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The Association of Reactive Oxygen Species with High-Risk Human Papilloma Virus Infection and Some Sperm Parameters in a Samples of Iraqi Idiopathic Infertile Males

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Abstract

Background Male infertility is a prevalent issue affecting couples worldwide. Human Papillomavirus (HPV) is known to cause cervical cancer in women, but its association with male infertility is gaining attention. High-risk HPV (HR-HPV) infection has been linked to oxidative stress (OS), an imbalance of reactive oxygen species (ROS) in the body. This OS may impact sperm function and fertility in men.

Objective To find out the association of ROS with HR-HPV infection and sperm parameters with group of infertile Iraqi patients with unknown causes.

Methods A case-control study of 68 seminal fluid samples (34 fertile and 34 infertile males) submitted to real time polymerase chain reaction (PCR) for detection of HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and the plasma of seminal fluid samples were tested for ROS by using enzyme-linked immunosorbent assay (ELISA).

Results The outcomes showed high statistically significant association of ROS level with sperm parameters and HR-HPV infection (P <0.001), twenty-nine (42.6%) of infertile men were with high level of ROS. Twenty-five (36.8%) of asthenozoospermia samples were had high ROS levels, while only 4 (5.9%) samples had low level of ROS. The abnormal sperm morphology was found in 34 (50%) of samples, 29 (42.6%) and 5 (7.4%) of them were with high and low levels of ROS, respectively. Furthermore, 17 of positive HR-HPV samples were showed 16 (23.5%) with high level of ROS.

Conclusion HR-HPV infection may cause elevated level of ROS, and this may potentially affect sperm parameters in idiopathic infertile males.

Keywords High risk human papillomavirus, idiopathic infertility, sperm parameter, reactive oxygen species

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List of abbreviations: HR-HPV = High-risk human papillomavirus, ROS = Reactive oxygen species, ELISA = Enzyme linked immunosorbent assay

Introduction

Human Papillomavirus (HPV) is the most common sexually transmitted virus worldwide, and over 180 genotypes of the virus have been reported [(1)]. HPV infection in men has been considered to be transient, with a main clinical presentation being warts in the external genitals. However, the presence of HPV has also been documented in the testicles, epididymis, vas deferens, prostate, urethra, and semen [(2)]. HPV virions can bind to different sites including ones on the sperm head,

probably due to glycosaminoglycans [3] or on the sperm surface, due to other soluble factors of similar chemical structure [4]. HPV infection in males was often considered to be transient and not associated with significant health issues, so its presence in semen has not been extensively studied. However, research suggested the possible role between HPV and male infertility. Several studies indicated that HPV can be found in the seminal fluid of men with unexplained infertility, suggesting a possible role of the virus in this condition [5-8].

Infertility is the inability of a sexually active couple, not using contraception, to conceive within one year [9]. Male infertility factors play a role in 45-50% of these cases, with approximately 7% of men worldwide being diagnosed as infertile [10]. Recent efforts have been directed towards understanding and addressing the unknown causes of male subfertility both in clinical settings and research laboratories. One significant factor identified at the cellular level is oxidative stress (OS) [11]. OS is characterized by an imbalance between reactive oxygen species (ROS) and antioxidants in the body. It refers to the excessive production of ROS, which overwhelms the body's antioxidant defense system [12]. ROS are highly reactive molecules derived from oxygen that have one or more unpaired electrons [13].

In the right balance, ROS play a crucial role in sperm functions, contributing to capacitation, the acrosomal reaction, and the fusion of sperm and oocyte [13,14]. However, when an imbalance occurs, it can lead to pathological conditions. The imbalance of ROS in the body can be caused by various factors, such as excessive production of ROS from internal or external sources, a lack of sufficient antioxidants, reduced activity or production of antioxidant enzymes, or a combination of these factors [15]. Several examples of conditions or behaviors that may lead to an overproduction of ROS include infections, varicocele (enlargement of a vein in the scrotum), smoking, alcohol and drug use, as well as exposure to environmental pollutants [12]. ROS-induced damage to spermatozoa and reduced levels of total antioxidant capacity in seminal fluid may be responsible for 30-80% of male infertility cases [16,17].

Spermatozoa's plasma membrane contains high amounts of vulnerable polyunsaturated fatty acids, making them susceptible to lipid peroxidation and damage caused by ROS [18,19]. OS can also negatively impact the integrity of sperm DNA, leading to elevated levels of DNA fragmentation [20]. Additionally, OS is associated with increased apoptosis (cell death) [21-23]. These pathological pathways are linked to various adverse clinical outcomes, including damaged germ cells, impaired fertilization, increased miscarriages, and a higher risk of health issues in the next generation [24,25].

OS induced modifications and impairments in sperm function, such as motility, morphology and DNA integrity, can have significant implications for male fertility. OS can lead to an increase in tyrosine nitration and S-glutathionylation activity, which in turn affects sperm motility and capacitation ability [26]. Another factor contributing to decreased sperm motility is ROS-induced defects in the process of adenosine triphosphate (ATP) utilization or in the contractile apparatus of the flagellum [27]. Sperm motility is considered one of the earliest and most sensitive indicators of DNA damage [28].

Excess production of ROS, specifically nitric oxide (NO), hydrogen peroxide (H_2O_2)), and 8-isoprostanes (8-EPI), has been directly associated with morphological abnormalities, including teratozoospermia [29]. Studies have shown that these ROS are negatively correlated with the percentage of normal sperms and sperm count [30]. Men with conditions such as asthenozoospermia, asthenoteratozoospermia, and oligoasthenoteratozoospermia were found to have significantly lower levels of seminal plasma catalase activity and total antioxidant capacity (TAC), while having significantly higher levels of free 8-EPI, indicating an increased risk

of oxidative stress. Furthermore, the activity of plasma catalase and TAC showed positive associations with sperm motility and morphology, indicating their protective role against oxidative damage. On the other hand, higher levels of free 8-EPI were negatively correlated with sperm motility and morphology, indicating their adverse impact on sperm quality [29].

Human sperm cells express different aquaporins (AQPs) are a family of channel proteins that facilitate the transport of water and small solutes across biological membranes, which are localized both in the plasma membrane and in intracellular structures. Besides cell volume regulation and end stage of cytoplasm removal during sperm maturation, the role of AQPs extends also to ROS elimination. Moreover, OS has been shown to inhibit AQP-mediated H_2O_2 permeability. A decrease in AQPs functionality is related to a decrease in sperm cells number and motility [31]. A decrease in AQPs functionality is related to a decrease in sperm cells number and motility. Pellavio *et al*. (2020), found heavily reduced water permeability of sperm cells in normospermic samples, and L1 protein and AQP8 interaction was also found [31].

This research sought to investigate the association among ROS, High-Risk Human Papillomavirus (HR-HPV), and male infertility to uncover potential diagnostic and therapeutic approaches for couples facing fertility issues. Unraveling this correlation holds promise for enhancing the management of reproductive health concerns.

Methods

Patients and controls

This is a case control study was conducted in Baquba, the center of Diyala Province, to determine the HPV infection and its impact on sperm parameters. A Total of 68 semen samples were enrolled. These were collected as follows; 34 samples as case (infertile males) and 34 as control (fertile males) from private clinic and Infertility Unit in Al-batoul Teaching Hospital. Each male produced the semen by masturbation after an abstinence of 2-7 days and semen samples were collected in a sterile plastic container confirmed to be non-toxic for spermatozoa. A routine semen analysis was performed within 1 h of collection, according to the methods described by the World Health Organization (WHO) [32].

Ethical approval

The study protocol was approved by Institutional Review Board (IRB) in Al-Nahrain University College of medicine at (7 November 2021) under the code number 63.

Viral DNA extraction

The DNA extraction from seminal fluid was done using the DNA-Sorb-A (Sacace Biotechnologies- Italy. REF K-1-1/A/100) nucleic acid extraction kit following the manufacturer instructions.

HPV DNA amplification and typing

HPV Genotypes 14 Real-TM Quant kit (Sacace Biotechnologies-Italy, REF V67-100FRT) used for detection and genotyping of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) following the manufacturer instructions. This kit based on multiplex real time amplification of 4 polymerase chain reaction (PCR) tubes for each sample. HPV Genotypes 14 Real-TM Quant detects the most widespread 14 genotypes of HPV.

Detection of ROS

Human ROS detected by enzyme linked immunosorbent assay (ELISA) kit to assay ROS levels in seminal fluid. This ELISA kit uses Sandwich-ELISA as the method. The Microelisa strip plate provided in this kit has been pre-coated with an antibody specific to ROS. Standards or samples are added to the appropriate Microelisa strip plate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)-conjugated antibody specific for ROS is added to each Microelisa strip plate well and incubated. Free

components are washed away. The tetramethylbenzidine (TMB) substrate solution is added to each well. Only those wells that contain ROS and HRP conjugated ROS antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of ROS. The concentration of ROS in the samples calculated by comparing the OD of the samples to the standard curve.

Patients' inclusion criteria

Men with primary or secondary infertility, infertile patients were considered those subjects with altered sperm parameters, at least 2 years of unprotected sexual intercourse without conception, and normal female partners (tubal, uterine, cervical abnormalities, and ovarian disorders will be excluded).

Patients' exclusion criteria:

Patients with hormonal problem, history of cryptorchidism, testicular trauma, or post-mumps orchitis. Varicocele and seminal infections will be excluded, respectively, by testicular Doppler ultrasound and microbiological sperm culture.

Statistical analysis

Data are presented as numbers and percentages for categorical variables and compared by using Chi square test ($\chi 2$), while continuous data were tested for normality distribution and accordingly expressed as median and range in addition to mean±standard deviation, and compared using Mann Whitney U test. ROC curve was used to select the optimal cut-off value for ROS test. The significance was ($P < 0.05$) by using SPSS version 25.

Results

Table (1) shows the two study groups; 34 infertile and 34 fertile males were equally distributed (34, 50%) for each. Regarding to the age groups the highest numbers of participants were in the age of (≤30) years old (40, 58.8%). Two parameters of sperm namely sperm motility and sperm morphology were employed to assess the quality of sperm samples of participants. These parameters were determined according to (WHO, 2010) [32], for the sperm morphology, 34 (50%) and 34 (50%) of participants were with normal and abnormal sperm morphology, respectively. Furthermore, 34 (50%), 29 (42%), and 5 (7.4%) of participants were progressive actively motile sperms, asthenozoospermia and necrozoospermia, respectively.

Table 1. Clinico-pathological characteristics of study groups

Variables		N (%)
Study Groups	Infertile males	34 (50.0)
	Fertile males	34 (50.0)
Age Categories (years)	≤ 30 years	40 (58.8)
	> 30 years	28 (41.2)
Sperms Motility	Progressive active motile	34 (50.0)
	Asthenozoospermia	29 (42.6)
	Necrozoospermia	5 (7.4)
Sperms Morphology	Normal	34 (50.0)
	Abnormal	34 (50.0)

Table (2) shows the difference in median and range of ROS (as the data were not normally distributed) in the study groups (fertile and infertile males), which were significantly higher in infertile males compared to fertile males 253.55 (110.8-1646.32), 78.86 (25.26-99.54) respectively.

Table 2. Comparison of reactive oxygen species between two study groups by Mann Whitney U test

Oxidative Stress	N	Cases	Controls	P value
Mean±SD	34	494.14±490.64	69.33±24.07	<0.001
Median (Range)	34	253.55 (110.8-1646.32)	78.86 (25.26-99.54)	

According to the receiver operating characteristic (ROC) curve and area under the curve (AUC) data, the cut-off value of ROS at highly matched specificity and sensitivity, was 105.17 pg/ml (Table 3) (Figure 1).

Table 3. ROC curve and AUC data of reactive oxygen species in study groups

Variables	ROS (pg/ml)
Area Under the Curve	1.000
Optimal cut-off value	105.17
Sensitivity	100%
Specificity	100%

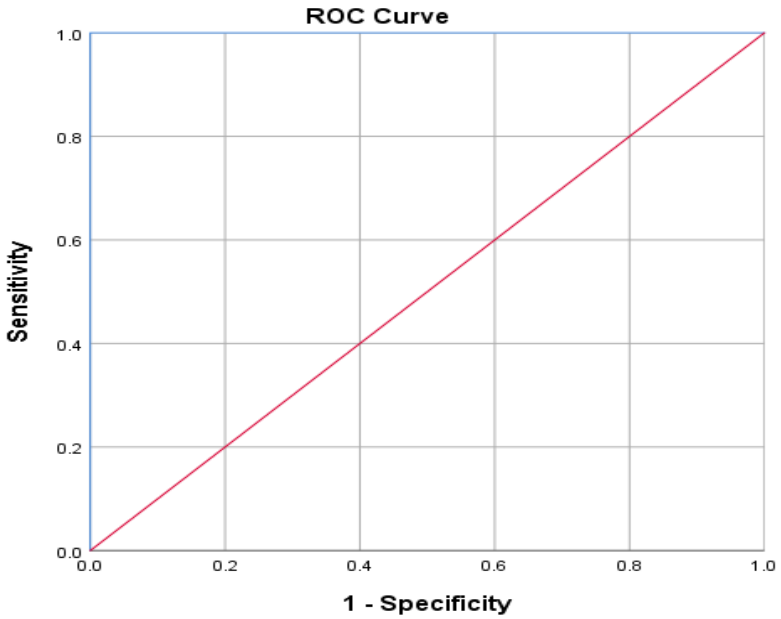

Figure 1. ROC curve of ROS cut-off value of reactive oxygen species in study groups

The results in table (4) shows highly significant differences (P<0.001) between study groups with ROS, as 36 (52.9%) samples were had low level of ROS; 5(7.4%) were in an infertile (case) male and 31 (45.6%) were in fertile (control) males. Meanwhile; 32 (47.1%) were had high level of ROS, 29 (42.6%) of them were in an infertile male and 3 (4.4%) were in fertile males.

Table (5) estimated highly significant association (P<0.001) of ROS with sperm motility. As 34 (50%) of progressive active motile males were distributed with 31 (45.6%) samples with low ROS levels and 3 (4.4%) samples were with high levels of ROS, while in asthenozoospermia 29 (42.6%), 25 (36.8%) were with high ROS levels and only 4 (5.9%) were with low levels of ROS. Five (7.4%) samples had necrozoospermia as 4 (5.9%) with high levels and only one with low levels of ROS.

Table 4. Association of reactive oxygen species with study groups

Study Groups		*ROS		Total
		Low <105.17 (pg/ml)	High ≥105.17 (pg/ml)	
Fertile men	Count	31	3	34
	% of Total	45.6%	4.4%	50.0%
Infertile men	Count	5	29	34
	% of Total	7.4%	42.6%	50.0%
Total	Count	36	32	68
	% of Total	52.9%	47.1%	100.0%

* P value <0.001

Table 5. Association of reactive oxygen species with sperm motility

Sperm motility		*ROS		Total
		Low <105.17 (pg/ml)	High ≥105.17 (pg/ml)	
Progressive active motile	Count	31	3	34
	% of Total	45.6%	4.4%	50.0%
Asthenozoospermia	Count	4	25	29
	% of Total	5.9%	36.8%	42.6%
Necrozoospermia	Count	1	4	5
	% of Total	1.5%	5.9%	7.4%
Total	Count	36	32	68
	% of Total	52.9%	47.1%	100.0%

* P value <0.001

Table (6) estimated highly significant association (P <0.001) of ROS with sperm morphology. As 34 (50%) of Normal sperm shape were distributed with 31 (45.6%) samples with low ROS levels and 3 (4.4%) samples were with high levels of ROS, while in Abnormal sperm shape 34 (50%), 29 (42.6%) were with high ROS levels and 5 (7.4%) were with low levels of ROS.

Table (7) revealed highly significant association (P <0.001) of ROS with HR-HPVs infection. Seventeen of positive HR-HPVs samples were showed 16 (23.5%) with high level of ROS. On the other hand, 51 (75%) samples were tested

as negative for HR-HPVs and the majority of them 35 (51%) with low level of ROS.

Table 6. Association of reactive oxygen species with sperm morphology

Morphology		*ROS		Total
		Low <105.17 (pg/ml)	High ≥105.17 (pg/ml)	
Normal	Count	31	3	34
	% of Total	45.6%	4.4%	50.0%
Abnormal	Count	5	29	34
	% of Total	7.4%	42.6%	50.0%
Total	Count	36	32	68
	% of Total	52.9%	47.1%	100.0%

* P value <0.001

Table 7. Association of reactive oxygen species with HR-HPVs infections

HR-HPVs PCR test		*ROS		Total
		Low <105.17 (pg/ml)	High ≥105.17 (pg/ml)	
Positive	Count	1	16	17
	% of Total	1.5%	23.5%	25.0%
Negative	Count	35	16	51
	% of Total	51.5%	23.5%	75.0%
Total	Count	36	32	68
	% of Total	52.9%	47.1%	100.0%

* P value <0.001

The result in table (8) showed highly significance association (P <0.001) between ROS and HR-HPVs co-infections. All ten (14.7%) samples of HR-HPVs single infection were with high level of ROS, while six (8.8%) samples of double infection showed five (7.4%) of them with high level of ROS and one sample of triple infection of HR-HPV was with high level of ROS.

Table 8. Association of reactive oxygen species with HPV co-infections

HPV test Co-infection		*ROS		Total
		Low <105.17 (pg/ml)	High ≥105.17 (pg/ml)	
Single infection	Count	0	10	10
	% of Total	0.0%	14.7%	14.7%
Double infection	Count	1	5	6
	% of Total	1.5%	7.4%	8.8%
Triple infection	Count	0	1	1
	% of Total	0.0%	1.5%	1.5%
Negative	Count	35	16	51
	% of Total	51.5%	23.5%	75.0%
Total	Count	36	32	68
	% of Total	52.9%	47.1%	100.0%

* P value <0.001

Discussion

This study estimated highly significant association of ROS with sperm motility. Also, multiple studies have demonstrated sperm motility can be negatively impacted by elevated ROS levels [33-36]. OS can lead to an increase in tyrosine nitration and S-glutathionylation activity, which in turn affects sperm motility and capacitation ability [18]. Another factor contributing to decreased sperm motility is ROS-induced defects in the process of ATP utilization or in the contractile apparatus of the flagellum [27].

Regarding morphological parameters; the present study demonstrated highly significant association of ROS with sperm morphology. This finding is consistent with some of the literature data [37,38]. Excess production of ROS, specifically NO, H_2O_2, and 8-EPI, has been directly associated with morphological abnormalities, including teratozoospermia [29]. There have been studies that have demonstrated that despite the negative impact on sperm quality and function, elevated ROS levels had no negative effect on in vitro fertilization or intracytoplasmic sperm injection (ICSI) rates [39,40]. Yeung et al. [40] proposed that ROS might have a positive effect on fertilization.

This study revealed highly significant association of ROS with HR-HPVs infection. HPV infection of sperms affects both AQPs expression and functionality.

In conclusions, the current study showed that HR-HPVs infection may cause elevated ROS levels and this may potentially affect sperm parameters in idiopathic infertile males. However, further research is needed to fully comprehend this relationship and its potential implications for clinical practice.

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Author contribution

Dawood: performed the laboratory works, statistical analysis and wrote the draft of this paper as part of his PhD thesis. Dr. Al-Shuwaikh designed, interpreted and supervised this work. Dr. Al-Kawaz supervised and contributed to sample collection and clinical aspects of this work. The final version of this manuscript was read, arranged and approved by all the authors.

Conflict of interest

There is no conflict of interest.

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