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Comparing the growth and the development of mouse pre-antral follicle in medium with PL (Platelet Layset) and with FBS

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Fetal Bovine Serum (FBS)

Abstract *Introduction:* In recent years a great deal of effort has been made to improve the culture medium which can be used for different cell culturing and Folliculogenesis. PL (Platelet Layset) with a high percentage of growth factors and also microelements can be effective on the growth of follicle and oocyte. *Material and method:* Preantral follicles were collected from 12 to 14 days female NMRI mice and cultured in culture medium with a different supplement as the serum for 12 days. The different serums were, 5% and 10% FBS as the control groups and the experimental groups were enriched by 5% PL, 10% PL and a combination of 5% PL and 5% FBS. The growth and development of follicle and oocyte was monitored and also the amount of Estradiol (E2) and progesterone (P4) was detected in different mediums on days 9 and 12 of culturing. *Result:* Oocytes had a significant growth in all medium compared to day zero and reached to the size of a mature oocyte ($p < 0.05$). Although survival rate after one day had significant decrease in all medium compared to 5% FBS ($p < 0.05$), after 12 days of culturing, the best survival rate belonged to 5% PL. Progesterone secretion increased in experimental groups significantly ($p < 0.05$) while Estradiol secretion in 5% and 10% PL decreased in half of control groups. *Discussion:* This result

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demonstrates that PL can be a considerable supplement that can be added to the culture medium or a good replacement for FBS as serum used in Folliculogenesis.

1. Introduction

The growth and development of preantral follicle has become an effective method in obtaining mature and fertilizable oocytes intended to be used in ART (Assist Reproductive Technology) (1). This method along with freezing methods plays a crucial role in restoring fertility in the human and other mammalians (2). In Vitro Folliculogenesis technique needs to be better improved because immature oocytes are potentially tentative to mature by In Vitro Folliculogenesis and reach MII phase. However, future development and growth cannot be promised. Culture medium can play an important role in the field of culturing. In recent years numerous studies have focused on improving culture medium (3–5).

In general, culture medium has three important parts including serum, additive supplement and growth factors. In appropriate conditions, preantral follicles with meiotic potential oocytes will be able to reach their final size and release mature oocytes (6). In cell culturing we need to use serum for enriching culture medium and the common serum between laboratories is Fetal Bovine Serum (FBS) (7). Using FBS has some advantages in cell culturing such as having stimulatory factors as well as low concentration of immunoglobulin, however, there are some inevitable disadvantages to it including its high cost, difficulty in purifying of the product and complication of this method on one side, and on the other side using unborn calves is an ethically questionable source (8). In recent years researchers have been trying to improve culture media to use in cell culturing and lots of research have focused on find an alternative supplement to overcome problems associated with the use of FBS (9).

PL (Platelet Layset) with high percentage of growth factors and microelements can be used as a suitable replacement for FBS in cell culturing and Folliculogenesis. Platelet is present in blood and its important role is to aggregate at the injury site and after clotting it starts the heeling process of the wound by releasing growth factors and other elements (10,11). For the first time, in 1970 a research showed that platelet is effective in restoring the wound and is reaching the source of growth factor such as PDGF, TGFβ, Fibronectin (12–14). In recent years, some other studies have been carried out to use PL as a good replacement for FBS, such as the study by Anna Alden et al., in 2007. They used PL for culturing hamster ovary cell and the result was impressive (15). In 2014 Pazoki et al. cultured preantral follicles in a medium enriched by PL for the first time and the survival rate and oocyte growth were acceptable (16).

The purpose of this study was evaluating the components of a platelet lysate medium to demonstrate its potential as a growth-promoting supplement in comparison with the Fetal Bovine Serum in Folliculogenesis.

2. Material and method

2.1. Production of platelet lysate

Platelet Layset (PL) was prepared from cord blood in Royan Institute in Tehran, Iran. Cord blood was transferred to laboratory at 2–8 °C during transferring. First, cord blood was centrifuged at 300*g* to get PRP (Plasma Riched Platelet) and after that PRP centrifuged at 3000*g* for separating platelet in high concentration from PPP (Plasma Poor Platelet). High concentration platelet was frozen at −70 °C. At the end, PRP was warmed at 37 °C to get PL and after freezing/warming platelet membrane was broken and platelet extraction (PL) was obtained.

2.2. Follicle collection

Preantral follicles were collected from prepubertal NMRI mice (aged 12–14 days). All mice were housed and bred according to national legislation for animal care. Animals were killed by cervical dislocated and then ovary were removed aseptically and placed on prewarmed isolation medium consisting α-MEM (sigma, USA) supplemented with 10% Fetal Bovine Serum (Gibco, Germany) and 100 IU/ml penicillin + 100 mg/ml streptomycin (sigma, USA). The ovaries were dissected mechanically by fine hypodermic needles (p-med china). Follicles with average size of 100–130 μm with two layers of granulosa cells and oocytes in normal condition were selected. The average number of collected preantral follicle was ∼30 in averages per ovary and 480 follicles were collected in total.

2.3. Follicle culture

Selected follicles were washed in isolation medium and then cultured individually in 20 μl droplets of culture medium overlaid with mineral oil (sigma, USA) in Petri dishes (60 × 15 Falcon, Germany), 16 droplets per dish. Culture medium consisted of α-MEM (sigma, **USA**), ITS (5 mg/ml Insulin, 5 mg/ml Transferrin, 5 mg/ml Selenium; (Gibco), 100 mIU/ml follicle-stimulating hormone (FSH)(Gibco) and 10 mIU/ml luteinizing hormone (LH)(Gibco) but the mediums were enriched by different amount of Fetal Bovine Serum (FBS)(Gibco) or platelet lysate (PL) as listed; first and second mediums were enriched by 5% and 10% FBS as control groups, third medium was enriched by 5% FBS and 5% PL together, and in last two mediums just 5% and 10% PL were added as experimental groups and without any serum. All fol-

licles were cultured in a humidified incubator under a 5% CO2 atmosphere at 37 °C. Refreshments were conducted every other day by removing and replacing 10 µl of medium. Follicles were assessed in term of follicle and oocyte diameter and antrum formation. The morphological characteristics of the follicles were recorded before refreshments. All assessment was done with an inverted microscope.

2.4. Sampling of conditioned medium for estradiol and progesterone measurements

10 µl spent medium was collected after each refreshment and was frozen at −80 °C to evaluate the amount of two steroids for further characterization of follicle growth and quality of development. Estradiol and progesterone level were measured by means of the Electrochemiluminescence immunoassay (ECLIA) kits and instrument (Elecsys 2010; Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim). Secretion of Estradiol and progesterone was measured at day 9 and 12 before the conduction of ovulation

3. Result

3.1. In vitro growth and development of pre-antral follicle

Oocytes had significant growth in all medium at the end of culturing compared to day zero ($P < 0.05$). As data shown in Table 1, after 12 days of culturing in medium just with PL and without any serum, oocytes could grow and reach to the normal size as a mature oocytes in medium with Fetal Bovine Serum.

Follicle's growth and granulosa cell proliferation were not normal in experimental groups compared to control groups. On the other hand, follicle could grow in control groups significantly compared to the first day of culturing ($P < 0.05$) but follicle in experimental groups did not have any significant growth compared to day zero.

Meanwhile, the number of follicle after one day significantly decreased in experimental groups compared to control group 5% FBS ($P < 0.05$). Whereas, at the end of the study, the best survival rate belonged to 5% PL and the number of follicles in control groups declined significantly compared to 5% PL ($P < 0.05$).

In addition, the data showed follicles in mediums with PL released their immature oocytes significantly between days 7 and 9 before conduction of ovulation compared to control groups ($P < 0.01$).

3.2. Estradiol and progesterone secretion

The secretion of Estradiol (E2) was measured at day 9 and 12 of culturing before conduction of ovulation. The data demonstrate that the secretion of Estradiol in experimental groups had significant decrease in comparison with the control group ($P < 0.05$). The amount of Estradiol was 4257 & 4039 pg/ml in 5% & 10% PL respectively while the level of E2 in control groups was >8000 pg/ml.

Progesterone secretion also was measured at day 9 and 12 before the conduction of ovulation. The secretion of progesterone had significant increase in all mediums with PL both in day 9 ($P < 0.01$) and 12 ($P < 0.05$). The level of P4 in experimental groups reached to 32.8 and 52.6 ng/ml for 5% and 10% PL respectively while the P4 level in control groups just reached to <22 ng/ml at the end of culturing.

4. Discussion

In Vitro Folliculogenesis is a very variable method in which not only could we obtain mature and fertilizable oocytes in order to use them in Assist Reproductive Technology (ART) but we are also able to study the essential factors for oocyte development (17–19). Follicle growth and development in vivo are including steroidogenic activity of theca layer, proliferation of the granulosa cells, increasing of oocyte size and subsequent of ovulation (20). The number of granulosa cells will be raised during culturing and these cells are responsible for the secretion of some factors that are necessary for oocyte development (21–23). In the present study, preantral follicles were cultured for 12 days in the culture mediums which were enriched by different concentrations of Platelet lysate. The survival rate, follicles and oocyte growth were monitored and also the level of E2 and P4 was detected at day 9 and 12 in different culture mediums. The aim of this study was to improve culture medium with Platelet Layset (PL) as a new additive supplement or a good replacement for common serum such as FBS to consider its effect on follicles and oocyte growth and development. The results in Table 1 show that oocytes could grow in all medium and reach the normal size as that of the mature size ($p < 0.05$). Increasing oocyte size can be considered as one of the major signs for the follicle growth and oocyte maturation in vivo (20). Therefore, this result shows that PL is effective on the growth of oocytes.

The secretion of hormones from granulosa cells is essential for oocyte maturation during Folliculogenesis (24). In fact, secretion of Estradiol (E2) from granulosa cells is interdepen-

Table 1 Data for oocyte growth from different experimental groups.

Groups	Oocyte mean diameter + SD in day zero (µm)	Oocyte mean diameter + SD in day 2 (µm)	Oocyte mean diameter + SD in day 4 (µm)	Oocyte mean diameter + SD in day 7 (µm)	Oocyte mean diameter + SD in day 9 (µm)	Oocyte mean diameter + SD in day 12 (µm)
5% FBS	60.31 ± 2.86	63.12 ± 2.5	65.31 ± 3.4	73.43 ± 6.25	80 ± 5.16	82.18 ± 2.56[a]
10% FBS	60.28 ± 2.39	62.16 ± 2.39	64.13 ± 2.21	73.26 ± 4.42	78.78 ± 2.86	81.12 ± 2.58[a]
5% PL	60.31 ± 2.86	61.87 ± 3.09	63.5 ± 3.16	68.43 ± 3.01	72.5 ± 4.08	80.62 ± 3.59[a]
10% PL	60.62 ± 3.09	61.25 ± 2.88	63.75 ± 3.87	68.75 ± 5.62	75.93 ± 4.55	80.31 ± 4.26[a]
5% PL + 5% FBS	60.31 ± 2.21	61.65 ± 2.39	63.75 ± 2.23	66.56 ± 2.39	70.62 ± 3.59	77.81 ± 3.63[a]

Mean diameter ± SD, diameter of oocytes and standard deviation in µm.
[a] Significant differences at day 12 compare to day zero; ($P < 0.05$).

Table 2 Morphological characteristics of follicle development of mouse early preantral follicles in vitro.

Groups	*N*	Follicle mean diameter + SD in day zero (μm)	Survival of follicles after one day (% of day 0)	Follicle mean diameter + SD in day 4 (μm)	Follicle mean diameter + SD in day 7 (μm)	Follicle mean diameter + SD in day 12 (μm)	Day 12 before conduction of ovulation		Number of MII released 16 h after conduction of ovulation (% of day 0)
							Follicles with antral cavity (% of day 0)	Survival of follicles (% of day 0)	
5% FBS	96	112.81 ± 4.81	96(100%)	214.06 ± 35.83	322.12 ± 102.24	469.37 ± 75.31[c]	75 (78.12 %)	88(91.66%)[d]	24(25 %)
10% FBS	96	114.68 ± 4.26	91(94.74%)[a]	237.18 ± 44.11	411.87 ± 72.31	540.62 ± 18.78[a,c]	72(75%)	87(90.62%)[d]	14(14.5%)[a]
5% PL	96	113.43 ± 4.36	90(93.75%)[a]	143.43 ± 26.56	204.37 ± 58.53	260.62 ± 74.15[b]	4(4.1%)[b]	90(93.75%)[a]	0%[b]
10% PL	96	112.81 ± 3.36	94(97.91%)[a]	168.12 ± 17.87	172.81 ± 47.57	105.93 ± 21.54[b]	2(2%)[b]	86(89.58%)[d]	0%[b]
5% PL + 5% FBS	96	110 ± 4.08	89(92.7%)[a]	144.37 ± 18.15	224.37 ± 48.16	250.62 ± 63.9[b]	2(2%)[b]	79(82.2%)[d]	0%[b]

Mean diameter ± SD, diameter of follicle and standard deviation in μm; *N*, number of follicles isolated; MII, metaphase II; FBS, Fetal Bovine Serum; PL, Platelet Layset.

[a] Significant difference to 5% FBS ($P < 0.05$).

[b] Significant difference to 5% FBS ($P < 0.01$).

[c] Significant difference from day zero ($P < 0.05$).

[d] Significant difference from survival rate on day one ($P < 0.05$).

Table 3 Estradiol (E2) and progesterone (P4) secretion analysis and the relation between amount of E2 & P4 and follicle growth and development.

Groups	Estradiol secretion at day 9 of culturing (pg/ml) mean ± SD	Estradiol secretion before conduction of ovulation (pg/ml) mean ± SD	Progesterone secretion at day 9 of culturing (ng/ml) mean ± SD	Progesterone secretion before conduction of ovulation (ng/ml) mean ± SD	Follicle with antral cavity before conduction of ovulation (% of total follicle)	Immature oocyte released before conduction of ovulation (% of total follicle)	MII released 16 h after conduction of ovulation (% of total follicle)
5% FBS	4448 ± 56.14	8573 ± 112.3	1.7 ± 0,65	19.8 ± 2.02	78.12%	0%	25%
10% FBS	4353 ± 45.09	8320 ± 165.22	1.92 ± 0.11	22.66 ± 2.51	75%	0%	14.5%[a]
5% PL	1849 ± 76.89[a]	4257 ± 39.51[a]	22.43 ± 1.401[b]	35.76 ± 3.1[a]	4.1%[b]	21%[b]	0%[b]
10% PL	729 ± 174.8[b]	4039 ± 61[a]	27.03 ± 1.001[b]	50.06 ± 2.33[a]	2%[b]	18.75%[b]	0%[b]
5% FBS + 5% PL	2565 ± 49.21[a]	3095 ± 95[b]	17.76 ± 1.56[b]	41.23 ± 2.67[a]	2%[b]	19.79%[b]	0%[b]

Mean diameter ± SD, diameter of follicle and standard deviation in μm; MII, metaphase II; FBS, Fetal Bovine Serum; PL, Platelet Layset.

[a] Significant difference to 5% FBS ($P < 0.05$).

[b] Significant difference to 5% FBS ($P < 0.01$).

dent with the production of androgen from theca cells in response to LH in medium (25). Estradiol secretion will begin from the earlier stage of follicle growth and this amount will increase dramatically up to the pre-ovulatory stage of Folliculogenesis (24). As the data demonstrated in Table 3, secretion of E2 is not enough for the oocyte maturation (less than <4300 pg/ml) in our experimental groups compared to the control groups (more than >8000 pg/ml) and there was a significant decrease compared to control groups ($P < 0.05$). Based on the data on Table 2, development of follicle and granulosa proliferation in experimental groups was not normal and there was a significant decrease in follicle growth and the number granulosa cell in comparison with control groups ($P < 0.05$). This result demonstrates that, in medium with PL, follicle did not have enough development and maturation was failed due to low concentration of E2.

Progesterone production will be increased in the culture medium after the addition of rHCG/rEGF into follicles culture mediums (26). Progesterone is responsible for the final maturation of oocytes and ovulation (26). In experimental groups the secretion of progesterone (P4) increased significantly both at day 9 ($P < 0.01$) and day 12 ($P < 0.05$). As the data shown in Table 3, the amount of P4 in medium with PL was extremely higher than control groups and at day 9 it was >22 ng/ml while progesterone secretion on control group was <2 ng/ml and at the end of culturing reached >35 ng/ml and P4 level in control groups reached to just 20 ng/ml. Based on the fact that progesterone is responsible for ovulation at the end of follicle's growth (26) the high level of progesterone seems to be responsible for immature oocyte release before the conduction of ovulation. The data demonstrated that PL in culture medium had a significant effect on P4 secretion from granulosa cells and this study suggested PL could be used in ovulation medium.

The results indicated that PL (Platelet Layset) with high percentage of growth factor and microelement is effective on the oocyte growth and progesterone secretion however, the follicle growth and granulosa cell proliferation failed in the medium with PL.

5. Conclusion

Platelet Layset with high concentration of growth promoting factors has positive effect on the oocyte growth and development, despite the fact that follicle growth and granulosa cell proliferation failed. This result suggested that PL maybe used as a good replacement for FBS but further investigation is needed to explore which factors are responsible for the failure of follicle growth and granulosa cell proliferation. It is assumed by the authors that by exploring these factors and removing them from the culture media, oocyte maturation and embryonic preimplantation development in-vitro can be improved.

Conflict of interest

There is no conflict of interest.

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