Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ DERGİSİ

Cukurova Med J 2017;42(3):413-418
DOI: 10.17826/cutf.323368

ARAŞTIRMA / RESEARCH

Malate dehydrogenase/malic enzyme: is it another NADPH generating enzyme in human follicular fluid?

Malat dehidrogenaz/malik enzim: insan folikül sıvısında NADPH üreten diğer bir enzim mi?

Hülya Leventerler[1], İbrahim Ferhat Ürünsak[1], Ümit Yaşar[2], Mehmet Turan Çetin[1], Nurten Dikmen[2]

[1]Cukurova University Faculty of Medicine, Department of Gynecology and Obstetric-Center of Assisted Reproduction, [2]Department of Medical Biochemistry, Adana, Turkey.

Cukurova Medical Journal 2017;42(3):413-418

Abstract

Purpose: The purpose of this study was to determine malate dehydrogenase and malic enzyme activities in human follicular fluid of patients who attended the assisted reproductive unit and to investigate their relation with oocyte and embryo development.

Materials and Methods: Thirty patients were classified into three groups with respect to their age. Changes in follicular fluid malate dehydrogenase and malic enzyme activity were analyzed and compared with serum follicle stimulating hormone and estradiol levels and other criteria (age, number of retrieved oocytes, number of developed embryos, number of grade 1 embryo and grade 2 embryo) to determine the possible effects on embryo development.

Results: A weak but significant correlation was detected between malate dehydrogenase-NADP activity and estradiol level. malate dehydrogenase-NAD activity and estradiol level were highest in the second group (age between 30-45). But no significant correlation was determined between malate dehydrogenase-NAD activity and estradiol level in this group.

Conclusion: NADPH is an important cofactor for lipid and steroidal hormon synthesis and enzymatic antioxidant defence systems in mammalian oocytes and embryos. According to our results, we believe that the presence of malate dehydrogenase activity in human follicular fluid may be an alternative supplier of NADPH in addition to glucose-6-phosphate dehydrogenase activity.

Key words: Enzyme, follicular fluid, oxidative stres, oocytes

Öz

Amaç: Bu çalışmanın amacı, üremeye yardımcı tedavi merkezine başvuran hastaların folikül sıvı örneklerinde malat dehidrogenaz ve malik enzim aktivitelerini inceleyerek oosit ve embriyo gelişimi üzerindeki olası etkilerini araştırmaktır.

Gereç ve Yöntem: Otuz hasta yaşlarına göre 3 gruba ayrıldı. Hastaların folikül sıvı örneklerinde malat dehidrogenaz ve malik enzim aktivite değişimleri incelenerek serum folikül stimülan hormon ve estradiol düzeyleri ve diğer kriterlerle (yaş, toplanan oosit sayısı, gelişen embriyo sayısı, 1. kalite embriyo sayısı, 2. kalite embriyo sayısı) ilişkisi ve embriyo gelişimi üzerindeki olası etkileri karşılaştırıldı.

Bulgular: Malat dehidrogenaz-NADP aktivitesi ve estradiol düzeyi arasında zayıf fakat anlamlı bir korelasyon saptandı. En yüksek malat dehidrogenaz-NAD aktivitesi ve estradiol düzeyi ikinci grupta (30-45 yaş aralığı) bulundu. Bu grupta malat dehidrogenaz-NAD aktivitesi ve estradiol düzeyi arasında önemli bir korelasyon saptanmadı.

Sonuç: NADPH, memeli oosit ve embriyolarında, lipit ve steroidal hormon sentezi ile enzimatik antioksidan koruma sistemleri için önemli bir kofaktördür. Sonuçlarımıza göre, insan foliküler sıvısında malat dehidrogenaz aktivitesinin varlığının gösterilmesi glukoz-6-fosfat dehidrogenaz aktivitesine ek olarak alternatif bir NADPH kaynağı olabileceğini düşündürmüştür.

Anahtar kelimeler: Enzim, folliküler sıvı, oksidatif stres, oosit

Yazışma Adresi/Address for Correspondence: Hülya Leventerler, Cukurova University Faculty of Medicine, Department of Gynecology and Obstetric-Center of Assisted Reproduction, Adana, Turkey. E-mail: hleventerler@cu.edu.tr
Geliş tarihi/Received: 14.10.2016 Kabul tarihi/Accepted: 30.11.2016

INTRODUCTION

Human follicular fluid (FF) is an important composition that contain biologically active molecules and proteins[1]. FF is produced during folliculogenesis and results from the transfer of blood plasma components and the secretory activity of the oocyte, granulosa and thecal cells[2]. It reflects the metabolism of the whole follicle contains regulatory molecules that are essential for the successful maturation of oocytes, fertilization and embryo development[1,3]. Investigation of follicular fluid composition as a medium by which signalling molecules are transported in and out of the follicle. Also importance of follicular fluid as a predictor of both oocyte developmental competence and embryo viability has increased in recent years[3-5].

The results of the recent studies provide strong evidence that preantral human follicles utilize both the glycolytic and the Krebs cycle for energy production when exposed to gonadotropins. It is proposed that cytosolic Malate Dehydrogenase (MDH)-1 is an essential factor for oocyte maturation and embryo development in mouse[6]. 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. MDH (E.C.1.1.1.37) catalyzes the interconversion of L-malate and oxaloacetate using NAD+ as a coenzyme[7,8].

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[9,10].

Mg+2 NAD(P)+ + L-malate⟷ NAD(P)H+pyruvate+ CO2

In our previous study, we examined MDH activity in seminal plasma and spermatozoa homogenates of normozoospermic, fertile and infertile males. MDH-NAD value in seminal plasma of asthenoteratospermic and azospermic groups were significantly lower than normozoospermic males[11]. So we thought that MDH activity may affect sperm motility, fertilization capacity of sperm and also male infertility.

The aim of the present study was to determine MDH and ME activities in human follicular fluid and to investigate their relation with oocyte and embryo development. Also serum Follicle Stimulating Hormone (FSH) and Estradiol (E2) levels were compared with enzyme activities and other criteria (age, number of collected oocytes, number of developed embryos, number of grade 1 embryo and grade 2 embryo) to determine the possible effects on embryo development.

MATERIAL AND METHODS

Sample collection

Follicular fluid samples were collected from 30 patients who attended the Assisted Reproductive Unit of Cukurova Medical School Department of Obstetrics for infertility evaluation. Written informed consents were obtained from all patients according to the criteria of the Ethical Committee of the Medical Faculty (Meeting date: 09.02.2012 decision-36). Only FF with no blood contamination from mature follicles was used in this study.

Patients either received a standard GNRH agonist (Triptorelin acetate) regime starting on day 21 of a spontaneous menstrual cycle or a standard antagonist regime (Ganirelix). Recombinant FSH stimulation was initiated once down-regulation was confirmed via ultrasound and serum E2 level or on day 2 or 3 for antagonist cycles. Further stimulation doses were determined according to Standard criteria for follicular maturation, assessed by ultrasound and serum E2 levels. Choriogonadotropin alfa (6500 IU) was administered when at least three follicles had reached a diameter of ≥18 mm. Oocytes were collected by transvaginal ultrasound-guided needle aspiration of the follicles under deep conscious sedation. Samples were centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was stored at -70°C until further analysis.

Thirty patients were classified into three groups with respect to their age. Group 1 (n=10): age under 29, group 2 (n=10): age between 30-35, group 3 (n=10): age over 36. 19 of the patients had male factor and 11 of the patients the cause of infertility remained unexplained. MDH (NAD/NADP) activities were determined in the FF samples and these activity

results were evaluated with the following criteria; age, number of retrieved oocytes, number of developed embryos, number of grade 1 embryo and grade 2 embryo. Serum FSH and E2 levels were measured on the day 3 of the menstrual cycle. Oocytes were examined for fertilization 16-18 h after Intra Cytoplasmic Sperm Injection (ICSI and cleavage of the oocytes were evaluated on day 2 (48 h) and day 3 (72 h) before transfer into the uterus. A grade 1(day 3) embryo is one in which all of the cells are the same size and there is no fragmentation in the embryo. Grade 2 embryo with blastomeres of equal size, minor cytoplasmic fragments or blebs12. 1 embryo was transfered to 17 patients and 2 embryos were transfered to 13 patients. All these results were compared with the clinical pregnancy rates.

MDH activity

MDH (NAD/NADP) activity (expressed as mU/ml) in FF samples were measured by the increase in absorbance at 340 nm resulting from the reduction of NAD/NADP with the presence of malate. Content of MDH was measured spectrophotometrically using EONTM microspectrophotometer (Malate dehydrogenase: wwwworthington-biochem.com).

FSH and E2 levels

FSH (expressed as mIU/ml) and E2 (expressed as pg/ml) levels were determined by chemiluminescence method and using the Beckman DX -800 device and commercially available kits.

Statistical analysis

All analyses were performed using SPSS 19 statistical software package (IBM SPSS Statistics). Categorical variables were expressed as numbers and percentages, whereas continuous variables were summarized as mean and standard deviation and as median and minimum-maximum where appropriate. For non-normal distributed data, Kruskal Wallis test was used to compare more than two groups. To evaluate the correlations between measurements, Spearman Rank Correlation Coefficient was used. The statistical level of significance for all tests was considered to be 0.05.

RESULTS

FF samples from 30 patients were used in this study and the characteristics of the study population are given in Table 1.

Table 1. Enzyme activities, hormone levels and other characteristics of the study population

	Group 1	Group 2	Group 3	p
Age (years)	25.36±0.69	31.40±0.92	39.82±1.29	
No. of oocytes retrieved	6.64±0.97	5.00±1.18	3.91±0.62	0.054
No.of embryo	3.82±0.81	3.60±1.20	2.36±0.45	0.237
No.of grade 1 embryo	2.36±0.56	2.80±0.86	2.09±0.41	0.711
No.of grade 2 embryo	1.45±0.49	0.80±0.49	0.27±0.19	0.090
MDH-NAD activity (mU/ml)	2.84±0,36	3.12±0.85	2.09±0.27	0.149
MDH-NADP activity (mU/ml)	0.92±0.64	0.36±0.36	0.83±0.66	0.020*
FSH (mIU/ml)	6.86±0.66	8.02±0.69	9.46±0.68	0.041*
E2 (pg/ml)	31.00±2.96	42.60±10.56	39.73±5.36	0.034*

Results were presented as mean ± SD *P<0.05; MDH: Malate Dehdyrogenase; NAD: Nicotinamide dinucleotide; NADP: Nicotinamide dinucleotide phosphate; FSH: Follicle Stimulating Hormone; E2: Estradiol

It is observed that the number of the retrieved oocyte and the number of the developed embryo was decreased with increasing woman age. Also FSH level was increased (p=0.041) and the number of the retrieved oocyte was decreased (p=0.054) with increasing woman age. In the study, a weak (r=0.444) but significant (p=0.020) correlation was detected between MDH-NADP activity and E2 level. Embryo transfer was performed to the 30 patients. 10 of the 30 patients were pregnant. B-hCG was measured for diagnosis of pregnancy 12 days after embryo transfer. However we found no relationship between pregnancy rate and other parameters. MDH-NAD activity was highest in the second group (age between 30-45). Also E2 level was detected highest in the second group. But no significant correlation was determined between MDH-NAD activity and E2 level in this group.

DISCUSSION

FF contains important molecules which have critical potential for oocyte growth and development and provides essential information about the growth and differentiation of the follicle. The analysis of the metabolites of the FF may also provide important information about the biochemical composition of the FF and the changes in the blood serum parameters. Changes of biochemical components mainly metabolic enzymes found in FF that relate to the folliculogenesis may help to investigate the events related with maturation of the follicle and oocyte[13].

MDH is an important enzyme of the Krebs cycle, most cells require this enzyme for their metabolic activity. Yoon et al. (2006) discussed the expression pattern of MDH and its role in mouse oocyte maturation and preimplantation embryo development. They explained that the cytosolic (Mor2) and mitochondrial (Mor1) MDH isozymes perform a key role in the passage of reduction equivalents through the internal mitochondrial membrane. Both isozymes are encoded by nuclear genes and synthesized in the cytoplasm. Cytosolic Mor2 remains in the cytosol after synthesis, whereas Mor1 is translocated to the mitochondrial matrix. Data from the study suggest that Mor2 plays an important role in oocyte maturation and embryo development. Ablation of Mor2 gene expression by microinjection of Mor2 dsRNA resulted in decreased oocyte maturation to the MII stage[14].

In our previous study, we determined MDH activity in seminal plasma and spermatozoa homogenates of normozoospermic, fertile and infertile males. MDH-NAD activity of seminal plasma of asthenoteratospermic and azospermic groups were significantly lower than the normozoospermic group. We found a positive correlation between MDH-NAD activity and sperm motility in seminal plasma of samples of asthenoteratospermic group[11].

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.

Normal reproductive development in mammals is depend on the properly regulated biosynthesis of sex steroids. Many diseases and conditions affecting fertility and general health are accompanied by aberrations in androgen or oestrogen metabolism. Estradiol synthesis requires the activity of members of the Cytochrome P450 enzyme family and a second family of enzymes, the hydroxysteroid dehydrogenases. Aromatization of androgens requires the sequential transfer of three pairs of electrons and consumes three moles of oxygen and three moles reduced NADPH in the synthesis of one mole of oestrogen[15].

Aim of the present study was to determine MDH activity in FF samples and to explore possible participation of the enzyme activity in oocyte and embryo development [16,17]. Also serum FSH and E2 levels were compared with enzyme activities and other criteria. In the study, a weak ($r=0.444$) but significant ($p=0.020$) correlation was detected between MDH-NADP activity and E2 level. Alan et al. (2001) reported that aromatization of androgens requires three moles reduced NADPH in the synthesis of one mole of oestrogen[18]. So MDH-NADP activity may effect oestrogen synthesis and may support follicle and oocyte development with the possible participation of E2.

Oxidative metabolism is important for gamete and embryo energy production and is related with the generation of reactive oxygen species (ROS). ROS may originate either directly from gametes and embryos or from their surrounding environment. Mouatassim et al.1999, Tarin et al.2000, Cetica et al. 2001, Guerin et al.2001, reported the presence of enzymatic antioxidant defences in mammalian oocytes and embryos. Also M.C. Carbone et al.,demonstrated the presence of the major antioxidant and detoxifying enzymes (Superoxide dismutase-SOD, catalase and glutathione peroxidase-GSH-Px) in human follicular fluid[19].

The antioxidant activity of glutathione (GSH) depends on its reduced form which is regenerated from oxidized glutathione (GSSG) through the action of glutathione reductase (Gardiner et al 1998). Glucose-6-phosphate dehydrogenase (G6PD) has been proposed as a cell enzyme aiding in survival against oxidative stress by generating NADPH required for GSH regeneration. Flint and Denton (1970) reported that NADPH can be

supplied by malate dehydrogenase and isocitrate dehydrogenase, which in rat ovary depicit an activity equal or higher to that shown by other tissues[20].

According to our results, we believe that the presence of MDH activity in human FF may be an alternative supplier of NADPH in addition to G6PD activity. NADPH is a cofactor for lipid, steroid, and nucleic acid synthesis. It is also used for regeneration of GSH. ROS in FF may affect the quality of oocytes, their development, fertilization potential and implantation rate. So continued embryo implantation failure implies that other factors affect oocyte quality in FF and also MDH activity may be effective as a NADPH supplier for the action of GSH[21].

Dorgan et al. suggested that in their study, there are age related changes in plasma estrogen and androgen levels in adult premenopausal women. The occurence of ovulatory cycles in women with regular menses increases with age during the teens and 20s, plateaus during the 30s, and falls of again in the 40s[22]. Musey et al. also reported higher levels of estradiol but not estrone during the follicular phase of the menstrual cycle in older (29-40 years) compared to younger women (18-23 years) and suggested that decreased hepatic metabolism may account for the elevated estradiol levels, but increased ovarian secretion could not be ruled out. The increases in follicular phase plasma estradiol and estrone levels that they observed with age suggest that the estradiol and estrone level might increase at least partly, due to increased ovarian secretion[23]. In our study we have an interesting determination. The following parameters were highest in the second group ; number of grade 1 embryo, MDH-NAD activity and E2 level. But there was no significant correlation in these parameters. We think that in the patients of the second group (30-35 years) elevated estradiol levels may cause an increase in MDH-NAD activity. So these hormon levels and enzyme activities may provide the optimum conditions for the development of grade 1 embryo.

In conclusion, this is the preliminary study to compare MDH enzyme activity with the following parameters; oocyte growth and development, embryo quality, serum hormon (FSH, E2) levels, presence or absence of pregnancy. Some limitations in the study exist such as the small sample size. Further studies with other parameters in larger study groups are required in order to validate current findings. Next studies may provide important contribution to the results of this study.

Acknowledgements

This project was supported by The Research Foundation of Cukurova University (Project number: TF2012BAP16).

REFERENCES

1. Bianchi L, Gagliardi A, Campanella G, Landi C, Capaldo A, Carleo A et al. A methodological and functional proteomic approach of human follicular fluid en route for oocyte quality evaluation. J Proteomics. 2013;90:61-76.
2. Hashemitabar M, Bahmanzadeh M, Mostafaie A, Orazizadeh M, Farimani M, Nikbakht R. A proteomic analysis of human follicular fluid: comparison between younger and older women with normal FSH levels. Int J Mol Sci. 2014;15:17518-540.
3. Revelli A, Piane LD, Casano S. Follicular fluid content and oocyte quality:from single biochemical markers to metaboolomics. Reprod Biol Endoc. 2009;7:1-13.
4. Zamah AM, Hassis ME, Albertolle ME. Proteomic analysis of human follicular fluid from fertile women. Clin Proteomics. 2015;1:1-12.
5. Wallace M, Cottell E, Gibney MJ. An investigation into the relationship between the metabolic profile of follicular fluid, oocyte developmental potential, and implantation outcome. Fertil Steril. 2012;97:1078-84.
6. Roy SK, Terada DM. Activities of glucose metabolic enzymes in human preantral follicles: in vitro modulation by follicle-stimulating hormone, luteinizing hormone, epidermal growth factor, insulin-like growth factor I, and transforming growth factor beta1. Biol Reprod. 1999;60:763-8.
7. Christopher RG, Nicholls DJ. Malate dehydrogenase: a model for structure, evolution, and catalysis. Protein Sci. 1994;3:1883-8.
8. Minarik P, Tomaskova N, Kollarova M. Malate Dehydrogenases-structure and function. Gen Physiol Biophys. 2002;21:257-65.
9. Yingwu X, Bhargava G, Wu H, Loeber G, Tong L. Crystal structure of human mitochondrial NAD(P)+-dependent malic enzyme:a new class of oxidative decarboxylases. Structure. 1999;7:877-89.
10. Yang Z, Zhang H, Hung HC, Kuo CC. Structural studies of the pigeon cytosolic NADP+ -dependent malic enzyme. Protein Sci. 2002;11:332-41.
11. Leventerler H, Taga S, Ürünsak İF, Arıdoğan İA, Solmaz S, Çetin MT et al. Malate dehydrogenase activity in human seminal plasma and spermatozoa homogenates. Cukurova Med J. 2013;38:648-58.
12. Dennis SJ, Thomas MA, Williams DB, Robins JC. Embryo morphology score on day 3 is predictive of

implantation and live birth rates. J Assist Reprod Genet. 2006;23:171-5.
13. Adiga SK, Kumar P, Bijoor SK. Lactate dehydrogenase estimation in follicular fluid: correlation with patient age, follicle size and super ovulation in ART cycles. Eur J Obstet Gynecol Reprod Biol. 2002;105:150-4.
14. Yoon SJ, Koo DB, Park JS, Choi KH, Han YM, Lee KA. Role of cytosolic malate dehydrogenase in oocyte maturation and embryo development. Fertil Steril. 2006;86:1129-36.
15. Conley A, Hinshelwood M. Mammalian aromatases. Reproduction. 2001;121:685-95.
16. Leventerler H, Taga S, Yaşar Ü, Ürünsak İF, Çetin MT, Dikmen N. Folikül sıvısında malat dehidrogenaz ve malik enzim aktivitelerinin incelenmesi. Abstracts of the IV. Meeting of the Society for the Reproductive Medicine, 26-29 September 2013, Belek- Antalya.
17. Leventerler H, Ürünsak İF, Yaşar Ü, Taga S, Sertdemir Y, Çetin MT et al. Study of the relation among malate dehdyrogenase activity of the follicular fluid and hormon level and oocyte quality of the patients who attended the assisted reproductive unit. 13th National Congress of Gynecology and Obstetric, 11-15 May 2015, Belek, Antalya
18. Carbone MC, Tatone C, Monache SD, Marci R, Caserta D, Colonna R et al. Antioxidant enzymatic defences in human follicular fluid: characterization and age dependent changes. Mol Hum Reprod. 2003;9:639-43.
19. Jana SK, Chattopadhyay R, Chakravarty B, Chaudhury K. Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. Reprod Toxicol. 2010;29:447-51.
20. Gardiner CS, Salmen JS Brandt CJ, Stover SK. Glutathione is present in reproductive tract secretions and improves development of mouse embryos after chemically induced glutathione depletion. Biol Reprod. 1998;59:431-6.
21. Camarillo CO, Gonzalez AG, Onofre MV, González-Padilla E, Avalos-Rodríguez A, Gutiérrez-Rodríguez ME et al. Changes in the glucose-6-phosphate dehydrogenase activity in granulosa cells during follicular atresia in ewes. Reproduction. 2009;889-986.
22. Dorgan JF, Reichman ME, Judd JT, Brown C, Longcope C, Schatzkin A et al. Relationships of age and reproductive characteristics with plasma estrogens and androgens in premenopausal women. Cancer Epidemiol Biomarkers Prev. 1995;4:381-6.
23. Musey VC, Collins DC, Brogan DR, Santos VR, Musey PI, Martino-Saltzman et al. Age-related changes in the female hormonal environment during reproductive life. Am J Obstet Gynecol. 1987;157:312-7.