minimized; (4) wide and well known distribution range; (5) existence of a resistance to pollutants, when a species is able to tolerate stress within several years; (6) well established dose-effect and cause-effect relationships; (7) well-known biology and ecology allowing clear differentiation between the signal and the background noise (Berhet 2013). Social insects, which dominate in their environments, and frequently are at the top of food chains, can be a proper group to indicate ecotoxicological effects in general and biomagnification effects in particular. However, they remain poorly studied in this case. Modern advances in studies of social insect ecology, biology, physiology, genetics, conservation and management practice, allow review directions for revealing potential biomarkers in this unique group of living organisms.

2 Biomarker Approach in Revealing Ecotoxicological Effects

The initial idea of using early warning signals for the description of the health state have been derived from human medicine and was closely related to biology of vertebrates (Amiard-Triquet et al. 2013). In the early 90s European Science Foundation (ESF) considered four review papers, published in the same year and concerning possible usage of early warning responses in invertebrates, vertebrates, terrestrial plants and communities of invertebrates. After that, ESF had come to conclusions to what extent biomarkers are useful to evaluate environmental change and recommended to use them in biomonitoring programs (Romeo and Giamberini 2013).

The biomarkers approach is quite new, thus several different definitions and terminological concepts are simultaneously in use. According to one of the most early raised and widely used definitions given by Depledge (1994), a biomarker is a biochemical, cellular, physiological or behavioral change which can be measured in body tissues or fluids or at the level of the whole organism that reveals the exposure at or the effects of one or more chemical pollutants". Some authors, for example Hansen (2003), restrict biomarkers only to biochemical parameters, but current understanding of a term «biomarker» is much wider. Biomarkers can be any biological responses towards chemical contaminant, which are measured in an organism or its products and not occurring in non-exposed organisms (Romeo and Giamberini 2013). Consequently, there are biochemical, physiological, morphological or behavioral biomarkers (Amiard-Triquet et al. 2013). As it can be seen in a review concerning history of biomarkers by Romeo and Giamberini (2013), some authors refer biomarkers only to sub-organismal level of biological organization, while changes occurring in a whole organism they propose to consider as bioindicators. Bartell (2006) proposes more functional approach and states that biomarkers indicate measures of exposure or dose, while bioindicators—measures of effects.

There are several classifications of biomarkers, the most widely used are one, proposed by Manahan (2003) in which there are biomarkers of exposure, effect and susceptibility, and the other one created by De Lafontaine (2000) with biomarkers of defense, signaling about adaptation capacities of the species to survive in polluted environments and biomarkers of damage, revealing biological impairments which may indicate detrimental effects on reproduction and survival. Biomarkers varies from those of high specificity, like enzyme aminolevulinic acid dehydratase (ALAD) inhibited only by lead to those, which can be caused by numerous chemical agents (Amiard-Triquet et al. 2013). Biomarkers are capable to reveal presence of environment contamination, however there is a significant lack of specificity and only a few of biomarkers are really specific (Amiard-Triquet et al. 2013).

3 Social Insects as Bioindicator Species

Among invertebrate insects comprise the largest class with the estimated number of extant species between six and 10 million (Chapman 2009). Insects from the orders Hymenoptera and Isoptera (ants, wasps, bees, bumblebees and termites) exhibit advanced level of the community organization—eusociality. Eusociality is characterized by three main features, which are: (a) division of labor within reproductive and non-reproductive individuals; (b) cooperative brood care; (c) simultaneous co-existence of several generations within one nest (Wilson 1971). Besides being unique representatives of the top-level social structures among animals, social insects play crucial and complex ecological role in many terrestrial ecosystems. They are generalist and specialist predators, pollinators, parasites, and they are significant as a prey in food webs. Social insects differ from solitary insects in that case, that they possess additional organizational level—colonial. Their colonies can be either annual (bumblebees, social wasps) or multiannual (ants, honeybees, termites). The colony stays in a certain place (except in army ants) and occupies one (monodomy) or several nests (polydomy). Nests of social insects provide excellent, environmentally buffered, habitat opportunities for numbers of other organisms (parasites, commensals, mutualists) and so function as biodiversity hotspots for particular ecosystem (Hughes et al. 2008; Härkönen and Sorvari 2014). All points, mentioned above, make them proper bioindicator species of the habitat and environmental conditions they live, and excellent model organisms to study the general effects of pollution on animals.

Ants are widespread insects. Occupying high trophic levels and in many cases specialized niches, they possess numerous opportunities to be used as environmental, ecological and biodiversity indicators (Ellison 2012). Ants are predators, omnivores and fungivores as well as significant ecological engineers (Frouz and Jilková 2008; Sanders and van Veen 2011; Frouz et al. 2016). They are highly sensitive and quickly reactive to various environmental changes. For terrestrial ecosystems ants can provide information about ecotoxicological effects as a significant part of the ground-layer indicator sets, also they can be indicators of foliage-inhabiting communities and open habitats (Gerlach et al. 2013). Ants were used as bioindicators of mining site rehabilitation (Majer 1983; Andersen and Majer 2004).

Bees and bumblebees are important ecosystem providers and they can be widely used in agricultural ecosystems to monitor biological responses towards pesticides and other agricultural chemicals spreading in non-target species (Gerlach et al. 2013). Honeybees (*Apis mellifera*) have been shown to respond to biopesticide spinosad biochemically within first 24 h of exposure (Rabea et al. 2010). It has been shown that such economically important pollinators as bumblebees are suffering from neonicotinoid insecticides, widely used for herbivore pests. Thus, neonicotinoid exposure results in reduced colony growth rate and decreased queen production (Whitehorn et al. 2012).

Due to specific biological features, social wasps are promising bioindicators. Many species build their nests in urban or suburban areas close to human dwellings. Social wasps collect and use many types of food from nectar, pollen and honey dew, to many herbivorous pests, caterpillars and other insects. Thus, social wasps are widely involved into food chains. Although colonies in most cases possess annul life cycles new queens frequently stay in the location of their maternal colonies and this phenomenon have been known as philopatry. Therefore, effects on a population can be monitored over several years. Yet, there are not so many studies about wasps as environmental indicators, but interestingly, larval fecal mass of *Polistes dominulus* paper wasp have been shown to lead concentrations (Urbini et al. 2006).

4 Biochemical and Physiological Biomarkers in Social Insects

Being invertebrate arthropods, social insects possess several specific morphological, physiological and biochemical features. First, adults (imagos) through the process of metamorphosis always acquire a three-part-body, consisting of the head, thorax and abdomen. In some cases, semi-independent physiological processes can arise in these body parts, allowing search for biomarkers in a direct place. Second, insects have resilient external chitin skeleton, supporting and protecting their organs, and numerous physiological and biochemical processes are involved in its formation and functioning. Consequently, the reflection of these biological processes can be revealed in the surface of an insect body. Finally, due to the interior fluid hemolymph (analogous to blood in vertebrates), which is in direct contact with tissues, insects are able for immediate physiological and biochemical responses towards various external stresses, at least at the cellular level. Details of current knowledge about insects' physiology and biochemistry are systematically organized by Nation (2015), and should be considered before search for biomarkers in social insects.

Biochemical biomarkers in social insects are in their developmental stage. There are several studies concerning biological responses in these organisms to various toxic substances, however the majority of them report the need of validation that is more precise and the necessity for future studies.

Perfluorinated organic chemical perfluorooctane sulfonic acid (PFOS) is considered to be the most widespread of the perfluorinated organic compounds. It is released into environment mainly from plastic waste. After that as a particulate matter it can enter terrestrial ecosystems, affecting soil properties and even transporting from soil to crops and penetrating into nectar and pollen (Stahl et al. 2009). Being semi-volatile, phthalates are easily adsorbing to atmospheric particles, and then trapped by insect cuticle, so these toxic substances can affect social insects both via terrestrial and atmospheric pathways. Mitochondrial electron transport

activity and lipid amounts of the bumblebee *Bombus terrestris* showed significant decrease in respond to (PFOS) chronic exposure via drinking or treated sugar (Mommaerts et al. 2011). Decreased mitochondrial membrane potential can lead to quick cell death and be a significant reason for an organism mortality.

Cuticular hydrocarbons (CHCs) act as water insulator and protection against pathogens (Martin and Drijfhout 2009; Ortiz-Urquiza and Keyhani 2013). In addition, CHCs are significantly responsible for chemical communication, e.g., nestmate recognition in social insects (Thomas et al. 1999; Sorvari et al. 2008). The cuticular wax layer and body fat reserves of ants have shown to easily absorb phthalates that are commonly used in plastic products (Lenoir et al. 2012, 2014, 2016). Black garden ant (*Lasius niger*) showed activation of immune genes *defensin*, *vitellogenin*, *histone-2A*, and *superoxide dismutase 1* in workers and degreased egg-laying in queens (Cuviller-Hot et al. 2014). Many of the phthalates are harmful for health, also for humans (Tickner et al. 2001; Matsumoto et al. 2008). In social wasp *Polistes dominulus* CHCs were shown to correlate with fertility of foundresses, conveying valuable information about their fecundity (Izzo et al. 2010). However, the effects of environmental pollutants on CHC-layer of social wasps remain unstudied. In addition to phthalates, the binding of other compounds on CHC layer of ants and social wasps might be worth of further research.

Although modern insecticides are developed far away from the harmful DDT, they still tend to affect non-target species and cause sub-lethal and lethal effects to economically important insects. Biochemical studies on honeybees (*Apis mellifera*), concerning early warning responses towards chemical exposure, showed significant inhibitory effects of insect growth regulators chlorfluazuron and oxymatrine and biopesticide spinosad on acetylcholine esterase (AChE) and adenosine triphosphatase (ATPase) (Rabea et al. 2010). Consequently, enzymes acetylcholinesterase (AChE), which is the nerve cell enzyme responsible for the acetylholine destruction, and adenosine triphosphatases (ATPases), which represent enzymes, catalyzing decomposition of ATP to ADP and a phosphate ion, can be promising biomarker to insecticide exposure.

Heavy metal poisoning also is an important ecotoxicological problems, and responses of social insects towards various environmental stresses can be quickly visible at the cellular level as well. Thus, encapsulation rate, which is an indicator of immune defense, of the red wood ant *Formica aquilonia* was elevated at the moderate levels of heavy metals, but suppressed in high levels of contamination (Sorvari et al. 2007). Encapsulation rate can be measured with a very simple method, by inserting a rubbed nylon mono-filament implant (Fig. 1) into the haemocoelon of insect and allow the circulating cells, including melanocyte cells, to encapsulate the object for certain time period. After the removal the implant can be photographed and its darkness refers the amount of cells participating the encapsulation reaction (Fig. 1). Encapsulation reaction is one of the major component in insect immunity, targeted especially against parasites and bacterial and fungal pathogens.

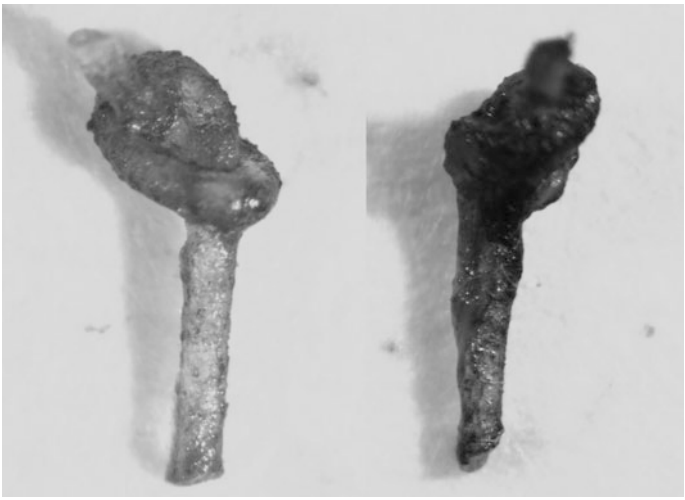

Fig. 1 Nylon monofilament implants used for encapsulation rate assay in insects. The darker the implant the stronger is the encapsulation reaction

Another cellular marker, hepato-nephrocitic system (HNS) has recently been found to be a promising biomarker. It has been shown that the HNS of the bumble bee *Bombus morio* responds to cadmium pollution and possible possessing broader practical application in the other bumblebees and bees (Abdalla and Domingues 2015).

5 Morphological Biomarkers in Social Insects

While search for biomarkers of ecotoxicological effects, insect morphology can be assumed in a broad sense, so many aspects of the outward appearance like size, shape, structure, pattern or colour can be considered. Social insects show visible size variation, which is typically measured as lengths or widths of different body parts as well as dry body weight. As it was mentioned before, social insects possess segmented body structure, thus some body parts or appendages may be more sensitive to various environmental stresses and influences of the toxic substances than the others may.

Generally, social insects are able to tolerate heavy-metal pollution and maintain their individual body resources. Thus, in workers of red wood ants (*Formica* s. str.) morphological characters, such as body mass and head width were not associated with the level of heavy metals: Al, Cu, Cd, Ni, Zn, As, Pb and Hg (Eeva et al. 2004). However, an individual body size can respond in long-term effects, as it was

shown by Fedoseeva (2011) or can react in a biased frequency. Black garden ant (*Lasius niger*), which colonies were located along a metal pollution gradient, demonstrated the bias towards the higher frequencies of small workers in more heavily polluted areas (Grześ et al. 2015a). This was suggested to be a consequence of increased proportion of young colonies with small first generation workers in polluted environment, but physiological stress could also play a role in growth.

Although fluctuating asymmetry is considered to be a reliable measure of developmental instability for many vertebrate and invertebrate taxa (De Anna et al. 2013), it has not yet detected in ants (Rabitsch 1997; Grześ et al. 2015b). In addition to asymmetry, allometry, i.e., the size/shape relationship, could be used as a biomarker. Allometries are outcomes of growth of different body parts in a different rate. Typically allometric relationships are similar between colonies in ants (e.g., Tschinkel et al. 2003), but sometimes such relationships, can differ between colonies as Perl and Niven (2016) found differences in size of compound eyes between red wood ant *Formica rufa* colonies. The different scaling can reflect differences in environmental conditions, however, associations between scaling differences and environmental pollutants has not been studied in social insects.

Melanin-based color polyphenism is a promising direction of future search for morphological biomarkers in social insects. The key enzyme, responsible for the melanin production, is phenoloxidase, and it is present in insect cuticle, midgut as well as in the hemolymph (Sugumaran 2002). Therefore, for insects melanin play an important role in numerous physiological processes, such as physical protection, thermoregulation, regulation of water balance and immunity. Consequently, coloration can reflect biological responses of an organism and is a promising candidate to be an indicator for heavy-metal pollution, because melanin could bind metals and store them in pigmented cells (McGraw 2003). Among social insects, social wasps and many ant species show variable color patterns (Fig. 2) that could be promising early warning biomarkers of environmental pollution.

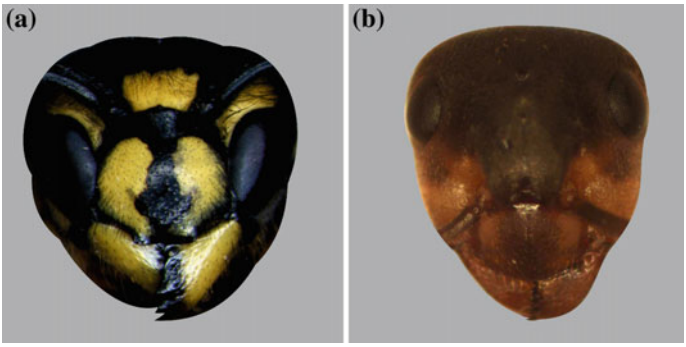

Fig. 2 Facial colour patterns of **a** a social wasp *Vespula vulgaris* and **b** a *red* wood ant *Formica lugubris*

6 Behavioral Biomarkers in Social Insects

Behavior is an integral part of any animal organisms and behavioral changes may indicate possible biochemical or physiological impairments such as dysfunction of a sensory system, endocrine disruption, metabolic disorders, neurotoxicity or some other adverse processes. Review of recent studies concerning behavioral biomarkers have shown that they are particularly responsive to nanoparticles contamination, as well as to antibiotics, brominated flame retardants and nonylphenols (Amiard-Triquet and Amiard 2013). That is why exactly behavior can be a key connecting link between responses at the infra-individual and supraorganismal levels (Amiard-Triquet and Amiard 2013).

Concerning social insects, behavioral biomarkers of ecotoxicological effects not systematically organized, yet there is a strong background allowing the rapid development of this direction. Thus, well-elaborated ethograms exist, like for example developed for the *Formica* ants (Wallis 1962). Social hymenopterans exhibit various behavior types concerning nest building and brood care, cooperative task performance and foraging activity, social organization and nestmate recognition, colony defense and grooming, etc. Impairments in these and the other types of behavior may indicate the presence of environmental stress. Behavioral tests were recommended to be used in risk assessment programs for toxic pesticides (e.g. neonicotinoids); as it was proved that chemical concentrations that might be considered safe, would cause adverse effects on foraging behaviour in bumblebees (Mommaerts et al. 2010) and pollen collecting efficiency in bees (Gill et al. 2012).

Such behavioral characteristics as passivity, aggressiveness and repeatability of various behavioral patterns worth considering when search for behavioral biomarkers in social insects. Heavy metal pollution has shown to be associated with passive behavior in territorial ant *Formica aquilonia* (Sorvari and Eeva 2010). Interestingly, repeatability as an independent parameter can be studied in different types of behavior: mating displays, male-female preferences, parental care, foraging, learning for different types of food, nest-building behavior, nest mate recognition and grooming behavior (Boake 1989).

7 Conclusions

Biomarkers of ecotoxicological effects in social insects are still in the stage of their development and systematization. To create efficient and informative biomarkers one should consider specificity of biological responses to toxicant in a particular species and determine the level of a biomarker specificity. Simultaneous assessment of biological responses at the different levels of biological organization may help to create biomarkers with higher specificity.

Practical application of biomarkers in social insects in terrestrial ecotoxicology requires simple and efficient protocols, which are easy in use and meet the needs of

environmental experts and land managers. Concerning terrestrial invertebrates as bioindicators, the existing gap between scientific data and their real functionality is a general problem, so the development of relevant but simplified techniques are needed (Andersen et al. 2002).

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