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Malate Dehydrogenase Activity in Human Seminal Plasma and Spermatozoa Homogenates

İnsan Seminal Plazma ve Spermatozoa Homojenatlarında Malat Dehidrogenaz Aktivitesi

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ABSTRACT

Purpose: Malate Dehydrogenase is an important enzyme of the Krebs cycle, most cells require this enzyme for their metabolic activity. We evaluated the Malate Dehydrogenase (NAD/NADP) activity in human seminal plasma and sperm homogenates in normozoospermic, fertile and infertile males. Also glucose and fructose concentrations were determined in the seminal plasma samples.

Material and Methods: Malate Dehydrogenase (NAD/NADP) activity in human seminal plasma and sperm homogenates of normozoospermic and infertile males was determined by spectrophotometric method. Semen analysis was considered according to the WHO Criteria.

Results: Malat Dehydrogenase-NAD value in seminal plasma (the mean ± SD, mU/ml) of asthenoteratospermic (40.0±25.7) and azospermic (38.0±43.6) groups were significantly lower than normozoospermic, (93.9±52.1) males. Malat Dehydrogenase-NAD value in sperm homogenates (the mean ± SD, mU/ $20x10^6$ sperm) of teratospermic group (136.8±61.8) was significantly higher compared to the normozoospermic (87.3±26.5) males. Glucose concentration (mg/dl) in asthenoteratospermic (4.0±1.4) and azospermic (15.4±6.4) groups were significantly higher than fertile (2.0±2.1) males. Also fructose concentration (mg/dl) in asthenoteratospermic (706.6±143.3) and azospermic (338.1±228.2) groups were significantly high compared to the normozoospermic (184.7±124.8) group.

Conclusion: Sperm may be some part of the source of Malat Dehydrogenase activity in semen. Malat Dehydrogenase activity in seminal plasma has an important role on energy metabolism of sperm. Intermediate substrates of Krebs cycle might have been produced under the control of Malat Dehydrogenase and these substrates may be important for sperm motility and male infertility.

Key Words: Malate dehydrogenase, male infertility, semen analysis, seminal plasma, spermatozoa homogenates.

ÖZET

Amaç: Malat Dehidrogenaz, Krebs siklusunun önemli bir enzimi olup pek çok hücre metabolik aktiviteleri için bu enzime ihtiyaç duymaktadır. Normozoospermik, fertil ve infertil erkeklerin seminal plazma ve sperm homojenatlarında Malat Dehidrogenaz (NAD/NADP) aktivitesi ölçüldü. Ayrıca seminal plazma örneklerinde glukoz ve fruktoz konsantrasyonları tesbit edildi.

Materyal ve Metod: Normozoospermik, fertil ve infertil erkeklerin seminal plazma ve sperm homojenatlarında Malat Dehidrogenaz (NAD/NADP) aktivitesi spektrofotometrik yöntemle ölçüldü. Semen analizi WHO kriterlerine göre değerlendirildi.

Bulgular: Seminal plazmada MDH-NAD değeri (ortalama ± SD, mU/ml), astenoteratospermik (40.0±25.7) ve azospermik (38.0±43.6) gruplarda, normozoospermik, fertil erkeklerden (93.9±52.1) anlamlı düzeyde düşük

bulunmuştur. Sperm homojenatlarında MDH-NAD değeri (ortalama ± SD, mU/ $20x10^6$ sperm) teratospermik grupta (136.8±61.8) normozoospermik, fertil erkeklerden (87.3±26.5) anlamlı düzeyde yüksek bulunmuştur. Glukoz konsantrasyonu (mg/dl), astenoteratospermik (4.0±1.4) ve azospermik (15.4±6.4) gruplarda fertil erkeklerden anlamlı düzeyde (2.0±2.1) yüksek bulunmuştur. Benzer şekilde fruktoz konsantrasyonu (mg/dl), astenoteratospermik (706.6±143.3) ve azospermik (338.1±228.2) gruplarda fertil erkeklerden anlamlı düzeyde (184.7±124.8) yüksek bulunmuştur.

Sonuç: Sperm, semendeki MDH aktivite kaynağının bir bölümü olabilir. Seminal plazmadaki MDH aktivitesi spermin enerji metabolizmasında önemli bir rol oynamaktadır. Krebs siklusunun ara substratları MDH'ın kontrolünde üretilmiş olabilir ve bu substratlar sperm motilitesi ve erkek infertilitesi için önemli olabilir.

Anahtar Kelimeler: Erkek infertilitesi, malat dehidrogenaz, semen analizi, seminal plazma, spermatozoa homojenatı.

INTRODUCTION

Infertility is defined as inability to conceive a child 12 months of unprotected intercourse without pregnancy. This problem affects approximately 15 % of the couples and male factor is responsible for 30-40 % of the cases[1,2]. Problems related to the production and maturation of the spermatozoa are the most common causes of male infertility. Sperm may be immature, produced in low numbers, abnormally shaped or unable to move properly[1,2,3]. The sperm cell compartmentalized into head, basal body (or midpiece), and tail. The basal body contains a large concentration of mitochondria that provides energy for the sperm motility through the production of ATP[4] .

Malate Dehydrogenase (MDH) and Malic Enzymes (ME) are found in mitochondria and cytoplasm. They are important enzymes of many metabolic pathways, including anaerobic respiration, tricarboxylic acid cycle, gluconeogenesis, maintenance of oxidation/reduction balance, NADPH production and fatty acid biosynthesis[5]. MDH is found in all eukaryotic cells as two isozymes: with mitochondrial and cytoplasmic forms. MDH (E.C. 1.1.1.37) catalyzes the interconversion of L-malate and oxaloacetate using NAD^+ as a coenzyme [5,6].

L-malate + NAD^+ ⟷ oxaloacetate + NADH + H^+

ME catalyzes the oxidative decarboxylation of malate to pyruvate together with the reduction of the cofactor NAD^+ or $NADP^+$ [7,8].

$$NAD(P)^+ + \text{L-malate} \leftrightarrow \overset{Mg^{+2}}{\text{NAD(P)H + pyruvate + } CO_2}$$

Oxidative phosphorylation, tricarboxylic acid cycle and aerobic glycolysis are energy supplying pathways for spermatozoa. Although MDH-NAD does not regulate Krebs cycle activity, due to the presence of cytosolic and mitochondrial enzymes in somatic cells, this enzyme plays a key role in the transport of reduction equivalents across the internal mitochondrial membrane. By biochemical analyses, MDH is reported as localized in the midpiece tail fractions of bovine spermatozoa. Histochemical studies indicated that MDH-NAD+ has the same localization in spermatozoa from the ram, boar and water buffalo. The mitochondrial activity enables bull spermatozoa to utilize the malate-aspartate shuttle as well as the lactate-pyruvate shuttle to transfer reducing equivalents from NADH (produced by glycolysis in the cytosol) to the mitochondria for complete oxidation by O2[9].

Fructose in human seminal plasma is derived from the seminal vesicles and is therefore a suitable marker for the secretory function of this accessory sex gland. Fructose is also an energy source for spermatozoa while staying in semen. In spite of low glucose concentration in seminal plasma, it may still supply almost half of the sugar consumption by spermatozoa at the average level of seminal fructose (12.4 µmol/mL) and glucose (0.3 µmol/mL) 10. The energy requirements of mature spermatozoa are primarily met through the utilization of glucose. Glucose is required for hyperactivated motility of sperm in mice and other

species, including humans. The absence of glucose significantly decreases human fertilization rates in vitro 11.

The aim of this study is to determine the MDH activity in the seminal plasma and sperm homogenates of normozoospermic, fertile and infertile men with the cofactor NAD^+ or $NADP^+$ and to examine the possible role of this enzyme on sperm motility. Also we determined the concentration of glucose and fructose in seminal plasma and we investigated the possible relation between MDH activity and these substrates.

MATERIALS and METHODS

Semen samples

Semen samples were provided from patients who attended the Assisted Reproductive Unit of Cukurova University, Medical School Obstetric Department for infertility evaluation. Written informed consents were obtained from all patients, according to the criteria of the Ethical Committee of the Medical Faculty. Specimens were collected by masturbation after 3-5 days of abstinence. Following liquefaction of the samples at 37˚C, sperm cell concentration, morphology and motility was determined according to the WHO criteria using light microscopy and Makler camera[12]. Samples were classified into normozoospermic, fertile (Group A, n=40) and four infertile groups. Infertile groups were; 1)oligoteratospermic (Group B, n=25), 2)teratospermic (Group C, n=25), 3)asthenoteratopermic (Group D, n=12), 4)azospermic (Group E, n=16) groups. Semen samples with more than $1x10^6$/ml leucocytes were excluded from the study.

Preparation of Seminal Plasma

Seminal plasmas were separated from the spermatozoa by centrifugation at 1500-2000 rpm for 10 min at room temperature. Samples were stored at -70˚C until the day of the study. Sperm contents were stored after swim up process.

Sperm Homogenisation

On the day of the study, spermatozoa were thawed at room temperature and treated with the mixture prepared from (1/1 (1/1 ratio) digitonin (% 0.02) and and phosphate buffer (pH 8.0 and 0.1M) with with a mean concentration of $20x10^6$/ml. ml. Spermatozoa samples were prepared in this way way and then homogenized with ultrasonic homogenisator (VIRTIS Virsonic 300 300 and 6 micro probe stroke) for 1-1.5 minute in ice. Sperm homogenates were were controlled with light microscopy to confirm the homogenisation process and and fragmentations were observed at the middle and tail compartments of the spermatozoa.

MDH Activity

MDH activity in seminal plasma and spermatozoa homogenates was measured by the increase in absorbance at 340 nm resulting from the reduction of NAD/NADP with the presence of malate [13]. Content of MDH was measured spectrophotometrically using Shimadzu (UV-260) spectrophotometer. The results for sperm homogenates were expressed as mU/ml MDH/$20x10^6$ sperm and mU/ml MDH for seminal plasma.

Glucose Concentration

Glucose concentration was measured by glucose oxidase (GOD-PAP) Enzymatic Trinder Method which is only specific for glucose as prescribed in the kit by Glucon.

Fructose Concentration

Fructose concentration was measured by Seliwanoff test **(**Bauer 1982)**.**

Statistical Analysis

Since semen parameters and MDH activities in seminal plasma and spermatozoa were not normally distributed, Mann-Whitney U-test (SPSS-12.0 and Excel program) was applied to compare the values between normozoospermic, fertile and infertile groups.

RESULTS

The mean values of the examined semen parameters in the normozoospermic, fertile and infertile groups are shown in Table 1. The mean ±

standart deviations (SD) of MDH (NAD/NADP) activities, glucose and ructose concentrations of seminal plasma in normozoospermic, fertile and infertile groups are shown in Table 2. MDH (NAD/NADP) activities of sperm homogenates in normozoospermic, fertile and infertile groups are shown in Table 3.

As shown in table 2 the mean ±SD of MDH-NAD activity in seminal plasma of asthenospermic group (p= 0.001) and azospermic group (p= 0.000) was significantly lower when compared with the normozoospermic cases. Also the mean ±SD of glucose concentration in seminal plasma of oligoteratospermic (p<0.05), asthenoteratospermic (p<0.05) and azospermic (p=0.000) groups were significantly higher when compared with the normozoospermic men. Additionally the mean ±SD of fructose concentration in seminal plasma of oligoteratospermic (p<0.05), asthenoteratospermic (p=0.000) and azospermic (p<0.05) groups were significantly higher when compared with the fertile cases.

According to the table 3 the mean ±SD of MDH-NAD activity in sperm homogenates of the teratospermic group (p=0.01) was significantly higher when compared with the normozoospermic men. There was a positive correlation (r=0.703 p=0.01) between sperm motility and MDH-NAD activity in seminal plasma samples of asthenoteratospermic group. A negative correlation (r=-0.616 p=0.03) was found between glucose concentration and MDH-NAD enzyme activity in this group. Also a negative correlation (r=-0.630 p=0.02) was detected between glucose concentration and sperm motility in seminal plasma samples of asthenoteratospermic group.

There was no significantly difference in MDH-NADP activity of seminal plasma and sperm homogenates.

Table 1. Basic parameters of semen samples in normozoospermic, fertile and infertile groups

Groups	Volume (ml)	Sperm concentration ($x10^6$ ml)	Motility	Normal morphology
Nodmozoospemia A Group (n=40)	3.3±1.1	53.1±31.6	71.3±6.6	17.8±4.3.4
Oligoteratospermia B Group (n=25)	2.8±0.9	12.4±3.9*	64.0±10.0*	3.9±2.1*
Teratospermia C Group (n=25)	3.5±1.6	46.4±24.5	68.4±7.9	6.5±2.6*
Asthenotarospermia D Group (n=12)	3.7±1.0	27.1±22.9**	18.8±14.3*	2.5±1.8*
Azospermia E Group	2.8±1.5	-------	------	------

Results are presented as mean±SD

*P=0.000 **P=0.001

Table 2. MDH (NAD/NADP) activities of seminal plasma in normozoospermic, fertile and infertile groups

Groups	MDH-NAD (mU/ml)	MDH-NADP (mU/ml)	Glucose (mg/dl)	Fructose
Nodmozoospemia (n=40)	93.9±52.1	155.1±77.7	2.0±2.1	184.7±124.8
Oligoteratospermia (n=25)	111.8±72.1	129.4±76.5	2.8.0±2.1***	272.1±104.3***
Teratospermia (n=25)	104.0±46.8	149.2±104.4	1.8±1.6	229.9±79.9
Asthenotarospermia (n=12)	40.0±25.7**	141.0±117.1	4.0±1.4***	76.6±143.3*
Azospermia (n=16)	38.0±43.6*	138.0±84.3	15.4±6.4*	338.1±228.2***

Results are presented as mean±SD

* P=0.000 **P=0.001 ***p<0.05

Table 3. MDH (NAD/NADP) (Mu/ML) activities of sperm homogenates in normozoospermic, fertile and infertile groups

Groups	MDH-NAD	MDH-NADP
Nodmozoospemia (n=40)	87.3±26.5	6.0±9.3
Oligoteratospermia (n=25)	88.3±49.3	20.0±26.7
Teratospermia (n=25)	136.8±61.8***	21.0±29.2
Asthenotarospermia (n=12)	103.0±24.6	7.9±27.4
Azospermia (n=16)	------	-------

Results are presented as mean±SD

* P=0.01

DISCUSSION

In this study, our aim was to compare MDH enzymes, in semen samples of fertile and infertile males in order to evaluate male infertility with a biochemical way.

In animal experiments MDH activity is studied in semen and spermatozoa. The importance of this enzyme is explained related to energy metabolism and fertilization capacity of spermatozoa. Lahnsteiner et al., studied the semen quality of the rainbow trout in accordance to sperm motility, seminal plasma parameters, and spermatozoal metabolism. They declare that sperm metabolism is characterised by key enzymes and metabolites of the most important spermatozoal metabolic pathways including oxidative phosphorylation (ATP,ADP, NADH, respiration activity), citrate cycle (malate dehydrogenase, NADH), lipid metabolism, glycolysis and ATP metabolism. They explained that seminal plasma LDH and MDH activity are correlated with the fertilization rate. MDH and aspartate aminotransferase enzymes were also correlated with sperm motility parameteres[14]. Zilli et al., reported that intracellular triglyceride concentration and MDH activity of sea bass sperm have a quadratic relation with fertilization rate. Low MDH activity is indicative of poor oxidative phosphorylation, but high enzymatic activity indicates low semen quality which requires an increased energy demand[15].

Prasad et al., studied MDH electrophoretically in a total of 99 semen samples obtained from normal, vasectomized, oligospermic, and infertile males and enzymatic patterns were compared with total sperm count and infertile males. They showed that the patterns of MDH did not differ in individuals with zero, low, or normal sperm counts[16]. But different MDH activity and different sperm counts were encountered in this study with enzymatic method.

MDH-NAD is found in cytoplasmic and mitochondrial forms and has an effective role on energy metabolism of sperm. Cordoba et al., studied with cryopreserved bovine spermatozoa and explained that MDH-NAD(P) and IDH-NAD(P) enzymes play a major role in supplying reduction equivalents and/or energy required for capacitation and acrosome reaction in spermatozoa[17].

Kohsaka et al., showed that in goat spermatozoa, lactate dehydrogenase was associated mainly with the inner acrosomal membrane in the head, the mitochondrial matrix in the midpiece and with flagellar fibrils in the tail, whereas malate dehydrogenase was present only in the mitochondrial matrix[18]. Matsuzawa et al., demonstrated two major MDH isozymes (MDH-A and MDH-B) in the rat sperm in an electrophoretic study[19].

Novak et al. investigated the global proteome of sperm and seminal plasma of fertile stallions to determine whether associations with relative in vivo fertility exist. They explained that when the proteins in spermatozoa are considered, all five identified proteins had a positive relationship with fertility and three of these proteins (citrate synthase, fumarate hydratase, and malate dehydrogenase) are involved in the TCA cycle. It is likely that increased sperm metabolism and the ability to use carbohydrates as energy may have a positive effect on the fertilizing ability of sperm[20].

Glucose supports functional modifications of sperms such as capacitation. Glycolysis plays a major role to supply ATP for sperm motility. In the absence of enough glucose, if there is a block on the glycolytic pathway or if the GLUT's are not functional then mitochondrial respiration may be effective to produce ATP in the use of the substrate. In this condition, intermediate substrates of the semen would be important for sperm motility. Then we can think about some metabolic pathways like malate (malate-aspartate shuttle) and pyruvate. If the pyruvate participates to the pathway, enough amount of ATP will not be gained. If pyruvate participates to the Krebs cycle then more ATP would be available . At this point we suggest that in the absence of glucose or if the use of glucose for sperm is not possible, then pyruvate in the semen can be utilised with a special transporter to use for gluconeogenesis. MDH/ME enzyme system is essential in the semen to supply this pyruvate.

Glucose metabolism plays a significant role in the hyperactivation mechanism of the sperms. The absence of glucose in the capacitating medium causes decreased ATP levels and failure of protein tyrosine phosphorylation. These changes appear to suppress hyperactivation whereas, pyruvate alone or glucose plus an uncoupler of oxidative phosphorylation can maintain about 90% of ATP levels in mouse sperm [4]. Miki explained that although the glycolytic pathway is essential for mature sperm function, substrate use seems to be optional. Sperms appear to adapt to their microenvironment by adjusting the ratio of glycolysis or oxidative phosphorylation depending on the availability of the substrate and oxygen. If the medium is poor in hexose, such as glucose or fructose, more lactate or pyruvate would be incorporated into sperm to compensate the shortage of hexose. ATP generated by the mitochondria in the middle piece may be distributed along the flagellum by adenylate kinase and other shuttles. Some of the mitocondrial ATP may also be used for glyconeogenesis, which provides substrates for re-extraction of ATP by the glycolytic pathway in the principal piece as observed in dog sperm [21]. Also Miki et al., reported that a monocarboxylate transporter (MTC2) is

localised in the sperm flagellum and should enable sperm to metabolize exogenous substrates (like pyruvate) via the mitochondrial tricarboxylic acid cycle [22].

In our study, MDH-NAD activity of seminal plasma of asthenoteratospermic and azospermic groups were significantly lower than the normozoospermic group (Figure 1). Glucose and fructose concentrations of asthenoteratospermic and azospermic groups were significantly higher than the normozoospermic group (Figure 2,3). Sperms use both glucose and fructose as an energy source. According to these results because asthenoteratospermic group has not enough motile sperm and azospermic group is presented with the absence of sperm there is no consumption of glucose and fructose in these groups. Also a positive correlation was found between sperm motility and MDH-NAD activity in seminal plasma samples of asthenoteratospermic group. Our results indicate that, the presence of pyruvate in the semen might be formed as a consequence of MDH/ME function . In asthenoteratospermic group a negative correlation was found between glucose concentration and MDH-NAD enzyme activity. Also a negative correlation was detected between glucose concentration and sperm motility. We thought that if MDH-NAD enzyme activity is low in seminal plasma then sperms would not express motility. So immotile sperms cannot use glucose and the level of glucose will increase in the seminal plasma. We found a positive correlation between MDH-NAD activity and sperm motility in seminal plasma samples of asthenoteratospermic group. Since the asthenoteratospermic group has lower sperm motility, it is postulated that the increase of MDH activity in seminal plasma might provoke the increase of sperm motility. Sperm cells need an effective energy metabolism to perform their functions and motility. We suggest that MDH activity has an important role on energy metabolism of sperm and intermediate substrates of Krebs cycle might have been produced under the control of MDH enzyme and these intermediate substrates are important for sperm motility.

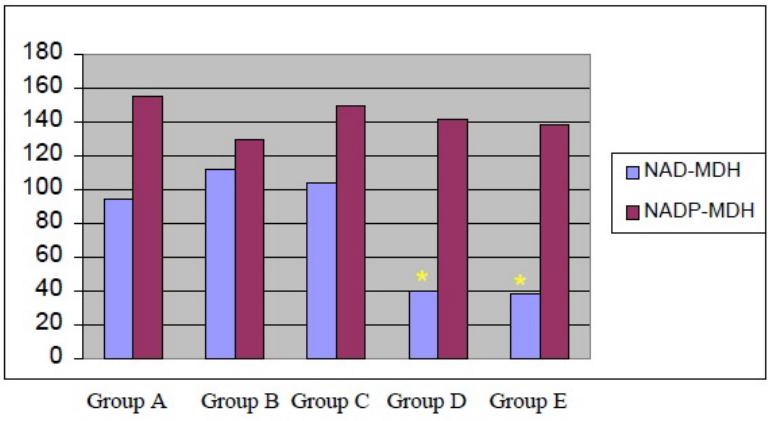

Figure 1. MDH (NAD/NADP) (mU/ml) activities of seminal plasma in normozoospermic, fertile and infertile groups

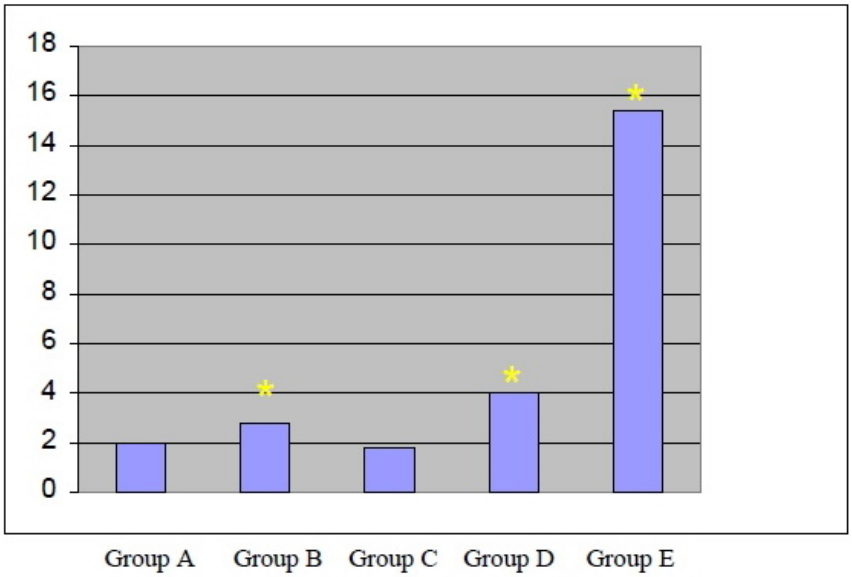

Figure 2. Glucose concentration (mg/dl) in seminal plasma of normozoospermic, fertile and infertile groups

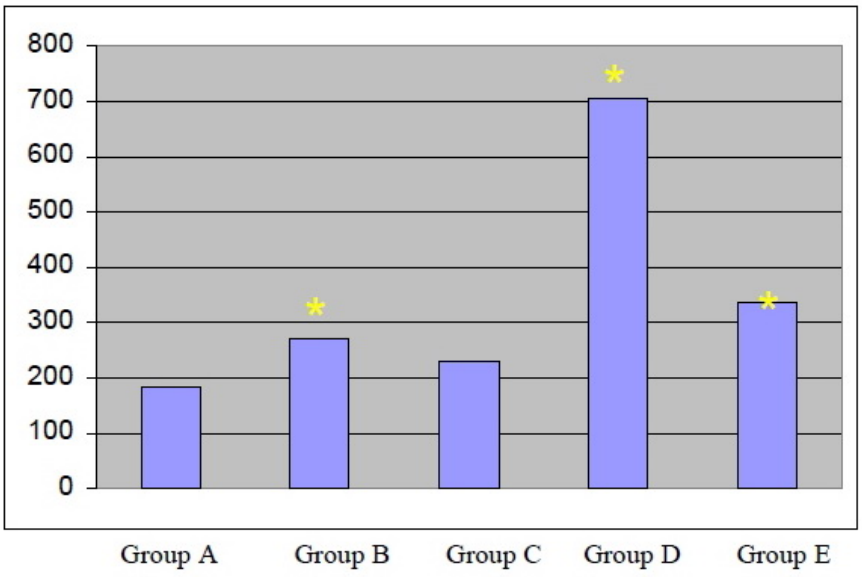

Figure 3. Fructose concentration (mg/dl) in seminal plasma of normozoospermic, fertile and infertile groups

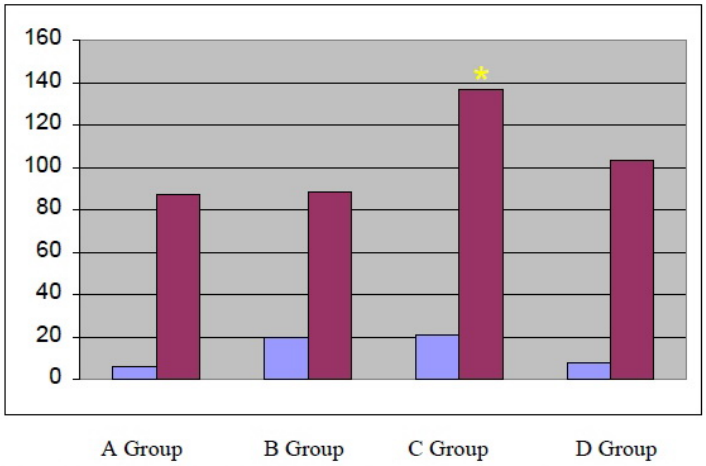

Figure 4. MDH (NAD/NADP) (mU/ml) activities of sperm homogenates in normozoospermic, fertile and infertil groups.

We believe that two pathways may be present in semen as a source of MDH. The first source might be the cytoplasm shedded during sperm maturation and the second one might be a secretion product that containes MDH. Also MDH-NAD activity in seminal plasma of azospermic group was significantly lower than fertil group. So we believe that one or both of these sources may be the source of MDH in seminal plasma. Mature human testes and spermatozoa possess a unique lactate dehydrogenase (LDH) isoenzyme, LDH-C4[23]. Clausen J et al., explained that the LDH-C4 isoenzyme can be determined in seminal plasma owing to the outward diffusion of the enzyme from the spermatozoa or to their destruction[24]. Garcia Diez et al., reported that the presence of LDH activity in the seminal fluid of the azospermic subjects suggest that the prostate gland and the seminal vesicles contribute to the secretion of that enzyme and higher LDH activity in the semen of normospermic subjects might have originated both from the testicles and the spermatozoa [25]. Also we found that normozoospermic group has higher MDH-NAD activity in their seminal plasma. In accordance with Carcia Diez et al., we also think that the prostate gland and the seminal vesicles may contribute to the secretion of MDH enzyme in seminal plasma and MDH might have been obtained in a similar way with LDH-C4[25].

Another interesting point that we found in sperm homogenates of the teratospermic group was significantly high MDH-NAD activity compared to the normozoospermic group . We thought that there may be a problem concerning the transport of the cytoplasmic MDH to the semen. Or if there is a problem on metabolic pathways, enzyme activity might have increased as a defense mechanism. We suggest that further studies on MDH activity of seminal plasma and sperm will be usefull to explain the changes of sperm morphology.

We found that NAD-dependent MDH enzyme activity in seminal plasma and sperm homogenates were more significantly different than NADP-dependent MDH form.

In conclusion, we believe that sperm may be the source of MDH activity in semen and MDH-NAD activity in seminal plasma has an important contribution to energy metabolism of sperm. So this enzyme may be an important infertility marker to evaluate sperm functions related to male infertility in clinical biochemical analysis. Surely this study should be supported with further studies.

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