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Research Article

Improving the Quality of Pre Implantation Human 2-Cells Embryos by Sphingosine-1 Phosphate (S1P)

Shahrzad Aghajani[1], Mohammad Hadi Bahadori[2], Ali Asghar Pilevariyan[1] and Mostafa Ashrafi osalou[3,4]*

[1]*Department of Biology, Isfahan Payame Noor University, Iran*

[2]*Department of Medicine, Cellular and molecular research center, Guilan University of Medical Sciences, Iran*

[3]*Department of Histology & Embryology, Dokuz Eylu L University, Turkey*

[4]*Mehr medical institute, Iran*

***Corresponding Author:** Mostafa Ashrafi Osalou, Department of Histology & Embryology, School of Medicine, Dokuz Eylu L University, inciralti 35340 Izmir/Turkey.

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Abstract

Background: Fragmentation is a common phenomenon in pre implantation human embryos. Fragmentation is a state that some particles of cells are smaller than natural and well size of embryo blastomeres. It is result of apoptosis. This phenomenon that approximate is in 50 percent of IVF and ICSI embryos and in the following step of develop fragmented embryos often resulting be stop cleavage and damage embryos. Very soon after transferring this embryo decrease there's developmental potential and decrease pregnancy rate. The goal of this study is inspection sphingosine-1-phosphate ability such as an anti-apoptotic material on decreasing fragmentation degree in pre implantation human embryos until blastocyst.

Methods: In this study patient that instigated with long protocol with using from GnRh analog with pure FSH and human monopos gonadotropin (hMG) entered the study. After ejecting follicles and washing them with culture follicles kept in CO_2 6% incubator for 2-4 hours and then denuded for ICSI and injected sperm and observing 2PN on next day and fertilization confirm.

In this step put on incubator and embryos with 25 to 50 percent of fragmentation put on two group of culture drop with sphingosine-1-phosphate (20 and 40 ml mol/l) and instigate there's growth until blastocyst step and compare with control group of embryos.

Results: Finding in this study showed that increasing sphingosine-1-phosphate caused expressive increasing arriving and grad of blastocyst embryos in group with 25% fragmentation (30 oocyte) in compare with control group embryos (40 oocyte) and in 50 percent group embryos didn't have any expressive different but caused stopping fragmentation degree ($P < 0.05$).

Conclusion: By attention to findings in this research showed sphingosine-1-phosphate can be effective on improving fragmentation degree and it is necessary to instigate more in this connection on pregnancy degree in embryos.

Keywords: *Apoptosis fragmentation; Pre implantation embryos; Sphingosine-1-phosphate*

Introduction

Despite significant advances in reproductive biology, the implantation rate of human embryos remains very limited. The reproductive potential of pre implantation embryos has been correlated, among many other factors, to their morphological characteristics as assessed by the embryologist in the laboratory. One important criterion of morphological quality is the fragmentation rate detected microscopically. The appearance of cellular fragmentation during early embryo development in vitro is a common feature in pigs [1], cows [2] and

humans [3]. Fragmented embryos often become rested with subsequent degeneration and are also associated with decreased pregnancy rates following transfer into the uterine cavity [3,4]. Fragmentation is a state that some particles of cells are smaller than natural and well size of blastomeres. It is result of apoptosis and relationship between apoptosis and embryo fragmentation is far from being established, so that apoptotic processes were linked to the fragmentation of pre implantation mammalian embryos [3,5,6] Moreover, fragmented bovine embryos were shown to exhibit typical morphological changes that conform to the general criteria of apoptotic cell death.

Sphingosine-1 phosphate (S1P) is a sphingolipid metabolite presents abundantly in blood. Through the interaction with a family of five G-protein-coupled receptors, it triggers diverse cellular responses, including cytoskeletal changes, Proliferation and migration. S1P is also a known potent anti-apoptotic substance [7,8] that acts via the suppression of ceramide, an intracellular mediator of apoptosis [9]. Several studies have shown that S1P protects cells and tissue [10] such as, oocytes [11,12], male germ cells [13], embryos [14,15] and ovarian tissue [16] against the apoptotic damage of many stressors including radiotherapy [17] and chemotherapy [6]. It was also possible to obtain good-quality embryos in mice from tissue protected by S1P that yielded a mice progeny with no genomic defects [17]. Briefly it was hypothesized that the addition of S1P to the embryos with 25 to 50 percent of fragmentation would reduce the severity of fragmentation and improves morphological embryo quality and reduce the rate of fragmentation and the aim of this study was to investigate this hypothesize that decrease of fragmentation would actually improve embryo quality in humans as well.

Materials and Methods

Women undergoing assisted reproductive techniques at the Azahra IVF center of Guilan-Medical Center between May 2011 and August 2013 were asked to participate in the study and a written informed consent was obtained. The study was approved by the institutional review board following the guidelines in accordance with the Helsinki Declaration of 1989 on human experimentation. Women with six or more mature oocytes retrieved were asked to donate one or more oocytes to the study. Exclusion criteria included age of female partner > 41 years, six or more previous assisted reproductive cycles and cycles with less than six oocytes collected upon retrieval. Donated oocytes constituted the experimental group. The remaining oocytes were handled according to standard protocol of oocyte handling, fertilization and culture and they constituted the control group.

Ovarian stimulation was carried out following pituitary down-regulation utilizing the gonadotropin-releasing hormone agonist triptorelin acetate (GnRh; Decapeptyl 0.1; Ipsen Beaufour, France) administered subcutaneously at a dose of 0.05 mg daily. Following complete ovarian suppression (serum estradiol < 50 pg./ml), follicular stimulation was initiated using intramuscular injections of human menopausal gonadotropin (HMG; Menogon 75; Ferring, Denmark) or subcutaneous injections of recombinant follitropin (rFSH; Puregon 50; Organon, Netherlands). The dose was managed in tandem with ovarian follicular development monitored mostly by serial vaginal ultra sonography in conjunction with serial serum estradiol concentrations.

When at least two leading follicles reached 18 mm in diameter, GnRHa and HMG/rFSH were discontinued and 10,000 IU of human chorionic gonadotropin (HCG; Pregnyl; Organon) were administered. Follicular fluid collection and oocyte retrieval were performed via ultrasound-guided transvaginal needle aspiration, 35-36 h after HCG administration.

All mature oocytes retrieved were identified under an Olympus stereomicroscope and cultured in G-IVF culture medium (Vitrolife, Sweden). Following hyluronidase denudation, they were then inseminated by intracytoplasmic sperm Injection (ICSI) in SP HEPES-buffered medium (Medicult, Denmark) using a Nikon inverted microscope and Narashigi micromanipulator. Inseminated gametes and embryos were then cultured in G-1 Plus (Vitrolife) culture medium in 50 ll droplets under embryo-tested mineral oil (Vitrolife) fewer than 6% CO2 in air at 37_C. A maximum number of four embryos were cultured together in the same droplet. Fertilization and cleavage were evaluated by the same embryologist 18-24 h post insemination, and zygotes obtained were transferred to fresh medium. Daily evaluation of developing embryos was performed until transfer into the uterine cavity on the third day following oocyte retrieval. Transfers were performed in G-2 culture medium (Vitrolife) using a Wallace transfer catheter under abdominal sonographic guidance. In the experimental group, donated oocytes were randomly taken from the original pool of oocytes, and cultured immediately following ICSI

in a modified G-1 Plus medium supplemented with S1P to a concentration of 20 l mol/l as described below. Developing embryos in the experimental group were never transferred into the uterine cavity and were left to degenerate in culture.

One milligram of sphingosine-1-phosphate (D-erythro-sphingosine-1- phosphate; Calbiochem, EMD Bioscience Inc., CA, USA) was dissolved in 2 ml methanol. Following evaporation of the solution at 65_C, 4 mg/ml solution of human serum albumin was used to make 125 l mol/l S1P stock aliquots.

The 125 l mol/l aliquots were diluted in G-1 Plus culture medium to prepare the 20 l mol/l incubation medium. This concentration was chosen arbitrarily from animal data and constitutes a preliminary suggestion that needs to be completed ultimately by a dose finding study.

All oocytes and embryos were evaluated by the same embryologist who was blinded at the time of assessment to the control/experimental groups. Embryos with different percent fragmentation were examined on the day of embryo transfer in 20 and 40 nmol S1P added medium, i.e. day 3 after oocyte retrieval, under a Nikon inverted microscope and scored according to an embryo grading system modified from [18]. It consisted of three main embryo fragmentation grades where a developmental rate of 4-6 cells on day 2 was considered optimal development. Embryos were labeled on the basis of fragmentation rate in relation to the total embryo volume, as follows: Grade I, less than 15% fragmentation; Grade II, 15 to 50% fragmentation; and Grade III, more than 50% fragmentation. All uterine transfers were made on day 3 and involved exclusively embryos derived from the control group. Abdominal ultra sonography with a half-full bladder was also performed to guide the advancement of the transfer catheter into the uterine cavity. No embryo transfers were made from the experimental group.

The primary outcome measure was the proportion of embryos in each fragmentation grading category in both experimental and control groups. The secondary outcome measure was the fertilization rate in either group, which closely parallels the influence of S1P supplementation on egg activation following intracytoplasmic injection of sperm. As the two groups of embryos were obtained from the same women, they were matched with respect to female partner age, causes of infertility and ovarian stimulation protocols.

Statistical analysis the sample size was calculated by Fisher's exact sample size estimates per group for groups of unequal size. The following parameters were included in the calculation: control oocyte number/study oocyte number ratio was taken as 4/1 and the estimated percentage of grade I embryos in the control group were considered 65%. For a power of 90% and type 1 error of 0.05, it was calculated a sample size of 125 study oocytes and 499 control oocytes in order to detect a 15% increase in the percentage of grade I embryos, up to a total percentage of 80%. The data from both control and experimental groups were analyzed using Statistical Package for Social Sciences version 18.0 (SPSS, Chicago, IL, USA). The distributions of various embryo fragmentation grades between the two groups were compared using Student's t-test. The mean ranks of embryo quality were compared using Wilcoxon signed-rank test.

Results

A total of 46 women consented to donate 334 mature human oocytes and 214 embryos for the experimental group, which were compared to 124 oocytes and 214 embryos in the control group. Patient characteristics and reproductive data were collected in the study are shown in (Table 1).

On day 3 of fragmented embryos culture, (70.6%) of embryos in the experimental group with 40 nmol S1P and (4.82%) of embryos in the control group improved and fragmentation decreases as shown in Fig.2 , On day 5 of fragmented embryos culture, (70.6%) of embryos in the experimental group with 40 nmol S1P and (4.82%) of embryos in the control group improved and fragmentation decreases as shown in Fig.3 reflecting a significant decrease in the mean improved rates in the S1P group in third day (70.6% vs. 29.4%; $P < 0.007$) and fifth day (72.2% vs. 27.8%; $P < 0.0001$). The mean fragmentation grade also differed significantly between the two groups ($P < 0.0001$), with less fragmentations associated with S1P supplementation.

Age of Mother	Duration of Infertility	Type of Infertility	Assisted Reproductive Method of Choice	Visit ivf	Sperm Count	Sperm Motility
Sperm morphology	The number of mature oocytes	No. of embryos	No. of transfers	Status makeup pronuclear	Arrangement at the core of the polar body	Congestion status of embryos
Ovarian stimulation protocols	Progesterone level at the time of injection	No. of embryos examined	S1P dose	Fragmentation of Day 1	Fragmentation of Day 3	Fragmentation of Day 5

Table 1: *Data were collected in the study.*

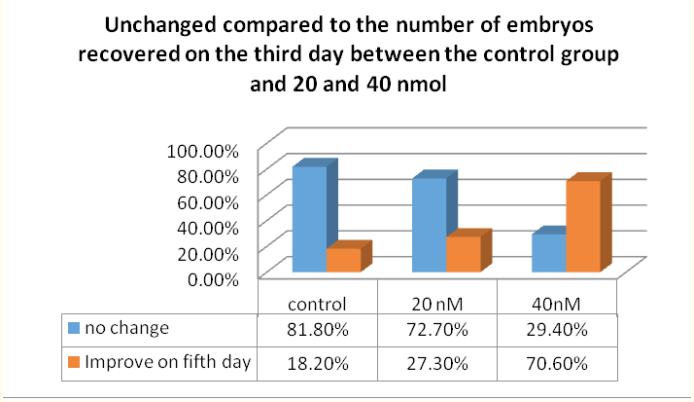

Figure 1: *Unchanged compared to the number of embryos recovered on the third day between the control group and 20 and 40 nmol.*

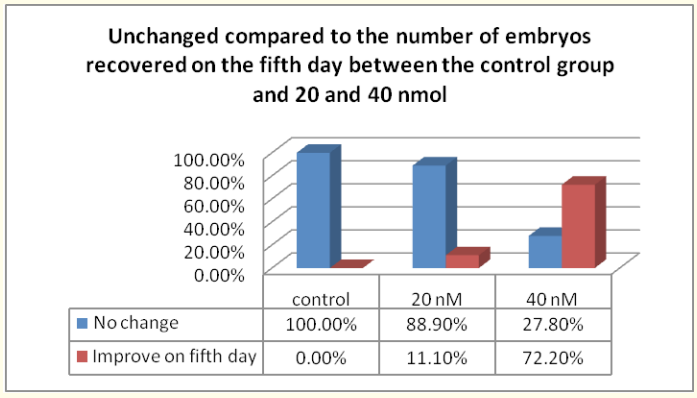

Figure 2: *Unchanged compared to the number of embryos recovered on the fifth day between the control group and 20 and 40 nmol.*

Also shows that the mean proportion of embryos with minimal fragmentation was significantly higher in the S1P supplemented group, and that the mean proportion of embryos with excessive Fragmentation was significantly lower in the same group.

Discussion

This study showed that sphingosine-1-phosphate, when added to the fragmented 2 cells pre implantation embryos culture medium of human oocytes, decreased significantly the severity of fragmentation rate in pre implantation embryos, as more in embryos with under 50 percent fragmentation were obtained in the S1P study group (70.6% vs. 29.4%; $P < 0.007$) in third day and (72.2% vs. 27.8%; $P < 0.0001$) in fifth day in 40 n mol dose. There was no visible change in 20 n mol dos of sphingosine-1-phosphate in the S1P study group (3.72% vs. 37%; $P < 0.05$) in third day and (11.1% vs. 88.9%; $P < 0.05$) in fifth day in 40nmol dose. Today's many researchers have focused on studies that increase pre implantation embryos qualify and decrease disrupting components such as fragmentation [19]. Edwards and colleagues in early 1970 described fragmentation in human IVF embryos and said that is kind of cell division without nuclear division during which cells lost partial or total of its integrity. But this Phenomenon is not limited to human IVF embryos and also it has shown in others mammalian's embryos [20]. Many studies indicate there are potential factors of fragmentation and cells arrest such as abnormal chromosomes, inappropriate conditions of follicles, In vitro culture conditions and also ROS [20]. In our study we saw that fragmentation rate in control groups 20% and 80% at days3 and 5 respectively and it suggests that it cannot make improvement spontaneously. It is clear that fragmentation is an obstacle to efficiency of somatic cells nuclear transfer technique because a large number of eggs followed by nuclear exit or after the formation of a donor nucleus are fragmented [20]. Furthermore there is some evidence that shows a direct link between fragmentation rate and apoptosis in pre implantation embryos [3,5,21]. Identifying dense nucleus and labeled with TUNEL in human fragmented embryos lead to this suggestion that fragmentation is due to apoptosis [20]. Therefore, if apoptosis is truly responsible for the increase in fragmentation rates, then it makes perfect sense to assume that anti-apoptotic factors could play a positive role in reducing fragmentation rates.

Sphingosine-1-phosphate (S1P) is a sphingolipid metabolite presents plentifully in blood and has known as an anti-apoptotic potent [20]. Previous studies have already shown the anti-apoptotic effect of S1P in some tissue and cells such as ovarian tissue, men germ cells, oocyte [14,15] and hepatocyte cells [22]. Although it was identified as an important molecule for regulating immune responses, inflammatory processes and S1P is able to create high-quality embryos in mice treated with it [17], therefore we selected S1P for reach to this purpose. So it used in 2 doses: 20 and 40 nm and observed in days 3 and 5. Our results showed that embryos have improvement in day3 that it was not significant in compare with control while it was significant in 40 n ml groups. Although according to our findings fragmentation has a significantly improvement in day 5 whit 40 n ml S1P in compare whit 20 n ml and control groups. It is emphasized that S1P could improve development arrest and fragmentation rate from day 3 to day 5 and this experience showed clearly that 40 n ml S1P more effective than 20 n ml. Ebner and colleague in a cohort study in 2001 reported that pregnancies derived from bad-quality embryos had a significantly higher rate of malformations [23]. Previously studies has shown that use of an anti -apoptotic factors in addition to reducing apoptosis leading to decrease fragmentation such as insulin-like growth factor-I. It is hoped that improved culture setups will lead to improve embryos quality. Hannoun and colleague in 2010 used 20 µ ml S1P and reported effect of S1P on embryos quality [19]. This study parallel with previous mentioned study emphasizes that S1P can be effective for decrease fragmentation rate and increase embryos quality.

Conclusion

Despite significant progresses in ATR science, it is still many failure reports in ART and pregnancy rate. So in this study we followed this purpose that with improve media of culture we can improve severity of fragmentation and embryos qualify. Thus we added S1P into media and we can say that S1P especially in 40 n ml dose decrease severity of fragmentation and increase embryos quality.

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