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Age associated variations in human neutrophil and sperm functioning

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ABSTRACT

Objective: To determine the functional and biochemical variations in sperm and the neutrophil with the progression of age. **Methods:** Ninety healthy male subjects were selected in the age group 26–40 for the collection of semen and blood samples were collected. Basic semen analysis, hematogram, differential count serum analysis, seminal plasma and serum biochemistry was performed. Mitochondrial isolation from sperm and neutrophil was done to ascertain mitochondrial markers. **Results:** Our data shows a significant age-dependent reduction in the levels of mitochondrial Superoxide Dismutase (SOD) and Catalase (CAT) in sperm and the neutrophil. The functional attributes of sperm and neutrophil did not show any specific trend. **Conclusion:** The decreasing trend of the mitochondrial antioxidants enzymes in the sperm and the neutrophil is an indicative of the reduction in the functioning of sperm and the neutrophil. The antioxidants enzymes of sperm and neutrophil shows similar declining trend with the progression of age suggesting its possible role as a prognostic marker for age related deformities and even in male fertility.

1. Introduction

Diabetes, hypertension, hyperlipidemia, and atherosclerosis are some of the lifestyle-related diseases that develop in parallel with aging and are closely related to senescent change[1]. Vulnerability to infection in the elderly could result from an age-related decline either in neutrophil supply and/or mitochondrial functional efficiency. The gradual loss of the capability of animals to cope with oxidative stress is one of the characteristics of aging[2]. The abilities of human cells to respond to endogenous and exogenous oxidative stress may be compromised by alterations of gene expression of antioxidant enzymes[3,4]. An imbalance of the free radical scavenging enzymes is thought to enhance oxidative stress and to evoke oxidative damage to tissue cells during the aging process[3].

A progressive and irreversible accumulation of oxidative damage caused by ROS impacts on critical aspects of the aging process and contributes to impaired physiological function, increased incidence of disease, and a decreased life span[5]. Mitochondrial oxidation is the major cause of oxidative lesions that accumulate with age[6]. Oxidative stress is related to mitochondrial dysfunction, as mitochondrion is the generator and target for reactive species[7].

Mitochondrial turnover is dependent on autophagy, which declines with age and is frequently dysfunctional in neurodegenerative diseases[8]. Maianski *et al*.[9] have shown that the mitochondria are limited in number, do participate in the Neutrophil apoptosis. The production of reactive oxygen species (ROS) is one of the principal mechanisms by which neutrophils destroy pathogens; it is not surprising that seminal leukocytes have the potential to cause oxidative stress[10]. Because of the great number of mitochondria present in spermatozoa, antioxidant mechanisms are crucial for the maintenance of sperm motility, the rate of hyperactivation, and the ability to undergo the acrosome reaction during sperm preparation techniques, especially in the absence of seminal plasma[11].

Mammalian spermatozoa being rich in polyunsaturated fatty acids, are more susceptible to ROS attack, resulting in a decrease in sperm motility, presumably by a rapid loss of intracellular ATP, causing axonemal damage[12], a decrease in sperm viability, and an increase in the morphological defects in the midpiece, with decreased effects on sperm capacitation and the acrosome reaction. Lipid peroxidation (LPO) of sperm membrane is considered to be the key

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mechanism of this ROS induced sperm damage[13] leading to infertility.

Aged males have reduced testosterone production, low sperm motility and a higher incidence of erectile dysfunction and prostate disorders, and altogether contribute to reduced fertility rate. Sperm mitochondrial energy metabolism is very important for male reproductive system to function properly and mitochondria may therefore constitute a common link between aging and fertility loss[14]. In neutrophil, mitochondria hardly participate in ATP synthesis, and have a very low activity of the tested marker enzymes. Two important mitochondrial enzymes, glutamate dehydrogenase (GDH) and fumarase, which are often used as markers of mitochondria, displayed a borderline low activity in neutrophils. It has been shown that the mitochondria possess additional functions in cell death. These organelles contain a number of proteins in the inter membrane space, which, once released into the cytosol, induce and/or amplify the activation of apoptotic caspases[9]. It is also find out that presence of mitochondrial network in neutrophils participates in regulation of cell shape and chemotaxis[15]. Mitochondria are a second, less prominent source of ROS in neutrophils[16]. Recent studies have shown that Neutrophil mitochondrial activity, although not essential for phagocytosis or respiratory burst initiation, does affect chemotaxis[15] and apoptosis[17,18].

Succinate dehydrogenase (SDH) related human diseases, the enzyme not only plays a central role in the Krebs cycle and the respiratory chain, but it also differs from other mitochondrial dehydrogenases due to its unique redox properties. In partnership with ubiquinone, SDH would represent a crucial antioxidant enzyme in the mitochondria controlling superoxides scavenging activity of the respiratory chain[19]. Tomar *et al*.[20] have shown that dispersed expression of mitochondrial SDH in head and midpiece of spermatozoa of asthenozoospermia subjects indicates mitochondrial damage because of metabolic or genetic factors, affecting energy production and leading to disturbed sperm motility, which could be pathologically significant. Detailed mitochondrial SDH immunolocalization studies are warranted to establish a role of deranged tricarboxylic acid cycle in causation of male infertility.

There has been fewer findings about the human sperm and neutrophil mitochondrial malate dehydrogenase (MDH), however, in rats suggested that during sperm passage through the epididymis a compensatory increase in cytosol MDH activity occurred in the sperm but mitochondrial MDH activity decreased[21]. In C57BL/6 mouse testes, the lactic-acid-dehydrogenase (LDH) and malic-acid-dehydrogenase (MDH) exhibited age related changes[22].

The main objective of the present investigation was to correlate age with the neutrophil and the sperm functioning with the progression of age.

2. Materials and methods

Healthy male subjects were identified at the Sun Pathology Laboratory and May Flower Hospital, Ahmedabad. The inclusion criteria for collecting the samples were healthy, fertile men while subjects with any kind of reproductive disorders, urinary tract infection (UTI), infertility and genetic disorder were excluded at the time of sample collection. The proposal was approved by Institutional Human Ethical Committee (IEC/NU/I/IS/04). Ethical standard in accordance with the declaration of Helsinki was strictly followed.

2.1. Semen collection and analysis

Semen sample were collected into sterile plastic containers by masturbation after an abstinence period of 3-5 days, and were analyzed within 1 h of collection. Liquefied semen samples were centrifuged at 1 000 g for 1 min to separate seminal plasma and sperm for further evaluation.

2.2. Semenology

Fresh, uncentrifuged semen samples were subjected to semen analysis to ascertain its fertility state. Following tests were performed i.e., sperm count, motility, morphology, determination of sperm vitality assay, hypo-osmotic swelling test (HOS) and acrosome intactness test (AIT)[23].

2.3. Seminal plasma biochemistry

The seminal plasma obtained after centrifugation was used for the seminal plasma biochemistry to determine the functional status of the reproductive organs. Fructose as a marker of seminal vesicle[23], acid phosphatase as a prostate marker[24] and α-glucosidase as a marker enzyme for the epididymis[23] and Lactate Dehydrogenase (LDH)[25] for testis was performed. The calcium in the seminal plasma was performed using diagnostic kits (Accucare Ltd. Mumbai, India).

2.4. Blood collection and analysis

A total of 5 mL of blood was collected from each of the subject which was then transferred EDTA coated and non-EDTA coated vials.

2.5. Hematological analysis

EDTA blood was subjected to hematocrit parameters. Haematology of the blood samples were carried out in order to detect the variation in the hemoglobin (Hb), total red blood corpuscles (RBC), total white blood corpuscles (WBC) counts, WBC differential count. Hematocrit analysis included Packed Cell Volume (PCV), Mean Corpuscular volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) [26,27].

2.6. Neutrophil isolation

EDTA coated vial blood samples were further used for neutrophil isolation[28].

2.7. Serum biochemistry

Non-EDTA blood was centrifuged at 3 000 rpm for 10 minute at room temperature. The serum samples were used for biochemical assay of total protein assay, cholesterol, triglyceride, creatine kinase-MB (CK-MB), urea, lactate dehydrogenase (LDH), serum glutamate oxalate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) using diagnostic kits (Accucare Ltd. Mumbai, India). Serum testosterone assay was performed by chemiluminescence method using immunoassay kit (Beckman Coutler, Inc).

2.8. Isolation of mitochondria

The isolated neutrophil and sperm were subjected for ultrasonication for isolation of the mitochondrial fraction. Neutrophil and sperm were suspended in PBS (pH 7.4). This suspension was according to the protocol of Mclean *et al*.[29].

2.9. Confirmation of the mitochondrial fraction

Confirmation of the mitochondrial fraction in each sample was done by qualitative estimation of mitochondrial JC-1 kit (Genscript, USA).

2.10. Mitochondrial marker enzymes

The mitochondria in the cell suspension were further subjected for the mitochondrial marker enzyme assay. In the present investigation, the level of the major scavengers of the spermatozoa *i.e.*, superoxide dismutase (SOD)[30] and catalase [31], NAD^+ malate dehydrogenase was used as marker for the inter membrane matrix activity and functionality[32] and succinate dehydrogenase as a marker for the inner membrane of the mitochondria were estimated [33].

2.11. Statistical analysis

Data were expressed as median ± SD.

3. Results

A total of 90 subjects were involved for the sample collection. These subjects were to categorize into three age slabs i.e., 26-30, 31-35 and 36-40. The number of subjects in each age slab was 30, 30 and 30 respectively. All the values are expressed in Mean ± SD.

3.1. Semenology

The sperm count and motility were calculated and all the samples of various age groups had good sperm count as well as motility pattern is desirable of a fertile sample. There was notable fluctuations observed among the various age groups but these fluctuations remained within the reference range (Table 1). However, in the age group 31-35, sperm count (86.0±21.00) and motility (73.0±19.00) was slightly higher as compared to the other age groups.

Table 1
Sperm count and motility in the various age groups.

Age group (yrs)	Sperm count (millions/mL)(%)	Sperm motility(%)	Sperm morphology(%)
26-30	60.0±22.0	62.0±12.0	46.5±12.8
31-35	86.0±21.0	73.0±19.0	54.4±12.6
36-40	76.4±26.0	67.0±18.3	49.2±18.1

Values are expressed in Mean±SD.

The morphological abnormalities observed in the present study were in the head, neck and midpiece and in tail. More profound abnormalities were observed in the age group of 36-40 years. The morphological abnormalities were found to increase with the increase in the age of the individual ranging from (46.5±12.8)% to (49.2±18.1)%.

Viability test and Hypo-Osmotic Swelling (HOS) test were performed to assess the fertilizing potentials of the sperm. There was no trend found for both the above mentioned tests in the present investigation. The values were found to be fluctuating from 53% to 70% and 73% to 83% for viability and HOS test respectively. Acrosome Intactness test (AIT) is generally carried out to ascertain the intactness of the sperm acrosome as evident by the formation of halo around the acrosomal head. In our findings, AIT showed fluctuations among various age groups and did not show any specific trend with values ranging from 65%-69%.

Although, the values for semenology was fluctuating among all the age groups but were within the range as per the WHO guidelines[23].

Table 2
Various sperm functional tests in the various age groups.

Age group (yrs)	Sperm viability (%)	HOS (%)	AIT (%)
26-30	70.00±3.00	73.00±1.55	65.00±5.25
31-35	53.00±2.11	83.00±7.00	58.40±3.00
36-40	64.00±9.19	76.00±3.00	69.20±5.00

Values are expressed in Mean ±SD.

3.2. Seminal plasma biochemistry

α-Glucosidase and fructose decreased with the increase in the age but remained within the normal range. Acid phosphatase increased in 31-35 age groups, which also corresponded to proposed elevated prostate function in this age group. There was an increased LDH activity observed in the 31-35 age group (6.00±0.24) comparable to that of the other two groups. A significant decreasing trend in the calcium level was also observed among the age slabs. The age group 26-30 has shown the highest level (11.00±3.23) which dropped to (8.10±2.27) in the age group of 36-40 (Table 3).

3.3. Hematological analysis

There was no significant change observed in any of the parameters of blood profiling. Hematocrit values of the various age groups did not show much change with respect to increasing age. However, the values fluctuated within the normal range among the age groups (Table 4a)

3.4. Blood cell differential count

No remarkable change was observed among all the age groups. Values were within the normal range Table 4b.

3.5. Serum biochemistry

None of the serum biochemical parameters show any remarkable trend is any of the parameters. However, total protein, SGPT and SGOT levels show a decrease with increase in age. Serum triglyceride, cholesterol and urea level increased with the progression of age. The values of glucose and creatine fluctuated within the normal range among all the age groups. The serum testosterone levels did not show any specific trend among the age groups, though it was found to be elevated in the age group 31-35 as compared to the other age slabs (Figure 1). Serum calcium has shown a decrease in the age group 36-40 (Table 5).

3.6. Confirmation of the mitochondrial fraction by JC–1 Kit

No significant trend was observed in the number of the apoptotic and non apoptotic mitochondria of both the sperm and the neutrophil (Figure 2).

3.7. Mitochondrial marker enzymes

In neutrophil and sperm mitochondria, the superoxide dismutase and Catalase levels showed a gradual decrease with the progression of age. There was a significant decline in the mitochondrial antioxidant levels with the increase in age.

The succinate dehydrogenase (SDH) levels were found to be fluctuating but the sperm mitochondrial levels were highest in the age group of 31–35 while the neutrophil mitochondria showed highest activity in the age group of 36–40.

Sperm mitochondria malate dehydrogenase (MDH) activity has shown a notable increase in the 31–35 age groups as compared to the other age groups where as in neutrophil, the levels of MDH is fluctuating in different age group. There is increased value is seen in 36–40 age group (Table 6).

Table 3
Seminal plasma biochemistry in the various age groups.

Age(Yrs)	α –Glucosidase (Activity mU/ejaculate)	Fructose (μmol/ejaculate)	Lactate dehydrogenase (Activity of LDH (Unit/ min/mg of protein)	Acid Phosphatase (Activity (U/mL)	Calcium (mg/dL)
26–30	29.40±6.02	17.10±3.00	5.10±0.29	0.020±0.004	11.00±3.23
31–35	28.10±4.15	16.30±2.23	6.00±0.24	0.050±0.005	10.00±3.00
36–40	28.00±3.38	16.00±2.02	6.00±0.04	0.020±0.006	8.10±2.27

Values are expressed in Mean ±SD.

Table 4a
The hematocrit values for the various age groups.

AGE(yrs)	Hb(%)	R.B.C ($\times 10^6$/c.mm)	W.B.C ($\times 10^3$/c.mm)	Platelets ($\times 10^5$/c.mm)	PCV(%)	MCV(fL)	MCH(pg)	MCHC(%)
26–30	15.0±1.00	5.1±0.35	8.0±3.00	3.0±0.32	43.0±2.02	83.0±3.00	29.0±1.01	35.0±1.12
31–35	14.3±1.07	5.0±0.26	11.0±2.00	3.0±1.00	42.0±2.49	84.0±4.07	29.0±2.24	35.0±1.26
36–40	15.0±1.24	7.0±1.00	7.0±1.05	2.0±1.00	43.0±3.00	81.4±6.37	28.0±2.33	34.1±1.44

Values are expressed in Mean ±SD.

Table 4b
WBC differential count for different age groups.

Age (Yrs)	Neutrophil	Leukocytes	Eiosinophil	Monocyte	Basophil
26–30	65.00±7.00	31.00±7.00	3.00±1.00	2.00±0.00	0
31–35	65.40±8.11	30.40±8.00	2.20±0.44	2.00±0.00	0
36–40	61.00±5.09	35.20±5.21	2.00±0.70	2.00±0..44	0

Values are expressed in Mean ±SD.

Table 5
Serum biochemical parameter values among all the age groups.

Age (Yrs)	Total protein (g/dL)	Triglyceride (mg/dL)	Cholesterol (mg/dL)	Urea (mg/dL)	SGPT U/I)	SGOT (U/I)	Glucose (mg/dL)	Creatine (mg/dL)	Calcium (mg/dL)
26–30	7.2±1.0	99.00±33.00	135.00±39.00	34.00±22.00	23.40±13.20	31.00±25.30	94.40±6.00	1.00±0.11	10.00±1.00
31–35	7.0±2.0	115.00±62.00	166.00±42.30	33.00±10.00	13.00±7.00	11.10±3.06	95.00±7.11	1.00±0.18	10.00±3.10
36–40	6.8±1.0	117.00±53.00	191.00±60.40	37.00±11.00	11.00±3.00	11.05±5.00	99.00±7.05	1.00±0.10	8.20±2.20

Values are expressed in Mean ±SD.

Table 6
Differential mitochondrial marker analysis in different age groups.

Age	SOD (unit/min/mg of protein)		CAT (unit/min/mg of protein)		SDH (Succinate Dehydrogenase (SDH)(concentration (pM DCPIP/ min/mg of protein)		MDH (Malate Dehydrogenase Activity (10^{-3}) unit/min/mg of protein)	
	Sperm	Neutrohil	Sperm	Neutrohil	Sperm	Neutrohil	Sperm	Neutrohil
26–30	0.15±0.02	1.10±0.18	0.170±0.080	1.50±0.27	1.12±0.08	0.36±0.04	25.07±14.70	1.98±0.70
31–35	0.09±0.02	0.93±0.41	0.140±0.020	1.06±0.29	1.96±0.66	0.35±0.03	43.93±15.80	3.28±1.60
36–40	0.08±0.02	1.37±0.24	0.097±0.007	0.62±0.13	0.97±0.09	0.55±0.05	32.80±4.80	2.11±0.60

Values are expressed in Mean ±SD.

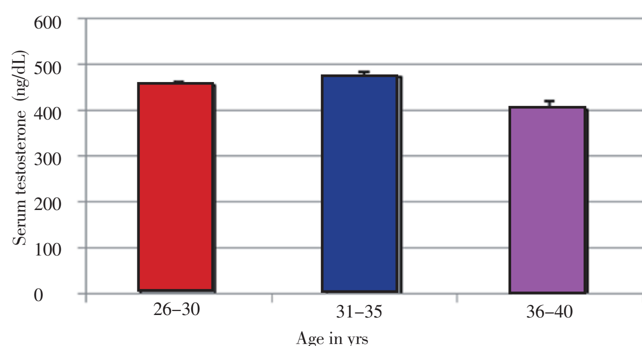

Figure 1. Serum testosterone levels in various age groups.
Values are expressed in Mean ± SD.

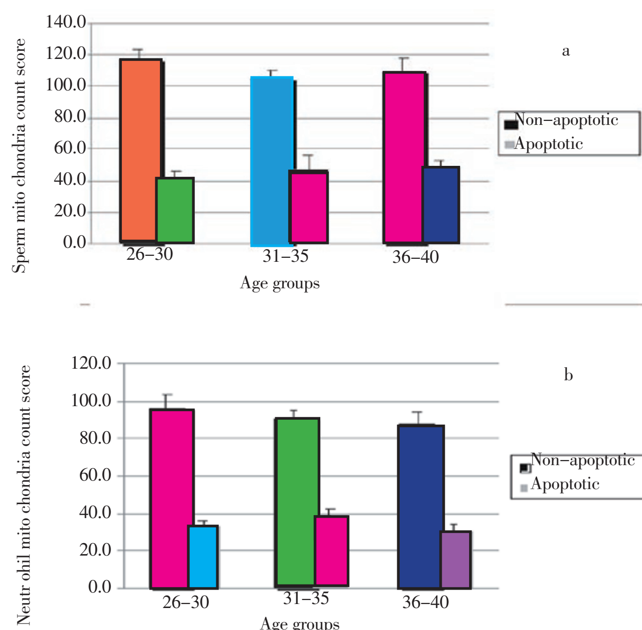

Figure 2. Mitochondria confirmation assay by JC–1 kit isolated from (a) sperm and (b) neutrophil from various age groups.
Values are expressed in Mean ±SD.

4. Discussion

Oxidative stress has been reported to increase in elderly subjects, possibly arising from an uncontrolled production of free radicals by aging mitochondria and decreased antioxidant defenses and is a common pathology seen in approximately half the population of infertile men. There is abundant experimental and observational evidence supporting the idea that aging is the sum of all free radical reactions throughout all cells and tissues, or that they are at least a major contributor to it[2,34]. Oxidative damage of biomolecules increases with age and is postulated to be a major causal factor of cellular biochemical senescence[35].

There are two main sources of ROS in semen: leucocytes and sperm. The production of ROS by sperm plays a positive role in fertilization at low levels, when produced at high levels it can lead to potential toxic effects on sperm quality and function. Additionally, environmental and lifestyle factors as well as pathologies of the reproductive system and chronic diseases are sources of sperm oxidative damage[36]. Although seminal plasma is well endowed with an array of protective antioxidants (Superoxide dismutase (SOD), Catalase (CAT), and glutathione peroxidase (GPX), these defenses are less abundant in sperm and seem to be impaired in cases of male infertility[10,36].

The main ROS generated in the neutrophil respiratory burst are superoxide anion (O_2^-). While neutrophil–derived ROS play an important role in breaking down damaged fragments of host tissue[37], when produced in excess, these toxic molecules may also contribute to oxidative stress. This involvement of neutrophils could have important implications for the development of a variety of pathological conditions that have been linked with oxidative stress and age including atherosclerosis, cancer, rheumatoid arthritis and post–ischaemic organ injury[38].

Our investigation revealed that basic semen analysis and sperm functional tests didn't show any trend with the progression of age. However, an investigation by Eskenazi *et al*.[39] has shown that there are significant age–dependent reductions in several aspects of semen quality among a cohort of healthy workers and retirees; the sperm motility was severely affected followed by moderately low effect on semen volume and sperm count. The number of men with abnormal percent motile and total progressively motile sperm also increased significantly across age decades. Mukhopadhyay *et al*.[40] have reported significant correlation of the age–specific changes on semen parameters and its gradual detoriation of these parameters in two decades time. Gopalappa and Malini[41] have demonstrated lower values of sperm functional test in infertile males as compared to the control subjects. In the last two decades, due to a combined effect of environmental changes in the lifestyle and dietary factor, there has been a decline in the semen quality[42,43].

The marker enzymes of various glands of male reproductive system analysed were acid phosphatase as prostate marker, α– glucosidase as a marker for epididymis and the level of fructose as a marker for proper functioning of seminal vesicles. The activity of acid phosphatase shows fluctuations on an average. Its activity increases in the age group of 31–35 years and again deteriorates in the age group of 36–40 years. Prostatic acid phosphatase is secreted during ejaculation to aid in liquefaction of semen. The prostate gland tissue is known to be a testosterone–dependent organ and, consequently, when the testosterone level decreases as a result from an increasingly pronounced metabolic syndrome, the growth–stimulating effect on the prostate gland by other aberrations might possibly be reduced[44]. The testosterone level along with the acid phosphatase activity is proportional to the functionality of prostate. So, it may be indicative of an increased prostate activity in 31–35 age group.

Intracellular concentration of calcium (Ca^{2+}) in the sperm membrane is an important marker of the sperm quality due to its direct relationship with sperm morphology and fertility potential[45]. An influx of Ca^{2+} is required to initiate the acrosomal reaction, with its attendant release of enzymes and membrane alterations necessary for sperm–egg interaction[46]. There is also evidence that this ion may be involved in sperm motility[47]. Fakih *et al*.[48] reported that Ca^{2+} increased the sperm motility and velocity. These reports support the association of Ca^{2+} content of seminal plasma and motility observed in their study. Calcium was reported as essential for sperm physiology including motility[49], metabolic

function[50], acrosome reaction, and fertilization[51, 52].

In our investigation, we obtained a gradual decrease in the calcium level in each age group considered along with the decrease in the fructose concentration. Seminal fructose concentration is directly correlated with the motile sperm concentration, suggesting that only the motile sperm consume fructose after ejaculation[53]. More is the sperm concentration, more fructose will be utilized as a source of energy and so lesser will be the amount of fructose present in the plasma.

Aging has been associated with a change in the levels of testosterone levels. In healthy, Aging groups of men testosterone levels decline with age on the order of 100 ng/dL per decade[54]. These hormonal fluctuations begin around the age of mid 40s and are well established by the age of 50 years as the individual enters into a phase of andropause. The normal reference interval for total testosterone levels in a male range from 300 to 1 000 ng/dL[55]. Decreased levels may cause a decreased libido and complications in reproduction. As an adult male becomes older, the level of testosterone may decrease[56]. Our results show a higher level of the serum testosterone in the age group of 31–35 and a subsequent decrease in 36–40 age group.

In the present investigation, a decrease in the total protein level was indicated which could lead to protein energy malnutrition is a factor in susceptibility to infectious disease, poor wound healing, and negative outcomes to interventions for chronic and acute conditions[57]. Triglyceride, cholesterol and urea levels indicated an increase in their levels with increasing age. Serum cholesterol and triglycerides exhibited a rise proportional to degree of examination stress[58]. In our study, decreased levels of SGPT and SGOT are an indicative of improper functioning of the liver. Schmucker[59] has reported several age–related changes that included diminished liver volume and poor response to oxidative stress.

According to the findings of Aono *et al.*,[60] the mean (Blood Urea nitrogen) BUN level in the elderly subjects was significantly higher than that in the control young subjects and a significant positive co–relation was found between the BUN levels and age in male elderly subjects. Serum glucose and serum creatine did not show any trend, but their levels fluctuated within the normal range among the age groups. Serum calcium has shown a steady decrease with increase in the age. According to the study of Fujita [61], Aging is associated with the progressive aggravation of calcium deficiency, such blunting also progresses with aging.

Antioxidant enzymes are considered to be a primary defence that prevents biological macromolecules from oxidative damage. Superoxide dismutase (SODs) rapidly converts superoxide radical to less dangerous H_2O_2, which is further degraded by catalase (CAT) to water. Thus, the steady–state level of antioxidant enzymes during Aging may protect some important tissues against free radical–mediated damage[62]. Changes in antioxidant capacities like hydrophilic radical scavengers (ascorbate, urate and glutathione), lipophilic radical scavengers (tocopherols, carotenoids), metal chelators and antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase activities have been demonstrated in aging[63].

In the present study, the mitochondrial antioxidants SOD and CAT from both the sperm and the neutrophil showed a significant decrease among the age slabs. As SOD and CAT are of great significance and constitute the antioxidant system in mitochondria along with the other enzymes. With increasing age CAT declines which can lead to an increase in ROS production and its accumulation in the cell. Eventually, this will lead to decrease cell activity, which may further result in apoptosis and cell death. Nandeslu and co–workers[64] have reported the redox status of human neutrophils during aging. Their study found age–related decline in activities of antioxidant enzymes in neutrophils from different age groups.

Succinate dehydrogenase (SDH) is known as the marker enzyme of mitochondria. The abnormal levels of SDH in mitochondria may be a sign of mitochondrial dysfunction or may lead to disorders related to mitochondria. Detection of sperm mitochondrial SDH has significance in evaluation of sperm mitochondrial function and may serve as an assisted marker of sperm viability[65]. Present study has shown that SDH activity in sperm and neutrophil differs in the individuals of different ages, which showed an increase in the age group of 31–35 and then in the subsequent age group *i.e.*, 36–40.

In general, malate dehydrogenase (MDH) is the final enzyme in the mitochondrial tricarboxylic acid (TCA) cycle[66]. MDH is one of the proteins present in the spermatozoa which has a positive relationship with fertility, and are involved in the TCA cycle. It is likely that increased sperm metabolism and the ability to use carbohydrates as energy may have a positive effect on the fertilizing ability of sperm[67]. Development and capacitation of mammalian sperm are accompanied by expression of several marker proteins including MDH[68]. The process of aging show significantly increased levels of MDH, SDH[69]. Our data showed a similar trend of MDH activity with the progression of age.

The transport of metabolites to and from mitochondria is also affected by Aging, as is the case of malate import, which has been demonstrated to decrease with age[70]. MDH catalyzes the NAD/NADH–dependent interconversion of the substrates malate and oxaloacetate. This reaction plays a key part in the malate/aspartate shuttle across the mitochondrial membrane, and in the tricarboxylic acid cycle within the mitochondrial matrix.

The stress one experiences vary from mild to severe depending on one's psychological, physiological and social make up is discussed by Chandraiah *et al.*,[71]. According to them, young adults (25–35 yrs) experiences more stress than middle aged (36–40 yrs). Uma Devi[72] has demonstrated major stress levels among the 35–39 age groups. High stress level can be attributed to individual's life styles, environmental and occupational exposure, temperature and radiation, drug treatment, addictions, dietary factors, growth factors, cytokines and hormones which directly induce oxidative stress in mitochondria leading to increased ROS level and cause altered mitochondrial functioning[73]. These reports support our data which shows a diminished mitochondrial enzymatic level in the age group 36–40 in the sperm and neutrophil indicating a direct negative relation between sperm and neutrophil functioning and progression of age.

It can be concluded that the decreased calcium level in the serum and the seminal plasma indicates that calcium can also be a potential marker for aging and male infertility. Mitochondrial antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) can be used as the potential markers for ageing in men as these antioxidants levels

decreased with the progression of age. Increased stress level is observed in the middle age. Hence, there is a decline in the mitochondrial enzyme activity (Succinate dehydrogenase (SDH) and malate dehydrogenase (MDH) there by affecting the sperm and the neutrophil function in the middle age group. The mitochondrial SOD, CAT, SDH and MDH can be used as the key markers for ageing and male infertility. The polymorphism studies of these enzymes along with other apoptotic markers needs to be further studied to co-relate with mitochondrial functioning with age as a parameter.

Declare of interest statement

There is no conflict of interest among the authors.

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