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ORIGINAL ARTICLE

The effects of platelet lysate on maturation, fertilization and embryo development of NMRI mouse oocytes at germinal vesicle stage

Hassan Pazoki[1,2] · Hussein Eimani[1,3] · Farah Farokhi[2] · Abdol-Hossein Shahverdi[1] · Leila Sadat Tahaei[1]

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Abstract
Purpose Improving in vitro maturation could increase the rate of pregnancy from oocytes matured in vitro. Consequently, patients will be prevented from using gonadotropin with its related side effects. In this study, the maturation medium was enriched by platelet lysate (PL), then maturation and subsequent developments were monitored.
Methods Oocytes at germinal vesicle stage with cumulus cells (cumulus–oocyte complex) and without cumulus cells (denuded oocytes) were obtained from mature female mice. The maturation medium was enriched by 5 and 10 % PL and 5 % PL + 5 % fetal bovine serum (FBS) as experimental groups; the control groups' media consisted of 5 and 10 % FBS. After 18 h, the matured oocytes were collected and, after fertilization, subsequent development was monitored.
Results The rates of maturation, fertilization and 2-cell embryo development for the denuded oocyte groups in experimental media 5 % PL and 5 % PL + 5 % FBS were significantly higher than those of the control groups ($P < 0.05$), while the results for the cumulus–oocyte complex groups were similar between the experimental groups and control groups.
Conclusions The results of this study indicated that platelet lysate could improve the maturation rate in the absence of granulosa cells compared to media with FBS. This extract also had positive effects on fertilization and embryo development.

Keywords Cumulus–oocyte complex · Denuded oocyte · In vitro fertilization · In vitro maturation · Platelet lysate

✉ Hussein Eimani
Hussein.eimani@yahoo.com

[1] Department of Embryology at Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

[2] Department of Biology, Faculty of Science, Urmia University, Urmia, Iran

[3] Department of Anatomy, Faculty of Medicine, Baqyiatallah (a.s.) University of Medical Sciences, Tehran, Iran

Introduction

In vitro maturation (IVM) has been considered to be the first and most important step for in vitro embryo production. This technique can not only obtain oocytes for assisted reproductive technologies (ART) such as in vitro fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI) [1], but can also produce embryos for carrying out basic biotechnology research like cloning [2]. The culture medium is an important factor in oocyte maturation in vitro. The maturation medium has a profound effect on oocyte maturation and can influence fertilization and subsequent embryo development [3, 4].

Several studies have been conducted on different aspects of IVM and on improving the developmental competence of oocytes by supplementing the maturation medium with different additives [5–8], including gonadotropins, serum and growth factors [9–11]. Most of the media were enriched by different sera because of their hormones, proteins and nutrients. On the other hand, serum contains amino acids, hormones, growth factors, cytokines, vitamins and many other substances which have an effect on maturation, fertilization and embryo development [12, 13]. Recently, there has been a trend to improve the maturation medium by adding factors such as epidermal growth factor (EGF),

insulin-like growth factor (IGF) and different concentrations of gonadotropin, and also using protein or bovine serum albumin (BSA) [14–16]. Platelet lysate (PL) is not known to have previously been used as a good source of protein and growth factors in culture media for IVM.

Platelets are blood cells and will aggregate at the injury site, and have an important role in healing the wound by releasing growth factors and other elements after clotting [16, 17]. In 1970, for the first time, some studies showed that platelets are effective in wound healing and indicated that platelets are rich in growth factors like pituitary differentiation growth factor (PDGF), transforming growth factor beta (TGFβ) and fibronectin [18, 19]. Other studies demonstrated that PL is replete with proteins and microelements along with growth factors such as fibroblast growth factor (FGF), TGFβ, PDGF, IGF and EGF as well as attachment factors like fibrinogen and serotonin which are vital for cell culturing and folliculogenesis [20, 21]. In recent years, some projects have been carried out using PL as a new additive supplement to improve media for cell culturing, and the results were impressive [22–24].

The present study was conducted to explore the possibility of using PL in the maturation medium in the presence and absence of granulosa cells as a maturation promoting factor, and to consider its effects on the maturation, fertilization and subsequent development of oocytes at the germinal vesicle (GV) stage.

Materials and methods

PL preparation

Platelet lysate was prepared from human umbilical cord blood (UCB) in the Royan Institute in Tehran, Iran. To obtain PL, first the collected cord blood was transferred to the laboratory at 2–8 °C, then 350–400 ml of UCB was centrifuged at 300*g* for 22 min at room temperature and plasma-rich platelets (PRP) were obtained for further processing. Collected PRP was centrifuged again at 3000*g* for 30 min at room temperature to separate platelet concentrate (PC) from plasma-poor platelets (PPP). The PPP was assessed for infections such as hepatitis or HIV and high PC was frozen at −70 °C for 48–72 h. The PC, which contained approximately 2×10^9 platelets/ml, was then rapidly thawed at 37 °C and centrifuged at 3000*g* for 30 min at 4 °C to remove the platelet bodies. Finally, prepared PL was frozen at −20 °C until use.

Oocyte collection

Oocytes at germinal vesicle stage (GV) were obtained from Naval Medical Research Institute (NMRI) female mice aged 6–8 weeks. The mice were housed in controlled light (light/dark cycle of 12:12 h) and temperature (20 °C). Humidity was maintained at a minimum of 50 % within the animal house of the Royan Institute. Animal care was in accordance with institutional guidelines and was approved by the local authorities. The mice were killed by cervical dislocation and then their ovaries were removed aseptically and placed on pre-warmed isolation medium consisting of Minimum Essential Medium (α-MEM) (Sigma, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco, Germany) and 100 IU/ml penicillin + 100 mg/ml streptomycin (Sigma). Oocytes from ovarian follicles were obtained by puncturing with fine hypodermic needles (P-med, China) under a stereomicroscope with cumulus cells (cumulus–oocyte complex) and without cumulus cells (denuded oocytes). A total of 720 oocytes were collected from 16 mice (∼25 oocytes from each ovary on average).

In vitro maturation

The maturation medium was α-MEM (Sigma), which consisted of 100 mIU/ml follicle stimulating hormone (FSH) (Gibco), 1.5 IU/ml luteinizing hormone (LH) (Gibco) and 5 ng/ml EGF (Sigma). The medium was enriched by different concentrations of FBS and PL as maturation-promoting factors; groups I and II as control groups had 5 and 10 % FBS, groups III and IV were enriched by 5 and 10 % PL and a combination of 5 % PL and 5 % FBS was added to the last group as experimental groups. The collected oocytes at GV stage with cumulus cells (cumulus–oocyte complex; COC) and denuded oocytes (DO) were each divided into five groups. Each group was placed in different 25-µl micro-drop maturation media overlaid with mineral oil (Sigma) for 16–18 h in an incubator with a humidified atmosphere of 5 % CO_2 at 37 °C. Matured oocytes were collected for IVF (an oocyte which released the first polar body was considered as a matured oocyte).

In vitro fertilization

Collected spermatozoa from epididymides of NMRI male mice (aged 12 weeks) were capacitated for 2 h in 500 µl capacitation medium. The capacitation and fertilization media were human tubal fluid (HTF) supplemented with 15 mg/ml BSA. The matured oocytes from each treatment group were placed into 500-µl droplet fertilization medium and capacitated spermatozoa were added and incubated for 6 h at 37 °C.

Cleavage

Fertilized oocytes at the two pronucleus stage (2PN) were collected from fertilization medium and after washing in

HTF medium they were cultured in 25-µl droplet cleavage medium (HTF supplemented with 5 mg/ml BSA). They were assessed for cleavage to the 2 cells after 24 h.

Statistical analysis

Collected data were analyzed by the χ^2 test. The differences in the values of maturation, fertilization and embryo developmental rates were considered significant when $P < 0.05$.

Results

In vitro maturation

The maturation rates for DO groups in media with 5 % PL and 5 % PL + 5 % FBS were significantly higher than that of the control group with 5 % FBS ($P < 0.05$), while the maturation rates for COC groups were similar between the control group with 5 % FBS and the experimental groups with 5 % PL and 5 % PL + 5 % FBS (Table 1). The maturation rates in media with 10 % PL and 10 % FBS for both DO and COC groups were significantly decreased compared to the control group with 5 % FBS ($P < 0.05$) (Table 1).

In vitro fertilization and embryo development

The number of fertilized oocytes (2PN) for the DO group in media with 5 % PL and 5 % PL + 5 % FBS increased significantly compared to 5 % FBS after 6 h of fertilization ($P < 0.05$), but the number of fertilized oocytes showed a significant decline in media with 10 % PL and 10 % FBS ($P < 0.05$) (Table 2).

The data show that the numbers of fertilized oocytes (2PN) in DO groups which developed into 2-cell stage were significantly higher than those in the control groups in all media enriched by PL ($P < 0.05$), while this rate in COC experimental groups decreased significantly compared to the control group 5 % FBS ($P < 0.05$) (Table 2).

Discussion

This study evaluated the effect of platelet extract on the maturation of NMRI mouse oocytes at germinal vesicle stage and derived embryo. Maturation in vitro is the most important step towards increasing the rate of IVF and embryo development [14]. Several studies have proved that the constituents of the media and additive supplements like serum have profound effects on maturation and embryo development [25]. The medium has important biological roles as a resource of vital components such as amino acids, vitamins, growth factors, energy and microelements [26]. In addition, there is some evidence that growth factors, cytokines, IGF and EGF can affect maturation and follicle development [27, 28]. In recent years, some studies have been conducted to improve the culture medium by adding different supplements like PL [22–24]. In this study, the maturation medium was enriched by different

Table 1 Morphological characteristics of oocyte maturation after 16-18 h in different maturation media

Groups		*N*	16–18 h after conduction of ovulation				
			GV (% of oocyte)	MI (% of oocyte)	MII (% of oocyte)	GVBD (% of oocyte)	Degenerated oocytes (% of oocyte)
5 % PL	COC	72	17[a]	8	68	76	7
	DO	72	10[b]	11	75[a]	86[a]	4
10 % PL	COC	72	30[a]	13	43[a]	56	14[a]
	DO	72	34	12	49[a]	61	5[a]
5 % FBS	COC	72	23	3	65	68	9
	DO	72	32	6	60	66	2
10 % FBS	COC	72	45[b]	8	42[a]	50	5[a]
	DO	72	41[a]	8	46[a]	54	5[a]
5 % FBS + 5 % PL	COC	72	19	11	63	74	7
	DO	72	15[b]	12	70[a]	82[a]	3

Degenerated oocytes, number of degenerated oocytes 16–18 h after induction of ovulation in the ovulation medium

N number of oocytes, *COC* cumulus–oocyte complex, *DO* denuded oocytes, *GV* germinal vesicle, *GVBD* germinal vesicle breakdown, *MII* oocytes with polar body, *MI* oocytes which resumed meiosis but could not release first polar body

[a] Significant difference from control group 5 % FBS ($P < 0.05$)

[b] Significant difference from control group 5 % FBS ($P < 0.01$)

Table 2 Statistical characteristics of fertilized oocytes in different fertilization media and embryo development in different cleavage media

Medium	Groups	Matured oocytes (MII)	6 h after IVF		24 h after IVF	
			2PN (%MII)	Degenerated oocytes (%MII)	2 Cells (% of 2PN)	Degenerated embryo (% of 2PN)
5 % PL	COC	53	(37) 69.9	(16) 30.1	43	57
	DO	55	(41) 75[a]	(14) 25	48[a]	52
10 % PL	COC	38	(21) 55.2[a]	(17) 44.8	33[a]	67[a]
	DO	43	(25) 58.1	(18) 41.8	45[a]	55[b]
5 % FBS	COC	47	(32) 68	(15) 32	51	49
	DO	43	(22) 51.2	(21) 48.8	36	64
10 % FBS	COC	37	(17) 46[a]	(20) 54	47	53[a]
	DO	35	(15) 42.8[a]	(20) 57.2	35	65
5 % PL + 5 % FBS	COC	45	(30) 66.6	(15) 33.3	53[a]	47[a]
	DO	51	(35) 68.6[a]	(16) 31.4	46[a]	54

N number of oocytes at GV stage, *COC* cumulus–oocyte complex, *DO* denuded oocytes, *2PN* fertilized oocytes (two pronucleus), *degenerated embryo* number of degenerated fertilized oocytes after 24 h in cleavage medium

[a] Significant difference from control group 5 % FBS ($P < 0.05$)

[b] Significant difference from control group 5 % FBS ($P < 0.01$)

concentration of PL and maturation, fertilization and subsequent development were monitored.

In general, cumulus cells improve nuclear and cytoplasmic maturation by secreting factors such as gonadotropin-releasing hormone (GnRH), pituitary adenylate cyclase-activating polypeptide (PACAP), EGF, IGF, activin, inhibin and follistatin through the gap junction [29–32]. Oocytes matured in the presence of growth factors like EGF and IGF show an acceleration of nuclear and cytoplasmic maturation as well as a higher fertilization rate [33]. Therefore, it seems that the presence of cumulus cells is essential for oocyte maturation and subsequent fertilization. However, some studies have shown that some factors which play important roles in maturation, like EGF and IGF, have their own receptors in oocytes. In addition, transcripts for IGF and EGF receptor proteins have been found in oocytes [34, 35]. Moreover, some growth factors like PACAP and EGF have a direct biological action on oocytes and have been demonstrated to affect meiotic maturation even in denuded oocytes. Consequently, it is suggested that specific receptors are present on the oocyte surface and if the culture medium is enriched by necessary growth factors and microelements, maturation and embryo development will be obtained even in the absence of cumulus cells [36, 37].

PL has the components which are necessary for maturation, like EGF, IGF, cytokines and microelements [17–21] and seems to be a good source of these factors for improving maturation and embryo development. Anna et al. in 2007 and Lohmann et al. in 2010 added PL to the cell culture media and the results showed that PL had good potential to support cell culturing and proliferation [22, 23]. Pazoki et al. in 2014 also used PL in pre-antral follicle culture medium for the first time and the result demonstrated that PL has an acceptable effect on oocyte growth [24]. The present study was carried out to consider whether PL has any positive effect on oocyte maturation at the GV stage. The results demonstrated that PL has a significant impact on oocyte maturation (Table 1), fertilization and embryo development (Table 2), and confirmed the outcomes of previous studies on the positive effects of PL in cell culture and maturation.

Previous studies such as Nishi et al. [38] and Mahmoudi et al. [39] showed that the presence of cumulus cells or murine cells is necessary for maturation, fertilization and embryo development, and that these rates in the presence of cumulus cells (COC) are higher than denuded oocytes (DO) because of essential factors which are produced by cumulus cells and transferred to oocytes by gap junctions [29]. By adding PL to the maturation, fertilization and cleavage media, the present study managed to obtain outcomes in DO groups significantly different from previously conducted research in which the media were not enriched.

In addition, in the previous studies, the best results were obtained when the PL concentration was 5 %, and in this study too the maturation rate was significantly increased in media which had 5 % PL and 5 % PL + 5 % FBS (Table 1). However, maturation was decreased in media with 10 % PL and 10 % FBS compared to control groups ($P < 0.05$). This result showed that the concentration of serum in the culture medium could be vital.

This study suggested that PL with high percentages of growth factor and nutrition may be used as a good substitute for common serum used in cell culturing and

cumulus cells, especially when it is necessary to remove cumulus cells.

In conclusion, this study indicates that maturation, fertilization and embryo development in the presence of PL can be improved and seems to be a good replacement for granulosa cells, especially when we need to remove granulosa cells. Further studies should be carried out to explore the factors in PL contributing to these results. It is our belief that by exploring and extracting these factors from platelet cells in blood and using them in culture media, maturation and further development will be improved.

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Compliance with ethical standards

Conflict of interest Hassan Pazoki, Dr Hussein Eimani, Dr Farah Farokhi, Dr Abdol Hossein Shahverdi and Leila Sadat Tahaei declare that they have no conflict of interest.

Human rights statements and informed consent This article does not contain any studies with human subjects performed by any of the authors.

Animal rights All institutional and national guidelines for the care and use of laboratory animals were followed.

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