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ORIGINAL ARTICLE

Hypoxia Induced Infertility in Males: Role of Transcription Factor Hypoxia Inducible Factor -1 Alpha

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ABSTRACT

Aim: To assess concentrations of HIF-1 α and correlation between HIF-1 α in healthy and sub fertile males' population in our study.

Methodology: In this study we investigated the concentrations of Hypoxia Inducible Factor 1-α (pg/mL) in the seminal plasma of healthy fertile males and sub fertile males using Enzyme-Linked ImmunoSorbent Assay (**ELISA**). Our study population (n=54) consisted of healthy fertile controls (n=18), sub fertile males without varicocele (n=22) and sub fertile males with varicocele.

Result: Our results showed significantly ($p < 0.001$) elevated levels of HIF 1-α in both sub fertile groups ($p < 0.001$) as compared to healthy fertile group. A significant ($r = 0.975$, $p<0.001$) positive correlation was noticed between the concentrations of Caspase 3, an apoptotic marker and HIF 1-α in the healthy fertile and sub fertile groups.

Conclusions: Our study results suggest that hypoxia induced apoptosis maybe an important factor in causing testicular dysfunction in sub fertile males. In conclusion HIF 1-α is an important hypoxic factor that can be used to predict apoptosis in testes. HIF 1-α can be used as a clinical marker that can facilitate scientists to predict the degree of apoptosis in spermatozoa.

Keywords: HIF-1, Subfertile, Apoptotic marker, Spermatozoa

INTRODUCTION

Infertility is a serious clinical problem affecting couples psychologically, socially and medically1 (Makker et al. 2009). Subfertility is described as any type of decreased fertility after a prolonged time of non-conception in a couple who is trying to conceive but fails to do so 2 (Gnoth et al. 2005). World Health Organization, (WHO), describes infertility as the inability of a couple to achieve pregnancy during 12 months of regular sexual intercourse. 13–20% of couples in the world are affected by infertility. Male factor is responsible in 25% to 50% of couple infertility cases[3]. According to WHO almost half of infertility cases have male factor to be responsible for the problem, where an alteration in sperm count, morphology or motility is observed in at least one sample of the two, taken two weeks apart[4](Agarwal et al. 2014). Etiology of male infertility can be described as anatomical malformation, for example semen outflow obstruction, varicocele or ejaculation disorders, a great number of cases are due to deranged spermatogenesis and disturbed sperm function. In spite of scientific progress and high sophistication in diagnostic procedures, the pathogenesis and etiology of male infertility is not known and are discussed under heading of idiopathic infertility. Disorders of male fertility are related to different factors of environment like chemical toxicity, heat, heavy metals and pesticides, or electromagnetic radiation[5]. Smoking, obesity, alcohol abuse, urogenital trauma, chronic stress, and inflammation in the male reproductive system are linked with male subfertility[6]

Oxidative Stress: A disproportion among raised reactive oxygen species (ROS) levels and lower levels of total anti-oxidant capacity concludes in a condition referred as oxidative stress which has deleterious effects on spermatozoa. Reactive oxygen species are free radicals which are highly reactive substances having damaging effects on many cellular organelles7 (Agarwal et al. 2006). Two principle sources of free radicals in semen are leukocytes and spermatozoa; leukocytes being more responsible for producing free radicals[8] (Tremellen 2008). Males with idiopathic infertility usually possess significantly raised ROS levels and decreased anti-oxidant as compared to healthy fertile males. High ROS levels are found in 25-40% of infertile males (Cocuzza et al. 2007). Oxidative stress results when ROS levels exceed the naturally occurring 9 anti-oxidant as defense in the body causing cellular damage. Oxidative stress may be due to exogenous sources as lifestyle factor, smoking, alcoholism, obesity, environmental pollution by heavy metals along with medical problems as spinal cord injury, genito-urinary tract infections and varicocele[10]. ROS has both detrimental and positive effects on sperm activity. During its journey through epididymis, sperm improves its motility progressively and their ability to pass through female genital tract to fertilize the ovum by facing different physiological changes called 'capacitation'. Superoxide anion plays main role in capacitation and acrosomal reaction. Studies show male germ cells can generate ROS during their differentiation[11]. Well controlled and low ROS levels have quite beneficial effects in sperm physiological processes as capacitation, hyper activation, signaling process and acrosomal reaction, which are mandatory for fertilization[12]. Raised reactive oxygen species damage the internal and external membranes of mitochondria resulting in the activation of caspases and induction of apoptotic sequence in response to emancipation of cytochrome C protein from the power house of the cell[13]. Antioxidants maintain the levels of reactive oxygen species within normal range in the semen. These scavenge the free radicals of oxygen and prevent damage to developing spermatozoa. They include glutathione peroxidase (GPX), catalase and superoxide dismutase (SOD) along with non-enzymatic antioxidant molecules for example vitamin E, vitamin C, pyruvate, carnitine and glutathione[14].

Varicocele: 15% of adult males suffer from subclinical or clinical varicocele. The incidence of varicocele in infertile males is 40%[15]. Varicocele is found in 15-20% of general population, while 25-40% in males with primary infertility and 70-95% in secondary infertility cases16 (Jarow 2001). Many hypotheses have been postulated regarding the mechanism of varicocele in male infertility such as endocrine and testicular paracrine imbalance, hyperthermia, hypoxia[17] and backward flow of adrenal blood but none of them could explain the mechanism completely[18].

Hypoxia is stated as low levels of oxygen content and pressure within the environment, tissue of the organisms, decreased oxygen exchanged or impaired supply of oxygen by vascular system[19]. Hypoxia leads to increased pulmonary ventilation, increased vascularization done by vascular endothelial growth factor (VEGF) which is mediated by HIF-1 α. Males exposed to hypoxic conditions often have low fertility rate and

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altered levels of sperm count, morphology and motility. Testicular oxygen distribution is determined by microvasculature of testis, diffusion of oxygen in testicular interstitium and seminiferous tubules (Reyes et al. 2012) Chronic hypoxia expresses different genes which cause non oxidative ATP generation and increased capillary irrigation by enhanced blood flow (Agarwal et al. 2006). HIF-1 α is ubiquitously expressed transcription factor, a master regulator of many genes which are responsible for oxygen homeostasis in mammals. Degradation of HIF-1 α is governed by the proteasomal enzymes during normoxia. In testis HIF-1-α is expressed in Leydig cells (Palladino et al. 2011). HIF-1-α has bimodal effect on cell physiology as it activates either cell survival or cell death depending upon type of cell and duration of oxygen debt (Piret et al. 2002).

METHODOLGY

The study was approved by Institutional Review Board of University of Lahore and Fatima Memorial Hospital Lahore, Pakistan. An informed consent was taken from participants in the study after explaining the whole purpose and procedure of study. Our study population consisted of a total of 54 males, further sub-divided into three groups: the healthy fertile males as controls from general population having child, sub-fertile males without varicocele and sub-fertile males with varicocele, while the sub-fertile group was classified after taking a thorough history, clinical examination and investigations of males married for more than one year coming to the Andrology Clinic, Fatima Memorial Hospital, Lahore for their fertility evaluation and treatment for failure in conception.

Subjects were given all the necessary instructions about collection of semen sample and obtained at collection center of Andrology Lab Department of Urology, Fatima Memorial Hospital Lahore, Pakistan after an abstinence period of 7-8 days by masturbation in a sterile environment. A manual semen analysis was performed after liquefaction at 37°C according to WHO (2010), Criteria. Both macroscopic and microscopic analysis was performed. The inclusion criteria was males between ages of 20-50 years, married for more than a year, married men as controls, who have normal healthy female factor who have their own children or children from a previous marriage or were declared healthy by a gynecologist after complete physical examination and investigation whereas exclusion criteria was azoospermic males and female factor seeking treatment for infertility from gynecologist. The quantitative sandwich ELISA method for HIF-1α assessment was done by Thermo Fischer- Catalog no. EHIF1A as manufacture instruction.

RESULT

Our study population included 3 groups of males categorized as healthy fertile controls and sub fertile groups. Sub fertile groups were further classified as sub fertile males without varicocele and sub fertile males with varicocele based upon the ultra-sonographic findings.

Age (years): Mean ± SEM age of fertile males was 32 ± 1.291, whereas the mean ± SEM age of varicocele negative males was 32.727 ± 1.289 while that of varicocele positive males was 33.928 ± 1.814.

Height (cm): Mean ± SEM height of fertile males was 167.15 ± 3.905, whereas the mean ± SEM height of varicocele negative males was 167.954 ± 1.602 while that of varicocele positive males was 166.964 ± 1.893.

Weight (kg): Mean ± SEM weight of fertile males was 72.027 ± 3.046, the mean ± SEM weight of varicocele negative males was 72.727 ± 1.870 and that of varicocele positive males was 75.785±2.447.

BMI (kg/m2): Mean ± SEM BMI of fertile males was 26.143 ± 0.922, the mean ± SEM BMI of varicocele negative males was 25.716 ± 0.317 and that of varicocele positive males was 27.198±0.782

Semen Characteristics: Mean ± SEM value of semen volume (mL) in healthy controls was 3.28±0.37, in sub fertile males without varicocele 2.65±0.11 and in sub fertile males with varicocele 2.47± 0.23. There was no significant difference observed between healthy controls and varicocele negative group (p1 = 0.335) and between healthy controls and varicocele positive group (p2 = 0.214).

Mean ± SEM value of sperm concentration (million per mL) in healthy controls was 67.22±4.80, in sub fertile males without varicocele 32.38 ± 0.52 and in sub fertile males with varicocele 15.66 ± 3.52. Significant decrease was observed in sperm concentration between healthy controls and varicocele negative group (p1 < 0.001***) and between healthy controls and varicocele positive group (p2 < 0.001***).

Mean±SEM value of motility(%) in healthy controls was 81.11± 2.50, in sub fertile males without varicocele 47.81±6.33 and in sub fertile males with varicocele 37.45±4.86. Significant decrease was observed between healthy controls and varicocele negative group (p1 < 0.001***) and between healthy controls and varicocele positive group (p2 < 0.001***).

Mean±SEM value of morphology (%) in healthy controls was 41.5±1.46, in sub fertile males without varicocele 20.06 ± 0.93 and in sub fertile males with varicocele 11.90±2.13. Significant decrease was observed between healthy controls and varicocele negative group (p1 <0.001***) and between healthy controls and varicocele positive group (p2 < 0.001***).

Mean ± SEM value of leukocyte count (millions per mL) in healthy controls was 0.46 ± 0.03, in sub fertile males without varicocele 1.94 ± 0.62 and in sub fertile males with varicocele 0.85 ± 0.23. No significant difference was seen between healthy controls and varicocele negative group (p1 < 0.095) and between healthy controls and varicocele positive group (p2 < 0.357).

Table 1: Mean ± SEM age, height, body weight and BMI of healthy fertile controls, varicocele negative and varicocele positive sub fertile patients

Parameter	Healthy Fertile Controls	Varicocele Negative	Varicocele Positive
Age (years)	32 ± 1.291	32.727 ± 1.289	33.928 ± 1.814
Height (cm)	167.15 ± 3.905	167.954 ± 1.602	166.964 ± 1.893
Weight (kg)	72.027 ± 3.046	72.727 ± 1.870	75.785 ± 2.447
BMI (kg/m^2)	26.143 ± 0.922	25.716 ± 0.317	27.198 ± 0.782

Table2: Mean ± SEM of semen volume, concentration, motility, morphology and leukocyte count of healthy fertile controls and sub fertile male subjects with and without varicocele

Semen Characteristics	Controls (18)	VAR-(22)	VAR +(14)	P1	P2
Volume (mL)	3.28 ± 0.37	2.65 ± 0.11	2.47 ± 0.23	0.335	0.214
Concentration (million/mL)	67.22 ± 4.80	32.38 ± 0.52	15.66 ± 3.52	**0.001*** **	**0.001*** **
Motility (%)	81.11 ± 2.50	47.81 ± 6.33	37.45 ± 4.86	**0.001*** **	**0.001*** **
WHO normal morphology (%)	41.5 ± 1.46	20.06 ± 0.93	11.90 ± 2.13	**0.001*** **	**0.001*** **
Leukocyte Count(million/mL)	0.46 ± 0.03	1.94 ± 0.62	0.85 ± 0.23	0.095	0.357

Values in parenthesis represent the number of subjects

P1 = P between controls and var – group, P2 = P between controls and var + group VAR - denotes varicocele negative, VAR + denotes varicocele positive.

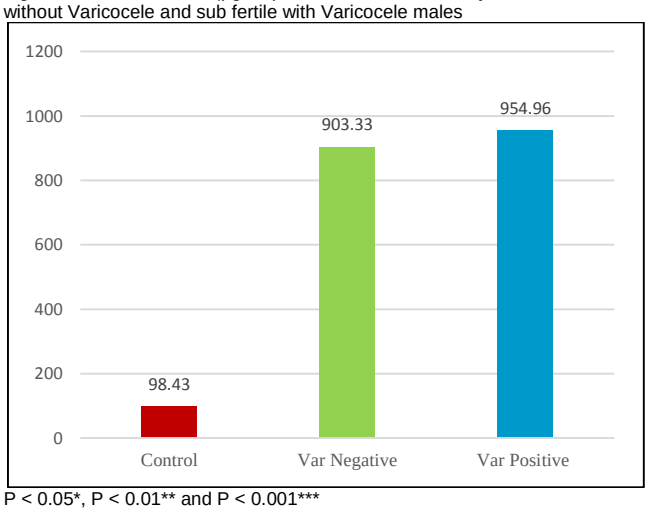
P <0.05*, P <0.01** and P< 0.001***

Table 3: Mean ± SEM of HIF-1 α of healthy fertile controls and sub fertile male subjects with and without varicocele

Biomarker	Controls(18)	VAR -(22)	VAR + (14)	P1	P2
HIF- 1 α (pg/mL)	98.43 ± 2.97	903.33 ± 18.62	954.96 ± 22.24	**0.001*** **	**0.001*** **

Values in parenthesis represent the number of subjects
P1 = P between controls and var – group, P2 = P between controls and var + group VAR - denotes varicocele negative, VAR + denotes varicocele positive.
P < 0.05*, P < 0.01** and P <0.001***

Figure 1: Mean HIF 1 α (pg/mL) concentration in healthy fertile, sub fertile without Varicocele and sub fertile with Varicocele males



P < 0.05*, P < 0.01** and P < 0.001***

HIF 1-α (pg/mL): Mean±SEM value of hypoxia inducible factor 1 α (pg/mL) in healthy controls was 98.43±2.97, in sub fertile males without varicocele 903.33±18.62 and in sub fertile males with varicocele 954.96±22.24. Significant increase was observed between healthy controls and varicocele negative group (p1 < 0.001***) and between healthy controls and varicocele positive group (p2 < 0.001***).

DISCUSSION

Our study groups were also categorized on the basis of semen characteristics, physical examination and ultra sound reports for diagnosis of varicocele. Healthy controls in our study had all the seminal parameters within normal range including semen volume, sperm concentration, motility, morphology and leukocyte count as was also seen by20 (Aleisa 2013). No significant difference was observed in the semen volume of healthy controls and varicocele negative group (p = 0.335) and between healthy controls and sub fertile males with varicocele (p=0.241). In contrast to our findings, a rise in the seminal volume in the ejaculate of sub fertile males was reported 21 (Benoff et al. 2004).Sperm concentration in semen was significantly decreased in sub fertile group as compared to healthy controls (p < 0.001***) which is similar to the results shown by22 (Feki et al. 2009). Significant decrease in sperm motility was observed in sub fertile without and with varicocele in comparison to healthy controls (p < 0.001***). Our results match with those found by23 (Jensen et al. 2002). Sperm morphology also showed a significant decrease in normal morphology from healthy controls to sub fertile males with and without varicocele (p < 0.001***). Our results coincide with the results of [24].

Varicocele which represents as convoluted and excessively dilated veins of spermatic cord is observed in 15% of normal healthy males and 40% of males seeking treatment for infertility (Wang et al. 2010). Varicocele has always been a controversial topic as far as etiology of male infertility is concerned. Varicocele has significant part in infertility[25].

Our present study found significantly (p < 0.001***) elevated levels of HIF 1 α in the sub fertile varicocele males and sub fertile without varicocele patients as compared to control group i.e. healthy males. This finding in our study suggests that intra-testicular hypoxia in varicocele males maybe a result of elevated levels of HIF 1 α and this intra-testicular hypoxic environment might be a contributing factor in causing hypo spermatogenesis resulting in male infertility.An increase was observed in Caspase 3 (µg/mL) levels in sub fertile varicocele patients and sub fertile non varicocele patients in comparison to healthy fertile group.

CONCLUSION

Although varicocele is usually diagnosed in men with infertility but the actual cause of ailment in these specific patients is multi-factorial. Data from the recent studies shed new light on the understanding of pathophysiology of varicocele with new diagnostic approach from these findings. These factors include androgen deprivation, oxidative stress, heat stress etc. Percutaneous aspiration of testicular fluid and seminal fluid may be utilized in future to identify apoptosis in males associated with heat stress. Some men with varicocele may present with damaged sperm parameters based upon Kruger's strict criteria.

A new era of andrology is rising in the field of medicine including application of clinical knowledge based upon genetic and molecular information. It is anticipated that varicocele patients will benefit from new protocols and treatment options from molecular/genetic knowledge. Last of all, current literature strongly supports a hypothesis about varicocele as being a cofactor with molecular/genetic problems in varicocele men. These may lead to infertility in combination and determine the potential and possibility for reversibility.

Conflicts of interest: Authors do not have any finical or personal conflicts exist during the course of study.

Authors Contribution: AZ: conceived, designed, manuscript writing and data analysis, **ZIS**, did data collection and analysis, review and final approval, **AA,SN, SR and FH** review the project, analysis and final manscript

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