STATUS OF ANTIOXIDANTS MOLECULES AND LIPID PEROXIDATION IN THE DIABETIC TESTES

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INTRODUCTION

Diabetes mellitus (DM) is a major concern of the global health due to its high prevalence and its serious complications. Besides being a metabolic and endocrine disorder, diabetes mellitus has also been associated with reproductive impairment in men [1] and its impact on reproduction can be profound, as seen by diminution in fertility and increase in reproduction losses [2-5]. A very common pathology experienced by diabetic men is the consistent inability to achieve and maintain penile erection (erectile dysfunction) sufficient for adequate sexual relations [6,7]. Erectile dysfunction (ED) and retrograde ejaculation is well recognized and examined in diabetic men [8-10].

It has been proved that DM alters structural and functional changes in the testes [11-13] but how the DM alters the normal organization of testis is not clearly established. There could be some molecular changes in the testes of diabetic men which is responsible for the histopathological changes in the testis and results in the decline of fertility. Further, time factor also play an important role in the pathology of any disease. The complete evaluation of whole parts of gonads in diabetic men is a challenging factor. Therefore, this study was designed to evaluate the activity of antioxidants molecules and status of oxidative stress in the testis of diabetic rats.

The body constantly interacts with oxygen during various physiological processes in the cell. Most of the body’s energy is produced by the enzymatically controlled reaction of oxygen with hydrogen during oxidative phosphorylation occurring within the mitochondria. Due to this enzymatic reduction of oxygen a highly reactive molecules are produced as free radicals which are known as oxidants. A free

ABSTRACT

Introduction: According to the epidemiological studies, diabetes mellitus has become a potential cause of male infertility. Knowledge regarding how diabetes mellitus interferes with the process of spermatogenesis and results in infertility needs the molecular study in the testis in diabetic condition. Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important role in the pathogenesis of chronic diabetes mellitus. So, this study was established to investigate the activity of enzymatic antioxidants and oxidative stress in the testis of diabetic model rats.

Material & Methods: Diabetes mellitus was induced in the rat by intraperitoneal injection of Streptozotocin. The rats were sacrificed and the dissection was done to take out the testis. The testes were processed for the activity of enzymatic antioxidants.

Results: It was found that oxidative stress was increased in the testes of diabetic rats. The sperms were also affected by the chronic hyperglycemia.

Keywords: Antioxidants, oxidative stress, diabetes mellitus, testes.
A radical is an oxygen containing molecule that has one or more unpaired electrons [14] making it highly reactive with other molecules. Since they are highly active and derived from oxygen, they are also called reactive oxygen species (ROS). ROS includes superoxide anion ($O_2^-$), hydroxyl (OH), hydrogen peroxide ($H_2O_2$), organic hydroperoxide (ROOH), hypochlorus acid (HOCL) etc. [15]. Aerobic environment is a constant source of ROS through in vivo mechanisms such as electron leakage during biologic oxidations and by physical activation of oxygen by external agents such as radiation e.g. UV sunlight.

Reactive oxygen substance can chemically interact with other cell components such as DNA, protein and lipid. Depending on their cellular concentration they can either exert beneficial physiologic effects (e.g. killing invading pathogens or microbes) or pathological damage to cellular components including gene, amino acids and lipids [16].

To counteract the destructive action of oxidants, the body produces other molecules known as antioxidant. These molecules present in the cells, prevent the oxidative damage done by oxidants. Antioxidants can be enzymatic such as catalase, superoxide dismutase, and glutathione peroxidase/reductase or non-enzymatic antioxidants such as vitamin C, vitamin A, vitamin E, pyruvate and glutathione [17]. Superoxide Dismutase (SOD) is an enzymatic antioxidant that repairs cells and reduces the damage done to them by superoxide, the most common free radical in the body. SOD catalyzes the reduction of superoxide anions ($O_2^-$) to hydrogen peroxide and oxygen [18-20]. This is the first line of defense to protect cells from the injurious effects. Catalase is another important antioxidant molecule which is used by cells to decompose hydrogen peroxide a very reactive molecule, into oxygen and water molecules [21].

Oxidative stress is a pathological state that arises when free radicals chemically damage biological molecules and the decrease in the ability of the body to counteract their harmful effects through neutralization by antioxidants. It is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases [22-24] such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, myocardial infarction, cardiovascular diseases, chronic inflammation, aging and other degenerative diseases in human [25,26]. Enhanced oxidative stress and changes in antioxidant capacity are considered to play an important role in the pathogenesis of chronic diabetes mellitus [27,28]. Lipid peroxidation is a well-defined mechanism of cellular damage in animals. Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as malondialdehyde (MDA). Measuring the end products of lipid peroxidation is one of the most widely accepted assays for oxidative damage [29-31].

Analysis of the sperm reflects the state of ongoing spermatogenesis in the testis and the sperm analysis is the initial standard test to evaluate the status of male fertility [32]. Semen analysis provides useful information concerning sperm count, motility and morphology as well as ejaculation and emission. Although the semen analysis reveals useful information for the initial evaluation of the infertile male, it is not a test of fertility [33]. However, it reflects some basic preliminary guidelines regarding fertility.

Most of the research in diabetes mellitus has been focused on the metabolic disorder rather than its effect on the reproductive system. The onset of diabetes mellitus (usually type 2 which accounts for 90% prevalence) is seen in later stages of life and by this time fertility of the individual is reduced naturally. However, in recent years, diabetes mellitus is occurring rampantly in the young age group due to stressful lifestyle [34] and also DM has become a potential cause of male infertility. With this view, this study has been designed to investigate the activity of enzymatic antioxidants and lipid peroxidation in the testis of diabetic model rats. Besides oxidative stress, this study also analyzed sperm of diabetic rats.

**MATERIAL AND METHODS**

Adult male Wistar rats weighing 200-250 grams were included in this study. Only the euglycemic rats were taken for the study. They were maintained in captivity in cages under natural light conditions, laboratory chow and water ad libitum were available. On the basis of duration of study; one week, one month and six months, the rats were divided into three groups. Each group contains control and diabetic rats. Diabetes mellitus was induced in the rat by intraperitoneal injection of Streptozotocin (STZ) at a dose of 50 mg/kg dissolved in freshly prepared citrate buffer (pH 4.5). The control group were given the same volume of citrate buffer. After 72 hrs of STZ injection, the blood sample of STZ group rats were
taken from the tail vein to measure the fasting blood glucose level by automated glucose analyzer. The fasting blood glucose level above 200 mg/dl was considered as diabetic rat. The fasting glucose level of control group was also measured to confirm the non diabetic. The fasting glucose level was monitored periodically to confirm the diabetic and non diabetic rats. The rats were sacrificed after one week, one month and six months according to the duration of study. The dissection was done to take out the testis and epididymis.

The testes were quickly excised, washed in sodium phosphate buffer (pH 7.2) and stored at -80˚C. The testes were decapsulated and the tissue was homogenised by the tissue homogenizer at the concentration of 50 mg/ml in 0.1M of ice cold phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 r/min at 4˚C for 5 minutes separately. Each supernatant was measured for the estimation of catalase, superoxide dismutase and MDA. Estimation of superoxide dismutase (SOD) was done by Marklund and Marklund method [35]. Estimation of catalase activity was done by Sinha method [36]. Estimation of malondialdehyde (MDA) was done by Sathoh’s method [37]. The sperms taken out from the epididymis were examined for count, motility and morphology following standard methods [38].

**OBSERVATIONS AND RESULTS**

The SOD activity in the diabetic rats after one week increases significantly. Thereafter, SOD activity decreases markedly in the diabetic rats when compared to the non-diabetic rats (Table 1, Fig. 1).

Table 1: Descriptive statistics of superoxide dismutase activity

| Study Group | Duration of study | Mean ± SD | SEM | t-stat | df | P value |
|-------------|------------------|-----------|-----|--------|----|---------|
| Control     | One week         | 2.98 ± 0.11 | 0.04 | -4.11  | 10 | 0.001*  |
| Diabetic    | One week         | 3.19 ± 0.05 | 0.02 | 8.26   | 10 | <0.001**|
| Control     | One month        | 2.77 ± 0.11 | 0.04 | -8.65  | 10 | <0.001**|
| Diabetic    | One month        | 1.56 ± 0.34 | 0.13 | 5.91   | 10 | <0.001**|
| Control     | Six months       | 2.47 ± 0.17 | 0.07 | 9.12   | 10 | <0.001**|
| Diabetic    | Six months       | 1.34 ± 0.43 | 0.17 | -3.5   | 10 | 0.001*  |

The mean value is expressed in Units/mg; *Significant, **highly significant.

The mean value of catalase activity in diabetic rats was less than the non-diabetic rats except in the first group where the catalase activity was increased but not statistically significant (Table 2, Fig. 2).

Table 2: Descriptive statistics of catalase activity

| Study Group | Duration of study | Mean ± SD | SEM | t-stat | df | P value |
|-------------|------------------|-----------|-----|--------|----|---------|
| Control     | One week         | 2.9 ± 0.21 | 0.08 | 0.86   | 10 | 0.2     |
| Diabetic    | One week         | 3.01 ± 0.21| 0.08 | 18.73  | 10 | <0.001**|
| Control     | One month        | 3.32 ± 0.17 | 0.07 | 25.66  | 10 | <0.001**|
| Diabetic    | One month        | 1.72 ± 1.31| 0.53 | 0.91   | 0.12| 0.05    |

The mean value is expressed in Units/mg of protein; *Significant, **highly significant.

The MDA level was increased in the testis of diabetic rats when compared with the non-diabetic
rats. At the end of the one week, there was no significant change in MDA level between the diabetic and control rats. However, statistically significant change was there after one month and six months of diabetes (Table 3, Fig. 3).

Table 3: Descriptive statistics of MDA

| Study Group | Duration of study | Mean ± SD | SEM | t-stat | df | P value |
|-------------|------------------|-----------|-----|--------|----|---------|
| Control     | One week         | 1.48±0.13 | 0.05| -0.9   | 10 | 0.19    |
| Diabetic    | One week         | 1.56±0.15 | 0.06| 0.05   | 10 | 0.96    |
| Control     | One month        | 1.52±0.13 | 0.05| -5.82  | 10 | <0.001* |
| Diabetic    | One month        | 2.26±0.28 | 0.11| -5.82  | 10 | <0.001* |
| Control     | Six months       | 1.76±0.14 | 0.06| -15    | 10 | <0.001**|
| Diabetic    | Six months       | 3.6±0.26  | 0.1 | 6      | 10 | <0.001**|

The mean value is expressed in nmol/ml; *Significant, **highly significant.

The number of sperms in diabetic rats decreased significantly in comparison to control rats except in the group of first week study. A significant decrease in the count was seen after a long term of diabetic conditions (Table 4, Fig. 4). The motility of the sperms in the diabetic group decreased as compared to non-diabetic groups (Table 5, Fig. 5). The abnormal morphology of the sperm of diabetic rats increased significantly than the non-diabetic rats in one month and six months of study (Table 6, Fig. 6).

Table 4: Descriptive statistics of sperm count

| Study Group | Duration of study | Mean ± SD | SEM | t-stat | df | P value |
|-------------|------------------|-----------|-----|--------|----|---------|
| Control     | One week         | 152.73±14.1 | 5.75| 1.71   | 10 | 0.05    |
| Diabetic    | One week         | 141.72±6.92 | 2.82| 4.15   | 10 | <0.001**|
| Control     | One month        | 164±18.95  | 7.74| 3.55   | 10 | <0.001**|
| Diabetic    | One month        | 77.18±47.52 | 19.4| 6.54   | 10 | <0.001**|
| Control     | Six months       | 138.8±28.66 | 11.7| 6.54   | 10 | <0.001**|
| Diabetic    | Six months       | 35.1±26.22 | 10.7| -6.54  | 10 | <0.001**|

The mean value is expressed in million/ml; *Significant, **highly significant.

Table 5: Descriptive statistics of motility of the sperms

| Study Group | Duration of study | Mean ± SD | SEM | t-stat | df | P value |
|-------------|------------------|-----------|-----|--------|----|---------|
| Control     | One week         | 75.08±7.5  | 3.06| 0.43   | 10 | 0.33    |
| Diabetic    | One week         | 77.16±6.99 | 2.85| 2.74   | 10 | <0.010  |
| Control     | One month        | 75.33±9.82 | 4.01| 6.54   | 10 | <0.001**|
| Diabetic    | One month        | 57.16±12.86| 5.25| 6.54   | 10 | <0.001**|
| Control     | Six months       | 73.58±8.36 | 3.41| 6.54   | 10 | <0.001**|
| Diabetic    | Six months       | 38±9.13    | 3.73| 6.54   | 10 | <0.001**|

The mean value is expressed in percentage (%); *Significant, **highly significant.
DISCUSSION

Millions of sperms are produced per ejaculation, from the testis of male reproductive system. This suggests that there is a high cell division in the germinal epithelium of the seminiferous tubules of the testis. The high rate of cell division of the germ cells and the ability of the sperms to move forward implies high consumption of energy and oxygen by the mitochondria of germ cells. However, the vascularisation of the testes is very poor so that oxygen tension in the tissue of testes is low [39] and the competition for this oxygen within the testes is extremely intense. Both spermatogenesis [40] and Leydig cell steroidogenesis [41,42] are vulnerable to oxidative stress. However, the presence of low oxygen tension may be an important mechanism to protect the tissue from reactive oxygen species. In addition, the testes also contain a number of antioxidant enzymes by which the testis protects itself from free radical-mediated damage. These antioxidant defence mechanism are of major importance because peroxidative damage is currently regarded as the single most important cause of impaired testicular function in wide range of pathological conditions [43-50].

Reactive oxygen species (ROS) or free oxygen radical are normally generated by Sertoli cells that cause alteration in cellular structures and induces morphological changes in spermatids during spermiogenesis [51] and controlled amount of ROS is essential for capacitation and acrosome reaction [52]. But increased free radical induce lipid peroxidation and DNA fragmentation of the spermatozoon, disrupting both the motility of these cells and their ability to fertilize the ovum [53-59]. At the level of the testes, oxidative stress is capable of disrupting the steroidogenic capacity of Leydig cells [60] as well as the capacity of the germinal epithelium to differentiate normal spermatozoa [61].

Superoxide Dismutase

In this study, after one week of induction of diabetes SOD activity was increased significantly in diabetic group when compared to control group. Since SOD is the first line of defence against the free radical, its production is accelerated during the early phase of diabetes i.e. after one week. Thereafter, SOD activities are markedly decreased after one month and six months group. This decline in SOD activity was due to tremendous increase in free radical where SOD may not be able to counteract destructive action of radical. There are reports of decreased antioxidant enzyme
Various observations indicate that spermatozoa may be more exposed and vulnerable to oxidative stress than germ cells. Spermatozoa found in the epididymis and vas deferens is not protected like germ cells in the seminiferous tubules by the microenvironment provided by the Sertoli cell barrier. Furthermore, the membranes of spermatozoa may be particularly susceptible to free radical attack because of their high level of polyunsaturated fatty acids [66]. Several studies have demonstrated that, in contrast to spermatogonia, primary spermatocyte and round spermatid, elongated spermatids and spermatozoa have a reduced capability or are even unable to repair DNA damage [67,68].

**Catalase**

We found the decrease in the activity of catalase in long term diabetic (six months) than one month diabetic rats. This shows negative correlation of catalase activity with the severity of diabetic. The increase activity of catalase in one week of diabetic rats is not significant (p>0.05). Other studies done by Singh et al., 2013 [69] and Adewole et al., 2007 [64] are also in accordance with our results in which catalase level were reduced in the testes of diabetic rats than the non-diabetic rats. When catalase activity is decreased, as in the present study, H$_2$O$_2$ is reduced to a very highly oxidizing OH radical in the presence of Fe$^{2+}$ or other transition metals. The OH radical cannot be enzymatically removed from the cells but a free radical scavenger can detoxify it. The activity of catalase was lowered in diabetic rats, probably due to glycation of the enzyme due to hyperglycemia. Kaleem et al., 2006 [70] proposed that decrease in activities of SOD and CAT in diabetic state may be due to over-production of reactive oxygen species in diabetic animals.

Catalase works closely with superoxide dismutase to prevent free radical damage to the body. SOD converts the dangerous superoxide radical to hydrogen peroxide, which catalase converts to harmless water and oxygen [71]. Hydrogen peroxide is a naturally occurring but destructive waste product of all oxygen-dependent organisms. It is produced in the human body when fatty acids are converted to energy, and when white blood cells attack and kill bacteria. Catalase, which is located in the cell’s peroxisome, prevents this naturally occurring hydrogen peroxide from harming the cell during these processes. It also helps prevent the conversion of hydrogen peroxide to hydroxyl radicals, potentially dangerous molecules that can attack and even mutate DNA. Catalase also uses hydrogen peroxide to break down potentially harmful toxins in the body, including alcohol, phenol and formaldehyde. Catalases are some of the most efficient enzymes found in cells; each catalase molecule can convert millions of hydrogen peroxide molecules into water and oxygen every second.

**Malondialdehyde**

The result of the present study showed an increase in the level of MDA in all diabetic groups which are consistent with results of Shaikh et al., 2014; Rabbani et al., 2009; Adewole et al., 2007; Ozkaya et al., 2002 [62-65]. Increase in MDA resulted from hypoinsulinemia that increases the activity of fatty acyl coenzymes A oxidase, which initiates β-oxidation of fatty acids, resulting in lipid peroxidation. Also, protein glycation and glucose auto-oxidation can lead to the formation of free radicals, and this can equally induce lipid peroxidation. In this study, there is non-significant increase in the level of MDA after one week. This may be due to significant increase in the level of SOD (after one week) which prevent the lipid peroxidation by neutralizing the toxic effect of superoxide radical.

The spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFAs) and their cytoplasm contains low concentrations of scavenging enzymes. OS–mediated damage to the sperm plasma membrane may account for defective sperm function observed in a high proportion of infertility patients [72]. Indeed, it has been shown that excess amounts of ROS and free radicals in spermatozoa and seminal plasma have adverse effects on sperm motility and fertility. It has also been reported that levels of plasma antioxidants in infertile men are significantly lower than those in plasma from controls [72]. Moreover, ROS-induced DNA damage may accelerate the process of germ cell apoptosis, leading to a decline in sperm counts associated with male infertility, and thus to the apparent deterioration of semen quality [73]. In fact, mitochondrial DNA mutations are associated with a decline of motility in human sperm, probably leading to male infertility. Additionally, mitochondrial respiration defects gave rise to meiotic arrest and abnormalities in sperm morphology, stressing the requirements of mitochondrial respiratory function in mammalian spermatogenesis [74].

Spermatogenesis is the integral part of the male reproductive system which cannot remain untouched.
by the effects of diabetes mellitus. For every cell of the testis, insulin is required for uptake of glucose from the blood to the cell. But insulin is deficient or resistance in diabetes mellitus, which affects the homeostasis of glucose in the testis which ultimately affects the spermatogenesis.

The findings regarding semen quality in diabetes mellitus have not been consistent, showing variations from normal to altered sperm count, motility, morphology or a combination of both. In a study conducted by Bartak et al., 1975 [75] in a young generation (16–22 years old), involving 25 diabetic and 24 control individuals showed that juvenile diabetics presented lower sperm values and significant differences in sperm motility and morphology. A few years later, another study by the same group compared the ejaculated semen of 65 diabetic and 77 control men and it was reported as a negative effect of diabetes mellitus on the ejaculate. The parameters mostly affected were sperm motility, morphology, volume and count [76]. These findings are concordant with our result. In our study, the sperm count and motility were decreased in diabetic group when compared with the control group. The total percentage of abnormal morphology of sperms was increased in the diabetic group. The effects of diabetes on sperms parameters in long term diabetic conditions are more than the short term diabetic. Though there is a divergent in semen parameters in all diabetic groups from control group but the result of one week study is statistically not significant. The decrease in sperm count, motility and increase abnormal morphology of sperms in diabetic conditions were also reported similarly by Seethalakshmi et al., 1987 and Kim and Moley, 2008 [77,78]. Slightly different results were reported by Pardron et al., in 1984, where diabetic adolescents presented a minor, non-significant, decrease in sperm count relatively to control individuals. The semen from these juvenile diabetic patients had lower volume and motility, as well as altered morphology, and presented significantly higher fructose and glucose levels, evidencing that an ineffective metabolic control can be deleterious or responsible for the observed alterations in the semen [79].

CONCLUSION

The study was conducted in order to clarify the relationship between oxidative stress originated by a diabetic condition and parameters related to testicular function in the rat. We concluded that the induction of diabetes in rats causes increase in the oxidative stress in the testis. Diabetes increases in the level of MDA in the testis. Furthermore, significant reduction in the activity of the antioxidant enzyme was a common feature in all the samples, which is suggestive of the ongoing oxidative disturbances. Prolonged hyperglycemia leads to decrease in the level of SOD, catalase. Decreased activity of these antioxidants might be due to its inactivation caused by excessive ROS production. Initial stage of diabetes mellitus increases the production of SOD since it is the first line of defence against free radical. Since the production of free radical is highly accelerated as the diabetes becomes chronic with time, the level of SOD decreases leading to increase oxidative stress in testis.

The sperms are severely affected in long term diabetes than in short term diabetes. Diabetes mellitus affects significantly in all the parameters of the sperms: count, morphology and motility. Besides decrease in number of spermatozoon, diabetes mellitus also alters structural (morphology) and functional (motility) aspects of spermatozoon. This result shows that diabetes mellitus does not cause only erectile dysfunction and retrograde ejaculation but also affects directly on spermatogenesis leading to infertility.

The consequences of oxidative stress may be a factor for errors in spermatogenesis which leads to loss of motility, count and increased abnormal morphology of sperms in diabetes rats. These findings now support a role of oxidative stress as a significant cause of male infertility in diabetes mellitus.

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