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Platelet-rich plasma promotes the development of isolated human primordial and primary follicles to the preantral stage

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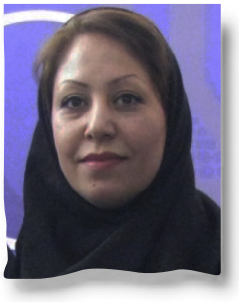

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KEY MESSAGE

Growth and survival of human primordial follicles isolated from fresh and vitrified-warmed ovarian tissue in a three-dimensional in-vitro culture were improved by supplementation of the culture media with platelet-rich plasma. This culture system has the potential to treat infertility in cancer patients when there is a risk of introducing malignant cells.

A B S T R A C T

This study aimed to assess the effects of platelet-rich plasma (PRP) on growth and survival of isolated early human follicles in a three-dimensional culture system. After fresh and vitrified-warmed ovarian tissue was digested, isolated early preantral follicles and ovarian cells were separately encapsulated in 1% alginate (w/v). The encapsulated follicles and ovarian cells were cultured together in a medium supplemented with foetal bovine serum (FBS), PRP, PRP + FBS, or human serum albumin (HSA) for 10 days. Growth and survival of the follicles were assessed by measurement of diameter and staining with trypan blue. Follicular integrity was assessed by histological analysis. After culturing, all follicles increased in size, but growth rate was greater in follicles isolated from fresh samples than those from vitrified-warmed ones ($P < 0.001$). Similarly, follicular viability of fresh samples after culturing was higher than that of vitrified-warmed ones. The growth and survival rates of follicles from both fresh and vitrified groups cultured in PRP supplemented media were significantly higher than those of other groups (growth $P < 0.001$ and survival $P < 0.05$, in both groups). In conclusion, media supplementation with PRP can better support viability and growth of isolated human early preantral follicles *in vitro*.

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Introduction

Common cancer treatments, including chemotherapy and radiation therapy, are often effective and life-preserving but may have a profound negative impact on a woman's reproductive health by damaging the reserve of ovarian follicles, which can ultimately lead to premature ovarian failure (Wo and Viswanathan, 2009). Standard methods for preserving fertility include cryopreservation of both oocytes and embryos (Bedoschi and Oktay, 2013; Cobo et al., 2010). To do so, the ovaries must be stimulated to retrieve mature oocytes. Such strategies are not recommended for patients with aggressive cancers because delaying treatment is not advisable. Moreover, ovarian stimulation is not applicable in prepubertal girls. An alternative for these patients is ovarian tissue cryopreservation and transplantation, a promising technique that has been shown to restore ovarian function and has resulted in more than 60 live births (Donnez and Dolmans, 2015; Loren et al., 2013). Despite these positive results, some studies have demonstrated a large percentage of follicle loss in grafted tissues as a result of post-grafting ischaemia (Demeestere et al., 2009). Moreover, transplantation of cryopreserved ovarian tissue is not advised in cases of blood-borne malignancies and ovarian cancers because of the risk of reintroduction of malignant cells (Dolmans et al., 2010; Rosendahl et al., 2010).

In-vitro culture of preantral follicles is an alternative fertility preservation approach for both reproductive-age women and prepubertal girls without hormonal stimulation or risk of reintroducing cancer cells. This procedure has produced successful results in several species, including sheep, goats, cows and non-human primates (Arunakumari et al., 2010; Saraiva et al., 2010; Sun and Li, 2013; Xu et al., 2013). However, attempts in humans have been less successful (Abir et al., 1999; Telfer et al., 2008). This could be due to the lack of growth factors and hormones necessary for the survival and development of human preantral follicles. However, supplementing media with numerous growth factors is not practical or affordable. Moreover, their addition in the appropriate culture periods and required concentrations can be very challenging.

Platelet-rich plasma (PRP), a blood by-product containing a significant concentration of platelets suspended in a small volume of plasma, can successfully replace foetal bovine serum, supporting the growth and viability of different cell types (Hemeda et al., 2014; Rauch et al., 2011). The positive effect of PRP is probably due to the high concentration of growth factors stored within platelet α-granules, which include platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), vascular endothelial growth factor, epidermal growth factor, fibroblast growth factor (FGF) and insulin-like growth factor (IGF) (Wang and Avila, 2007). Although PRP has never been used for in-vitro culture of isolated preantral follicles, evidence indicates that the factors in PRP have a positive effect on survival and growth of follicles (Danforth et al., 2003; Gutierrez et al., 2000; Matos et al., 2007; Nilsson et al., 2006; Zhou and Zhang, 2005). Therefore, the goal of this study was to evaluate the possible effects of PRP supplementation on survival and development of isolated human early preantral follicles *in vitro*.

Materials and methods

All experimental procedures were reviewed and approved by the Research Ethics Committee of the Avicenna Research Institute on 20 September 2011 (reference number 90/270) and by the Iranian National Committee of Ethics in Medical Research on 19 November 2011. All chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA), unless otherwise indicated in the text.

Experimental design

In our experiments, in order to clarify the effect of media supplementation on follicle development we first applied FBS, PRP and FBS + PRP in fresh samples. Because in practice we have to preserve the samples for future applications, we considered more experimental groups for vitrified samples, which were comprised of FBS, PRP, FBS + PRP and HSA groups. Each experiment included three replicates.

Collection of ovarian tissue

Ovarian tissues were obtained from three females under 35 years of age whose ovaries were donated after brain death following written informed consent. Ovaries were removed and transported to the laboratory at 4°C in a medium consisting of α-MEM + 1% FBS (Gibco) +100 µg/ml penicillin and 50 µg/ml streptomycin. The ovarian medulla was removed using a scalpel, and the cortex was cut into small cubes (1 × 1 × 1 mm) with a McIlwain Tissue Chopper.

Ovarian tissue vitrification and warming

Vitrification of ovarian cortical strips was performed as previously described (Amorim et al., 2012). First, ovarian tissues were exposed to 6.5% ethylene glycol (EG) and 2.5% DMSO in α-MEM supplemented with 20 mg/ml BSA as a base medium (equilibration solution) for 5 min. The samples were then transferred into vitrification solution 1 containing 13% EG and 5% DMSO in base medium; kept for 5 min; transferred into vitrification solution 2 with 26% EG, 10% DMSO, 2.5% polyvinylpyrrolidone (PVP, MW 10,000), and 1 mol/l sucrose in base medium; and kept for 1 min. All samples were maintained at 4°C. The tissue pieces were then transferred into a cryovial with a minimum volume of medium and immersed in liquid nitrogen.

For warming, the vitrified ovarian cortical pieces were transferred into warming solution 1 containing 1 mol/l sucrose in base medium for <15 s and then transferred to different concentrations of sucrose (0.5 mmol/l and 0.25 mmol/l, respectively) for 5 min each at 37°C. After each step, the samples were rinsed with the base medium.

Ovarian follicles and cell isolation

Follicles were isolated from fresh and vitrified-warmed ovarian cortical tissues as previously described (Rice et al., 2008). The tissue was enzymatically digested in Krebs ringer bicarbonate buffer supplemented with 1 mg/ml collagenase IA and 1 mg/ml DNase (Roche) in a shaking water bath at 37°C for 50–60 min. Digestion was terminated by adding an equal volume of bicarbonate tissue culture medium (BTCM) supplemented with 10% FBS at 4°C. The resulting suspension was centrifuged at 50*g* for 10 min at 4°C. The supernatant was discarded, 5 ml of warm HEPES tissue culture medium (HTCM) containing 4 mg/ml BSA and 3 mg/ml polyvinyl alcohol was added, and the suspension was pipetted to mechanically disrupt the digested tissue then filtered through a 70-µm filter. The isolated follicles were picked up under a stereomicroscope and washed three times with α-MEM containing 10% serum. Next, the rest of the cell suspension was filtered through a sterilized 40-µm filter. The filtered solution was centrifuged at 260*g* for 5 min, and the pellet was resuspended in

α-MEM supplemented with 10% serum to obtain a concentration of 50,000 cells (Luyckx et al., 2014).

Matrix preparation

Sodium alginate was dissolved in deionized water at a concentration of 1% (w/v) and mixed with activated charcoal, 0.5 g of charcoal per gram of alginate, to remove organic impurities. The alginate solution was then sterilized by filtration and lyophilized. Finally, the lyophilized alginate was mixed with culture medium to obtain a final concentration of 1% (Xu et al., 2009).

Encapsulation of isolated ovarian follicles and cells and in-vitro culture

Morphologically intact isolated follicles were selected for encapsulation. The follicle diameter was measured; 5–7 follicles were used to evaluate follicular viability after isolation. The remaining follicles were transferred to alginate droplets as previously described (Xu et al., 2009). To form the beads, the droplets of alginate (5 µl) containing 5–7 ovarian follicles or 50,000 ovarian cells were separately released into a crosslinking solution of 0.1 mmol/l $CaCl_2$. After 2–3 min, the beads were removed from the $CaCl_2$ solution and washed in α-MEM containing 1% serum to remove the $CaCl_2$. One bead containing 5–7 follicles and one bead containing 50,000 ovarian cells (consisting of granulosa, theca, stromal and endothelial cells) were then transferred into each culture drop containing α-MEM culture medium supplemented with 1% (v/v) ITS, 50 µg/ml ascorbic acid, 50 µg/ml penicillin–streptomycin, 100 ng/ml FSH (Merck Sereno) and 100 ng/ml growth differentiation factor-9 (GDF-9) supplemented with either 10% FBS, 10% PRP, 5% PRP + 5% FBS or 10% HSA (vitrified samples only). The encapsulated follicles from fresh and vitrified samples and beads containing ovarian cells were incubated at 37°C in a 5% CO_2 atmosphere for 10 days. Half of the medium was refreshed every other day.

PRP preparation

To prepare PRP, 5 ml of human blood was drawn into tubes containing 0.5 ml of acid citrate solution (Merck) as an anticoagulant. The blood was centrifuged at 200*g* for 20 min at 20°C. The top and middle layers were transferred to new tubes and centrifuged at 3000*g* for 15 min. The supernatant plasma was discarded and the remaining 0.5 ml of plasma containing precipitated platelets was mixed evenly and considered to be PRP. The PRP was clotted to release the growth factor from platelets by adding 20 IU/ml thrombin and incubating for 1 h at 37°C. Finally, the clot was centrifuged at 4000*g* for 5 min at 4°C to remove the platelet fragments. It was stored at −20°C until use (Kazemnejad et al., 2016; Xie et al., 2012).

Follicle growth and viability assessment

Follicles with an intact basement membrane, bright and homogeneous granulosa and theca cells, and a clear boundary between oocyte and granulosa cells were classified as morphologically normal (Xu et al., 2009). The follicular diameters were determined using image processing with Image J Software version 1.48.

Trypan blue staining was used to evaluate the viability of the human ovarian follicles. Follicles were assessed for viability immediately after isolation and after encapsulation and in-vitro culture. To release the follicles, the alginate beads were transferred in α-MEM containing 10 IU/ml alginate lyase for 30 min at 37°C. The recovered follicles were then stained with 0.4% (v/v) trypan blue in PBS for 20 min at room temperature and their viability was assessed using a stereomicroscope. Follicles with a dead oocyte or with >50% dead granulosa cells, stained blue, were classified as dead (Sanfilippo et al., 2011).

Histological analysis of isolated human follicles

The encapsulated follicles in 1% alginate were fixed in 10% buffered formaldehyde for 2 h. Samples were dehydrated by incubation in ethanol and xylene and impregnated with melted paraffin. Encapsulated follicles embedded in paraffin were prepared in 5-µm serial sections. The sections were mounted and stained with haematoxylin and eosin and analysed by light microscopy.

Follicles were classified as morphologically normal when an intact oocyte was surrounded by two or more layers of well-organized granulosa cells with no pyknotic nucleus. Atretic follicles were defined as those having disorganized granulosa cells with pyknotic or defragmented nuclei (Lunardi et al., 2015).

Statistical analysis

Data were statistically analysed by one-way ANOVA followed by Tukey's post hoc test using the Statistical Package for Social Sciences (SPSS) version 16.0 (SPSS Inc., USA). Non-parametric data were analysed by the Kruskal–Wallis and Mann–Whitney tests. Values were considered statistically significant when $P < 0.05$.

Results

Viability and growth of human early preantral follicles isolated from fresh ovarian tissue after in-vitro culture

A total of 60 primordial follicles isolated from fresh ovarian tissues were cultured (**Figure 1A and B**). As shown in **Table 1**, the size of follicles significantly increased ($P < 0.001$) after 10 days of culture in each of the different medium supplements. The increases in follicular size ($P < 0.001$) and viability ($P < 0.05$) were greater in the PRP supplemented group compared with the FBS and PRP + FBS supplemented groups.

Viability and growth of human early preantral follicles isolated from vitrified-warmed ovarian tissue after in-vitro culture

A total of 240 primordial follicles isolated from vitrified-warmed ovarian tissues were cultured. On day 0, the isolated follicles were at the primordial stage (**Figure 1C**) with no significant differences in their diameters (**Table 2**). After in-vitro culture, most of the follicles appeared to be morphologically normal and had developed into more advanced stages (**Figure 1D**). PRP supplementation had the greatest positive impact on the growth and viability of isolated follicles from vitrified-warmed tissue (**Table 2**).

Comparing growth and viability of human isolated follicles from fresh and vitrified-warmed ovarian tissues after 10 days of culture

After in-vitro culture, the vast majority of follicles isolated from both fresh and vitrified-warmed samples appeared healthy (**Figure 1B and D**) and their diameters were significantly increased (**Tables 1 and 2**). However, the growth rate was significantly higher in follicles

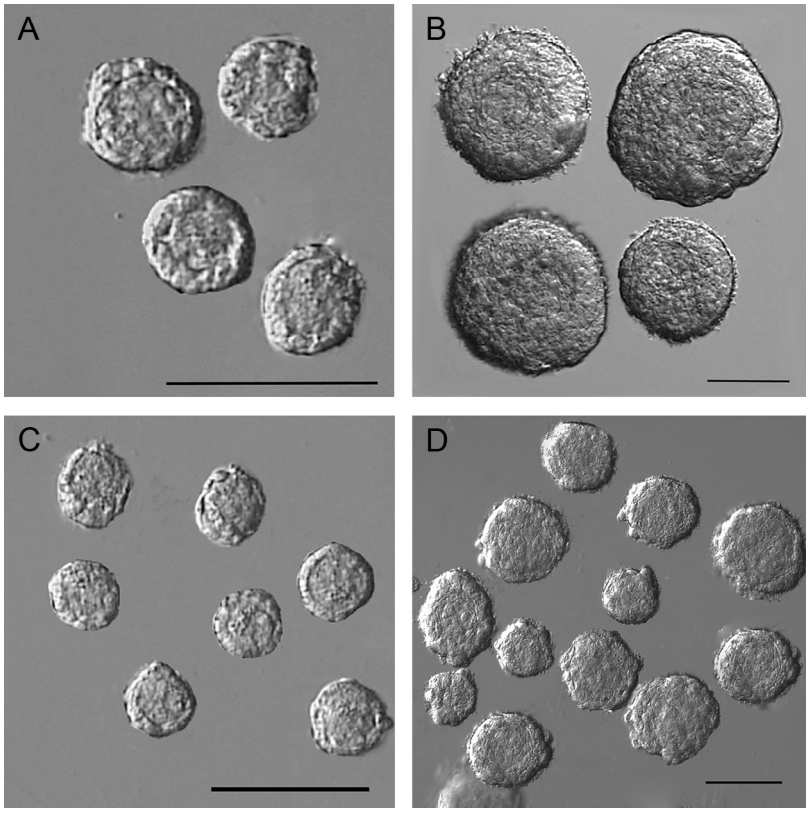

Figure 1 – Primordial follicles isolated from fresh ovarian cortex before (A) and after 10 days of culture in PRP supplemented group (B). The corresponding follicles isolated from vitrified-warmed samples before (C) and after 10 days of culture in the presence of PRP (D). Bar = 100 μm.

isolated from fresh samples than in those isolated from vitrified-warmed samples ($P < 0.001$; **Table 3**). The viability rate of isolated follicles decreased after culture in both groups of follicles. However, the decrease was significantly higher in vitrified-warmed follicles than in their fresh counterparts ($P < 0.001$; **Table 3**).

Histological analysis of isolated human follicles

Normal follicles with regular basal membranes are composed of follicular cells, the zona pellucida and a spherical oocyte with uniform cytoplasm (**Figure 2A**). Degenerated follicles are characterized by disorganized granulosa cells with differing degrees of nuclear changes such as pyknosis and nuclear fragmentation (**Figure 2B**).

Discussion

To the best of our knowledge, this was the first study to represent the development of human primordial and primary follicles isolated from fresh and vitrified-warmed ovarian tissues to the preantral stage after culture in 1% alginate. In both fresh and vitrified-warmed ovarian tissues, media supplementation with PRP better supported the survival and growth pattern of human isolated follicles compared with media supplemented with FBS, FBS + PRP or HSA (tested in vitrified-warmed tissues only).

A 3D system was used during follicular culture to prevent the loss of cell communication between granulosa cells and the oocyte, which

Table 1 – Size and viability of human follicles isolated from the fresh ovarian tissue before and after 10 days of in-vitro culture.

Experimental groups	Number of follicles	Follicle diameter (μm)*		Viability rate (%)**	
		D0	D10	D0	D10
FBS	20	52.40 ± 1.27^{a}	$104.82 \pm 2.58^{b,A}$	100^{a}	$61.00 \pm 0.89^{b,A}$
PRP	20	56.25 ± 0.95^{a}	$176.44 \pm 3.74^{b,B}$	100^{a}	$91.40 \pm 1.86^{b,B}$
PRP + FBS	20	54.30 ± 0.74^{a}	$132.94 \pm 3.43^{b,C}$	100^{a}	$71.00 \pm 0.89^{b,C}$

$^{a–b}$ For each parameter, numbers with different superscript letters in the same row differ significantly (*$P < 0.001$, **$P < 0.05$).
$^{A–C}$ Numbers with different superscript letters in the same column significantly differ (*$P < 0.001$, **$P < 0.05$). Data are expressed as mean ± SEM.
D0 = Day 0; D10 = Day 10; FBS = foetal bovine serum; PRP = platelet-rich plasma.

Table 2 – Size and viability of human follicles isolated from the vitrified-warmed ovarian tissue before and after 10 days of in-vitro culture.

Experimental groups	Number of follicles	Follicle diameter (μm)*		Viability rate (%)**	
		D0	D10	D0	D10
FBS	60	51.35 ± 0.98[a]	88.33 ± 1.63[b,A]	100[a]	59.00 ± 1.00[b,A]
PRP	60	53.83 ± 0.71[a]	111.25 ± 1.85[b,B]	100[a]	82.00 ± 1.70[b,B]
PRP + FBS	60	52.25 ± 1.01[a]	99.16 ± 1.75[b,C]	100[a]	66.40 ± 1.12[b,C]
HSA	60	51.50 ± 0.93[a]	70.58 ± 1.86[b,D]	100[a]	54.20 ± 1.31[b,A]

[a–b] For each parameter, numbers with different superscript letters in the same row differ significantly (*$P < 0.001$, **$P < 0.05$).
[A–D] Numbers with different superscript letters in the same column significantly differ (*$P < 0.001$, **$P < 0.05$). Data are expressed as mean ± SEM.
D0 = Day 0; D10 = Day 10; FBS = foetal bovine serum; HSA = human serum albumin; PRP = platelet-rich plasma.

are necessary for subsequent oocyte development and maturation. To do this, we chose alginate, a matrix that supports normal morphology, function and development of cultured follicles (West et al., 2007). Although ovarian tissue culture has been a method of choice for in-vitro activation of primordial follicles (Telfer and McLaughlin, 2012; Telfer and Zelinski, 2013), heterogeneity between individuals and even within the human ovary makes it difficult to predict whether a specific piece of ovarian tissue contains follicles (Schmidt et al., 2003). This issue is more problematic in older individuals, in whom the total follicle numbers have decreased (Faddy and Gosden, 1996). One solution is isolation and culture of follicles.

In humans and other mammalian species, despite the ability of isolated secondary follicles to develop up to the antral stage (Arunakumari et al., 2010; McLaughlin et al., 2010; Saraiva et al., 2010; Xiao et al., 2015; Xu et al., 2009), in-vitro culture of isolated primordial and primary follicles has been notably challenging because of the rapid degeneration of these follicles in culture (Laronda et al., 2014). Our study found that isolated human primordial follicles in the presence of growth factors have the potential to develop to more advanced stages without support from the native ovarian cortex. Nonetheless, in tissue culture, the physical environment of the follicles and the cell–matrix signalling, which are critical regulators of follicle development, affect the growth of follicles. In a pilot study, we found that 7 days' culture of isolated follicles in the absence of ovarian cells led to death of the majority of follicles (data not shown). To partially compensate the lack of ovarian cortex, one alginate bead comprised of ovarian cells was added to each culture drop. The results of Amorim et al. (2009) support our findings, indicating that small human preantral follicles isolated from cryopreserved ovarian tissue can survive and develop after 1 week of culture in an alginate matrix.

Table 3 – Size and viability of human follicles isolated from fresh versus vitrified-warmed ovarian tissue after 10 days of in-vitro culture.

Experimental groups	Number of follicles*	Follicle diameter (μm)		Viability rate (%)	
		D0	D10	D0	D10
Fresh	60	54.30 ± 0.74[a]	138.07 ± 4.26[b,A]	100[a]	80.06 ± 2.61[b,A]
Vit/warmed	180	52.47 ± 0.53[a]	99.58 ± 1.22[b,B]	100[a]	70.41 ± 2.61[b,B]

[a–b] For each parameter, numbers with different superscript letters in the same row differ significantly (t-test; $P < 0.001$).
[A,B] Numbers with different superscript letters in the same column differ significantly (t-test; $P < 0.001$). Data are expressed as mean ± SEM.
D0, Day 0; D10, Day 10.
* The numbers are total follicle numbers in FBS, PRP and FBS + PRP groups.

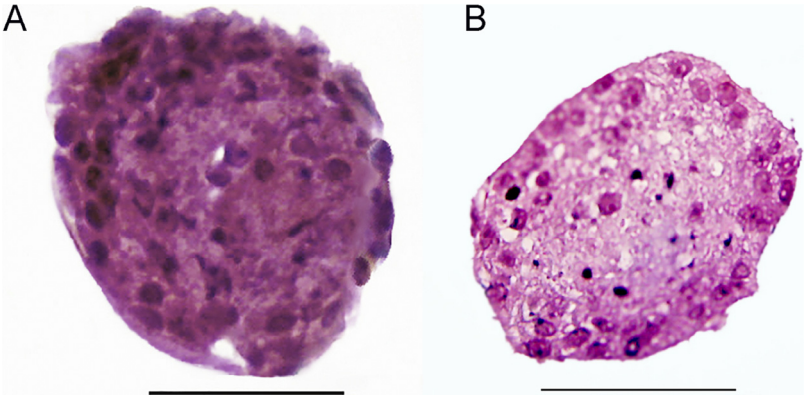

Figure 2 – Histological sections of human follicles isolated from vitrified-warmed ovarian tissue. The healthy (A) and degenerated (B) early preantral follicles after 10 days of culture in PRP supplemented group. Bar = 50 μm.

However, the growth of follicles was lower in their study than in ours, which might be due to the lack of appropriate growth factors in their culture condition (such as GDF9) or the co-culture of ovarian cells that was added to our culture medium. Laronda et al. (2014), however, reported that alginate encapsulation did not support the growth of isolated human primordial follicles, as they observed a complete dissociation of the oocyte from the surrounding granulosa cells after a 3-day culture. One possible reason for this discrepancy could be inappropriate enzymatic digestion during follicle isolation, which in turn negatively affected subsequent development.

Numerous studies have shown a positive effect of PRP on growth and differentiation of many cell types and supported its use as an alternative for FBS in cell culture (Cho et al., 2011; Hemeda et al., 2014; Rauch et al., 2011). Autograft of frozen/thawed human ovarian tissue treated with PRP has led to live births (Callejo et al., 2013). Moreover, a study by Rajabzadeh et al. (2015) showed that platelet lysate (PL) improves the survival rate of preantral mouse follicles after follicle implantation. However, PL had no effect on follicular growth, which might be related to the reduction of growth factors during 14 days of implantation (Rajabzadeh et al., 2015). Pazoki et al. (2015) also compared PL with FBS in in-vitro culture of isolated mouse secondary follicles and observed a higher follicular survival with no effect on follicle development in PL compared with FBS. The difference between our results and the previous studies (Pazoki et al., 2015; Rajabzadeh et al., 2015) is probably due to different supplements (PRP versus PL). Indeed, the increased follicular growth in our study might be related to the difference in the type and concentrations of growth factors, such as G-CSF, GM-CSF, HGF, MIP-1α and MIP-1β, in our culture media supplemented with PRP as well as the presence of ovarian cells compared with the studies devoid of those supplements (Amable et al., 2013). As previously stated, the positive effect of PRP on follicular growth might be related to the presence of various growth factors that support the early stage of follicle development. In a study by Wang et al. (2014), media supplementation with bFGF during 8 days of 3D culture of early human follicles supported follicle development to the preantral stage. However, the final diameters of developed follicles in their study were less than corresponding diameters in our study, indicating the media supplementation with PRP, which is rich in different growth factors, had more advantages over addition of only one growth factor (bFGF).

The reduced effect of the 5% PRP + 5% FBS group compared with the 10% PRP group might be related to the lower concentration of PRP in the former culture medium.

In our study, the growth and viability of isolated follicles were higher in fresh than in vitrified-warmed ovarian tissue. In agreement with our results, Ting et al. (2011) showed that secondary primate follicles isolated from fresh ovarian samples increased in size twice as much as follicles isolated from vitrified-warmed tissues. Additionally, follicular growth in vitrified groups is delayed compared with fresh ones, as cultured follicles isolated from cryopreserved samples did not grow to the size of fresh follicles even after 10 weeks of culture (Ting et al., 2011). In agreement with this, Schubert et al. (2008) showed that fresh and cryopreserved ovarian cortex grafted into SCID mice could sustain ovarian tissue function, although the follicular growth was lower in frozen/thawed tissue compared with fresh tissue.

In conclusion, our results showed that PRP better supports in-vitro viability and growth of encapsulated/isolated human primordial and primary follicles compared with other supplements and is a more favourable source of growth factors. We believe that our findings on the culture of small preantral follicles constitute an important initial step for in-vitro development of human follicles, which may be a promising tool for female fertility preservation.

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