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IMPLICATION OF *8*-HYDROXY-2' -DEOXYGUANOSINE (8-OHdG) AND GONADAL AXIS PROFILE IN AGEING MALES: STUDY FROM BAHAWALPUR-PUNJAB-PAKISTAN

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ABSTRACT

For a wide range of diseases, age itself is a single and important risk factor. During the process of aging, there is gradual and constant accumulation of oxidized proteins take place inside the cells because of reduction in tolerance to physiological stress. Aging is also related to changes in hormonal levels of HPG axis. In aged males, reduction of testicular functions along with a decline in reproductive functions arises year by year. Oxidative stress has a main role in process of aging. The study is carried out on a total of healthy male subjects, ages between 21 years to 100 years. This is a comparative study between younger group and older group. All the samples were collected from Bahawalpur, Pakistan. Physical measurements and blood collection was done according to WHO criteria. To study the changes and effects, serum levels of LH, FSH, SHBG, testosterone and 8OHdG were used.The mean values of Hemoglobin (14.62±1.44 g/L), lymphocytes (32.97±9.16 %), eosinophils (5.13 ±4.28 %) and HCT (46.19 ±4.40 %) were significantly increased in age group (41-60) as compared to other age groups. On the other hand,

the mean values TLC (9.43± 7.35 %), monocytes (11.45 ±1.90 %) and MCV (91.00 ±3.67 %) were higher in older age groups (81-100) as compared to other age groups. Whereas, the mean values of RBC, MCHC and platelets were significantly increased (5.83±0.66 s/L, 32.00 ±1.65% and 295.55±79.70) in the age group (21-40) respectively, as compared to other age groups.The mean value of SHBG was significantly increased (53.25±27.24 nmol/l) in older age group (81-100) as compared to other groups with P-value of (0.02). Apart from that, the mean values of LH (9.79±8.27 mIU/ml) and FSH (18.23±29.43 mIU/ml) and 8-OHdG (1.15±0.058 ng/ml) were also higher in older age group (81-100) as compared to other age groups. On the other hand, the mean value of testosterone was significantly increased (530.60±168.74 mIU/ml) in age group (21-40) as compared to other groups. In the present study, 8-OHdG has been used not only as a biomarker for the estimation of endogenous oxidative DNA damage, but also a major risk factor to cause ageing in males. From the gonadal axis profile, the sex hormone binding globulin (SHBG) has a significant role in the ageing process. Alteration of SHBG has strongly linked with obesity, prostate hypertrophy, sexual activity, cardiovascular disease and HDL-cholesterol. In conclusion, SHBG is a potential physiological biomarker to control aging.

Keywords: Ageing, FHS, LH, SHBG, Total Testosterone, 8-OHdG

INTRODUCTION

Aging is a physiological phenomenon which involves changes in structure and function of the organs of the body along with behavioral, physical, structural modifications. It has also involved changes in energy expenditure and endocrine environment of the body **[1]**. Biological functions at molecular, cellular and tissue levels are influenced by aging. It causes decline of metabolic processes, decreases tolerance to physiological stress and ultimately body becomes vulnerable to disease and death **[2]** Biological aging is related to a decrease in the repairing and reforming power of tissues and organs. This decrease exhibit in the form of reduced physiological reserve under stress termed as homeostasis and the gradual collapse of complex molecular mechanisms that eventually results in different kinds of disorders. All organisms certainly aged with time **[3]**. Aging in men is completely different as compared to women. The endocrinological alteration occurs in both men and women. As men grow old, there is little and gradual decrease in sex hormones particularly, testosterone and dehydroepiendrosterone coupled with the

increase in luteinizing hormone, follicle stimulating hormone and sex hormone binding globulin. While in women the hormonal changes are abrupt and the ovarian cycle suddenly stops. The reduction of testosterone in men with age is mainly due to testicular dysfunction **[4]**. Aged humans experience decrease in sex hormone secretion due to reduction in functional pool of endocrine organs. The reduction in testosterone causes intrinsic aging because it has great association with male body, skin and mood. Aged men may face reduction in sexual drive, intelligence, lean body mass, erectile function, body hair and skin function **[5]**. Sexual hormones secreted by gonads such as testosterone in men and estradiol in women, are main hormones for modulating sexual demarcation and growth. These hormones are also influenced by physiological functions such as metabolism of lipids, carbohydrates, behavior and bone mineral density **[6]**.

It is important to investigate the natural changes in sexual hormones to understand the normal process of aging. Hypothalamic-pituitary-gonadal axis is a dynamic system to adjust sexual hormones **[7]**. The negative feedback loop in the healthy brain controls the hypothalamic pituitary gonadal axis. The gonadotrophin releasing hormone is released by hypothalamus into the median eminence, and then transported through hypophysial portal system to the anterior pituitary where it binds to its receptor and causes the production and secretion of gonadotropins, including luteinizing hormone and follicle stimulating hormone **[8]**. Gonadotropins stimulate the secretion of sex steroids, androgens and estrogen. In men, LH and FSH act onlaydig cells, Sertolicells and germ cells in the testes. Laydig cells are testosterone producing cells and Sertoli cells secrete inhibin B[9]. These hormones send the negative feedback to the anterior pituitary and the hypothalamus. Once testosterone is released in the peripheral circulation, it converts into estradiol by the enzyme aromatase, which influences fertility. Any alteration in these steps has great effect on male fertility **[9]**.

FSH is a glycoprotein made up of alpha and beta subunits. The beta subunit present in FSH, while the alpha subunit is locatedin TSH, hCG, and LH.Gonadotrophin releasing hormone (GnRH) is released in an episodic way. FSH is produced at low pulse frequencies and LH is synthesized at high pulse frequencies. Inhibin B is produced by sertoli cells has a negative feedback effect to stop FSH secretion **[10]**. In children, the testicular volume is increased by the sertoli

cells under the influence of FSH. Another hormone produced by the sertoli cells is the anti-mullerian hormone, which prevents the formation of female sex organs by involuting the mullerian ducts. The normal sperm count and function can be regulated by secretion of FSH and testesterone. According to studies, the normal sperm count and quality of the surviving sperms is lowered by the reduction of FSH levels **[11]**. Along with FSH, the gonadotrophs of anterior pituitary secretes another glycoprotein hormone, called luteinizing hormone. Luteinizing hormone is a constituent of hypothalamic-pituitary-gonadal axis. In this axis, the gonadotropin releasing hormone triggers the secretion of LH and intern inhibited by testosterone in men and estrogen in women. LH in men acts on Leydig cells in testes to stimulate the secretions of testosterone **[12]**. Hypothalamus releases two hormones for the regulation of luteinizing hormone by the pituitary gland. These two hormones are gonadotropin-releasing hormone and kisspeptin, which triggers the release of luteinizing hormone in a pulsatile manner from anterior pituitary. Kisspeptin is the hormone that leads the secretion of gonadotrophin releasing hormone in pulsatile manner from hypophyseal portal blood system which inturn stimulates pulsatile release of LH. Under LH influence, testosterone releases from leydig cells into spermatic vein **[13]**.

Male and female hormones bind with a high affinity binding globulin in the blood called sex hormone-binding globulin (SHBG). Free hormone hypothesis states that sex hormone binding globulin regulates the activity of sex hormones into the target cells by controlling their movement into the tissues **[14]**. In serum, the main sex hormone carrier protein is Sex Hormone Binding Globulin (SHBG). There is approximately 70% of the testosterone hormone in the serum bind with SHBG with high affinity bond in physiological state of body. While, 20- 30 % of testosterone is bound to albumin and 1-2% is found in free form **[15]**. It is proved that many intracellular and biological functions are performed by SHBG that absorbed into the cells. At plasma membrane, steroid hormone signal transduction is also mediated by SHBG Plasma contains free albumin bound and SHBG bound testosterone. SHBG is a glycoprotein present in blood formed by liver cells. Higher the levels of SHBG, greater are the proportion of SHBG bound testosterone **[16]**. A number of hormones including estrogen, insulin, steroid hormone and thyroid hormones, increase the production of SHBG from liver cells.

Androgen in the presence of hypothyroidism and insulin resistance reduce SHBG. With increasing age SHBG level in plasma increase, but it decreases with plasma insulin and triglyceride without any association with age **[17]**.

MATERIAL METHODS

The study was carried out on fifty (n=50) male subjects ages between 21 years to 100 years. The present study has been approved by the institutional review board and research ethical committee of Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore. Before recruitment for data collection, written informed consent is taken from the participants. All the participants were informed that their clinical data will beused for scientific purposes. They were also explained about the method of collection of samples before drawing the sample. All the samples were collected from Bahawalpur division Punjab, Pakistan. The data obtained used for cross- sectional prospective study.

INCLUSION CRITERIA

Male age 18-100 years was included in this study.

EXCLUSION CRITERIA

Following male were excluded from the study; having acute or chronic illness. Any infectious disease that affects the hypothalamic-pituitary-gonadal axiswas excluded from this study. Exogenous supplementations or medications known to effect body growth and metabolism were also not included. Subjects with age less than 18 years and those data and laboratory reports had been lost were not willing to take part in this research.

BLOOD COLLECTION

Blood samples of the subjects included in study were drawn by BD 10cc syringe from cubital vein. A sample of 6ml of blood was drawn and collected in disposable sterile BD vacutainer serum separator tube made in Korea. Care was taken to follow the standard protocol with specific tools and methods for collection of blood samples. Within two hour after collection, the blood samples were centrifuged at 3000 rpm for 5-7 minutes. After centrifugation, the clear serum obtained was placed in eppendrof tubes and stored at -80ºC till the biochemical and hormonal assays were done. Blood complete examination and serum sugar random was performed on the same day. CBC Counter Boule Medonic AB machine manufactured by MERCK SWEDEN was used for blood complete examination. Serum sugar random was tested by using ACCU-CHEK Performa glucometer manufactured by Roche diabetic

care, Indiana United Stat and the results were noted on subject's Performa.

ASSAYS

Sample processing for all assays were conducted in standardized manner and according to recommendations of manufacturer. All assays were carried out by skilled technical personals. For measuring the levels of Follicular stimulating hormone, Latinizing hormone, sex hormone binding globulin (SHBG) and serum total testosterone frozen serum samples were used. These assays were done by competitive chemiluminescence enzyme immunoassay technique on Alinity Ci machine. This is a compact immunoassay system that maximizes throughput utilizing CHEMIFLEX chemiluminescence technology which is manufactured in Chicago United State of America. The kits used for hormonal assays were made by ABBOTT. Lot number of kit used for testosterone was (107850P00) and reference number was (07D68-22), for luteinizing hormone lot number (90017U100) and reference number (7P91-20), for follicular stimulating hormone lot number (91274U100) and reference number (07P49-30) and for Sex Hormone Binding Globulin lot number was (00231L818) and reference number was (09P38-20). All the results of assays entered in excel sheet.

STATISTICAL ANALYSIS

Statistical analysis was performed by SPSS statistics 17.0. The results of all variables were evaluated by using one way ANOVA.

RESULTS

DEMOGRAPHIC PROFILE OF HEALTHY MALE INDIVIDUALS IN VARIOUS AGE GROUPS

In **Table 1**, demographic profile of healthy male individuals divided into different age groups, i.e., Group (21-40), Group (41-60), Group (61-80) and Group (81-100), which explain significant differences in mean values of all age groups. The mean values of age and height were significantly higher (81.0 ± 0.00yrs and 169.51 ±7.58cm) in Group D (81-100) and Group B (41-60) respectively. Meanwhile, the mean values of weight (71.76± 12.41kg) and BMI (25.05± 3.91) were significantly increased in the age Group A (21-40) as compared to other age groups.

HAEMATOLOGICAL PROFILE IN HEALTHY MALE INDIVIDUALS WITH DIFFERENT AGE GROUP

According to **Table 2**, the hematological profile (Hemoglobin, TLC, neutrophils, lymphocytes, monocytes, eosinophils, RBC, WBC, HCT, MCV, MCHC and platelets) was measured in

different age groups. The mean values of Hemoglobin (14.62 ±1.44 g/L), lymphocytes (32.97±9.16 %), eosinophils (5.13 ±4.28 %) and HCT (46.19 ±4.40 %) were significantly increased in age group (41-60) as compared to other age groups. On the other hand, the mean values TLC (9.43± 7.35 %), monocytes (11.45 ±1.90 %) and MCV (91.00 ±3.67 %) were higher in older age groups (81-100) as compared to other age groups. Whereas, the mean values of RBC, MCHC and platelets were significantly increased (5.83±0.66 s/L, 32.00 ±1.65% and 295.55±79.70) in the age group (21-40) respectively, as compared to other age groups. The mean value of neutrophils (49.62± 25.88 %) was significantly high in age group (61-80) as compared to other age groups.

GONADAL AXIS PROFILE OF HEALTHY MALE INDIVIDUALS WITH DIFFERENT AGE GROUPS

According to table 03, the hormonal profile of healthy male individuals divided into different age groups. The mean value of SHBG was significantly increased (53.25±27.24nmol/l) in older age group (81-100) as compared to other groups with P-value of (0.02). Apart from that, the mean values of LH (9.79±8.27 mIU/ml) and FSH (18.23±29.43 mIU/ml) and 8-OHdG (1.15±0.058 ng/ml) were also higher in older age group (81-100) as compared to other age groups. On the other hand, the mean value of testosterone was significantly increased (530.60±168.74 mIU/ml) in age group (21-40) as compared to other groups.

DISCUSSION

The great body of evidence confirmed the increased number of diseases related to age and their association with mitochondrial abnormalities. One can prevent or improve the process of aging; while advanced therapeutic applications can also be made to prevent age related degenerative diseases by properly understanding the oxidative stress response and mitochondrial dynamics **[18]**. Reactive oxygen species (ROS), oxidative stress and mitochondrial dysfunction are the three main elements that involve in the aging process. The importance of mitochondria in the process of aging is demonstrated by a factor that is involved in the process of aging because of their ability to cause oxidative damages to DNA, proteins and lipids. Various free radicals such as superoxide anion (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2) are the main chemical elements produced by reactive oxygen species by reacting with different cellular molecules under stressful conditions **[19]**. It has been believed that free proton and ROS are highly reactive molecules with great

affinity for normal cellular constituents. Oxidative stress primarily attacks on mitochondria, because ROS are produced as a by-product during aerobic respiration of mitochondria. The stress conditions that affect mitochondria play an important role in aging. Inside the cell various anabolic and catabolic processes in addition with mitochondrial respiration are the main source of generation of intrinsic ROS **[20]**. Among all cellular organelles mitochondria is the main sourceof generation of ROS and it is produced considerably during electron transport chain when electrons leaks directly to oxygen that in turn produces free radicals which further converted to non-radical derivative like H_2O_2. These radicals are short lived because within the cells enzymes like catalase, glutathione peroxidase and other antioxidants deactivates these radicals and removes them from the cell **[21]**. Growth factors, cytokines responsible for inflammatory reactions, ultra violet radiations, hyperaxia and drugs used in chemotherapy are the main causes which are responsible for the production of reactive oxygen species. After their production lipids, proteins and nucleic acids within the cells are the main target of these species **[22]**. ROS cause oxidative damage of cells by reacting with these molecules. ROS can also cause genomic instability by reacting with DNA. Reactions of ROS with DNA causes oxidation of DNA bases and breaking of DNA strands. One of the most formed and well recognized product of DNA damage caused by ROS is 8-hydroxy 2-deoxyguanosine and it is also used as a biomarker of DNA damage **[23]**. More than fifty years back Denham Harman suggests a theory called free radical theory, according to it gathering of harmful effects of oxidative damage by ROS results in ageing. It also suggests that the lifespan of an organism is defined by the capability of organism to fight and handle with the cellular damage caused by ROS. Enhanced released of ROS by mitochondria and increased mitochondrial DNA contents of 8-hydroxy 2-deoxyguanosine in aged tissues proved this hypothesis **[24]**. Many studies also suggests that in process of aging gradual accumulation of oxidative DNA damage plays an important role. It is also stated in many studies that aging itself is a cause of increased production of ROS and oxidative damage **[25]**.

Oxidative stress is commonly defined as an imbalance among the production of oxidants and defensive mechanism of antioxidants against oxidants. In normal physiological conditions the production of reactive oxygen species is controlled in

highly organized manner. During cellular respiration and as a result of reaction of cytochrome p450 in mitochondria, components of mitochondrial electron chain, generation of these oxygen species occurs **[26]**. In signaling pathways some of these species act as second messenger for example H_2O_2. Among these oxygen species $HO^{\cdot}$ are more reactive and near their production side they react rapidly with any target. ROS can negatively regulates the cellular functions by unfolding and impairment of proteins **[27]**. Phospholipids, proteins and nucleic acids are the biomolecules which are the main target of oxidants. When hydroxyl group reacts with nucleic acid, 8-hydroxy 2-deoxyguanosine and 8-hydroxyguanosine are formed. So these two products are used as biomarkers of nucleic acid oxidation. It is noted that with increasing age the production of thesebiomarkers increases **[28]**. For the regulation of physiological sperm functions for example capacitation, acrosomal reaction and sperm motility, the reactive oxygen species are required which are produced during natural cellular metabolism and perform these functions effectively when produced in normal physiological amounts. These species can cause sperm damage and ultimately male infertility as shown by many studies and it is observed that in 30-80% males this is the cause of infertility **[29]**. Because these species contain oxygen molecules with unpaired electrons so they have the ability to react with other intracellular molecules and can endanger cell survival if produced in extra amount. By damaging sperm membrane or by damaging sperm DNA, ROS can cause male infertility **[30]**. Because of insufficient cell repair system and due to little amount of cytoplasmic contents inadequate antioxidant defense system, spermatozoa are more susceptible oxidative stress (Sulagna Dutta 2019). In spermatozoa of infertile male the levels of 8-hydroxy 2-deoxyguanosine is found elevated **[31]**. Several studies explains that nucleotide guanosine converts into 8=hydroxyl 2-deoxyguanosine if the oxidative damage occurs at the level of DNA. This molecule causes transition mutagenesis in the daughter cell during replication process after fertilization by binding with thyamine instead of guanine. 8-hydroxy 2-deoxyguanosine is also used as a biomarker of DNA damage **[32]**. Male fertility is also effected by advancing age. Researchers observed that oxidative stress is also related with increasing age. Epididymal spermatozoa of aging males are more prone to oxidative stress damage. So in the reproductive system of aged males elevated

oxidants can lead to defaulted sperms which ultimately interfere men's fertility **[33]**. It is also observed that the major cause of sperm DNA damage is related to oxidative stress. It is proved by the elevated levels of 8-hydroxy 2-deoxyguanosine, product released during DNA damage, in seminal fluid of infertile males supposed to have oxidative stress. Studies suggests that age effects male fertility because older males have elevated levels of reactive oxygen species which attack sperms to alter their quality or because of reduced antioxidants in semen to prevent sperm from oxidants **[34-47]**.

Table 1: Demographic Profile of Healthy Male Individuals With Various Age Group

Parameters	Groups	Mean±Std	P-value
Age	21-40	29.93 ±4.73	0.000
	41-60	46.48± 5.40	
	61-80	67.43 ±3.85	
	81-100	81.0 ±0.00	
Weight (kg)	21-40	71.76± 12.41	0.063
	41-60	66.50± 10.23	
	61-80	64.82±10.67	
	81-100	65.00 ±0.00	
Height (cm)	21-40	169.18± 6.64	0.904
	41-60	169.51 ±7.58	
	61-80	169.00±8.22	
	81-100	164.00 ±0.00	
BMI	21-40	25.05± 3.91	0.034
	41-60	23.01± 3.34	
	61-80	22.76± 3.27	
	81-100	24.00±0.00	

Table 2: Haematological Profile In Healthy Male Individuals With Various Age Group

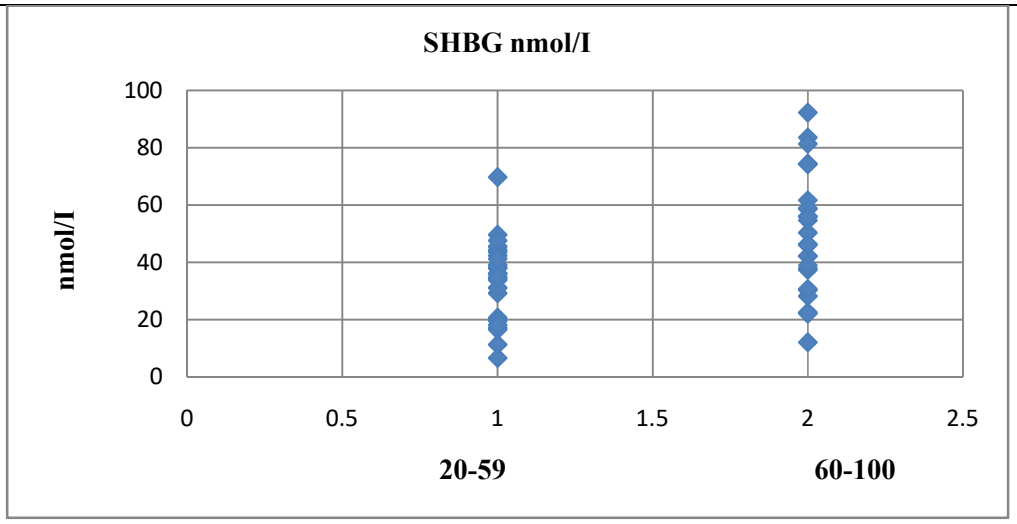
Parameters	Groups	Mean±Std	P-value
HB g/L	21-40	14.32±1.26	0.000
	41-60	14.62 ±1.44	
	61-80	12.44± 3.46	
	81-100	11.75± 0.63	
TLC %	21-40	7.28± 2.04	0.011
	41-60	9.25±3.03	
	61-80	8.46± 2.89	
	81-100	9.43± 7.35	
Neutrophils %	21-40	44.78± 20.95	0.752
	41-60	46.62± 22.39	

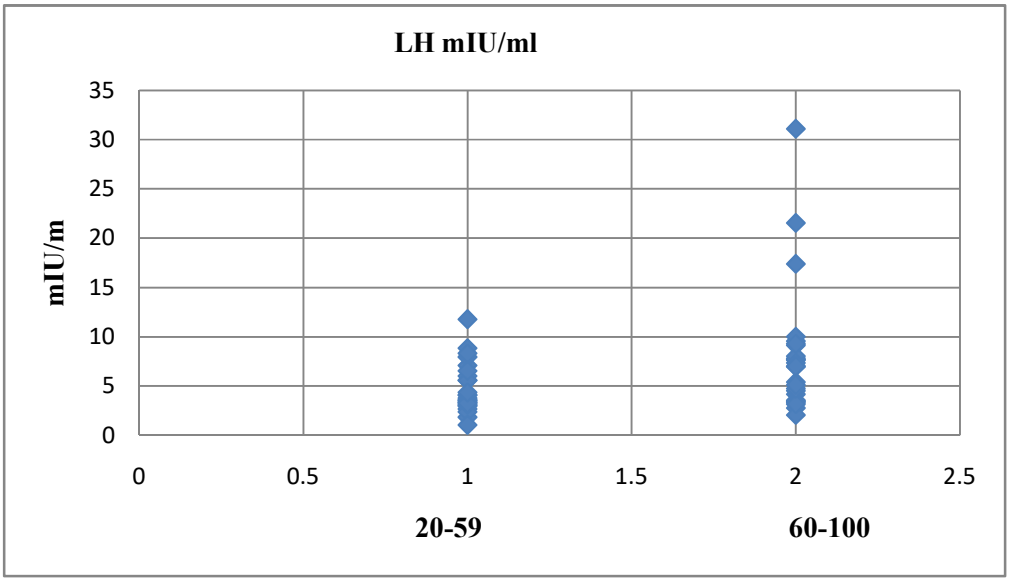
	61-80	49.62± 25.88	
	81-100	35.70± 50.80	
Lymphocytes %	21-40	32.41±7.81	0.004
	41-60	32.97±9.16	
	61-80	26.59± 7.75	
	81-100	20.35± 8.13	
Monocytes %	21-40	7.28 ±2.63	0.118
	41-60	6.75 ±2.35	
	61-80	7.57 ±3.20	
	81-100	11.45 ±1.90	
Eosinophils %	21-40	5.41 ±4.07	0.411
	41-60	5.13 ±4.28	
	61-80	3.97 ±4.21	
	81-100	2.35± 2.05	
RBC s/L	21-40	5.83±0.66	0.014
	41-60	5.74± 0.68	
	61-80	5.29± 1.24	
	81-100	4.41± 0.48	
HCT	21-40	44.81 ±3.91	0.002
	41-60	46.19 ±4.40	
	61-80	40.73 ±8.35	
	81-100	40.10 ±2.82	
MCV %	21-40	78.02 ±11.27	0.205
	41-60	81.09 ±8.69	
	61-80	78.06± 12.11	
	81-100	91.00 ±3.67	
MCHC %	21-40	32.00 ±1.65	0.054
	41-60	31.67 ±1.22	
	61-80	30.94 ±2.80	
	81-100	29.35±0.49	
Platelets	21-40	295.55±79.70	0.543
	41-60	294.48 ±79.65	
	61-80	293.70 ±73.28	
	81-100	213.50 ±4.94	

Table 03: Gonadal Axis Profile Of Healthy Male Individuals With Different Age Groups

Parameters	Groups	Mean±Std	P-value
SHBG(nmol/l)	21-40	35.76±14.80	0.020
	41-60	30.78±13.04	
	61-80	47.41±16.51	
	81-100	53.25±27.24	
LH(mIU/ml)	21-40	4.29±1.99	0.084
	41-60	5.40±3.14	
	61-80	7.18±5.36	
	81-100	9.79±8.27	
FSH(mIU/ml)	21-40	3.40±1.41	1.54
	41-60	7.29±5.38	

	61-80	**12.88.18.33**	
	81-100	**18.23±29.43**	
Testosterone (mIU/ml)	**21-40**	**530.60±168.74**	**0.14**
	41-60	**428.69±184.88**	
	61-80	**445.83±181.56**	
	81-100	**254.85±246.51**	
8-OHdG (ng/ml)	**21-40**	**0.78±0.021**	**0.011**
	41-60	**0.99±0.032**	
	61-80	**1.025±0.015**	
	81-100	**1.15±0.058**	





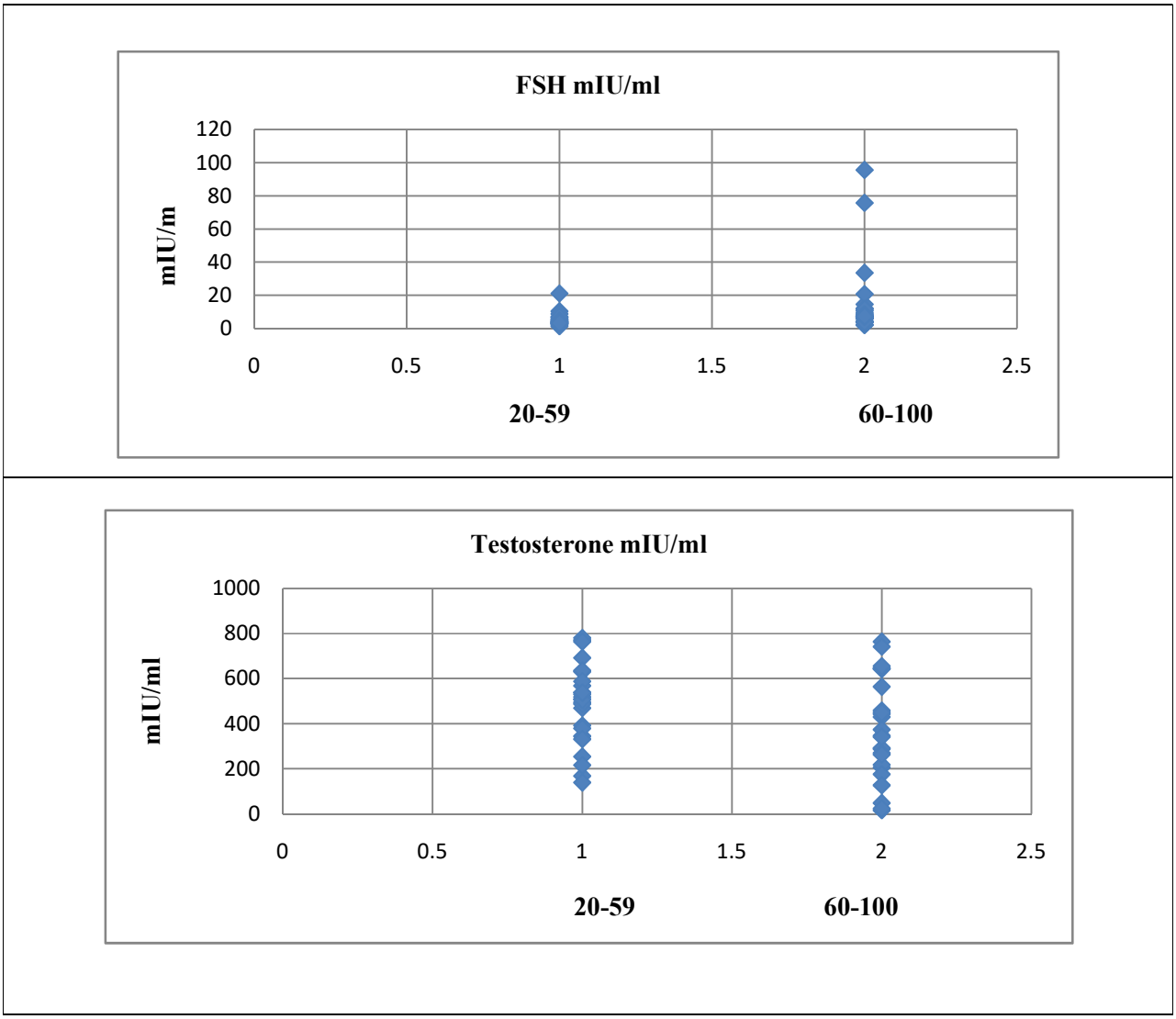


Figure 1: Levels of Gonadal Axis Profile In Healthy Male Individuals With Different Age Groups

CONCLUSION

The aim of the study is to examine the age related natural alterations in hypothalamic-pituitary-gonadal axis in men and the effect of oxidative stress on male aging and male infertility. To study these changes and effects serum levels of testosterone, LH, FSH, SHBG and serum levels of DNA stress marker 8-hydroxy 2-deoxyguanosine were used. It has been concluded that, oxidative damage occurs during ageing process which can be measured by analysing various products. Sulfhydryl and carbonyl residues are the products of protein oxidation, while lipid oxidation tends to produce malondialdehyde.

The oxidative DNA is damaged leading to production of 8-OHdG. In the present study, 8-OHdG has been used not only as a biomarker for the estimation of endogenous oxidative DNA damage, but also a major risk factor to cause ageing in males. From the gonadal axis profile, the sex hormone binding globulin (SHBG) has a significant role in the ageing process. Alteration of SHBG has strongly linked with obesity, prostate hypertrophy, sexual activity, cardiovascular disease and HDL-cholesterol. In conclusion, SHBG is a potential physiological biomarker to control aging.

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CONFLICT OF INTEREST

Authors declare no conflict of interests.

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