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The Effect of Melatonin on Izumo1 Gene Expression, Sperm Motility and In Vitro Fertilization in Mice

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Abstract

Objective: IZUMO 1 is one of the most important and the most recent known proteins of sperm-oocyte fusion function. The present study aims to investigate the expression of *IZUMO1* gene with melatonin injection in in-vitro conditions. Moreover, the sperm-oocyte fusion rate in in vitro fertilization (IVF) condition was examined.

Materials and Methods: In this study, 30 female and 45 male mice were divided into control and experimental groups. To investigate Izomo1 gene expression and sperm motility, 10 µM melatonin was added to the culture medium in the experimental group for 1 hour and to examine the fertilization rate 10 µM melatonin was added to culture medium in the experimental group for 1 hour. Then, sperms of the control and experimental groups were added to the oocyte collected and the fertilization was examined on embryo formation.

Results: Examinations showed a significant increase in *IZUMO1* gene expression and sperm motility in the experimental group (receiving melatonin) compared to the control group. Counting formed embryos showed that 83% of the oocytes have evolved to the fetus.

Conclusion: We conclude that melatonin may be a good alternative for rising oocyte fertilization success by sperm in IVF.

Keywords: Melatonin, In vitro fertilization, IZUMO1, Gamete fusion, RT-PCR

Introduction

Today, infertility is one of the main problems of human societies that affects about 10%-15% of young couples. According to the definition of the World Health Organization (WHO), infertility means failure to pregnancy within a year among couples who are sexually active and do not use any contraception method (1,2). About 30%-50% of infertility is related to males' factors that may be due to azoospermia, oligospermia, abnormal sperms in terms of morphology and motility and fertility (3,4). One of the factors that could affect fertility is oxidative stress (5). Oxidative stress is clearly associated with male factor infertility by inducing lipid peroxidation, DNA damage and problem related sperm-oocyte interaction (2,6).

Assisted reproductive technology (ART) is the most common treatment for infertility. In vitro fertilization (IVF) is one of the common methods in ART (2). The membrane fusion of spermatozoon and oocyte cells is the central event of fertilization. Fertilization occurs only in the case of interaction between protein receptors located on the surface of sperm and oocyte that recognizes and binds sperm and oocyte (7,8).

The new science of molecular and biology by comparing fertile and infertile men's surface sperm proteins have identified potential markers of fertility involved in male infertility (9). Several protein receptors involve in the fertilization process. But, clearly 2 proteins have been proven in in-vivo fertility, including IZUMO1 in sperm and Cd9 in the oocyte. Izumo1 is expressed at the plasma membrane of acrosome-reacted sperm (7,9,10). During the acrosomal reaction that is carried out in 3 stages, the protein is moved from the anterior of the sperm head to the site where fusion will take place(9). Men with inadequate IZUMO1 are infertile and even sperms without IZUMO1 are not able to bind to the oocyte (7,11). In fact, infertility of oocytes extracted from the in-vivo after fertilization with sperms without IZUMO1 showed that sperms passed through the zona pellucida (ZP), but accumulated in the perivitelline setting and could not combine and pierce the oocyte membrane (9). These results were shown in an in vitro environment through an IZUMO monoclonal antibody by preventing the sperm-oocyte fussing and were approved. Furthermore, intracytoplasmic sperm injection (ICSI) without IZUMO1 into the normal oocyte was associated with fertility of female mice. These results provide evidence that IZUMO1 is an important factor for fertility (7,12,13). Recently Juno has been identified as IZUMO1 receptor that is known as membrane-tethered folate receptor also known as Folr4. Female mice with

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the lack of Juno are infertile and oocytes with insufficient Juno are not able to fuse with normal sperms (14,15).

Due to the high cost of ART techniques and low rates success of these techniques, to find a way to increase fertilization rates seems necessary. One of the ways to increase the fertilization rate is the use of antioxidants in IVF (2,16). Antioxidants are compounds that prevent the formation of reactive oxygen species (ROS) and lipid peroxidation or destroy the formed ROS (6).

Biological compounds with antioxidant properties are capable to protect cells and tissues against impairment of ROS and free radicals (5,6,17). Melatonin is the hormone secreted by the pineal gland with an antioxidant specification that prevents the cells and tissues damage against the oxidative stress due to the antioxidant activity (18,19). Previous studies have been shown; melatonin can regulate the expression of some genes in rat epididymis through G protein receptors, and has possessed genomic actions (20,21). In this study the effect of melatonin on *IZUMO1* gene expression, sperm motility and IVF in mice has been investigated.

Materials and Methods

Animals

In this study, 30 female mice and 45 male mice of the BALB/c were used. The mice were obtained from the animal house of Tabriz University of Medical Sciences (TUMS) and were kept under standard conditions (temperature of 22-24˚C with the cycle of light/dark for 12 hours).

Preparation of Melatonin

Melatonin was produced from Sigma Company and its stock solution was prepared in ethanol/TCM199 system. For this purpose, 23.23 mg of melatonin was solved in 1.0 mL of absolute ethanol and the diluted by TCM199 and 10 µM melatonin stock solutions were prepared. The stock solution was refrigerated at 4°C (less than 2 weeks).

IZUMO1 Gene Expression and Sperm Motility

Thirty male mice were used to study the expression of *IZUMO1* gene and motility of the sperm. Mice killed by cervical dislocation and their cauda epididymis was removed and placed at the culture medium of Ham's F-10 at 37°C CO2 incubator for 30 minutes. The samples collected were divided into two control and experimental groups. In the experimental group 10 µM melatonin was added to the culture medium and it was incubated for 1 hour. Then, sperm motility and *IZUMO1* gene expression were studied in both control and experimental groups.

Real Time Reverse Transcription-Polymerase Chain Reaction

Gene expression analysis with real time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from mouse spermatozoa using RNeasy Micro kit according to the manufacturer's recommendations. Table 1 shows the primer sequences of *IZUMO1* gene. Result was normalized with use of internal control gene (*GAPDH*).

Sperm Motility

To determine the percentage of sperm motility 10 µL of sperm suspension of each control and experimental groups were placed on a microscope slide and 10 fields were selected randomly in the sample from each animal to determine the sperm motility. Then, the average of motile sperm on total average of visible sperms in the 10 field of view was recorded as the motility percent.

In Vitro Fertilization

Thirty female and 15 male mice were used for studying the fertilization rate. To induce ovulation in female mice 10 IU hMG was injected and after 2 days 10 IU hCG was injected intraperitoneally. 24 hours after hCG injection, mice were killed and their ovaries were removed. Then the mice ovaries were placed in sterile phosphate-buffered saline (PBS); the surrounding stromal tissue was removed; and the oocytes were collected by flashing method (n = 100). Sperms were collected from the caudal epididymis male mice and then incubated in 37°C CO2 incubator for 30 minutes. Collected sperms were divided into 2 experimental and control groups. In the experimental group 10 µM melatonin was added to the sperm culture medium for 1 hour. Then, sperms of the control and experimental groups were added to the collected oocyte and the fertilization rate was evaluated based on the formed embryos.

Data Analysis

All statistical analysis was performed by SPSS software version 22 and independent sample *t* test. *P* values ≤0.05 were considered statistically significant.

Results

The Effect of Melatonin on the Expression of *IZUMO1* Gene in Sperm

The results of the expression of *IZUMO1* gene is displayed by Figure 1. *IZUMO1* gene expression on sperm was performed via real time RT-PCR method. According to the studies, there is a significant increase in the level of

Table 1. Process of Primer for Real-Time RT PCR

Gene	Sequence	Product Length	Melting Temperature	Guanine-Cytosine content%
IZUMO1	F:5GGGATGACCGGTGACTCTTGG3 R: 5CTTTCCAATTCCGCCCTC3	165	62.45 61.99	61.90 60.00
GAPDH	F: 5CGGGGTCCCAGCTTAGGTTC3 R:5GCCCAATACGGCCAAATCCG3	103	62.25 62.07	65.00 60.00

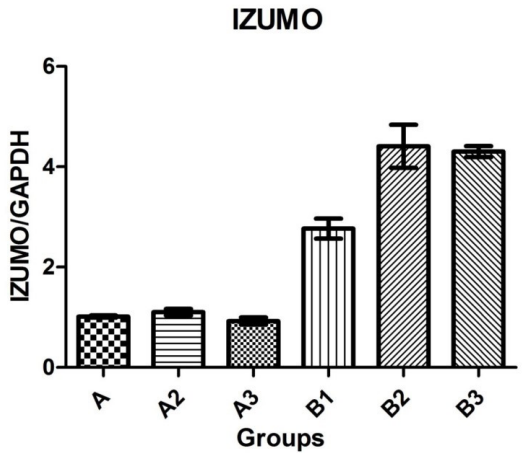

Figure 1. Rate of IZUMO1gene Expression (A: control group, B: experimental group).

expression of *IZUMO1* gene in the experimental group (receiving melatonin) compared to the control group ($P = 0.001$).

The Effect of Melatonin on Sperm Motility

Melatonin had a positive effect on sperm motility. Statistical analysis showed that the addition of 10 μM melatonin to the sperm culture medium makes a significant increase in sperm motility compared to the control group ($P < 0.05$; Table 2).

The Effect of Melatonin on Fertilization Rate

IVF was performed in both groups and 100 oocytes were used for each group. Counting the number of formed embryos in the control group indicated that 77% of oocytes developed to the embryos whereas in the experimental group adding melatonin to the culture medium, 83.01% of the oocytes developed to the embryos (Table 3). Developed embryos in the experimental group are shown in Figure 2.

Discussion

Sperm-oocyte interaction mechanism is in a way that many molecular factors may be involved in. The most important of these factors are proteins such as ADAM3 (while the sperm passes through the ZP layer), IZUMO1 and CD9 (in connection of the sperm membranes and oocyte) that have been mentioned by many studies (7,10,14,22,23). In the connection of sperm –ZP, acrosin is one of the most important proteins. Some results suggested that sperm is capable to fertilize oocytes without the acrosin (24,25). In general, ADAM3 molecules of the sperm and ZP of the ZP layer play an important role for the passage of sperm from the ZP (9). After passing through the ZP layer, the sperm requires a membrane interaction to fertilize oocytes. Various proteins play a role during sperm-oocyte fusion function including fertilin (ADAM1B/ADAM2), cyritestin (ADAM3), CD46, CRISP (cysteine -rich secretory protein) and MN9 antigen (9). But in the meantime, IZUMO1 protein is in the final point of the fusion, which establishes the possibility of a successful fertilization (7,9). Ellerman et al showed that IZUMO consists of 4 protein families (IZUMO1-4). Types 1, 2 and 3 are simply expressed only in the testis tissue and constitute molecular structures of dimer, trimer and tetramer (26). It should be noted that among isomers of this molecule, type 1 has a fundamental role and acts as the central axis. Extracellular domain of IZUMO1 protein with a place of N-linked glycosylation is the functional place of the protein to fuse with other cells membrane protein (11). Obviously, changes in the structure of this protein, its receptor or associated molecules could reduce the level of sperm fertilization in in-vitro and in-vivo conditions through any factor. However, level reduction of environmental factors such as free radicals improves capacity-taking and the sperms fertilization power (27,28). In IVF, the negative effects of ROS is not negligible because consequently will have the destructive effects on embryo quality (16). Therefore,

Table 3. Percent of Fertilization Rate in Control and Experimental Groups

Group	Experimental	Control	*P* Value
Fertilization rate	83.01%	77%	<0.05

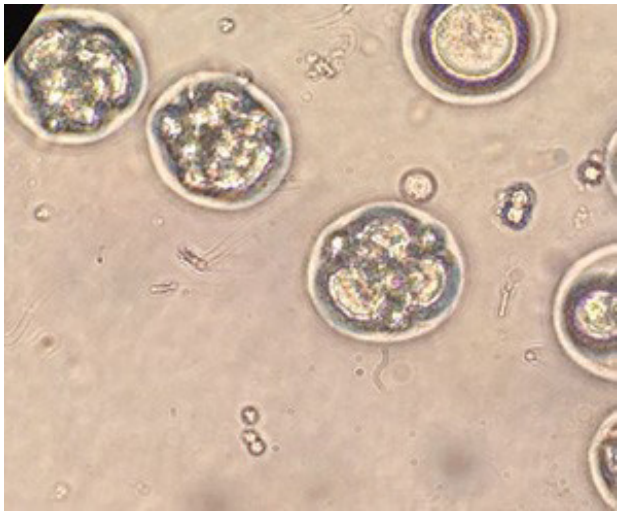

Figure 2. Photomicrograph of Inseminated Oocytes in Experimental Group.

Table 2. Average Percentage of Moving Sperm

Group	Average Percentage of Moving Sperm				Viability	*P* Value
	Rapid	Moderate	Slow	In Situ		
Experimental	53.4	8.9	6.8	13.6	82.5	≤0.05
Control	35.5	28.1	2.5	13.2	79.3	≤0.05

hypothesis of the present research was as follows: The increase of this protein expression level and reduction of the negative effects of ROS in vitro conditions using melatonin can enhance the rate of fertilization in IVF. Therefore, the use of antioxidants such as melatonin may prevent oxidative stress and improve quality of the technique (29). Although, the use of antioxidants to prevent damage to sperm DNA is suggested in many studies, some results have shown that certain doses of these compounds can be constructive (29). It is believed that in physiological conditions melatonin regulates the release of gonadotropins from hypothalamic-pituitary axis and adjusts ovarian function (30,31). In-vivo studies have shown that due to the antioxidant properties, melatonin may play a protective role in the sperm cell membrane lipid structure and be effective in the mitochondria function of the sperm flagella (32). Melatonin because of the small size and high fat-friendly properties penetrates the cell membrane easily and spreads over the whole cell. Its concentration is very high in the nucleus and protects DNA against malicious agents (32,33). Studies have shown that in in-vitro conditions melatonin affects the number of CAMP molecules in the epididymis cells of the rat and could phosphorylate the promoter region of the DNA, resulting in the expression of some genes (21).

According to the studies, melatonin receptors are also present on spermatozoa and this hormone affects on the cells through the stimulation of G proteins receptors and then activation of the adenylate cyclase, synthesis of CAMP, enabling pKA and phosphorylation of the protein (20,21). Furthermore, a research conducted in 2014 showed that adding melatonin to IVF culture medium can increase cleavage and blastocyst rate by reducing oxidative stress (16). However, in another study it was shown that melatonin at a dose of 1000 nmol damages the sperm DNA in the IVF (29). This result was not consistent with our data because in our research, melatonin led to the increase of the fertility rate as well as enhancement of IZUMO1 protein expression. In addition to the effects of melatonin on spermatogenesis, it has also been used to improve the process of oogenesis. High melatonin concentrations in follicular fluid show that it plays an important role in mammalian oocyte maturation (18,34,35). Normally, melatonin concentration in granulosa cells gradually increases along with the development of follicles. According to the study of Reiter et al, due to the effect of melatonin, implantation and pregnancy rates have increased compared to the control group; hence, it is suggested that to use melatonin causes stimulation of immature oocytes (19,36). Based on the results of the previous researches and the current study, the increase of IZUMO1 protein expression leads to the increased motility and sperm fertilization power at in vitro conditions. Therefore, one can assume that melatonin may develop successful fertilization rate through the improvement of performance in both sperm and oocytes cells; thus, many studies should be conducted in this regard in order to understand the molecular mechanisms.

Conclusion

The results show that melatonin as an antioxidant increases IZUMO 1 protein expression in sperm, sperm motility and oocyte fertilization rate. Therefore, it is concluded that melatonin can improve the rate of fertilization due to the increase of IZUMO 1 activity and sperm motility.

Ethical Issues

Animal handling and all related procedures were confirmed by Tabriz University of Medical Sciences, Ethical Committee.

Conflict of Interest

All authors have no conflicts of interest.

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