Original Article

Comparative Study of Enzymatic Markers in Seminal Liquids of Normospermic and Azoospermic Samples at Pasteur Institute of Cote D'Ivoire (IPCI)

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ABSTRACT

This study is initialized to assess the enzymatic activity link to the presence or absence of spermatozoa.
In fact, thirty nine ejaculates from men aged 31 to 42 years were collected. 17 normospermia and 22 azoospermia were found. Seminal fluid was obtained by centrifugation of the samples and freeze at 4˚C. Those tests revealed there is no significant difference in term of the age, the bit with the enzyme markers; the statistical analysis show significant variations in certain parameters. Thus, for the Aspartate aminotransferase (AST), we notice a difference between normospermia with the values of 224.17 ±17.30 UI/l and azoospermia with the values of 143.13 ± 13.69 UI/l. These remarks were made for the γ-Glutamyl Transferase (GGT) with 16938 ± 1795 UI/l corresponding to the normospermia which are different from the azoospermia with the following values of 11357± 1326 UI/l.
The consideration of these parameters could be envisaged after an assessment on a broad sampling in order to confirm the effectiveness of the presence of the spermatozoa according to the activity of these enzyme markers.

Keywords: Seminal fluid, Enzyme activity, Normospermia, Azoospermia.

INTRODUCTION

The seminal fluid is the part of the sperm ensuring metabolism, survival and transport of spermatozoa in the female genital tract[1]. The study of the biochemical characteristics of the sperm allows the assessment of the testicular activity and post-gonadal events[2,3]. According to the World Health Organization procedures[4], the

methods of analysis of the seminal fluid are mainly cytological and focused on macroscopic and microscopic analysis of the sperm. Currently, the evaluation of biochemical markers is more restricted in case of azoospermia. Indeed, investigations helped highlight some biochemical markers which, associated with the endocrine assessments, allow to appreciate the type of azoospermia[5,6]. In addition to the compounds currently used as markers that are fructose, zinc, citrate, alpha-glucosidase and carnitine[4], the seminal fluid is also composed of several elements (carbohydrates, organic acids, whey proteins, minerals, vitamins, growth factors, enzymes) whose actions in the sperm and upon spermatozoa are not yet accurately identified[7,8].

In addition, the biochemical assessment of the testicular activity (sperm production) seems necessary to better assess the functioning of the tissues and organs actively involved in the production, the maturation and the excretion of the sperm. Therefore, even if the application of these results is not yet effective in assessing fertility and curing patients, the study of the biochemical parameters of the seminal fluid in azoospermia and normospermia appears to be a fundamental approach for the research of biochemical markers associated with pathologies in the investigation and treatment of male infertility[9-11]. Moreover, the assessment of the activity of some enzymes in the seminal fluid, showed their relationship to the metabolism of sperm cell which is driven by the energetic activity, especially at the mitochondrial level[12-14,1]. In this experimental study, the enzyme markers measured in the seminal fluids are Aspartate aminotransferase (AST), Alanine amino-transferase (ALT), Lactate dehydrogenase (LDH), Creatine phosphokinase (CPK), γ-glutamyl transferase (GGT), Alkaline phosphatase (ALP) and α-amylase (AMYL). Several studies showed that Alkaline phosphatase (ALP) in the seminal fluid could be from epididymal and testicular origin[15,16]. In humans, the seminal activity of LDH was assessed in 2487 ± 1384 IU/l[17,18]. This enzyme activity is higher among oligozoospermia than among the normospermia and may also be associated with teratozoospermia related to the acrosome[19,20].

Correlations have been established between the activity of creatine kinase and the morphological anomalies and the maturation of the spermatozoa[20-23]. The studies have indicated the importance of the activities of this enzyme in the seminal fluid which is 661 ± 360 IU/l[24,25]. The assessment of the sperm or the seminal activity of the Aspartate aminotransferase (AST) indicates that it is in touch of the sperm membrane integrity[26-29]. This reminds that its liberation in the seminal plasma could be in favor of the cell lysis. In addition, the ratio AST/ALT could be a discriminating factor between excretory and secretory azoospermia[30].

The γ-glutamyl transferase (GGT) intervenes in the fight against stress[31-33,29]. The bovementioned work indicates a positive correlation between the seminal activity of this enzyme with testosterone production and that of the α-amylase which intervene in the liquefaction of the seminal fluid[34,35].

MATERIAL AND METHODS

Material

The biological material was the sperm of patients collected in the Reproductive Biology Unit of the Pasteur Institute of Côte d'Ivoire for a spermogram, or a spermocytogram, or a survival test capacitation for the duration of the study. Only sperm samples obtained by masturbation after three days of sexual abstinence and with either azoospermia or a

normospermia were selected. This study was conducted on 39 ejaculates with 22 azoospermia and 17 normospermia. This study was approved by the National Council of Ethics and Research (Reference number: 36/MSLS/CNER/TB).

The equipment was composed of a Cobas Integra 400 spectrophotometric automaton, a binocular optical microscope, a cell Mackler, a Jouan® centrifuge and an oven, comprises an additional set of indispensable elements to the spectrophotometric and the routine microscopy. The pH-meter strip, eosin 0.6 %, nigrosine 5 %, and RAL kit 555® were used to achieve the test. In addition to these elements, there are cassettes for determining the activity of the transaminases Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) of Alkaline Phosphatase (ALP), Creatine phosphokinase (CPK), Lactate dehydrogenase of (LDH) of γ-Glutamyl transferase (GGT) and α-amylase (AMYL).

Methods

The methodology of this work used a cross-sectional study conducted at IPCI. The sperm is collected by masturbation into a sterile collection pot and immediately placed in an oven at 37 °C for liquefaction for 30 minutes to 1 hour. The cytological analysis of the sperm, that is routinely made, consisted in examining spermogram and spermocytogram with a binocular optical microscope. The concentration of spermatozoa was determined with a cell Mackler. The spermocytogram was performed according to the modified method of David[4].

The ejaculates with azoospermia or normospermia were identified and selected for a biochemical analysis. For this purpose, a volume of 0.5 to 1 mL of the ejaculate liquid was transferred to a hemolysis sterile tube and centrifuged at 900 g for 10 minutes. The seminal plasma, aliquoted into a sterile hemolysis tube, was cleared of cellular moiety and immediately stored in a freezer at -4°C for enzyme dosages by using a spectrophotometric automaton. Thus, by the method of enzyme kinetics and to a wavelength l of 340 nm, the activities of the AST and ALT were determined following the disappearance of NADH while those of LDH and CPK were determined by the disappearance of $NADH + H^+$. At the wavelength of 405 nm, the activities of ALP, of GGT and amylase were determined by enzyme kinetics following respectively the appearance of PNP, training of phosphonitroaniline and training 2-chloro-4-nitrophenol.

Statistical

The statistical analyses in this study allowed to compare the values obtained for every parameter between both groups of samples by means of the graph pad software Prism 5 with the use of the Mann-Whitney U test and a precision P of 0.05.

RESULTS AND DISCUSSION

This study on the enzyme markers of the seminal fluid allowed to compare the age of the two selected groups that samples in figure 1 represented 56 % for azoospermia and 44 % for normospermia.

There was no statistically significant difference for ALT, CPK, LDH, ALP and AMYL as indicated in table 1. On the other hand, in the same table, significant difference was identified for AST means (143.14 ± 13.69 IU/l) for azoospermia, and (224.17 ± 17.30 IU/l) for normospermia, and also with the means of γ-GT (11357 ± 1326 IU/l) for azoospermia and (16938 ± 1795 IU/l) for normospermia. In addition, the ratio AST/ALT being significant, it showed values of 3.8 ± 0.31 % for azoospermia, and a value of 6.52 ± 1.19 % for normospermia.

The enzyme activities of the parameters, presenting a significant difference, represented on the figures1 and 2, corresponded to the enzyme activities of AST and GGT respectively.

Male infertility is subjected to many scientific investigations with the assessment of the enzyme activities of the seminal fluid that could permit to better appreciate the abnormalities of this biological product. In this study carried out at IPCI, the selected patients were adult males aged 31 to 42 years. Mean age was 37.65 ± 0.97 years in normospermia group, 39.23 ± 0.87 years in azoospermia group. The statistical analysis of the age of both populations indicates that there is no significant difference between the age of azoospermia and normospermia men with a significativity P = 0.2334. This explains that the azoospermia is not a pathology associated with age for this segment of the population. However, this pathology is diagnosed after puberty since the test is performed by adults, consulting for an assessment of fertility. This study was therefore conducted in a homogeneous population.

Alkaline phosphatase is an enzyme which catalyzes the reactions of the hydrolysis of mono phosphate ester bonds at alkaline pH. It is linked to the cellular membranes and is thus involved in the transport of the metabolites across the cell membrane.

The profiles of ejaculates indicate an average of 275.12 ± 53.18 activities IU/l for the normospermic ejaculates and 308.30 ± 53.54 IU/l for the azoospermic ejaculates. This non-significant variation of the activity of the Alkaline phosphatase between azoospermic and normospermic seminal plasma with P = 0.668 allows to consider that the activity of this enzyme in the seminal fluid is of an epididymal and testicular origin as confirmed by previous studies[12,16]. In the case of azoospermia, its activity could therefore be used to differentiate the excretory azoospermia from a secretory type[16]. The non-significant change in Alkaline phosphatase activity between the azoospermic and normospermic seminal plasma indicates that this enzyme could be a seminal marker which expression is not specific to the spermatogenesis quality. The activity of the α-amylase in normospermia is 22.55 ± 6.09 IU/l compared to azoospermia whose average activity is 15.569 ± 1.44 IU/l. The obtained data indicate a non-significant variation between the normospermia and azoospermia ejaculates with P = 0.219. The comparison of these results with previous data shows that the action of α-amylase on the liquefaction of the seminal fluid is not related to the presence of sperm in the ejaculate[35]. Therefore, evaluation of the seminal activity of α-amylase could not inform about the quality of the spermatogenesis.

The seminal enzyme activity of CPK assessed was 681.35 ± 199.83 IU/l within the normospermic ejaculates and 548.50 ± 135.92 IU/l in the azoospermic ejaculated with a P value which is equal to 0.5733. These values indicate no significant change in the seminal CPK activity in the two kinds of ejaculate. Moreover, the results are consistent with those of the previous work that showed 661 ± 360 IU/l seminal activity [25]. On the contrary, its usage as a marker of maturation on morphology and to the potential fertilization of spermatozoa could be envisaged for a comparative study of previous data[20-22]. The profile of the LDH activity of the normospermic ejaculates indicated 1812.20 ± 174.48 IU/l average activity and 1579.60 ± 158.5 IU/l average activity of the azoospermic ejaculates with a value of P equal to 0.3325, indicating a non-significant variation. This profile of the seminal LDH activity is comparable to the one obtained from the infertile patients with

2487 ± 1384 IU / l average activity[17]. The results show variability in the expression of the seminal markers in the seminal plasma[11]. The comparative statistical analysis of the mean enzyme activity of the seminal markers indicates a non-significant variation of the seminal enzyme activity between normospermia and azoospermia for LDH, CPK, ALP and AMYL (P > 0.05). The presence of these enzymes in the sperm helps to envisage a further study of the sperm parameters in order to identify the correlations between these markers. These elements of the qualification of the seminal fluid, compared to previous studies, showed that the enzyme activity could be associated with the morphological damage of the spermatozoa[26, 29,23] and the number of spermatozoa in the ejaculate[1] and the Alkaline phosphatase[16].

Significant parameters

The assessment of the seminal activity of the transaminases (AST and ALT) has permission to present a profile of the seminal activity of these enzymes. For the ALT, the profile of normospermic ejaculates indicated 41.49 ± 3.72 IU/l average activity which was substantially identical in the azoospermic ejaculates whose average activity is 40.77 ± 4, 40 IU/l. Moreover, the statistical analysis indicates a non-significant difference in the activity of this enzyme in these two types of ejaculates (P = 0.9058). The seminal activity of the ALT could therefore not be influenced by the presence of spermatozoa in the ejaculate. The exploration of the seminal fluid, taking into account this enzyme only, could therefore not provide information about the quality of the spermatogenesis. However, in the normospermic ejaculates, there is an important enzyme activity of the AST which is 224.17 ± 17.30 IU/l compared to the azoospermic ejaculates where the enzyme activity whose average is 143.14 ± 13.69 IU/l with P = 0.0007 is low. Besides, the ratio of seminal activities AST/ALT indicates a higher value in the normospermic plasmas than in the azoospermic plasmas. In this case, the activity of the seminal AST is from 2 to 20 times higher than that of the ALT. This allows to have in the normospermic ejaculates the average ratio AST/ALT = 6.52 ± 1.09 whereas in azoospermia, this ratio is 3.80 ± 0.31. Regarding the statistical analysis which indicates a significant difference, the ratio of the seminal activities AST and ALT could therefore serve as a specific marker of the spermatogenesis. This increased datum in the normospermic ejaculates prove an effective spermatogenesis. These results are in accordance with those of the previous studies (30), which indicate that in case of azoospermia, this ratio is higher in the excretory type than in the secretive type. The seminal activity of the Aminotransferase aspartate (AST) and the ratio of AST/ALT activities could serve as spermic markers that provide information about the effectiveness of the spermatogenesis and the spermatozoa production.

Furthermore, studies on rabbit indicate that the improvement of the quality of the sperm obtained from dietary supplementations with vitamin C and / or E leads to a decrease of the activity of the AST, ALT and LDH in the seminal fluid[29]. This sperm quality is related to the volume of secretion, vitality, mobility and the sperm morphology. The improvement of these parameters is correlated with the decreased activities of these enzymes in the seminal fluid. Indeed, some studies reported that the acrosome, damages are due to enzymes released in sperm[26].

Besides, the seminal liquid contains cellular flows of germ cells and their cytoplasmic content. So, the spermatic cytosolic enzymes are also part of the

detectable enzymes in the seminal plasma. Moreover, the enzymatic activity of AST is related to the integrity of the spermatic membrane[28]. In the seminal fluid, they found out that a correlation exists between the AST located in the intermediate part of the spermatozoa and the spermatozoa immobility. The reason is that the lysing of the spermatic membrane caused by the blockage of the synthesis of Acid Trinucleotide Phosphate (ATP) could lead to the release of the AST[28]. As the AST has a spermatic origin, it could therefore reflect a presence of spermatozoa metabolism in the seminal fluid. The high activity of the AST in the normospermic ejaculates could be explained by the important presence of spermatozoa in the seminal fluid of normospermia comparatively to the azoospermic seminal fluids that do not contain spermatozoa Forejtek and Navratil, 1984[27]. In addition, the energy metabolism of spermatozoa is largely performed by the mitochondria located in the intermediate part of the spermatozoa through the oxidative phosphorylation that follows the glycolysis and the Krebs cycle[13,14]. The proton transfer from glycolysis and the Krebs cycle in the mitochondria are achieved by exchange systems located in the mitochondrial membrane. The shuttle aspartate/malate is a system of exchanges at the mitochondrial membranes with two enzymes that are the Dehydrogenase malate and the Aminotransferase aspartate (AST). Therefore, the AST is actively involved in the transfer of protons from glycolysis to the respiratory chain; enabling the process of the oxidative phosphorylation that outcome is the production of energy in the form of ATP. The metabolism of spermatozoa, which requires an important activity of the mitochondria[13], suggests an important expression of this enzyme in this cell organelle, which, moreover, occupies most of the cytoplasm of the cell. Its release in the sperm could therefore result in the lysis of the cell membrane resulting in the release of the cellular organelles and their digestions. The assessment of the enzyme activity shows a significant increase of the activity of the γ-glutamyl transferase in the normospermic ejaculates averaging 16938 ± 1795 IU/ l, compared to the azoospermic ejaculates where the enzymatic activity is 11357 ± 1326 IU/l. The statistical analysis of the results indicates a significant difference with a precision P = 0.0147. Unlike azoospermia, the enzyme expression of the γ-glutamyl transferase is very important in the seminal fluid of normospermia.

The administration of antioxidant vitamins, such as vitamins C and E, results in a remarkable improvement of the parameters of the sperm quality[29]. In addition, this treatment causes a significant reduction of the production of the reactive oxygen species with an increase of the activity of the glutathione S-transferase. The enzyme activity of the GGT has a positive correlation with the number of the horse's spermatozoa[1]. Its action in the protection of the spermatic cells against the oxidative stress could explain the increase in activity in normospermia ejaculates (32, 33, and 29)[31,32,29].

Numerous studies have shown that the substances that generate the reactive oxygen species can induce GGT[33]. This induction of GGT of the oxidative stress facilitates Glutathion (GSH) metabolism, de novo synthesis of GSH and GSH conjugates detoxification. This phenomenon makes the cells more resistant against a future oxidative challenge.

Previous studies have shown a positive correlation between the testosterone and the seminal activity of GGT[34]. So, this seminal activity of GGT could provide information about the quality of the secretion of the testosterone. Therefore a

sufficient hormone secretion is necessary for a good spermatogenesis. The epididymal origin of the GGT suggests an involvement of this enzyme in the sperm maturation[31]. This confirms that a high expression of this enzyme would be required for a complete maturation of the spermatozoa and the improvement of its kinetic structure. Thus, the involvement of the seminal vesicle secretions in the metabolism of the spermatozoon suggests the importance of the components of these secretions in the metabolism of spermatozoon which contains the GGT[31]. The metabolism of the germinal cells and the secretory glands would require an important expression of GGT. This could support the argument that an important seminal activity of GGT would promote the proper functioning of the secretory glands that are associated with normospermia. This enzyme appears as a seminal marker that activity expression is influenced by the presence of spermatozoa in the seminal fluid. It could therefore serve as a marker of the spermatogenesis seminal.

CONCLUSION

The cytological analysis of the sperm still occupies an important place in the assessment of the sperm quality. However, the biochemical analysis of the seminal fluid is important for a better understanding of the functioning of the glands. This would include the gonadal and post gonadal events. In this study, the evaluation of the seminal enzyme activity indicates significant enzyme activities in the seminal fluid. The comparison of the seminal enzyme activity of azoospermia with that of normospermia indicates a variation of the enzyme activity in the seminal fluid. The production of these enzymes is influenced by the gonadal activity, especially in the spermatozoa production. The AST and GGT are mainly better specified. The assessment of the enzyme activities of the AST and GGT may therefore serve in the diagnosis of azoospermia. This study also reveals that the ratio of enzyme activities AST/ALT serves as a marker to distinguish azoospermia from normospermia. Indeed, the mitochondrial localization of the AST could highlight a spermatic metabolic activity. In addition, the involvement of the GGT in the protection of the spermatic cells against the oxidative stress, inflammation, and in the transfer of amino acids across the spermatic membrane, also helps assess the quality of the spermatogenesis and the spermatic metabolism. Taking into account these parameters are considered after an assessment on a broad sampling to confirm the effectiveness of the spermatozoa presence depending on the activity of these enzyme markers.

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Table 1. Ages and enzyme activities in the seminal plasma

	Groups		P < 0, 05 significativity	
	Normospermia	Azoospermia		
Age (year)	37.65 ± 0.97	39.23 ± 0.87	0.2334	No significant
AST (UI/l)	224.17 ± 17.30	143.14 ± 13.69	0.0007	Significant
ALT (UI/l)	41.49 ± 3.72	40.77 ± 4.40	0.9058	No significant
AST/ALT %	6.52 ± 1.09	3.80 ± 0.31	0.0113	Significant
CPK (UI/l)	681.35 ± 199.83	548.50 ± 135.92	0.5733	No significant
LDH (UI/l)	1812.20 ± 174.48	1579.60 ± 158.55	0.3325	No significant
GGT (UI/l)	16938 ± 1795	11357 ± 1326	0.0147	Significant
ALP (UI/l)	275.12 ± 53.18	308.30 ± 53.54	0.668	No significant
AMYL (UI/l)	22.55 ± 6.09	15.569 ± 1.44	0.219	No significant

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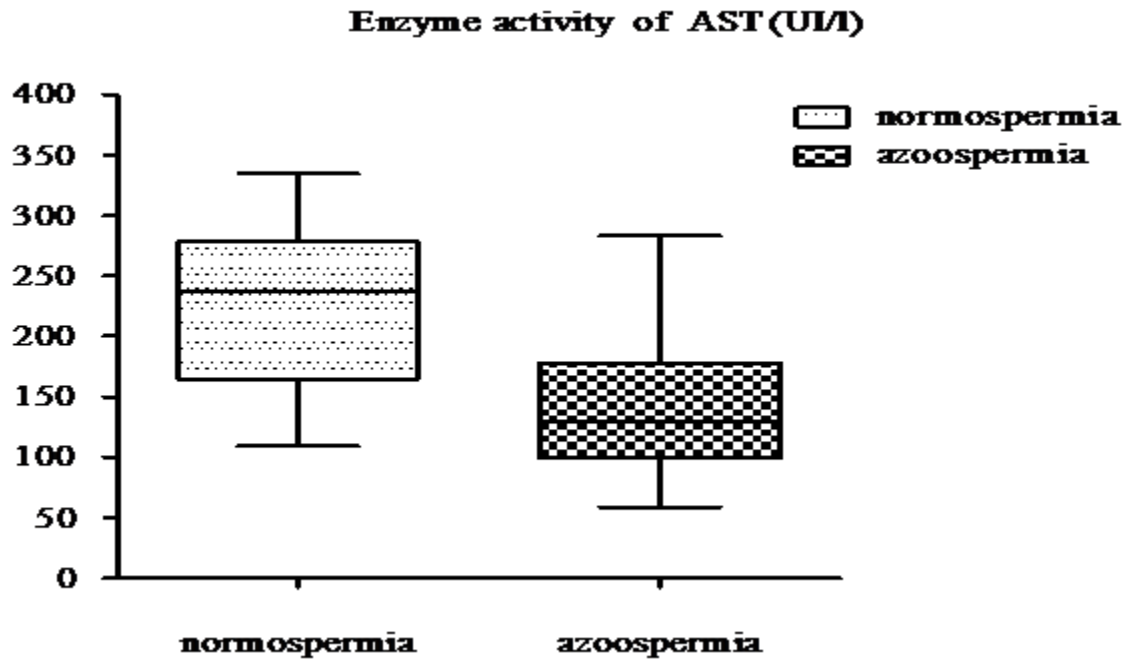

Figure 1. Comparison of mean enzymatic activity of AST (U/l)

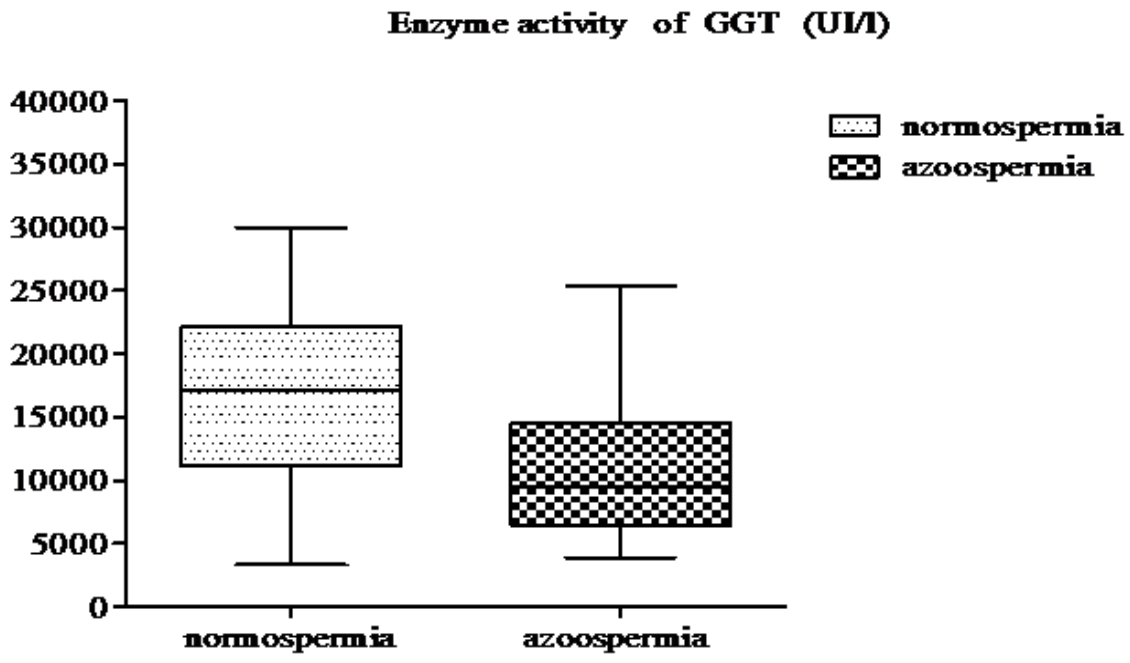

Figure 2. Comparison of mean enzyme activity of GGT