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Short Communication

Assessment of Seminal Plasma Creatine Kinase Activity and Malondialdehyde Concentration among Normozoospermia but Infertile Men

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Abstract: **Background:** Despite the considerable research efforts that have been made by Reproductive Biologists to understand the etiologies of male infertility, idiopathic male factor infertility still remains unexplained. It occurs in about 10% cases of infertility. Since the etiologies cannot be identified using routine semen analysis, it is important to identify cellular and sub-cellular sperm complications that may help to explain the cause(s) of the infertility, thus stimulate caregivers to direct the further work-up, diagnosis and counseling of affected individuals. The objective of this study was to determine seminal plasma creatine kinase (CK) activity and malondialdehyde (MDA) concentration among normozoospermia but infertile males. **Materials and Methods:** After routine semen analysis, seminal plasma CK activity and MDA were determined among 75 normozoospermia but infertile men and 50 men of proven fertility using spectrophotometric method. Unpaired Students-t-test and regression analysis were used to compare and associate CK activity and MDA concentration with sperm characteristics. **Results:** Significantly higher ($p<0.001$) level of MDA and CK activity were observed among infertile subjects than control group. Sperm count, percent motility, and viability were significantly lower ($p<0.05$) among study participants than control subjects. Similarly, percentage abnormal morphology was significantly higher ($p<0.05$) among infertile subjects than control group. The Odds of elevated CK activity to impair sperm motility, viability and morphology were 9.12(CI 102.6, 318.3), 3.18 (CI 129.9, 170.8) and 1.9 (CI 192.2, 208.1) times respectively higher among infertile group than controls. Similarly, the Odds of higher levels of MDA to impair sperm cell motility, viability and morphology were 5.02 (CI 2.14, 4.6), 2.26 (CI 2.34, 3.64) and 2.9 (CI 2.12, 3.73) times respectively higher among infertile group than control subjects. **Conclusions:** The seminal plasma CK activity and MDA constitute good indicators of functional metabolic activity and fertility potentials of spermatozoa among subjects with unexplained infertility.

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INTRODUCTION

Male factor is responsible for 40- 50% of infertility among married partners, and 10% of the causes of infertility are regarded as idiopathic or unexplained. Medical anxiety regarding infertility has risen recently because of declining reproductive capability of couple globally (Agarwal et al., 2021).

A large group of apparently healthy males have difficulty impregnating their wives even when their fertility standing by microscopic semen analysis is considered normal. The occurrence is classified as unexplained infertility. Idiopathic male infertility is classified as idiopathic oligoasthenotetratozoospermia which means that those men have an unexplained decline in sperm quality. Conversely, unexplained male infertility refers to infertility of unknown cause with normal semen parameters, and without physical or endocrine abnormalities and absence of female factor infertility (Sabanegh et al., 2012).

Creatine kinase enzyme (CK) is essential for sperm function because it accelerates the restoration of Adenosine triphosphate (ATP) from adenosine diphosphate (ADP) which are indispensable for the generation, transport, and effective use of energy within sperm cells (Paduch, 2008). The determination of CK in human sperm cells is an unbiased index of sperm maturity and fertilization potential. Raised CK values may be a manifestion of a high number of functional deformities and enlarged cytoplasmic residues (Micic et al., 2009). Although earlier researchers have revealed that total creatine kinase activity and isozyme spread are not determinants of male fertility (Rolf et al., 1998), it is believed CK activity may contribute to fertility potential in humans. It is therefore consequential to evaluate seminal plasma CK activity among Nigerians with unexplained infertility to work out its contribution to male infertility. Flaws in sperm cells maturation have adverse effects on enzyme functions in the ejaculate (Zeqiraj and Gashi, 2014).

Semen analysis form the lion's share of first line investigation conducted when evaluating men for infertility and studies have indicated it may not accurately determine the cause(s) of the disorder. Moreover, there are group of infertile men with normal sperm indices yet cannot achieve pregnancy (Uadia and Emokpae, 2015; Olatunbosun et al., 2018; Moronkeji et al., 2021). It is now clear that basic semen analysis is inadequate for the diagnosis of the fertility status of humans because spermatozoa are highly specialized cells that exhibit diverse series of biological effects to achieve fertilization. A rising occurence of male infertility and the difficulties in making accurate diagnosis necessitate biochemical investigations, like the assay of seminal fluid malondialdehyde (MDA), a biomarker of sperm membrane lipid oxidation and to aid semen analysis in identification of the infertile man and Creatine Kinase (Olatunbosunet al., 2018). The CK is essential for sperm obligation because it accelarates the reformation of ATP from the chemical alternate between creatine and creatine phosphate an indicator of sperm maturity that is closely associated with the sperm fertilizing capacity. A rise the level is closed linked with a surge in the rate of functional deformities and soared cytoplasmic retention (Hallaket al., 2001). Results of CK activity has not be consistent. Earlier studies have revealed that total CK activity and isozyme diffusion are not indicators of male fertility (Rolf et al., 1998). It is therefore essential to determine seminal plasma CK activity and MDA among

Nigerians to find out their contributions to unexplained male infertility. The aim of this study was to determine the CK activity, MDA level among normozoospermia but infertile males in order to determine their contributions to unexplained male infertility.

MATERIALS AND METHODS

Study design

This cross sectional study involved males with normozoospermia but partners of infertile couple presenting at the fertility clinic with established diagnosis of infertility after review by a clinician. Males with proven fertility served as controls. The study was conducted at the Fertility Clinic of a tertiary hospital in Benin City, Nigeria. Participants were selected after a detailed history, clinical examination and laboratory investigations. Structured questionnaire was used to collect socio-demographic data of study participants.

Sample size determination

The minimum sample size was calculated using Sample size determination formula in health studies by Lwanga and Lemeshow (1991);

$n= Z^2 pq/d^2$

Where
n= minimum sample size
Z= Standard normal deviate at 95% confidence limit (1.96)
p = Infertility prevalence rate in a previous study 5-8% (Okonofua, 2003).
q= (1-p)
I = the alpha level of significance (10%).
$n = (1.96)^2 \times 0.05 \times (1-0.05) = 72.99$
0.0025
A total of 75 participants were enrolled in the study.

Inclusion criteria

Supposedly wholesome men diagnosed with infertility but have sperm count within the normal reference (normozoospermia) who had regular unprotected sex for at least 12 months were recruited in the study. Subjects who have had history of infertility for more than a year, sperm count $<15x10^6$ cells/mL and few or no white blood cell count per field were enrolled. Individuals without any history of infertility with sperm indices within the normal reference range, (at least 50% motility and >30% normal sperm morphology and count of ≥15 x 10^6cells/mL) were enrolled as controls.

Exclusion criteria

Individuals with poor semen quantity and quality were excluded from the study. The subjects who are already using supplementary antioxidants or drugs for male infertility were not included in this study. Also, individuals with testicular varicocele, urogenital infection, increased white blood cells, chronic metabolic illness and serious systemic diseases, alcohol abusers, or smoke were left out of the study.

Ethical considerations

Ethical approval was sought and obtained from the Ethics Review Committee of the University of Benin Teaching Hospital, Benin City, Nigeria (ADM/E22/A/VOL.VII/14831269 dated 18th February 2022). Informed consent was duly obtained from the participants.

Sample collection

The ejaculate was collected by masturbation after at least three days of sexual abstinence into a sterile wide-mouth container and kept at ambient temperature (37 °C) to prevent significant alteration in the temperature that may upset the sperm cells. The semen was labeled with identification code, date, and time of collection. The specimen was thereafter placed in an incubator at 37 °C to allow for liquefaction.

Semen analysis

Liquefied sample was analyzed manually according to World Health Organization guidelines (WHO, 2010). The semen characteristics determined included sperm count, sperm motility, percent morphology and percent abnormal morphology.

Sample Preparation and Laboratory Analysis for Creatine Kinase Estimation

After liquefaction, the ejaculate was spuned at 4000 rpm for 5 min and the supernatant seminal fluid was decanted into another clean and sterile plastic container for the assay of CK activity.

Assay Method for Creatine Kinase Activity (Wicks et al.,1982).

Principle

After the inhibition of the M subunits of CK-MM and the single M subunit of CK-MB, the B subunit was determined. The CK-B activity was determined from the rate of NADPH formation, and the rate of change was measured at 340nm, using hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH) in a coupled reaction.

Creatine Phosphate +ADP ⟶ Creatine + ATP

ATP +Glucose $\xrightarrow{HK}$ ADP+ Glucose-6-Phosphate

Glucose-6-Phosphate + $NADP^+$ $\xrightarrow{G6P\text{-}DH}$ Gluconate -6-Phosphate + NADPH + H^+

Measurement of Malondialdehyde (Rao *et al.*, 1989).

Principle

Malondialdehyde reacts with thiobarbituric acid (TBA) to form a pink colored product. The intensity of the colour is proportional to the concentration of MDA in the sample.

Data analysis

Data from the result were organized and analyzed using Statistical Package for the Social Sciences software (version 20.0, IBM SPSS, Armonk, NY, USA). The differences between the means were compared using the Student t- test, ANOVA (Analysis of variance) while Pearson correlation coefficient was utilized to determine the correlation between the measured parameters, and clinical condition. A p-value less than 0.05 was considered as significance.

Table 1: Comparison of demographic data, sperm indices, Creatine kinase activity and malondialdehyde between normozoospermia but infertile men and control subjects.

Parameters	Normozoospermia but Infertile subjects	Control subjects	p-Value
Number of subjects	75	50	
Age (Years)	41.72±8.67	41.32±5.91	0.09
Weight (kg)	64.89±5.01	67.62±4.98	0.05
Height (m)	1.77±0.09	1.79±0.07	0.165
BMI (KG/m^2)	20.77±1.63	21.04±1.48	0.297
Malondialdehyde(nmol/L)	3.16±1.02	2.07±0.62	0.001
Creatine kinase(U/L))	210.56±107.83	100.38±52.42	0.001
Semen Volume (mL)	2.76±0.94	2.57±0.53	0.08
Sperm Count ($x10^6$/mL)	20.20±2.36	48.66±2.90	0.001
Percent Motility (%)	40.17±2.18	60.87±7.58	0.001
Viability (%)	50.58±15.20	65.64±6.79	0.001
Abnormal morphology (%)	15.17±2.96[c]	7.30±2.52[a]	0.001

BMI=body mass index, Kg=kilogram, m=meter

Table 2: Association between Creatine Kinase Activity, Malondialdehyde and Sperm Characteristics among Normozoospermia but Infertile Men

Biochemical parameters	Sperm Indices	Unadjusted Odd Ratio	95% Confidence interval	p- Value
Creatine Kinase	Percent Motility (%)	9.12	102.6, 318.3	0.001
	Viability (%)	3.18	129.7,170.8	0.005
	Abnormal morphology (%)	1.9	192.2,208.1	0.06
MDA	Percent Motility (%)	5.02	2.14,4.16	0.001
	Viability (%)	2.26	2.34, 3.68	0.02
	Abnormal morphology (%)	2.9	2.12,3.73	0.005

MDA=malondialdehyde

RESULTS

Table 1 shows no significant different in age, weight, height and BMI of Normozoospermia but Infertile subjects and the control subjects p> 0.05. However, there was a significantly higher

level of MDA and CK activity ($p<0.05$) when compare with the control group. Sperm count ($x10^6$/mL), percent motility (%), and viability (%) were significantly lower ($p<0.05$) when compared with the control subjects. Also, abnormal morphology (%) among the Normozoospermia but Infertile subjects was significantly lower ($p<0.05$) when compared with the control group.

Table shows the association between CK activity, MDA and sperm characteristics among normozoospermia but Infertile men. The Odds of elevated CK activity to impair sperm motility, viability and morphology were 9.12(CI 102.6, 318.3), 3.18 (CI 129.9, 170.8) and 1.9 (CI 192.2, 208.1) times respectively higher among normozoospermia but infertile group than controls. Similarly, the Odds of higher levels of MDA to impair sperm cell motility, viability and morphology were 5.02(CI 2.14, 4.6), 2.26(CI 2.34, 3.64) and 2.9 (CI 2.12, 3.73) times respectively higher among infertile group than control subjects.

DISCUSION

Despite the considerable research efforts that have been conducted to understand the etiologies of male infertility, most of the male factor infertility still remains unexplained (Punab et al., 2017). It is important to identify cellular and sub-cellular sperm dysfunctions that may help to explain the cause(s) of the infertility, thus stimulate caregivers to direct the further work-up, diagnosis and counseling of affected individuals (Hamada et al., 2012). The capacity of a spermatozoon to fertilize an ovum is of utmost importance in an instance of subjects with normozoospermia but infertile men, when semen analysis does not detect any significant alteration in sperm indices. It is therefore imperative to use other diagnostic methods such as seminal plasma creatine kinase and MDA to identify specific abnormality associated with sperm function. Sperm cells need enormous energy uptake for progressive movement, and CK is the major enzyme that provides the needed energy. The function of CK in mitochondria in the neck of spermatozoon is to provide the needed energy to navigate the female birth canal to reach the ovum. Creatine kinase catalyzes rephosphorylation of adenosine diphosphate (ADP) into adenosine triphosphate (ATP) (Zeqiraj and Gashi, 2014). Previous study has indicated that seminal plasma CK activity and MDA concentration are inversely associated with sperm quality parameters and positively with increasing body mass index (BMI) of participants (Babatunde and Emokpae, 2022).

In this study, we compared the CK activity and MDA level between normozoospermia but infertile men and BMI-matched control subjects in order to determine their contributions to unexplained male infertility. A significantly higher MDA level and CK activity were observed among normozoospermia but infertile men than control subjects with similar BMI. Previous authors have reported from a cytochemical study that significantly higher CK activity was observed in semen samples that contain immature sperm cells due to the presence of cytoplasmic residues (Huszar and Vigue, 1993; Celic-Ozenci et al., 2002). Naturally, mature spermatozoa do not have cytoplasm in the neck region and such cells are associated with low CK activity. Others have reported that immature spermatozoa with cytoplasmic residue do not have the ability of effective capacitation; the physiological changes undergo by spermatozoa to penetrate and fertilize an ovum (Aitken et al., 1994; Gomez, 1996). Seminal plasma enzymes may not be responsible for loss of sperm cell functions, but act as a biochemical indicator to differentiate viable sperm cells from non-viable cells. Determination of CK activity in seminal plasma may be an important pointer to identifying functional metabolic activity of the spermatozoa (Guerin et al., 1979). It is inferred that immature cells were counted during microscopic sperm cell counts. Physiologically, CK activity in seminal plasma is several folds higher than found in serum indicating that CK performs specific functions within the testes (Huszar and Vigue, 1993; Zeqiraj and Gashi, 2014).

Because of conflicting reports associating obesity with male factor infertility in literature, this study recruited study participants and control subjects with similar BMI (Ntuen et al., 2015; Al-Hail et al., 2019; Emokpae and Brown, 2021). Mean sperm count, percent motility and viability were significantly lower among normozoospermia but infertile subjects than control subjects. More importantly, significantly higher percentage of morphologically abnormal spermatozoa in ejaculate of normozoospermia but infertile subjects were observed. It therefore suggests that other factors aside of abnormal weight may be contributing to increasing incidence of male infertility, even though obesity has been implicated in the deteriorating male factor infertility in the recent times. Obesity has been associated with hypogonadotropic-hypogonadism and hyperestrogenism among infertile men leading to poor "sperm quality, sperm mitochondrial activity, enhanced sperm DNA damage and oxidative stress" (Guo et al., 2017; Baydilli et al., 2020).

Higher levels of seminal plasma MDA have been associated with poor fertilization potentials of spermatozoa. The enhance generation of lipid peroxidation product (MDA) in the seminal plasma has the potential to cause oxidative stress and harmful effects to sperm function. The spermatozoa are readily susceptible to free radical -induced damage due to the abundant polyunsaturated fatty acid (PUFA) present in the spermatozoa plasma membrane (Baydilli et al., 2020). Free radicals can damage cellular macromolecules, stimulating stress signaling and, at high levels, cell death. But the availability of cellular antioxidant defenses in the body can scavenge free radicals are important especially for the protection of developing sperm cells (Selvaratnam and Robaire, 2016).

CONCLUSION

The mean CK activity and MDA concentration were significantly higher among normozoospermia but infertile men than control subjects. Although, the diagnosis of male infertility has relied on microscopic assessment, other specific biochemical assays are important to identifying the cause of unexplained male infertility. The seminal plasma CK activity and MDA constitute good indicator of functional metabolic activity and fertility potentials of spermatozoa especially among subjects with unexplained infertility.

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