Alteration of Some Sperm Proteins, Anti-oxidants, and Prostate-specific Antigen in the Seminal Plasma of Bacteriospermic Infertile Males

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Authors' contributions

This work was carried out in collaboration among all authors. Author EASB designed the study and wrote the protocol. Author IE performed the statistical analysis. Author AMA wrote the first draft of the manuscript and managed the literature searches. Authors HB and ABC managed the analyses of the study. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aim: To investigate the alteration of some sperm proteins, anti-oxidants, and prostate-specific antigen in the seminal plasma of bacteriospermic infertile males.
Study Design: The study is a case-control design to investigate the semen parameters and some sperm proteins in infertile males with bacteriospermia attending urology or fertility clinics in Port Harcourt, Rivers State, Nigeria.
Place and Duration of Study: The study was carried out in the Department of Medical Laboratory Science, Rivers State University, Port Harcourt. However, some of the Laboratory investigations were done in the Chemical Pathology Unit and Medical Microbiology Unit of the University of Port Harcourt Teaching Hospital, Port Harcourt. The Study was between the period of September 2019 and Feb. 2022.
Methodology: A total 193 subjects were recruited of which 76 were fertile without bacterial infection, 24 were fertile with bacterial infection, 37 infertile without bacterial infection and 56 infertile with bacterial infections. Infertile males were further classified as teratozoospermic, asthenoteratozoospermic, and oligoasthenoteratozoospermic. HSP70, PKA, MDA, TAC, and GPX

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(in seminal plasma), PSA, and testosterone (in serum) were analyzed using ELISA while TAC was done using spectrophotometric methods. Microbial culture was done to isolate and identify bacteria in semen. Results obtained were analyzed using GraphPad Prism and SPSS.

Results: Culture results indicated that 52% of normospermic subjects had no semen infection at the time of the investigation. On the other hand, infertile male subjects without semen infections had 18.7%, while infertile males with semen infections were seen to be 29.2%. Likewise, subjects with bacteriospermia as scanty growth had 12.5%, 9.3% moderate, and 7.3% heavy growth. Significantly higher values of HSP*70*, PKA, PSA, and MDA were seen in infertile males with bacteriospermia compared with control and infertile males without infection while significantly lower values were seen in TAC, GPX, OPN, and testosterone were observed in the infertile males with bacteriospermia compared with control and infertile males without infection.

Conclusion: Bacteriospermia significantly increased lipid peroxidation cum oxidative stress leading to altered physiological sperm proteins in the seminal plasma. These alterations could also be responsible for the poor sperm qualities observed in the infertile subjects.

1. INTRODUCTION

Accurate assessment of male factor infertility is quite difficult due to multi-factorial etiologies or risks. Male factor infertility in Nigeria accounts for up to 50% of all infertility cases [1]. According to the international committee for Monitoring Assisted Reproductive Technology and the World Health Organization (WHO), infertility is a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse [2]. In African society, infertility comes with psychological stress and trauma due to the strong emphasis on childbearing [2,3].

Etiologies such as reduced number of spermatozoa (oligozoospermia), reduced sperm motility (asthenozoospermia), reduced sperm vitality (necrozoospermia), and abnormal sperm morphology (teratozoospermia) or any combination of these have been reported to induce male infertility [4,5,6]. More so, several factors such as genetic factors (deletion, inversion, mutation, aneuploidy, congenital abnormality (cryptorchidism) and translocation), environmental factors (exposure to chemical contaminants which are endocrine disruptors), oxidative stress, lifestyle (age, obesity, smoking, consumption of alcohol, use of recreational drugs) and bacterial infections have also been implicated in male infertility [1,2,7-9].

Bacterial infection of semen (bacteriospermia) has been observed as one of the major risks of infertility in couples. In males, asymptomatic or symptomatic bacterial infections in the seminal plasma have been reported to affect sperm parameters, viability, and motility resulting in male factor infertility. Several micro-organisms such as mumps virus -paramyxovirus (causative agent of mumps orchitis and related disorders), *Chlamydia Trachomatis, Streptococcus* species*, Staphylococcus aureus, Escherichia coli, Pseudomonas* sp, etc, have been implicated in male-factor infertility [10,11]. However, in this study, our interest is on bacterial infection in seminal plasma which has also been reported to contribute greatly to reducing sperm count, motility, speed, quality, and play a critical role in inducing morphological derangement such as asthenospermia and teratospermia [1,11].

In recent times, male factor infertility has been strongly linked with some sperm protein expression, reduction, or absence. Therefore in this work, the influence of bacterial infections on some sperm proteins associated with male infertility such as osteopontin (OPN), Heat Shock Protein 70 (HSP*70*), Protein Kinase A (PKA), and anti-oxidants such as glutathione peroxidase (GPX) and total anti-oxidant capacity (TAC) in seminal plasma will be our focus. These proteins have been reported to be involved in acrosomic reactions, maintenance of sperm–oocyte interaction, promote cell survival against environmental stress such as increased scrotal temperature, spermatogenesis, enhanced motility of sperm cells, and the elimination of reactive oxygen species (ROS) in the seminal plasma.

2. MATERIALS AND METHODS

2.1 Experimental Design and Subject Classification

The study is a case-control design to investigate the semen parameters and sperm proteins in

infertile males with bacteriospermia attending urology or fertility clinics in Port Harcourt, Rivers State. A total of 276 males indicated interest to participate in the study of which 193 male subjects were recruited. Of the 193 subjects recruited, 76 were fertile without bacteriospermia, 24 were fertile with bacteriospermia, 37 infertile without bacteriospermia and 56 were infertile with bacteriospermia. Infertile males with or without bacterispermia were further re-grouped as teratozoospermic (TZS), asthenoteratozoospermic (ATS), and oligoasthenoteratozoospermic (OAT). Those with normal morphology of ≤30%, progressive motility ≥50% were grouped as teratozoospermic while those with normal morphology of ≤30%, and progressive motility ≤50% were grouped as asthenoteratozoospermic. Finally, infertile males with a total count of <19 x 10^6 cells/ml, normal morphology of <30% with progressive motility of <50% were grouped as oligoasthenoteratozoospermic. Also, their semen leukocyte concentration was <1 x 10^6/ml of ejaculate.

2.2 Sample Size Determination

The sample size was determined using the equation as described by Claran & Biswas [12] with precision or absolute error of 5% and 95% confidence interval using the equation Sample size =Z^2 P(1-P)/d^2. A total of 93 was calculated as the sample size for infertile males with the expected proportion of male-factor infertility of 6.5% as reported by Sule et al. [13].

2.3 Inclusion and Exclusion Criteria

A well-structured questionnaire was issued to all the participants to obtain demographic information, medical history, and lifestyle after obtaining their consents.

2.3.1 Inclusion criteria

Subjects used in this study are those attending urology/fertility clinics, without a history of hypertension, cardiovascular disorders, osteoporosis, diabetes mellitus, or smoking. Omron digital blood pressure kit (Omron healthcare co., Ltd, Japan) was used to check blood pressure while the glucose oxidase method was used to determine their diabetic status. The control subjects were males that have established pregnancies within the period of this study with a total sperm count of ≥ 20 x 10^6 cells/ml, normal sperm morphology, and

without bacterial infection. The cases were infertile males that have failed to establish pregnancies after two years, been clinically diagnosed with infertility, with or without bacterial infections, and with a total sperm count of <19 x 10^6 cells/ml.

2.3.2 Exclusion criteria

Those excluded from the study include subjects that did not give consent, had semen leukocytes (pus cells)>1 x 10^6/ml, and semen specimens collected without masturbation. More so, subjects that did not abstain for at least 72 hours (3 days) and lifestyles like smoking and alcohol consumption were also excluded. In addition, those with a history of hypertension, cardiovascular disorders, immunological disorders, osteoporosis, diabetes mellitus, cryptorchidism, varicocoele, obstructive seminal ducts, and prostate-specific antigen (PSA) of >10ng/ml were also excluded.

2.4 Specimens Sampling and Preparation

2.4.1 Blood and semen collection

Following ethical approval, blood and semen samples were collected from eligible subjects. All semen samples were collected into universal sterile plastic containers by masturbation after an abstinence period of 72 hours. In addition, 5ml of venous blood samples were collected in fasting state from all the participants into plain bottles.

2.4.2 Sample preparation

Semen samples collected into universal sterile plastic containers were placed on the bench at room temperature of 25^0C for 40 minutes to liquefaction before analyses (culture, macroscopic, and microscopic) were performed within 1 hr of collection. Subsequently, the liquefied semen samples were centrifuged at 4500rpm for 15 minutes as described by Conquer et al. [14]. The supernatant seminal plasma was then carefully aliquoted into Eppendorf tubes and frozen at -70^0C pending biochemical analysis. In terms of the blood samples, the whole blood samples were collected into plain bottles and allowed to stand for 30 minutes to clot. Clotted blood samples were retracted and spun at 3500rpm for 10 minutes to obtain serum for the estimation of testosterone and total prostate-specific antigen.

2.5 Equipment and Reagents

Equipment used includes Olympus binocular microscope, Neubauer hemocytometer, and Auto Elisa P microplate reader (Labtech). Other equipment used includes a merment oven, universal bucket centrifuge model 320, Omron digital blood pressure kit (Omron healthcare co., Ltd, Japan), Haier thermocool deep freezer (China), pipettes, microscopic glass slides and cover slips. Reagents used include Osteopontin (OSP), Heat shock protein *70* (HSP70), Protein-kinase A (PKA), Malondialdehyde (MDA), Prostate-Specific Antigen (PSA), and testosterone (Testo) enzyme-linked immunosorbent assay (ELISA) kits purchased from Bioassay Technology Laboratory (Shangai, China). Glutathione peroxidase (GPX) ELISA kits and glucose oxidase reagent, as well as total antioxidant capacity (TAC) spectrophotometric kits, were purchased from Elabscience (Houston, Texas, USA) and Fortress Diagnostics (Antrim, United Kingdom) respectively.

2.6 Analysis of Sperm Parameters and Biochemical Parameters in Seminal Plasma and Serum

2.6.1 Determination of Sperm Volume, pH, and Viscosity

The semen ejaculatory volume was determined using a graduated glass Pasteur pipette while the pH of the semen was determined using a combi-9 strip, dipped into the semen, and matched against the combi-9 colour chart. The viscosity of the liquefied semen specimens was determined by observing the slimy thread formed when a glass rod is placed on the semen specimen and withdrawn from the specimen. The length of the slimy threads not less than 0.5cm but not exceeding 2cm was considered normal as described by Vasan [15].

2.6.2 Determination of Sperm Concentration

The sperm concentration (count) was manually determined using a Neubauer cell counter. The semen specimens were mixed and diluted 1 in 20 using semen diluting fluid consisting of sodium bicarbonate, formalin, and distilled water. The cell counting chamber was charged with the diluted specimen and allowed to settle for 15 minutes. The sperm count was made in the four secondary squares and five tertiary squares of the central secondary squares as described by WHO [16] and Ochei [17]. The total sperm concentration per ml was estimated by multiplying the number of sperm cells counted x dilution factor (20) x multiplication factor (50,000).

2.6.3 Microscopic Evaluation of the Sperm Cells

A wet preparation was made by placing a drop of semen with a Pasteur pipette from a well-mixed sample on a clean grease-free slide and covering it with a coverslip. The wet preparation microscopic fields were examined under high-powered objectives using x40 objective lens to estimate and quantitate the presence of pus cells and epithelial cells and also to determine the motility, morphology, and viability of the sperm cells as described by WHO [16]. At least a hundred cells were examined at a magnification of x1000, to determine the percentage of sperm cell motility as described by WHO [16]. In addition, the morphology of the sperm cells was determined using the methylene blue-eosin staining technique after incubation at 25^0C with trypsin for 10minutes.

2.6.4 Determination of Bacterial Quality and Identification

The presence and degree of bacterial load in the semen specimens were determined using the culture technique. The culture media used were McConkey and blood agar. Loopful semen specimens were collected under sterile conditions and inoculated into the culture plates and incubated at 37^0C for 48 hours. After the period of incubation, bacteria were isolated and identified biochemically.

2.7 Assay of Biochemical Parameters

Heat Shock Protein *70* (HSP*70*), Osteopontin (OPN) and Protein Kinase A (PKA), Glutathione Peroxidase (GPX), testosterone, and Prostate-Specific Antigen (PSA) were determined as described by Engvall & Perlmann [18] ELISA Quantitative Assay Method while Malondialdehyde (MDA) was determined using ELISA method as described by Moron et al. [19]. More so, Total Anti-oxidant Capacity (TAC) was done using Colorimetric Method as Described by Mencci et al. [20].

2.8 Statistical Analysis

Statistical packages used for data analysis were Graphpad Prism 8.0.2 (California, USA) and

Statistical Package for Social Science (SPSS) version 23.0. Descriptive statistics used were mean and standard deviation while inferential statistics used include students't-test, Chi-Square, and One-Way ANOVA with Post-Hoc done with Tukey's multiple comparison analysis tests. Results were presented as Mean±Standard Deviation. Statistical significance was set at $P=.05$.

3. RESULTS

3.1 Results on Prevalence and Microscopic Characteristics of Semen/Sperm Cells

Culture results indicated that 52% of normospermic subjects had no semen infection at the time of the investigation. However, during the interview, 39.6% reported never having a semen infection while 12.5% reported that been previously had semen infection and were treated accordingly. On the other hand, infertile male subjects without semen infections had 18.7%, while infertile males with semen infections were seen to be 29.2%. The Chi-square results also indicate no significant difference for both the various age intervals and infertile males. Likewise, subjects without any microbial infection had a prevalence of 70.8% those with scanty growth had 12.5%, moderate had 9.3% and heavy growth had 7.3%. The Chi-square results also

indicate no significant difference. The comparison of their respective % distribution also indicated no significant differences at p<0.05 (Table 1).

3.2 Results of Sperm Parameters and total sperm count in Infertile Males with Microbial Growth

When infertile males with varying degrees (scanty, moderate, and heavy) of bacterial infections were investigated, the results also indicated no significant differences in age, sperm volume, and pH. Although, significantly lower values were seen in % motility and %Active motility when control was compared with infertile males without infection, those with scanty, moderate, and heavy bacterial infections. Also, significantly lower values were seen in %normal sperm morphology of infertile males with moderate and heavy bacterial infections compared with normospermic, infertile males without infection and infertile males with scanty infections. In addition, significantly lower values of testosterone were seen in infertile males without bacterial infection, scanty, moderate, and heavy growth. However, infertile male subjects without infections had significantly higher values of testosterone compared to infertile scanty, moderate, and heavy bacterial infections at p<0.05 (Table 2).

Table 1. Microscopic Characteristics of Semen/Sperm Cells

Subjects/Specimens Characteristics	No		Prevalence		% Prevalence		Chi-Square	Pvalue
Microbial Quality (Culture)								
Fertile males, no growth	76	100	0.396	0.521	39.6	52.1	2.00	0.572
Fertile Males, infected before	24		0.125		12.5			
Infertile males, no growth	37		0.187		18.7			
Infertile males, with growth	56		0.292		29.2			
Bacteria Isolated & Identified								
Staphylococcus aureus	35		0.182		18.2		3.333	0.504
Escherichia coli	10		0.056		5.6			
Klebsiella Pneumoniae	4		0.020		2.0			
Pseudomonas aeruginosa	2		0.0104		1.04			
Mixed *S. aureus* + *E. coli*	5		0.026		2.6			
Degree of Microbial Quality								
No Growth	136		0.708		70.8		2.00	0.572
Scanty	24		0.125		12.5			
Moderate	18		0.093		9.3			
Heavy	14		0.073		7.3			

Table 2. One-Way ANOVA of Sperm parameters in Infertile Males with Varying Degree of Microbial Growth

Parameters	Control (No MG)	Infertile males (No MG)	Infertile Males (SMG)	Infertile Males (MMG)	Infertile Males (HMG)	Fvalue	pvalue	Remark
Age (years)	39.22±7.028	36.67±5.750	41.83±7.39	38.33±6.93	37.00±6.29	0.731	0.5730	NS
Sperm vol. (mL)	2.63±1.51	2.14±0.99	2.68±1.09	2.24±1.37	2.10±1.24	0.376	0.8252	NS
pH	7.95±0.28	7.97±0.12	7.88±0.23	7.94±0.30	7.92±0.34	0.281	0.8890	NS
% Motility	60.51±17.33[a]	28.00±17.63[b]	31.25±22.71[b]	28.89±16.16[b]	26.71±21.34[b]	13.13	<0.0001	S
% Active Motility	45.86±21.59[a]	17.28±11.27[b]	21.92±18.33[b]	15.00±13.23[b]	15.71±15.39[b]	9.468	<0.0001	S
% Norm. Morph.	82.55±25.21[a]	64.50±37.78[a]	75.00±32.86[a]	44.33±31.04[b]	28.86±21.06[c]	10.03	<0.0001	S
% Ab. Morph	17.49±25.71[a]	35.50±37.78[a]	25.00±32.86[a]	55.67±31.04[b]	71.14±21.06[c]	9.606	<0.0001	S
TSC (10^6cells/mL)	42.53±19.75[a]	16.56±6.519[b]	9.975±4.621[b]	8.656±7.385[b]	4.129±3.324[c]	24.79	<0.0001	S

Turkey's Post Hoc: Within the same row, values with different superscripts differ significantly at P=.05.

No MG= No Microbial Growth, SMG=Scanty Microbial Growth, MMG=Moderate Microbial Growth, HMG=Heavy Microbial Growth on culture media, TSC=Total Sperm Count. S= Significant, NS=Not Significant at P=.05.

Table 3. Results of One-Way ANOVA of Seminal Plasma Proteins, Anti-Oxidants, Enzymes, MDA, Testosterone, and PSA in Infertile Males with Microbial Growth

Parameters	Control (No MG)	Infertile males (No MG)	Infertile males (MG)	Fvalue	P value	Remark
HSP70 (ng/ml)	12.17±4.59[a]	15.77±3.51[b]	16.47±7.48[b]	6.554	0.0022	S
OPN (ng/ml)	4.39±2.14[a]	3.15±1.27[b]	3.05±1.27[b]	6.428	0.0024	S
PKA (ng/ml)	7.54±3.04[a]	9.73±2.14[b]	9.67±4.19[b]	5.093	0.0080	S
GPX (ng/ml)	8.32±1.07[a]	7.707±1.77[a]	6.16±1.79[b]	19.83	<0.0001	S
MDA (ng/ml)	52.15±17.29[a]	70.14±37.05[a]	102.6±36.74[b]	28.14	<0.0001	S
TAC (mmol/L)	7.49±2.0[a]	4.49±3.07[b]	2.16±0.78[c]	65.11	<0.0001	S
TESTO (ng/ml)	4.91±1.41[a]	3.06±1.58[b]	2.55±1.10[b]	30.55	<0.0001	S
PSA (ng/ml)	4.17±3.07[a]	4.82±3.0[ac]	5.96±2.59[bc]	3.390	0.0380	S

Turkey's Post Hoc: Within the same row, values with different superscripts differ significantly at p<0.05. No MG= No Microbial Growth. S= Significant at P=.05.

3.3 Results of seminal Plasma Proteins, Anti-Oxidants Enzymes, MDA, Testosterone, and PSA in Infertile Males with Microbial Growth

When sperm proteins in seminal plasma were considered in infertile males without or with infections significantly higher values of HSP*70*, PKA, and MDA were seen in the infertile males without infections as well as in the infertile males with infections compared to control. However, no significant differences were between infertile males without infections and infertile males with infections. With regards to PSA, significantly higher values were seen in infertile males with bacterial infections compared with normospermic subjects and infertile males without infection. However, no significant differences were seen in PSA values between control and infertile males without infection. Furthermore, significantly lower values were seen in TAC and Testosterone of infertile males without infection and infertile males with infection when compared with control subjects. There was also a significantly lower value of TAC in infertile males with infections compared with infertile subjects without infection. However, in OPN and GPX the significantly lower values were only seen in infertile males with bacterial infections when compared with control subjects and infertile males without infections (Table 3).

3.4 Results of seminal Plasma Proteins, Anti-Oxidants Enzymes, MDA, Testosterone, and PSA in Infertile Males with Varying Degree of Microbial Growth

When some sperm proteins in seminal plasma were considered in fertile (control) and infertile subjects with or without bacterial infection of the semen, the results of sperm proteins in seminal plasma indicated significantly lower values in HSP70, PKA, and MDA of normospermic (control, fertile) subjects compared to infertile subjects with scanty, moderate, and heavy bacterial infections. With regards to PSA, a significantly higher value was observed in the infertile subjects with heavy bacterial growth when compared to normospermic fertile subjects. However, no significant differences were seen between the normospermic subjects and the infertile subjects without infection, with scanty

and moderate bacterial infections. Furthermore, in MDA significantly higher values were seen in infertile subjects with heavy bacterial infection compared to those with scanty and moderate infection. Likewise, those with scanty or moderate infection had significantly higher values of MDA compared to those that are infertile without infection.

In addition, significantly lower values were seen in osp, gpx, tac, and testosterone of infertile subjects with scanty, moderate, and heavy bacterial infections compared to fertile normospermic subjects without seminal fluid infection at *p*=.05 (Table 4). However, in tac, significantly lower values were observed in the infertile subjects with scanty bacterial infections when compared with infertile subjects without infections at *p*=.05 (Table 4).

3.5 One-Way ANOVA of seminal Plasma Proteins, Anti-Oxidants Enzymes, MDA, Testosterone, and PSA in Infertile Males with Microbial Growth and Sperm Cells Deformities

More so, when some sperm proteins in seminal plasma were considered in infertile subjects with bacterial infection of the semen and subjects' sperm cell deformities were statistically analyzed, the ANOVA results of sperm proteins in seminal plasma indicated significantly higher values were seen in HSP*70*, PKA, PSA, and MDA of infertile subjects with scanty, moderate, and heavy bacterial infections of the semen and those with sperm cells deformities compared to normospermic fertile subjects. However, the MDA values with significantly higher values were seen in oligoasthenoteratozoospermic (OAT) subjects compared to infertile subjects with varying degrees of seminal plasma infections as well as those with sperm cells deformities such as teratozoospermic (TZS) and asthenoteratozoospermic (ATS) at *P*=.05.

Also, significantly lower values were seen in OSP, GPX, TAC, and testosterone of infertile subjects with scanty, moderate, and heavy bacterial infections of the semen as well as subjects with sperm cells deformities such as TZS, ATS, and OAT when compared to normospermic fertile subjects without seminal fluid infection at p<0.05 (Table 5).

Table 4. One-Way ANOVA of seminal Plasma Proteins, Anti-Oxidants Enzymes, MDA, Testosterone, and PSA in Infertile Males with Varying Degree of Microbial Growth

Parameters	Control (No MG)	Infertile males (No MG)	Infertile Males (SMG)	Infertile Males(MMG)	Infertile Males (HMG)	Fvalue	pvalue	Remark
HSP70 (ng/ml)	12.17 ± 4.59^a	15.77 ± 3.51^b	18.73 ± 10.48^b	18.22 ± 4.62^b	20.70 ± 2.90^b	7.545	<0.0001	S
OPN (ng/ml)	4.39 ± 2.14^a	3.15 ± 1.27^b	2.62 ± 0.74^b	2.40 ± 0.45^b	2.643 ± 0.72^b	5.705	0.0004	S
PKA (ng/ml)	7.54 ± 3.04^a	9.73 ± 2.14^b	11.13 ± 2.66^b	11.18 ± 4.02^b	12.07 ± 3.67^b	7.471	0.0001	S
GPX (ng/ml)	8.32 ± 1.07^a	7.707 ± 1.77^a	7.02 ± 1.52^b	5.74 ± 1.79^b	4.21 ± 1.0^b	19.08	<0.0001	S
MDA (ng/ml)	52.15 ± 17.29^a	70.14 ± 37.05^a	103.4 ± 38.97^b	80.19 ± 17.17^b	134.8 ± 28.78^c	21.59	<0.0001	S
TAC (mmol/L)	7.49 ± 2.0^a	4.49 ± 3.07^{bc}	1.79 ± 0.81^{bd}	2.60 ± 0.73^b	2.36 ± 0.57^b	31.93	<0.0001	S
TESTO (ng/ml)	4.91 ± 1.41^a	3.06 ± 1.58^b	2.89 ± 1.15^b	2.81 ± 0.91^b	2.04 ± 0.82^b	15.08	<0.0001	S
PSA (ng/ml)	4.17 ± 3.07^a	4.82 ± 3.0^a	5.84 ± 2.45^a	5.91 ± 2.56^a	7.84 ± 1.83^b	3.238	0.0157	S

Turkey's Post Hoc: Within the same row, values with different superscripts differ significantly at P=.05. No MG= No Microbial Growth, SMG=Scanty Microbial Growth, MMG=Moderate Microbial Growth, HMG=Heavy Microbial Growth on culture media, S= Significant at P=.05.

Table 5. One-Way ANOVA of seminal Plasma Proteins, Anti-Oxidants Enzymes, MDA, Testosterone, and PSA in Infertile Males with Microbial Growth and Sperm Cells Deformities

Parameters	Control (No MG)	Infertile males (No MG)	Infertile males (MG)	TZS	ATS	OAT	Fvalue	pvalue	Remark
HSP70 (ng/ml)	12.17 ± 4.59^a	15.77 ± 3.51^b	16.47 ± 7.48^b	18.11 ± 3.71^b	15.78 ± 4.11^b	18.20 ± 3.98^b	9.335	<0.0001	S
OPN (ng/ml)	4.39 ± 2.14^a	3.15 ± 1.27^b	3.05 ± 1.27^b	2.52 ± 0.76^b	2.59 ± 0.62^b	2.27 ± 0.87^b	11.28	<0.0001	S
PKA (ng/ml)	7.54 ± 3.04^a	9.73 ± 2.14^b	9.67 ± 4.19^b	10.34 ± 3.11^b	10.60 ± 2.77^b	13.57 ± 3.21^b	9.071	<0.0001	S
GPX (ng/ml)	8.32 ± 1.07^a	7.707 ± 1.77^b	6.16 ± 1.79^b	4.79 ± 1.25^b	4.56 ± 0.97^b	4.35 ± 0.86^b	37.50	<0.0001	S
MDA (ng/ml)	52.15 ± 17.29^a	70.14 ± 37.05^b	102.6 ± 36.74^b	109.2 ± 33.65^b	83.44 ± 31.63^b	129.1 ± 29.74^c	20.17	<0.0001	S
TAC (mmol/L)	7.49 ± 2.0^a	4.49 ± 3.07^b	2.16 ± 0.78^b	2.21 ± 1.14^b	2.43 ± 1.23^b	1.331 ± 0.27^c	62.25	<0.0001	S
TESTO (ng/ml)	4.91 ± 1.41^a	3.06 ± 1.58^b	2.55 ± 1.10^b	2.30 ± 1.14^b	2.08 ± 0.96^b	1.96 ± 0.69^b	31.36	<0.0001	S
PSA (ng/ml)	4.17 ± 3.07^a	4.82 ± 3.0^a	5.96 ± 2.59^b	5.82 ± 2.75^b	6.49 ± 2.71^b	7.67 ± 2.13^b	3.845	0.0028	S

Turkey's Post Hoc: Within the same row, values with different superscripts differ significantly at P=.05. No MG= No Microbial Growth, SMG=Scanty Microbial Growth, MMG=Moderate Microbial Growth, HMG=Heavy Microbial Growth on culture media, TZS= teratozoospermic, ATS= Asthenoteratozoospermic, OAT= Oligoasthenoteratozoospermic, S= Significant, at P=.05.

4. DISCUSSION

Bacterial infections in seminal plasma after culture indicated a prevalence of 29.2 % in infertile males of which 12.5%, 9.3%, and 7.3% indicate scanty, moderate, and heavy bacterial growth. The organisms isolated and identified were *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli,* and *Pseudomonas aeruginosa.* Of the organisms, *Staphylococcus aureus* had the highest prevalence with 18.2%, followed by *Escherichia coli* with 5.2%, *Klebsiella pneumoniae* with a prevalence of 2.0%, and *Pseudomonas aeruginosa* with 1.04% as well as mixed growth of *Staphylococcus aureus and Escherichia coli* with 2.6%. Our findings are similar to the observations made in Port Harcourt by Oyeyipo et al. [21]. They also reported microbial infections (and prevalence) such as *Staphylococcus aureus* (30%), *Klebsiella pneumoniae* (10%), *Escherichia coli* (2%), *Proteus mirabilis* (10%), and *Pseudomonas aeruginosa* (7%) isolated from seminal plasma.

The high prevalence of *Staphylococcus aureus* could be attributed to normal flora contamination of the glans penis or orifice of the urethra. However, their heavy presence (*Staphylococcus aureus*) in seminal plasma alongside scanty or moderate growth of *Escherichia coli* observed as mixed growth could suggest actual pathogenic bacterial infection of the seminal plasma with significance. Seminal plasma infection has contributed to male infertility affecting sperm cell viability and motility.

The non-significant differences observed in sperm volume and pH of the control subjects compared against the infertile subjects with bacterial infection (Table 2) is in line with the findings of Palani & Alahmar [22]. They also reported no significant difference in the ejaculatory volume of semen of bacterial-infected infertile males and control. However, the significantly lower values in sperm motility, active (progressive) motility, normal morphology, and total sperm counts were observed in the infertile subjects with bacterial infection (Table 2) are similar to previous works done in Port Harcourt by Oyeyipo et. al. [21] and Green & Nwachuku [6], they also gave similar findings as seen in our results in their respective studies. This further reveals that sperm volume and pH are never good indicators of microbial infections despite the severity of the infections, significantly lowered total sperm counts, sperm motility, active (progressive) motility, and morphological abnormalities.

When the degree of infection was considered, significant differences were not seen in the motility, and active motility amongst those infertile males with scanty, moderate, and heavy infections (Table 2). However, significantly lower values were observed in the normal morphology of sperm cells, sperm viability, and total sperm count in infertile males with heavy bacterial infection compared to infertile males with scanty and moderate infection (table 2). It was also observed that those (infertile males) with scanty infections had higher values of morphologically normal sperm cells compared to those with moderate and heavy bacterial infections. Our findings were not really in line with the reports of Oyeyipo et al. [21] and Green & Nwachuku [6] because the degrees of infection were not considered in their respective study. However, our results are similar to the reports of Liu et al. [23], who reported that the degree and type of bacterial infection in seminal plasma correlates negatively with the level of sperm cell viability, motility, and progressive motility. The significantly lower values of motility, active motility, normal morphology, and total sperm count could be as a result of the activities of the infectious agents directly or indirectly on the sperm cells. Toxins released by these organisms stimulate the defense mechanism of the host system releasing interleukins, tumor necrotic factors-alpha, and infernos. The activities of these inflammatory substances can result in the production of reactive oxygen species and subsequently oxidative stress. Furthermore, due to the susceptibility of sperm cells to oxidative stress as a result of their limited cytoplasm to tackle reactive species and high levels of polyunsaturated fatty acids (PUFAs), the bacterial-induced oxidative stress can lead to poor motility, sperm cells deformation, and eventually death. Infections affecting sperm cell motility have been reported as one of the major causes of infertility. Our results further suggest that the higher the degree of infection, the higher the levels of sperm cells deformities, the lower the levels of active motility, and the lower the sperm concentration in every ejaculate and vice versa. The cumulative effect of these, results in male infertility.

When the influence of bacterial infections on sperm proteins, GPX, and TAC in the seminal plasma as well as testosterone and PSA in the serum were considered (table 3 and table 4), the

significantly higher values of HSP*70* observed in the infertile males with bacterial infection are in line with findings of Kosecka-Strojek et al., [11], Yu et al., [24], and Njemini et al. [25]. They all reported in their respective study that bacterial infection of the seminal plasma such as *Staphylococcus aureus, Escherichia coli, klebsiella pneumoniae*, etc induced significantly higher values of HSPs family including HSP*70*. Kosecka-Strojek et al. [11], further reported that these changes were also observed in other mammals like chickens while Yu et al. [24], also explained viral infections have also been seen to induce increased levels of HSP*70*. In addition, the findings of significantly lower levels of OPN in the infertile males and those infertile with bacterial infections still support the results of Waheed et al. [26] who documented that higher levels of OPN are associated with fertility. More so, the significantly higher levels of PKA seen in infertile subjects are in line with the reports of Blommaert et al. [27]. They also reported significantly higher values of Anchor Kinase Activated Protein (AKAPs) and PKA in subjects with infertility. Again, the higher values of MDA and lower values of GPX and TAC in our findings are similar to the findings of Liu et al. [23], when values of anti-oxidants were compared in subjects with bacterial infections compared to controls. The levels of testosterone were also found to be lower in subjects with infection while PSA was observed to be higher in infertile subjects with bacterial infections. The higher value observed in HSP*70* in our study could also be related to the protected role of these proteins in mitigating infections. As infections trigger the defense system of the body, proteins such as HSP*70* alongside interferons, interleukins, and lymphocytes are usually released to eliminate or mitigate the activities of the infectious agent. HSP*70* could have been induced through changes in the ROS concentration and degradative enzymes such as coagulase, hyalinedurinase, etc produced by the infectious organisms. HSPs are known to conserve and protect spermatozoa and other cells against deformities and as such may be involved in the activation of cytotoxic T-lymphocytes without the assistance of T-helper lymphocytes. In 2010, Guo and colleagues [28] also reported that HSP70 in the cellular response to infections is actively produced when monocytes and macrophages are exposed to bacterial toxins. The significant reduction in GPX and TAC as well as the increase in MDA observed suggests oxidative stress induced by the infectious pathogens. Oxidative stress has been reported to

cause the death of sperm cells, deformities, and reduced sperm cell motility. These factors could be one of the major causes of infertility among males.

The significant fall in testosterone concentration in the infertile males could also be due to the influence of generated ROS as a result of the infections on the Sertoli and Leydig cells of the testis. Oxidative stress has been reported to influence negatively steroidogenesis and spermatogenesis in males. The increase in PSA among infertile males (table 3) especially among those with heavy bacterial infection (table 4) could also suggest inflammation of the prostate resulting in infectious prostatitis. Healthy prostate has been reported to contribute majorly to the seminal plasma. Therefore, higher values of PSA suggest inflammation of the prostate which in turn may affect sperm quality and fertility. Our results further suggest that though scanty and moderate bacterial infection could be complaisant in inflammation of the prostate, heavy bacterial infection elicits more inflammation of the prostate as reflected by the PSA results (Table 4).

Also, our findings further suggest that the degree of infection did not cause any significant change in the values of HSP*70*, PKA, OPN, GPX, and TAC in seminal plasma as well as testosterone in the serum among infertile males with bacterial infection (Table 4). This also implies that scanty bacterial infection can elicit a similar effect as moderate or heavy bacterial infection on sperm proteins such as HSP*70*, PKA, OPN, and GPX as well as TAC in the seminal plasma including testosterone in the serum. However, the values of MDA were significantly increased based on the degree of infection. This could be because MDA is a metabolic product of lipid peroxidation and the higher the degree of infection, the higher the tendency of lipid peroxidation viz-a-viz MDA concentration in the seminal plasma. Our findings contradict the findings of Cedenho et al. [29] and Coa et al. [30]. Though we agree that HSPs family including HSP*70* were altered significantly in infertility, they (HSPs) are not better predictors or markers of sperm motility dysfunction rather MDA is a better predictor because the changes in their concentration in the seminal plasma are proportional to the changes observed in the level of ROS/OS as indicated by the value of TAC.

When sperm abnormalities were considered, significantly higher values of HSP*70*, PKA, MDA,

and PSA while significantly lower values were seen in OPN, GPX, TAC, and testosterone in infertile males without bacterial infection, infertile subjects with bacterial infection, and subjects with sperm abnormalities (TZS, ATS, and OAT subjects) compared to fertile subjects (Table 5). However, significant differences were not observed in the comparison amongst the infertile without bacterial infection, infertile subjects with bacterial infections, and subjects with sperm abnormalities (TZS, ATS, and OAT subjects) except PSA and MDA (Table 4). Our findings suggest that both infection and sperm deformities may exert similar pathophysiological changes in the seminal plasma levels of sperm proteins HSP*70*, PKA, OPN, GPX, and TAC as well as in the values of testosterone but not necessarily the same for PSA and MDA. PSA values were observed to be significantly lower in infertile males without bacterial infection unlike the sperm proteins and anti-oxidant proteins in the seminal plasma. This result further suggests that infection of the prostate or inflammatory response of the prostate as indicated by the values of PSA in the serum play a significant role in exacerbating the degree or problem of infertility amongst males. Furthermore, in the case of MDA it was also seen that MDA values were significantly highest in OAT subjects compared to TZS and ATS subjects as well as infertile subjects with bacterial infections. Oligoasthenoteratospermic (OAT) has been previously reported by Agarwal et al. [31] and Cologar et al. [32] as one of the main causes of male infertility due to its three-fold negative dimension of sperm quality affecting the sperm concentration, progressive motility, and normal morphology. These MDA results could suggest that OAT subjects are more prone to oxidative damage than other sperm cell deformities and bacterial infections. These results further suggest MDA is a better predictor of sperm cells damages and infertility because the changes in their concentration in the seminal plasma are proportional to the changes observed in the level of ROS/OS as indicated by the value of TAC.

5. CONCLUSION

The results of bacterial infection of seminal plasma indicated significantly increased lipid peroxidation cum oxidative stress leading to altered physiology of the sperm proteins in the seminal plasma. In addition, the infections (especially those with heavy growth), induced a significant reduction and increase in plasma testosterone and PSA levels respectively. These alterations could also be responsible for the poor sperm qualities and their infertile state as observed in the case subjects.

CONSENT AND ETHICAL APPROVAL

Before the start of the study, ethical approval was sort and obtained from the Ethical Review Boards of the Rivers State Government, through the Rivers State Ministry of Health covering the Rivers State University Teaching Hospital and Primary Healthcare Centres in the State with approval no of MH/PRS/391/VOL.2/726 and MH/PRS/391/VOL.2/727 respectively. In addition, written informed consents were gotten from all the participants before being recruited for the study. We hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed following the ethical standards laid down in the 1964 Declaration of Helsinki.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/87689