RESEARCH ARTICLE

Improvement of the *in vitro* Growth and Maturation of Isolated Mouse Preantral Follicles in the Presence of Repaglinide

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ABSTRACT

The development of in vitro culture systems that result to preantral follicles growth and increasing of developmental competency of oocytes obtained from follicles has an important role in fertility preservation and assisted reproductive techniques. In this research, we evaluated the effect of repaglinide on in vitro growth and maturation of preantral follicles. Preantral follicles were isolated from 12–14 day-old female National Medical Research Institute (NMRI) mice ovaries and cultured for 12 days cultured in alpha minimal essential medium (α-MEM) (*Control*), α-MEM supplemented with 1μM of repaglinide. Follicles examined for development on 1, 3, 6, 9, 12 days of culture. At the end of the culture period, after HCG administration in vitro oocyte maturation was assessed. Results showed that in vitro follicle growth, survival, the density of granulosa cells, and steroidogenic activity were higher than the control group ($p < 0.05$). The in vitro maturation rate in oocytes derived from follicles in the treatment group was higher than the control group ($p < 0.05$). Therefore the supplementation of the culture medium with repaglinide can improve the ovarian follicle survival, growth, and subsequently, in vitro oocyte maturation.

Keywords: *In vitro* growth, *In vitro* maturation, Mice, Ovarian follicle, Repaglinide.

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INTRODUCTION

At any time of a woman's reproductive age, there may be problems, for example, cancer, neurological, and endocrine disorders that increased the risk of ovarian dysfunction and infertility (22; 32; 1). The culture of preantral ovarian follicles at the in vitro condition is an important option for assisted reproductive treatment investigation and fertility preservation in mammals with impaired reproductive activity (23; 6). In recent years, many researchers have been focusing on the designing and development a system for in vitro growth of ovarian follicles from the earliest stage to completely maturation and producing mature oocytes.[13] A complex of biological process is necessary to obtaining fully developmental competence of oocyte and subsequently fertilization and embryo production. This potential is gained gradually during of folliculogenesis, that involving several important steps and mechanisms.[41] Folliculogenesis and oocyte–granulose cell interactions is under the controlling of the autocrine, paracrine and endocrine factors that result to important changes in differentiation and proliferation of granulosa, theca cells and finally progresses competency of oocyte.[28,18] Oocytes from mammalian species undergo cell-cycle arrest, and changing in Ca^{2+}influx has an important role during meiosis resumption. The maintenance of intracellular Ca^{2+} homeostasis due to extracellular and intracellular calcium stores requires for oocyte maturation, fertilization and egg activation (31; 40; 7). ATP-sensitive K+ (KATP) channels are widely presented in tissues that expressed these channels; their fundamental role is the regulating of intracellular metabolic state and cellular electrical excitability and involving in insulin secretion, smooth muscle tone and others.[33] In many species of mammal's oocytes, KATP channels have been founded and maybe drugs that targeting KATP channels have a potential to affect on oocyte activity.[14] Repaglinide is one of the antidiabetic drugs that by inhibitory effect on KATP channels, opening the voltage gated calcium channels causes to depolarization of pancreatic beta cell membrane and enhancement of intracellular calcium, finally releasing of insulin from these cells. The improvement of the culture medium is an essential point for the manipulation of the oocytes enclosed in preantral follicles.[3] Therefore, this study aimed to evaluate the effect of repaglinide in the culture medium on in vitro preantral follicle growth and maturation as well as embryonic development in mice.

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MATERIALS AND METHODS

Chemicals were purchased from Sigma Chemical Corporation (St. Louis, MO) and Gibco (Grand Island, NY, USA) unless repaglinide purchased from Farabi Corporation, Iran.

Animals and ovarian collection

Female 12-14 day-old NMRI mice (n=40) were kept under standard conditions (20-25˚C temperature, 50% humidity, and 12 hours dark–light period) and fed with food and water. This experiment was performed according to the ethical guidelines for the Care and Use of Laboratory Animals at Razi University. The Animals were sacrificed by cervical dislocation, then their ovaries were harvested and immediately transferred to dissection medium that containing of α-MEM, supplemented with 5% fetal bovine serum (FBS), 100 IU/mL penicillin G and 100 µg/mL streptomycin.

Experimental design

The collected ovaries were categorized into control and repaglinide groups. One ovary from each mouse was used randomly for the culture in the presence of repaglinide and the other was reserved as the control group.

Isolation of pre-antral follicles

Ovaries were placed in 50 µL micro drops of the medium. The preantral follicles were mechanically isolated from ovaries by using a 27-gauge needle under a stereomicroscope. Usually, 20–25 preantral follicles were isolated from each ovary. Only the follicles with a diameter of 100–150 µm that centrally located round oocyte, intact basal membrane with one or two layers of granulosa cells and a thin layer of theca cells were randomly selected and transferred to new microdroplets (30 µL) of culture medium consisting of α-MEM medium supplemented with 10% FBS, 100 mIU/mL recombinant follicle-stimulating hormone (rFSH), (Gonal-f, Merck-Serono, Germany), 10ng/mL recombinant epidermal growth factor (rEGF) and 100 IU/mL penicillin and 100 µg/mL streptomycin. Follicles were cultured for 12 days individually in 30 µL micro drops of culture medium without repaglinide (control group) and with1µM concentration of repaglinide (repaglinide group) in 60 mm culture plate under sterile mineral oil (5% CO2 in the air at 37 °C). Media were changed by refreshing one-half (15µl) of the medium every other day with fresh pre-incubated culture medium.[5]

Follicle growth

For recording follicles growth, according to previously described method with some modifications,[35] follicle diameters were assessed by measuring two perpendicular diameters at 10× magnification using a calibrated ocular micrometer (Dino Digital Eyepiece: AM323, Taiwan), on days 1, 3, 6, 9, 12 of culture.

Follicular cell density

At the end of the culture period for the evaluation of follicular cells density, 10 µg/mL bisbenzamide (Hoechst 33258) were added to the droplets of culture medium. Follicles incubated for 15 minutes at 37˚C (38). After exposure to a fluorescent dye, follicles were examined under an inverted fluorescence microscope with excitation filters at 460 nm for blue fluorescence. and classified into four categories depending on the percentage of follicular cells, according to a classification established by our group: D1, maximally density with more than 100% increase in cell population; D2, moderately density with 50–100% increasing in cell population; D3 minimally density with less than 50% increase in cell population, D4, constant density without increasing in cell population

Follicle viability

The viability of follicles was determined based on visual examination of the integrity of the oocyte membrane and the normality of the follicle shape and form. The validity of morphological classification was confirmed by vital staining with propodium iodide as already used by 10. To perform vital staining, follicles were stained with propodium iodide at a final concentration of 20 µg/mL in α-MEM, which added to droplets of culture medium in the dark. The follicles were examined under an inverted fluorescence microscope (Olympus, IX71; Japan) with excitation filters at 560 nm for red fluorescence and classified into four categories depending on the percentage of dead follicular cells, according to a classification established previously described:

V1, follicles with the oocyte and all granulosa cells viable; V2, minimally damaged follicles with less than 10% dead follicular cells;

V3, moderately damaged follicles with 10–50% dead follicular cells;

V4, dead follicles with both the oocyte and all follicular cells dead.27

Follicle activity

Microscopic evaluation of lipid droplets and hormone assay in cultured follicles were used to assess granulosa cell activity. To investigate the detection of lipid droplets in follicles, they were stained with Sudan III (24; 11) with some modification. At the end of the culture period, the follicles were washed and fixed in 4% paraformaldehyde in α-MEM for 30 min and stained with Sudan III for 20 min. After washing three times with PBS, the follicles were examined under an inverted microscope (Olympus, IX71: JAPAN). The lipid droplet accumulation in follicles visualized by Sudan III staining. For the evaluation of the endocrine function of ovaries, the concentration of progesterone (P4), 17-β-estradiol (E2), and androstenedione (A4) were measured. On 12 day of culture period in both groups, 5 IU/mL human chorionic gonadotropin (HCG) was added to the culture medium and 24 h later, the conditioned medium removed and measured using ELISA kits (Cat No: ABIN1569496 and Cat No: ADI-900-011; Biocompare; USA, respectively) after extraction with methanol over a Sephadex (Pharmacia and Upjohn, Kalamazoo, MI) C-18 column and subsequent evaporation. The absorbance for each sample was measured by an ELISA reader (ELx800™, BioTek Instruments, Italy) and was interpolated with a standard curve.

In vitro oocyte growth and maturation

Oocyte diameters were assessed by measuring two perpendicular diameters in oocytes at 10× magnification using a calibrated ocular micrometer (Dino Digital Eyepiece: AM323, Taiwan), on days 1, 3, 6, 9, 12 of culture. On 12 day of culture period in both groups, oocytes maturation was induced by the addition of 5 IU/ml HCG (Human Chorionic Gonadotropin) to the culture medium and after 24 h, the oocytes mechanically isolated from follicles and assessed under an inverted microscope (Olympus, IX71: JAPAN) that graded for different stages of nuclear maturation such as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase II (MII) and degeneration (Deg).

Statistical analysis

Data were analyzed by using SPSS: Software Program (version 19: SPSS. Link., Chicago, IL) and results are represented as means ± standard error of the mean (SEM). Follicle viability and in vitro oocyte maturation rates were assessed by the chi-square test. Follicle and oocyte diameters analyzed by t-test and estradiol and progesterone levels analyzed by using the one-Way ANOVA and post hoc Tukey. A $p < 0.05$ was considered to be statistically significant.

RESULTS

Follicle growth

The developmental characteristics of cultured follicles are summarized in Table 1 and Figure 1. During in vitro culture, the follicle diameters increased from day 1 to day 12 of culture in both groups. The follicular sizes in the control and repaglinide groups at the Day 1 of the culture were 112.24 ± 0.02 and 112.23 ± 0.02 µm, respectively; the *follicular* sizes on Day 3 of culture were170.18±0.16 and 191.02±0.16 µm, respectively; and the follicular sizes on Day6 were 234.31 ± 0.17 and 261.71 ± 0.23 µm, respectively. The follicular sizes on Day 9 of culture were 331.22 ± 0.32 and 369.20±0.15 µm, respectively, and the follicular sizes on day 12 were 431.11 and 473.87 µm, respectively. Significant differences existed between the control and repaglinide groups from day six of culture ($p > 0.05$). Antrum formation was observed in some of the follicles from Day 6 onwards; however, there were no significant differences between the control and repaglinide groups.

Follicular cell density

Follicular cell density was evaluated by bisbenzamide staining under a fluorescence microscope. Repaglinide causes increase

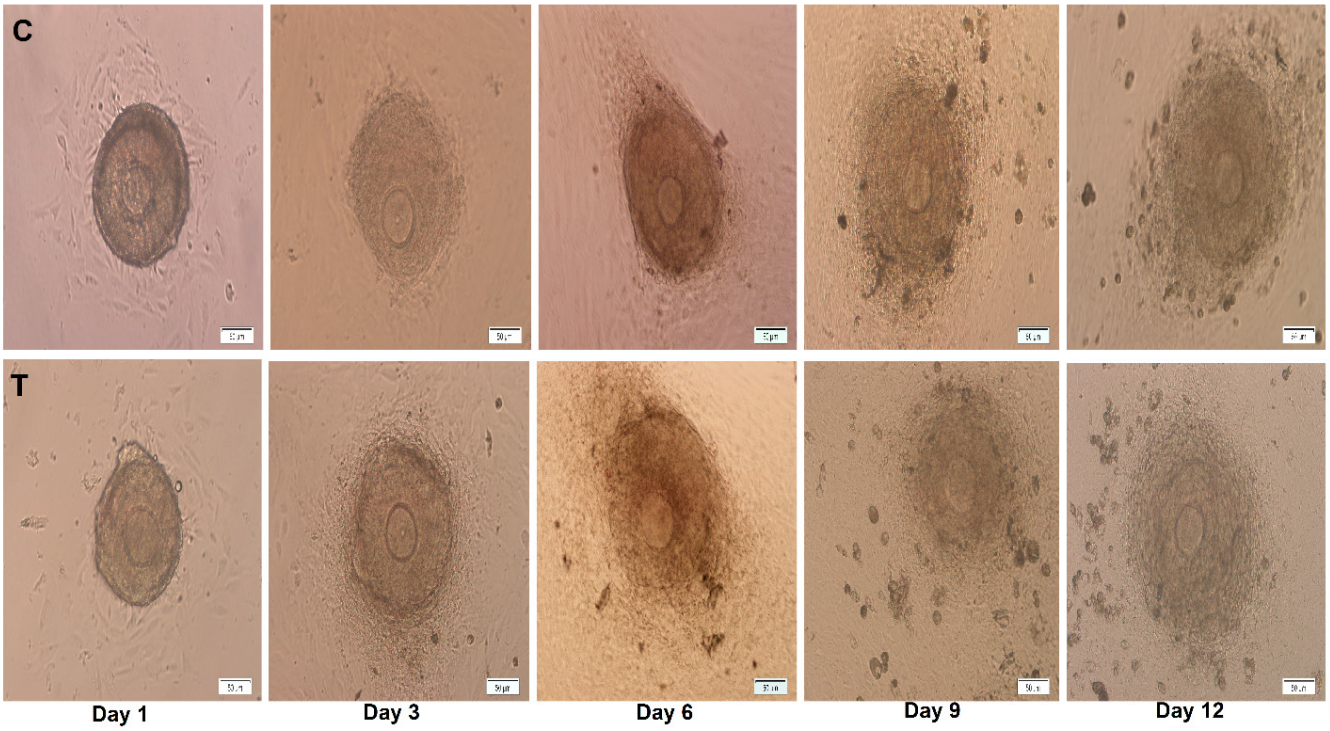

Figure 1: Follicular growth of the cultured of preantral follicles in the presence of repaglinide on days 1, day 3, day 6, day 9 , day 12 (Scale bar: 50 µm)

C: Control group in the absence of repaglinide in culture medium.

T: Repaglinide group in the presence of 1µM concentration of repaglinide in culture medium

Table 1: Follicle diameter (µm) of cultured follicles in the presence of repaglenid during different days of culture

Group	*N*	*Day 1*	*Day 3*	*Day 6*	*Day 9*	*Day 12*
Control	157	112.24 ± 0.02[a]	170.18 ± 0.16[a]	234.31 ± 0.17[a]	331.22 ± 0.32[a]	431.11 ± 0.20[a]
Repaglinide	164	112.23 ± 0.02[a]	191.02 ± 0.16[a]	261.71 ± 0.23[b]	369.20 ± 0.15[b]	473.87 ± 0.13[b]

Data are represented as means ± SEM. N: Total number. a/b Values within columns with different superscripts are significantly differences (t-Test, $p < 0.05$).

in the expansion of granulosa and theca cells as well as follicular cell count in comparison to the control group (Figure 2).

Follicle viability

After 7 days of culture, the viability percentage of ovarian follicles in repaglinide group (100%) was higher than in the control group (85.23%) ($p < 0.05$). Follicles analyzed from repaglinide group were in V1 (4.36%), V2 (80.43%), and V3 (15.21%) category. Follicles analyzed in the control group were in V2 (12.5%), V3 (72.72%) and V4 (14.77%) category (Table 2, Figure 3).

Microscopic evaluation of steroidogenesis by Sudan III staining

The results show that follicular cells store lipid differently depending on culture conditions. In the presence of repaglinide

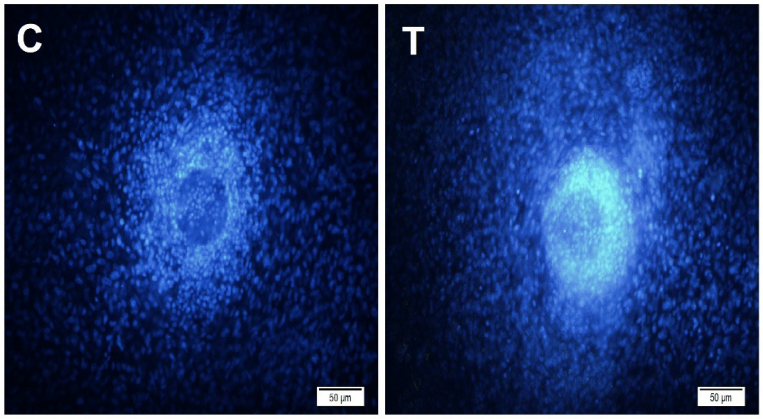

Figure 2: Follicular cell density by Bisbenzamide staining and fluorescence microscopy at the end of follicles culture (12 days), (Scale bar: 50 µm)
C: Control group in the absence of repaglinide in a culture medium. **T:** Repaglinide group in the presence of 1µM concentration of repaglinide in a culture medium.

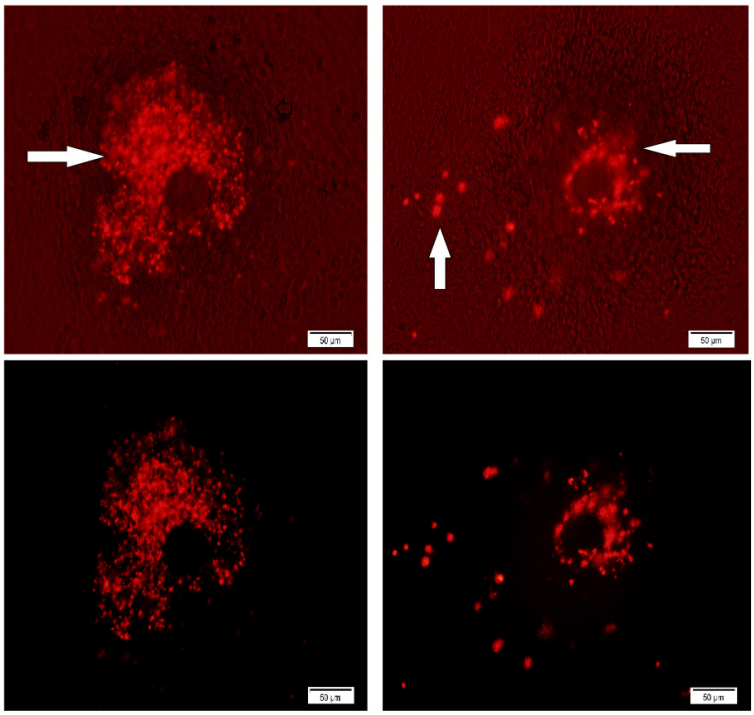

Figure 3: Follicle Viability by PI staining and fluorescence microscopy at day 12 of culture. (Scale bar: 50 µm)
C: Control group in the absence of repaglinide in culture medium. **T:** Repaglinide group in the presence of 1µM concentration of repaglinide in culture medium. Viable cells without staining and dead cells red–stained that determined by arrows.

Table 2: Viability of cultured follicles in the presence of repaglenid.

Groups	N	V1 (%)	V2 (%)	V3 (%)	V4 (%)
Control	88	0^{a}	12.51^{a}	72.72^{a}	14.77^{a}
Repaglinide	92	4.36^{b}	80.43^{b}	15.21^{b}	0^{b}

Data are presented as percentage. N: Total number. a/b Values within columns with different superscripts are significantly differences (chi-square test, $p < 0.05$).

the average follicular cells stores very more lipid as judged by the much lipid droplets seen per cell. When in untreated cells little storage of lipid takes place. On the other hand, folliullar cells contain red-stained vacuoles founded in both groups, but the more cells containing more lipid droplets in the repaglinide group seen in comparison to the control group (Figure 4).

Hormone production

The basal progesterone production (without hCG stimulus) remained was low in a culture medium. The production of progesterone increased after administration of hCG in culture medium. There was a significant difference in the level of 17-β-estradiol (E2) hormone between the two groups ($p < 0.05$). The level of 17-β-estradiol (E2) hormone decreased in the repaglinide-treated group in comparison with the control group ($p < 0.05$) (Table 3).

Evaluation of oocytes growth and maturation

During follicles culture, the diameter of oocytes increased from day 1 to day 12 in both groups. But, in the treatment group, at a comparison to the control only in 9 and 12 days there was significant differences in diameters (Table 4). After 24 h of HCG addition to the culture medium, the IVM rate of oocytes derived from follicles were assessed. In control the percentage of oocytes that remained in the GV stage was higher than the treatment group (21.30% and 10.28%, respectively), and the

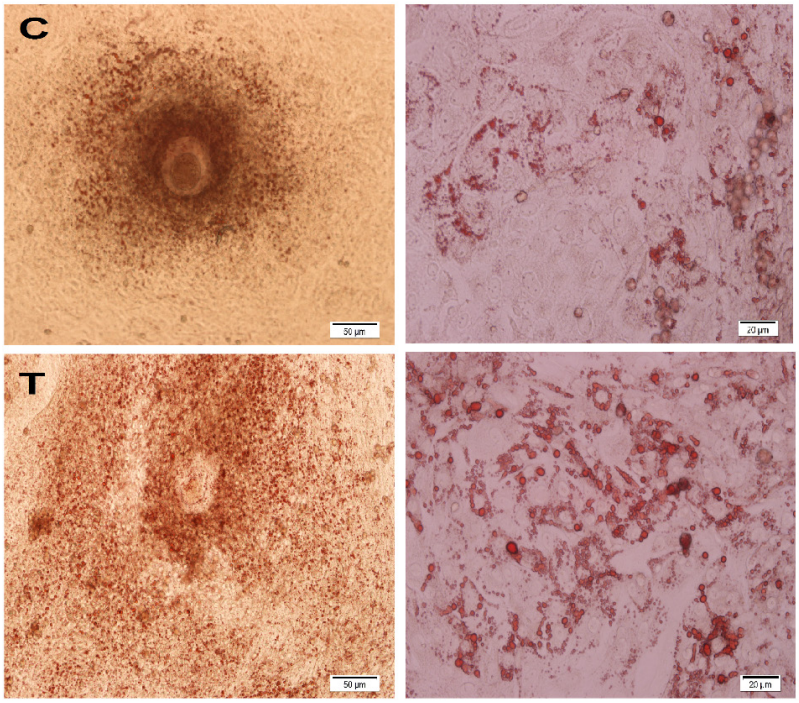

Figure 4: Storage of intracellular lipid in follicular cells cultured in the presence or absence repaglinide
Cells treated with repaglinide accumulated more lipid droplets. (Sudan III staining Scale bar: 50 and 20 µm). C: Control group in the absence of repaglinide in the culture medium.
T: Repaglinide group in the presence of 1µM concentration of repaglinide in the culture medium.

Table 3: Steroid hormone concentration in culture medium collected from follicles cultured in the presence of repaglinide with and without HCG in culture medium.

	Without HCG				*With HCG*			
Groups	*P4 (ng/ml)*	*E2 (pg/mL)*	*A4 (pg/mL)*	*P4/ E2*	*P4 (ng/mL)*	*E2 (pg/mL)*	*A4 (pg/mL)*	*P4/ E2*
Control	45.38 ± 0.07 [a]	1477.48 ± 6.50 [a]	330.20 ±1.30 [a]	0.030[a]	43.42 ± 0.06 [a]	1185.00 ± 5.00 [a]	442.95 ± 1.75 [a]	0.038[a]
Repaglinide	51.41 ± 0.11 [b]	1438.50 ±7.53 [b]	226.55 ±1.25 [a]	0.035[a]	64.04 ± 0.03 [b]	1103.00 ± 2.00 [b]	451.90 ± 0.30 [a]	0.058[b]

Progesterone (P4), 17-β-estradiol (E2), and androstenedione (A4). Data are presented as means ± SEM. a/b are different superscript that indicate significant differences (one-Way ANOVA test, $p < 0.05$).

Table 4: In vitro maturation of oocytes derived from cultured follicles in the presence of repaglinide

Groups	N	GV (%)	GVBD (%)	MII (%)	DEG (%)
Control	102	21.30 ± 1.05[a]	27.78 ± 0.92[a]	33.35 ± 0.87[a]	17.55 ± 0.72 [a]
Repaglinide	108	10.28 ± 0.71[b]	34.78 ± 1.00[b]	46.12 ± 1.05[b]	8.79 ± 1.14 [b]

GV: Germinal vesicle; GVBD: Germinal vesicle break down; MII: Metaphase II; DEG: Degenerate oocytes.
Data are presented as means ± SEM. N: Total number. a/b Values within columns with different superscripts are significant differences (Chi-square test, $p < 0.05$).

percentage of degenerated oocytes in control was more than the treatment group (17.55% and 8.79%, respectively). The percentage of oocytes that reached to the GVBD and MII stages in the treatment group significantly more than control (for GVBD 34.78% and 27.78 %, for MII 46.12% and 33.35%, respectively), (Table 4, Figure 5).

DISCUSSION

In the present study, we examined the effects of repaglinide on in vitro preantral follicle growth and maturation. Our results indicated that the follicle growth parameters such as diameters, the density of granulosa cells, hormone production and viability rates in the repaglinide-treated group

Improved in comparison to the control. Oocyte growth and nuclear maturation (passes from GV step and reaches to GVBD and MII stages) in the repaglinide-treated group was significantly higher than control. The progression of in vitro culture systems that result to increasing the growth of the preantral follicles and subsequently enhance of developmental competence of oocyte can improve in vitro embryos production.[2] In recent years, numerous in vitro culture systems for mammal's ovarian follicles and immature oocytes have been designed to study the regulatory mechanisms that are occurring during folliculogenesis and oogenesis. These studies contributed to the development of new reproductive biotechnologies, including clinical application in the treatment of human infertility. Many types of culture systems are used usually for in vitro follicles culture for example, usage of different culture mediums that supplemented with different growth factors and hormones to facilitating follicle culture and growth.[9,34] Main types of media used to culture follicles such as α-MEM medium supplemented with a different substance that is typically used for follicle growth and maturation.[17] In this study for follicle culture, we used the α-MEM medium that supplemented with 1μM concentration of repaglinide. The best-developed systems applied to mammalian follicles that can be translated to human follicle-culture systems and reproductive research have been achieved from mice model (39; 42; 15). In the present study, we evaluated the effect of repaglinide on preantral follicle were obtained from female NMRI mice ovaries. The improvement of culture medium continues and in the search for an ideal system, substances and intra ovarian factors have been studied, among which can mention the calcium.[29] Calcium signaling has a key role at different stages of oocyte maturation, fertilization and egg activation in all species.[30] During maturation organization of Ca^{2+} storage organelles and the ability of oocyte changes for release Ca^{2+} in response to various triggers changes in MII eggs is approximately 3-fold larger than that induced in GV oocytes, and the greatest change occurs between MI and MII.[20] It has been shown that oocytes express KATP channels.[14] KATP channels link intracellular metabolic conditions with the membrane excitability. KATP channel inhibitory drugs such repaglinide by an effect on voltage-dependent Ca^{2+} channel causes to depolarization of cells membrane and increasing of intracellular calcium.[37] Fernandes and his workers (16) when they study human MII oocyte indicated that human oocytes maintain Ca2+ homeostasis with difficulty when exposed to routine in vitro conditions, which could interfere with fertilization. Both inhibition and activation of KATP channels is useful for maintaining Ca^{2+} homeostasis in oocytes under in vitro conditions. It seems that inhibition of KATP channels is a particularly efficient strategy in protecting human oocytes against stress. Considering the inhibitory effect of repaglinide on KATP channels, previous study by Kalehoei and Azadbakht 21 showed that the improvement of in vitro oocyte maturation and subsequently embryo cleavage rate may be related to the increasing of intracellular calcium concentration. Folliculogenesis need to the bidirectional interaction between the oocyte and granulosa cells, which during this process, the oocyte progressively grows while the granulosa cells proliferate and differentiate.[25] At the starting of follicle growth,

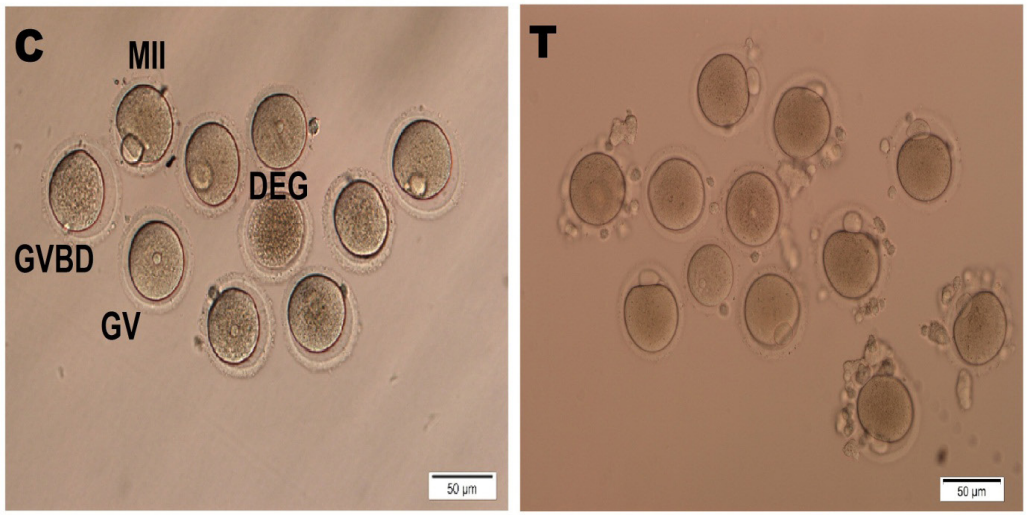

Figure 5: Nuclear maturation of mouse oocytes from follicles cultured in the presence or absence of repaglinide
GV germinal vesicle, GVBD germinal vesicle breakdown, MII metaphase II, DEG degenerated (Scale bar: 50 µm). C: Control group without repaglinide in the culture medium. T: Treatment group with 1µM concentration of repaglinide in the culture medium.

the number of granulosa cells significantly increased, and their shape changed from flattened to cuboidal, then columnar and formed multiple layers. When the second layer of granulosa cells start to develop, they reached a maximal packing density on the basal lamina that the increasing plating density of the cells induced a specific response as indicated by an altered gene expression profile and hormone production.[12] In this study, the result showed that in the treatment group with 1μM concentration of repaglinide in the culture medium the cell density increased significantly than the control group. Factors secreted from granulosa cells such as estradiol and insulin-like growth factor (IGF) are necessary for follicle growth, development and survival, by decreasing of survival-promotingg factors, granulosa cells lose their proper functions and undergo cell death. Morphologically, apoptosis is induced in granulosa cells located in the inner cell surface of the granulosa layer, but not in cumulus cells, oocyte and inner or extra theca layers in the early stage of atresia.[19] Many studies indicated that the granulosa cells are determining for continue follicle growth or undergo atresia.[28] In the present study, survival rate in the treatment group was significantly higher than the control, and this result shows that repaglinide maybe by increasing survival-promoting factors, lead to enhance survival rate during follicle growth. Ovarian granulosa and theca cells are the major source of estrogen production during reproductive age, and after induces with preovulatory luteinizing hormone (LH) their morphological, physiological, and molecular properties changed. Cell culture models are suitable tools for study of regulatory mechanism that involved in folliculo-luteal transformation and many researchers interested in it 4; 8. The study performed by Baufeld and Vanselow[4] indicated that appropriate condition are necessary to successful steroid active cell culture; for example, increasing the plating density of the cell causes changes in gene expression and hormone production. Steroid hormone concentration altered in granulosa cells cultured at different plating densities, for example when granulosa cells were cultured at a high cell density, the estradiol (E2) concentration significantly decreased, while the progesterone (P4) concentration only tended to be increaser study showed that in the treatment group at comparison to the control. In previously study, Lucidi and his workers[26] by cultured the pig granulosa cells grown as monolayers for 5 days that surrounded the oocytes, indicated that in different IVM condition, the oocyte after development from GV stage to completely maturity can produce conditioned media (OCM) that containing oocyte secreted factors which influenced on steroidogenesis of granulosa cells, by study of E2 and P4 production with radioimmunoassay method. Their results showed that the matured oocytes by inhibiting E2 and increasing P4 production could have an effect on granulosa cells steroidogenic activity,[36] by evaluation in vitro culture of ovarian mouse follicle in the oil-containing culture at comparison oil-free culture, measured E2 and P4 from conditioned medium during the antral follicle growth phase (Days 8 and 12 of culture). Their results showed that E2 concentration that was 7 times lower at the beginning of the antral phase (day 8) and 14 times lower in the preovulatory stage (day 12). Progesterone concentration was determined both before and after the hCG stimulus. In oil-containing and oil-free culture, there was a significantly higher progesterone concentration in the post-hCG conditions compared to the basal pre-hCG progesterone concentration. In this research, according to the previous studies after collection conditioned medium, the E2, and P4 concentrations were assessed in both groups. Our results showed that in group without HCG, E2 concentration decreased, and P4 concentration significantly increased in control and treatment with 1μM concentration of repaglinide in a culture medium, but after HCG administration in the treatment group, P4 concentration significantly higher than the control group.

CONCLUSIONS

In conclusion, this study demonstrated that the addition of Repaglinide to the follicle growth media, at 1 μM, could improve the growth, viability and activity of follicles in mouse after 12 days of culture. Furthermore, the present study updates our knowledge on application of repaglinide in culture medium of ovarian follicles that may have positive effects on cell function and stimulation of progesterone and 17-β-estradiol synthesis.

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