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EVALUATION OF PREDICTIVE VARIABLES OF MEDICAL IMPORTANCE AND THEIR POTENTIAL ROLE IN INFERTILE MALES

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ABSTRACT

INTRODUCTION: The prevalence rate of infertility is about 15% in which the couples are unable to conceive after unprotected intercourse and male factors are considered as one of the contributing factors in the progression of this disease. Oxidative stress is one of the major mediators for male infertility, which cause dysfunction in sperms. It is basically the raised level of oxygen and its related free radicals such as reactive oxygen species (ROS) that triggered the damaging of cellular membrane in intracellular environment. Throughout the process of oxidative stress, the increased production of reactive oxygen species overcomes the level of antioxidants in the body system. Whereas minimum amounts of reactive oxygen species (ROS) are necessary for the physiological process of sperm's functions.

MATERIAL AND METHOD: Patients clinically analyzed with male infertility were included in present study to evaluate the role of oxidative, inflammatory, HSPs, antioxidants and vitamin D in infertile patients.. Fifty infertile patients were taken in the age group of 20-50 years were eligible for inclusion in the study. Informed consent was obtained before been included in this

study. Fiftyclinically apparently healthy individuals were included as controls.In present study various types of inflammatory (MMP-9, IL-6) and oxidative biomarkers (8-OHdG, 4-HNE, SOD, GSH and CAT) were determine through commercially available ELIZA kits but the levels of MDA were estimated through spectrophotometery method.

RESULTS: Present study showed reveal a significant elevation of MDA (3.63±0.65), 8-OHdG (1.26±0.011), Isoprostanes (91.32±12.56) and 4-HNE (20.19±4.56)in subjects as compare to control group (0.95±0.056), (0.03±0.0023), (0.86±0.032) and (3.35±1.25) respectively. According to the results of present study levels of inflammatory biomarkers (IL-6 and TNF-α) also significantly increasedlike IL-6 (17.26±6.35), TNF-α (31.26±4.28) and MMP-9 (199.65±6.35) in subjects as compare to control group (6.35±1.88), (19.35±4.26) and (41.26±4.25) respectively. The levels of antioxidantsSOD (0.06±0.008), GSH (6.58±1.55) and CAT (4.26±1.09) were significantly low in subjects as compare to normal healthy individuals (0.14±0.034), (9.65±2.19), (6.35±1.88) respectively. Additionally, levels of vitamin D (13.28±2.09) disturbed in subjects as compare to control (36.55±4.19).

CONCLUSION: Conclusively, it is evident that vitamin-D exhibits positive correlation with the male fertility by maintaining the levels of sex hormones (LH, FSH, and testosterone), by up-regulating the antioxidant defense (SOD, CAT, and GSH), and down-regulating the oxidative stress (MDA and NO species) induced by ROS and RNS. Therefore, the levels of vitamin D are of prime important in maintaining the sexual health of human.

Keywords: male infertility, oxidative stress, antioxidants, HSPs, spermatogenesis, apoptosis

INTRODUCTION

Infertility has remained a worldwide hazard to human health as it is the most well-known health issue all over the world. The World Health Organization (WHO) defines infertility after having unprotected intercourse for at least 12 months [1]. Statistics show that 15% of all couples in the United States are infertile, and the male factor is 25% [2]. Infertility can be classified as being unable to conceive early in the absence of pregnancy or secondary if it occurs after one or more pregnancies. Nearly 15% of couples trying to make their first conception with a failure and 10% face second- type of infertility. Male and female represent one-third of cases of infertility cases, while the third is due to problems with both partners and unknown causes [3].Male reproductive endocrine system in majorly perform their biological function by

hypothalamus, anterior pituitary gland and testes axis. The male reproductive system is contain on epididymis, ductus deferens and testis. The commonly function of male reproductive system to produce the spermatozoa and secrete the specific hormone for the regulation of reproductive system. Anterior pituitary gland released the two type of hormone like luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the response ofhypothalamic gonadotropin hormone (GnRH) [4]. Both hormones are important for the secretion of testosterone from the testis by cascade of reaction. Different parts of male reproductive tract perform the different function in male reproductive system include testis involved for the production of sperms, epididymis involved in maturation and storage of sperms as well as duct deferens, accessory gland and penis is also involved in transportation of sperm into female tract [5]. Testis are involved for the production of steroid hormone and also associate with spermatogenesis. Steroidogenesis is occur in laydig cells, whereas spermatozoa take place in seminal tubules [6]. Some important micro and macro nutrients required for successful reproduction such as vitamin B12, Zinc, vitamin B9, vitamin E, vitamin A (retinol), folate, vitamin D, nickel, chromium, fatty acid, protein, carnitine, arginine, copper, manganese and selenium [7]. Antioxidants are consist in two form including enzymatic and non-enzymatic which play important role in defense system against the cellular oxidative stress condition. The type of antioxidants such as sodium dismutase (SOD), glutathione systems (peroxidase, transferase, and reductase), catalase (CAT), ascorbic acid and vitamins C and E act as a cofactors. Superoxide anion (O^{2-}) are produce in stress condition can be detoxify into hydrogen peroxide (H_2O_2) by the action of SOD, than H_2O_2 which is also toxic metabolite that converted in to neutralize form like H_2O and O_2 in the presence of catalase. The defense system of glutathione are directly bind to free radicals of ROS and oxidize it in the form of glutathione peroxidase (GSSH). Glutathione peroxidase are involve in the destruction mechanism of hydroxyl radicals and hydrogen peroxide. So the GSH and GSSH are regenerated by the action of GR in biological system. Thus according to this study GR and GPx are involved in the regeneration pathway of glutathione [8]. Deficiency of vitamin D (less than 20ng/mL) and insufficient of vitamin D (20-29ng/mL) are linked with skeletal diseases like osteoporosis, rickets, osteomalacia and

chronic illnesses such as autoimmune diseases like type I diabetes or multiple sclerosis, inflammatory bowel disease, hypertension lead to cardiovascular disturbance, heart failure, coronary artery disease, cancer include breast cancer, colon cancer, neurocognitive disorder including Alzheimer disease [9]. The process of spermatogenesis is a well organized by which the normal diploid cells transfer into sperm which present within seminiferous tubules. The process of sperm production can be divided into three phase like: diploid spermatogenesis proliferation, haploid spermatids occur through spermatocytes meiosis and haploid spermatids differentiation during the process of sperm production. At the final stage of spermatogenesis the round form of spermatids are differentiate into elongated spermatozoa with tail and mid piece. During spermatogenesis the condensed nucleus are formed by the remodeling and compacting of chromatin. After this process the basal compartment of seminiferous epithelium is filled with spermatozoa that released into seminiferous tubules, immature spermatocytes and spermatogonia. The seminiferous tubules is the main part of testis and also site of sperm maturation and production [10]. Several envirmental, genetic and physiological factors have been involved in the abnormal condition of sperm and infertility. It is very important to determinate the reason by sperm play their unusual or poor role in biological system. Mostly, the free radicals cause the oxidative stress and it influence on the sperm characteristics such as seminal volume, density, spermatozoal motility, morphological alteration as well as viability is also change [11].

MATERIALS AND METHODS

PLACE OF WORK

The entire experimental work conducted at the Institute of Molecular Biology and Biotechnology (IMBB). The University of Lahore. All chemical reagents of analytical grades were purchased from Sigma Chemicals Co (St. Louis, MO, USA).

EXPERIMENTAL DESIGN

Total one hundred (100) individuals were included in present study in which fifty (50) samples of diagnosed infertiles patients and other fifty (50) samples are normal healthy individuals. MalePatients with clinically diagnosed infertile and 5.0 ml of venous blood from healthy individuals (negative controls group) and male patients of infertility (positive control group).Patients suffering from diabetes, hypertension, myocardial infarction or any other hepatic, pulmonary pancreatic or renal diseases were

excluded from the present study.Sample Collection. 5.0 ml of venous blood was drawn from healthy individuals (controls) and cancer patients undergoing anticancer therapy.Blood was centrifuged a 4000 rpm for 10 minutes and serum was separated. Blood samples were collected into EDTA tubes.

BIOCHEMICAL ANALYSIS OF SAMPLES

The samples were processed and analyzed for the estimation of SOD (Kakkar method) [12], MDA (Ohkawa method) [13], Catalase (David and Richard method) [14] and GSH (Moron method) [15] by spectrophotometer method. Whereas Isoprostanes F2α (IsoP- F2α) (pg/ml), 8-Hydroxy-2′-deoxyguanosine (8-OHdG pg/ml), 4-Hydroxynonenal (4-HNE µmol/l), Vitamin-D ng/ml, Follicle stimulating hormone (FSH mU/ml), Leutenizing hormone (LH mU/ml), Testosterone (ng/dL), MMP-9 (ng/ml), inflammatory markers such as interleukins (IL-6) (pg/ml) and TNF-α, heat shock proteins (HSPs) (70 and 90) were analyzed by commercially available ELISA kits.

STATISTICAL ANALYSIS

Results have been expressed as mean±SD (Standard Deviation). Statistical significance was determined by one way analysis of variance and spearman correlation (Two Tailed) were used to correlate the different variables. The difference was considered significant at $p<0.05$.

RESULTS

Oxidative stress biomarkers malondialdehyde (MDA), isopeostanes, 8-OHdG, 4-HNE, AOPPs and AGEs were differed significantly (p=0.015, p=0.000, p=0.032, p=0.015, p=0.047 and p=0.031) in subject and control. (3.63±0.65 Vs. 0.95±0.056nmol/ml, 91.32±12.56 Vs. 0.86±0.032 pg/ml, 1.26±0.011 Vs. 0.03±0.0023 pg/ml, 20.19±4.56 Vs. 6.35±2.17 µmol/l, 1.99±0.06 Vs. 0.43±0.09mmol/L and 3.26±1.09 Vs. 1.09±0.22 AU). Decrease level of antioxidants SOD (0.06±0.008 U/ml), GSH (6.58±1.55 µmol/L), GPx (6.35±2.19 µmol/L), GR (3.26±1.88 µmol/L) and CAT (4.26±1.09 U/L) were observed in subjects as compared to healthy individuals SOD (0.14±0.03 U/ml), GSH (9.65±2.19 µmol/L), GPx (8.56±3.23 µmol/L), GR (6.35±2.17 µmol/L) and CAT (6.35±1.88 U/L). Proinflammatory cytokines such as TNF-α and IL-6 were significantly raised (p= 0.014and p=0.017) in male infertility (31.26±4.28 pg/ml and 17.26±6.35 pg/ml) as compared to controls (19.35±4.26 pg/ml and 6.35±1.88). Level of NO, iNOS, MMP-9,

HSP-70, and HSP-90 were increased (29.65±6.53 µmol/L, 19.66±2.19 µmol/L, 199.65±7.16 ng/ml, 29.35±2.18 and 19.65±3.77) significantly in male infertility as compared to healthy controls (14.23±1.89 µmol/L, 9.66±2.19 µmol/L, 41.26±4.25 ng/ml, 3.29±1.08 and 5.19±1.09). Whereas the level of vitamin D (ng/ml), was decreased significantly (p=0.04) in patients as compared to healthy individuals. The level of FSH, LH and testosterone were decreased significantly (p= 0.011, p=0.000 and p=0.023 respectively). As for as the levels of L-Arginine (91.65±6.58 Vs. 229.35±4.26 µmol/L) was decreased significantly (p=0.000) in subjects as compared to controls.

Table 1: Levels Of Circulating Variables of Medical Importance And Their Interplay In Male Infertility

VARIABLES	CONTROL (n=50)	SUBJECT (n=50)	P- VALUE (0.05)
MDA (nmol/ml)	0.95±0.056	3.63±0.65	0.015
Isoprostanes (pg/ml)	0.86±0.032	91.32±12.56	0.000
8-OHdG (pg/ml)	0.03±0.0023	1.26±0.011	0.032
SOD (U/ml)	0.14±0.03	0.06±0.008	0.014
GSH (µmol/L)	9.65±2.19	6.58±1.55	0.026
CAT (U/L)	6.35±1.88	4.26±1.09	0.00
IL-6 (pg/ml)	6.35±1.88	17.26±6.35	0.141
TNF-α (pg/ml)	19.35±4.26	31.26±4.28	0.026
MPP-9 (ng/ml)	41.26±4.25	199.65±7.16	0.031
NO (µmol/L)	14.23±1.89	29.65±6.53	0.047
iNOS (µmol/L)	9.66±2.19	19.66±2.19	0.016
GPx (µmol/L)	8.56±3.23	6.35±2.19	0.000
GRx (µmol/L)	6.35±2.17	3.26±1.88	0.011
4-HNE (µmol/l)	3.35±1.25	20.19±4.56	0.015
Vitamin-D ng/ml	36.55±4.19	13.28±2.09	0.041
HSP-70 (ng/ml)	3.29±1.08	29.35±2.18	0.012
HSP-90 (ng/ml)	5.19±1.09	19.65±3.77	0.000
FSH (mU/ml)	10.25±1.99	6.38±1.11	0.011
LH (mU/ml)	11.25±3.18	8.49±3.29	0.000
Testosterone (ng/dL)	24.55±1.77	17.88±4.16	0.023
L-Arginine (µmol/L)	229.35±4.26	91.65±6.58	0.000

DISCUSSION

Infertility has remained a worldwide hazard to human health as it is the most well-known health issue all over the world. The World Health Organization (WHO) defines infertility after having unprotected intercourse for at least 12 months. Statistics show that 15% of all couples in the United States are infertile, and the male factor is 25%. Infertility is a social and economic issue that is considered to be of public interest to health in Pakistan and many undeveloped countries. Young couples have become helpless, disappointed, and physically assaulted, physiological and emotionally stressed not only from their families, but also from friends and relatives [16]. This is a huge crisis for many couples.

Now with developments in the field of medicine, male's sperm counts are considered the same as the female factors infertility and play an equal role for infertility. Infertility can be classified as being unable to conceive early in the absence of pregnancy or secondary if it occurs after one or more pregnancies. [17]. Spermatozoon was the first type of the cells that show a strong susceptibility with oxidative damage. Whereas there are various situations in which pro-oxidant damaging process will be repair due various mechanism. But in case of spermatozoa, no repair systems were present due to the deficiency of cytoplasmic enzymes. This is the only reason in which spermatozoa can be believed as particular to the susceptibility of oxidative stress [18, 19]. There is a fact that cell membrane is composed of poly unsaturated fatty acid that is prone to oxidative damage by lipid peroxidation. Consequently, the loss of ATP by lipid peroxidation involved to diminish sperm motility, raised the morphological defects of sperm and axonemal damage [20, 21]. However, oxidative stress is a major concern for scientist and clinicians due to this fact that it has a major role in reduced embryonic growth, poor fertilization, pregnancy failure, and cancer in childhood and birth defects [22, 8]. Nearly 15% of couples trying to make their first conception with a failure and 10% face second- type of infertility. It can cause specific or multiple physical and physiological factors, including abnormalities, hormonal imbalances, and genetic disorders. Many biology and genetically engineered organisms have been linked to poor sperm functionality and infertility. Oxidative stress is caused by excessive generation that is primarily regarded as interfering with free radicals and the effectiveness of antioxidant protection. Antioxidants are a type of oxidative stress by extracting or overcoming the reaction of oxygen before they are responsible for the destruction of many biological molecules or preventing cell proliferation against oxidative damage by reducing the radical oxidative reaction of oxidation [23]. Vitamin D have the positive correlation with sperm count, physiologically activity, motility, density and improve the rate of fertility indirectly because the desire levels of vitamin D maintain the Ca+ levels that is very important for the synthesis of sperm in sertoli cells and it also maintain steroidal hormones secretion like LH, FSH. Both hormones have the significantly role for the synthesis of testosterone in leydig cells through the regulation of lipid metabolism and initiate the process of spermatogenesis in sertoli cells

respectively because the levels of testosterone are very important for spermatogenesis [4, 19].

The present study also show that ROS also have the significant negative relationship with male fertility because during OS condition the levels of free radicals are high with antioxidants defense system and lead to cause the damaging process, results in the levels of damaging biomarkers including (MDA, Isoprostanes, 8-OHdG) were high but levels of antioxidants (SOD, GSH, CAT) decrease. During OS condition the mitochondrial membrane also destroy and release the cytochrome C that is the major activator of pro-apoptotic protein and trigger to apoptosis by cascade of reaction which also lead to cause the male infertility [24]. Deficiency of Ca+ also cause the negative relation with protein synthesis and proper folding in ER because during ER stress HSPs were stimulate and involve in the proper folding of protein and it also increase the efflux of Ca+ from ER and influx in mitochondria that is the another factor for the releasing of cytochrome C and trigger to apoptosis. The higher levels of RNS (ONOO-) were trigger to CRH, ACTH and corticosterone axis. The corticosterone hormone is the stress hormone that have the inversely relationship with LH and FSH secretion, in this response the levels of testosterone and spermatogenesis reduce that lead to decrease sperm count, motility, density and volume.Deficiency, of folate and low levels of MTHFR might be indirectly associated with the methylation of DNA that is significantly important in biological system because both of there are have the directly proportional with the levels of SAM and SAH because deficiency of both product have the specific relationship with DNA methylation. Thus, defective methylation might be lead to cause the malignant diseases like abnormal fetal development [2, 8,13].

Free radicals of ROS and RNS were disturbed the cell homeostasis, in this response the levels of corticosterone were increased from the adrenal gland. In the response of corticosterone over production the levels of LH and FSH were disturbed. Indirectly the levels of testosterone and spermatogenesis decreased due to the abnormal production of LH and FSH. LH are regulate the biosynthesis of testosterone by the metabolism of cholesterol in the mitochondria. ONOO- are also directly inhibit the action of aromatase enzyme which is involve in the synthesis of testosterone in mitochondria. FSH directly effect on the sertoli cells and regulate the process of spermatogenesis to maintain the levels of

sperm as well as the process of spermatogenesis also depend at the concentration of testosterone [24, 25].According to current study, it has been reported that infertile patients with mature spermatozoa and elevated levels of reactive oxygen species (ROS) had significantly raised levels of apoptosis as compared to control individuals [26]. The process of apoptosis was stimulated in the results of calcium influx in mitochondria lead to generate the free radicals which was damage the mitochondrial membrane and released the cytochrome c. Additionally, it trigger to apoptotic protein such as cascade caspases reaction to stimulate the process of Apoptosis. The cytochrome C was activate to procaspase 9 to caspase 9 and inhibit the anti-apoptotic protein family like Bcl2 and Bcl-XL atc, After the activation of caspase 9 it further stimulate to caspase 3 and initiate the process of apoptosis. During in this condition the process of degeneration was increased and finally it cause the low production of testosterone and decreased the rate of spermatogenesis [27].

According to the previous work nature given the ability to normalize the any abnormalities that may be caused by exogenous and endogenous. In the response of abnormalities the oxidative stress occurred that generate the free radicals which were effected on the integrity of cell membrane (Aitken et al., 2014). But in the production of free radicals the body has developed the various types of antioxidants which involved to neutralize the effect of free radicals in which including SOD, CAT, GSH, GPx, GPr and vitamins etc [28]. Imbalance between the production of free radicals and antioxidant activity cause oxidative stress and generate the free radicals that damage the mitochondrial membrane and loss its integrity. SOD has ability to inactivate the more toxic reactive oxygen species like O_2^- into less toxic H_2O_2 but this product is also toxic for membrane so body have the other enzyme that also converted the H_2O_2 into water and oxygen. So in overproduction of free radicals the antioxidants defense mechanism not compete to normalize due to decreased the activity of antioxidants [29]. There are some semen characteristics including motility, morphology and concentration that are used to analyze the potential of fertilization in sperm. However, it provides the basic information related to sperm's quality. But these characteristics do not give any information regarding to DNA which is the major component of reproductive system. According to the research work, it has been suggested that

chromatin are involve in oxidative stress which lead to DNA fragmentation and base modification in infertile [30]**.** In human spermatozoa, the chromatin is highly organized and condensed structure which is covered by nucleosomes. The excess amount of NO is involved in severe damage of mitochondria and nuclear DNA by the process of oxidation, nitration and deamination [31]. The output of damage DNA is very unfavorable because by this damaging process of spermatozoon impaired conception rates is increase, defect in offspring and incidence of abortion too increased. Furthermore, the rate and frequency of damage may be high due to break down of single and double stands of DNA, suppress the respiration of mitochondria, synthesis and repair mechanism of DNA, rearrangement of chromosome, bases modification, cross-linking of chromatin as well as microdeletion of chromosome [32]. According to prior studies mitochondrial dysfunction lead to cause oxidative stress and generates the free radicals. This type of radicals were attack on the integrity of sperm DNA and altered the modification of DNA nucleotides, in this results DNA stands are breaks and occur chromatin cross linking. Since spermatozoa have the partial protective defense mechanism against the damaging process. The free radicals are attack on the DNA and released the 8-OHdG that is the DNA damaging stress markers. Due to free radicals the DNA double stands ware damage and it might be closely related with male infertility [33].

CONCLUSION

The levels of LH and FSH might be disturbed due to the excessive production of ONOO- in mitochondrial membrane through the activation of iNOS which lead to cause the oxidative stress in mitochondria and released the stress hormone called corticosterone through CRH-ACTH and corticosterone axis. The higher levels of corticosterone have the negative relationship with the secretion of LH and FSH. Additionally, the higher levels of ONOO- also inhibit the process of lipid metabolism for the synthesis of testosterone in mitochondria. Results in mechanism of spermatogenesis were disturbed and cause the infertility

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