Effect of Vitrification Technique and Different Biochemical Materials on *In Vitro* Maturation Outcomes

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Abstract

Background:

In vitro maturation (IVM) is a culture technology that enables the immature oocytes to reach metaphase II (MII). Vitrification of immature oocytes followed by *in vitro* maturation can offer advantages, such as to avoid the use of large doses gonadotropins and also be an alternative approach to the use of endocrine stimulation to obtain pre-ovulatory oocytes in all cycles.

Objective:

The objective of the study is to determine the effect of vitrification technique and several biochemical materials on outcomes of *in vitro* maturation.

Materials and Methods:

Normal and viable immature oocytes divided into two major groups including: control group and vitrification technique group. Post-thawing, each major group subdivided into four *in vitro* maturation (IVM) groups using SMART medium with special additives involving: (G1) group: contained gonadotropins (Gn) only, (G2) group: supplemented with Gn and sucrose (4%), (G3) group: consisted of Gn, sucrose (4%) and follicular fluid (FF) (5%), and (G4) group: contained Gn, sucrose (4%) and FF (10%).Then, assessed the results of *in vitro* maturation.

Results:

The results of *in vitro* maturation for control group shows non significant differences ($P>0.05$) among all groups. While, a significant increase ($P<0.05$) in percentage of IVM was achieved when compared G3 and G4 groups with G1 and G2 groups in vitrification technique. Therefore, comparison the results of IVM between control group vitrification technique group appeared a significant difference ($P<0.05$) in G1 groups and also between G2 groups. However, non significant difference ($P>0.05$) was appeared between G3 groups and also between G4 groups when comparing between control group and vitrification technique group.

Conclusions:

Vitrification of immature oocytes were appeared as a promise technique for preservation of oocytes. As well as, the addition of follicular fluid (FF) to maturation medium produced the best results for *in vitro* maturation (IVM).

Key words: Vitrification, *in vitro* maturation, follicular fluid, sucrose, gonadotropin.

Introduction:

Vitrification is a process by which liquid turns into solid without the formation of ice crystal [1]. Vitrification was achieved simply by plunging the sample into liquid nitrogen at -196ºC [2]. In 1985, the ultra-rapid freezing method through vitrification was introduced based on inducing solidification of the cells and the surrounding vitrification solution (glass-like or vitreous) without formation of any ice crystallization [3]. Vitrification involves exposure of the cell to high concentrations of CPAs for brief periods of time, usually at or near room temperature, follows by rapid cooling in liquid nitrogen. The high osmolarity of the vitrification solution rapidly dehydrates the cell and submersion into liquid nitrogen quickly solidifies the cell, so that the remaining intracellular water does not have time to form damaging ice crystals [4].

A large number of variables affect the outcome of vitrification of oocytes including the type and concentration of CPAs, stage of development of oocytes and the presence or absence of cumulus cells [5]. It has been observed that the immature germinal vesicle stage oocytes tolerate the cryopreservation damage more efficiently compared to oocytes at metaphase II and cumulus compact oocytes are less vulnerable to cryo-injuries compared to their denuded counterpart [6].

In vitro maturation (IVM) is an advanced embryology laboratory technique whereby immature oocytes, which are removed from ovarian follicles prior to completing their growth *in vivo*, are cultured from the germinal vesicle (GV) to the metaphase II (MII) stage. Upon reaching maturity *in vitro*, these oocytes can then be fertilized and the resulting embryos can be cultured using conventional *in vitro* fertilization (IVF) techniques and transferred or cryopreserved [7].

In vitro maturation (IVM) of oocytes is a promising technique to reduce the costs and avert the side effects of gonadotropin stimulation in *in vitro* fertilization (IVF) [8]. Therefore, immature oocyte retrieval followed by IVM has become a widespread treatment for infertile women with polycystic ovarian syndrome (PCOS) because there are numerous antral follicles within the ovaries in this group of patients [9]. IVM can also support fertility preservation (when it combined with cryopreservation) for women with malignant diseases or autoimmune disorder especially those with disseminated lupus erythematosus before administration of chemotherapy, because they have poor tolerance to hormonal stimulation [10].

Therefore, the aim of study is to determine the effect of vitrification technique and several biochemical materials on outcomes of in vitro maturation.

Materials and Methods:

Oocytes collection:

From all visible follicles on the ovarian surface with 2-6 mm diameter, oocytes were collected using aspiration technique. Oocytes with follicular fluid were aspirated using 20-gauge hypodermic needle attached with a sterile disposable 5 mL syringe contain 0.5 mL of culture medium supplemented with 20 IU/mL heparin to prevent clotting within follicular fluid. After oocyte retrieval, contents of each syringe were poured into Petri dish containing oocytes under dissecting microscope, the oocytes were collected using modified pasture pipette and washed for three times with CM [(11)].

Preparation of vitrification solution:

Two solutions were prepared for this procedure. Equilibration solution (ES) consisted of 7.5% (v/v) ethylene glycol (EG) with 0.25M sucrose and vitrification solution (VS) consisting of 15% (v/v) EG with 0.5 M sucrose. These two solutions were prepared by adding the corresponding volume of CPA to culture medium containing 10% Human Serum Albumin (HSA).

Thawing solution:

Thawing solution (TS) or (warming) solution (WS) consist of two descending solutions, solution one consisting of sucrose with concentration of 0.5M, and solution two consisting of sucrose with concentration of 0.25M, which were added to CM containing10% HSA.

Preparation of follicular fluid:

The follicular fluid was aspirated from the visible follicles. The fluid was centrifuged at 3000 rpm for 10 minutes and the supernatant was heat-inactivated at 55°C for 30 minutes. The heated fluid was cooled and sterilized with a filter (0.22 µm-pore-size filters). All FF samples were kept at -20° C until the time of the culture [(12)].

Vitrification Procedure:

Immature oocytes (three oocytes) were placed in 0.5 mL of ES at room temperature for 15 minutes. After that, they were placed into 0.5 mL of VS time. Then oocytes were placed on the Cryotop strip in a small drop (1mL) of VS(figure A) and the Cryotop immersed into LN2. The transfer of oocytes into the vitrification solution and the vitrification process were performed within 1minut and plunging into LN2.

Then, the strip was covered with the plastic tube within LN2 to protect it during storage. After 4 weeks thawing process was done [13].

Figure (A): Photograph of the cryotop.

Thawing procedure:

For thawing of vitrified oocytes, the protective cover was removed from the Cryotop while it is still submerged in LN2. Stepwise removal of the cryoprotectant was done by transferring the oocytes through a descending concentration of thawing solution at room temperature. The strip was immersed directly into the thawing solution of 0.5M sucrose solution for 3 minute, depending on the sugar concentration of the vitrification solution. Then, the thawed oocytes were transferred to 0.25M sucrose solution for 3 minutes and then washed twice with culture medium.

In vitro Maturation:

Fresh and post-thawed normal oocytes were culture in SMART medium with the addition of concentrations of follicular fluid (10%, 4%) and sucrose with concentration (4%) supplemented with 10 IU/mL PMSG, 5 IU/mL hCG and 2µg/mL Estradiol and cultured in four well Petri dish and covered with liquid paraffin and incubated for about 24 h in CO_2 incubator (5% CO_2) at 38.5°C with high humidity (95%) [14]. For confirmation of maturation after 24 hours the oocytes were evaluated for morphological changes and *in vitro* maturation performance under inverted microscope. The oocytes with an intact zona pellucida, plasma membrane and homogenous cytoplasm were considered as morphologically normal in the study. *In vitro* maturation performance was assessed on the basis of the presence of 1st polar body and on the expansion of cumulus cells surrounding the homogenous oocytes [15].

Results:

In the present study, (327) immature oocytes were used for IVM technique as (161) for control group and (166) for vitrification technique group. No significant difference (P>0.05) in the IVM percentages for the control group (Figure 1). The highest IVM percentage (65.52%) was occurred in G4 group which included sucrose (4%), FF (10%) and gonadotropins Gn. While, the lowest IVM percentage recorded in G1 group (54.35%) enriched with gonadotropins (Gn). Figure (2) shows a significant increase (P<0.05) in the IVM percentages for vitrification technique in G3 and G4 groups when compared with G1 and G2 groups. The addition of sucrose (4%), FF (10%) and Gn to SMART medium in G4 group given the highest percentage of IVM (50%). Whereas, lowest percentage of IVM (26%) occurred in G1 group contains only gonadotropins (Gn). Comparison the results of IVM between the control group and vitrification technique group shows a significant differences (P<0.05) was noticed in between G1 groups. Moreover, a significant difference (P<0.05) in IVM percent between control group and vitrification technique group occurred in G2 groups. Furthermore, the results of

IVM in G4 and G3 groups explained no significant difference (P>0.05) between the control group and vitrification technique group (Figure 3).

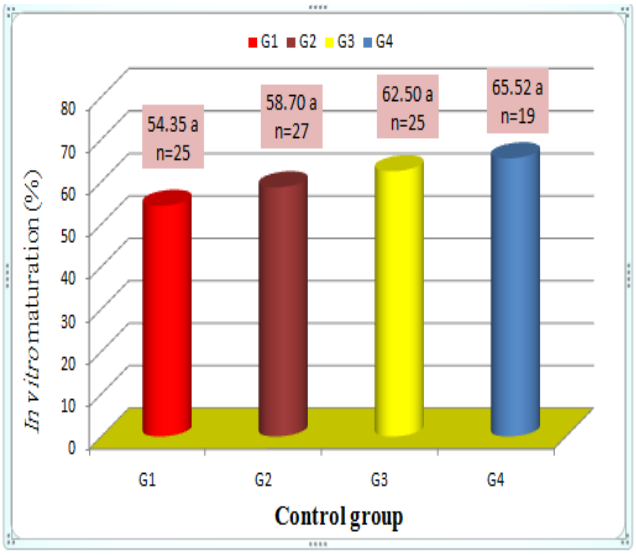

Figure 1: Effect of SMART medium additions on IVM percentage in control group.

- $Chi^2 = 1.379$
- P-value = 0.710

- G1: Gonadotropins (Gn).
- G2: Sucrose 4% and Gn.
- G3: Sucrose 4%, FF 5% and Gn.
- G4: Sucrose 4%, FF 10% and Gn.
- *Similar letters: No significant difference (P>0.05) between groups.

Discussion:

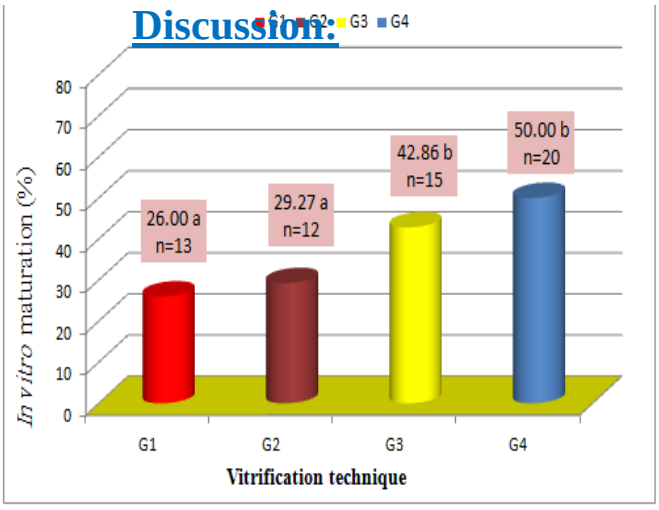

Figure 2: Effect of SMART medium additions on IVM percentage in vitrification technique group.

- $Chi^2 = 10.306$
- P-value = 0.016*

- G1: Gonadotropins (Gn).
- G2: Sucrose 4% and Gn.
- G3: Sucrose 4%, FF 5% and Gn.
- G4: Sucrose 4%, FF 10% and Gn.

*Different letters: Significant difference (P<0.05) between groups.

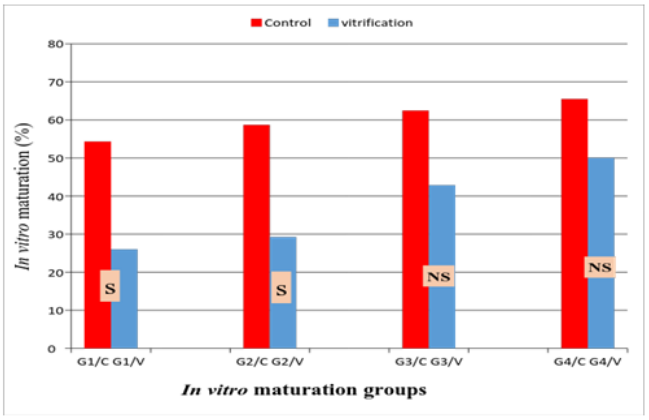

Figure 3: Comparison the IVM percentages between control group and vitrification technique.

- G1: Gonadotropins (Gn).
- G2: sucrose 4% and Gn.
- G3: sucrose 4%, FF 5% and Gn.
- G4: sucrose 4%, FF 10% and Gn.
- S: significant difference (P<0.05).
- NS: no significant difference (P>0.05).

The results of IVM for control group showed no significant differences (P>0.05) in percentages of IVM among all groups. The percentages of IVM for control group in this study was (54.35-65.52%) similar to those reported by Byrd et al. (72.5%) [16], Kochhar et al. (60-85.7%) [17], and Nazari *et al.*, [18] they reported a 59.4% maturation rate for IVM.

One of the most important factors regulating the number and quality of oocytes maturing *in vitro* is the culture system used for IVM. Culture media components and culture conditions can affect and even modulate the meiotic regulation of mammalian oocytes [19]. Therefore, its necessary to devise and optimize culture systems that take into account all the factors essential for the completion of oocyte maturation *in vitro* [20].Moreover, Moore and Trounson demonstrated that the addition of gonadotropins to culture medium found to be helpful in achieving ovine oocyte maturation *in vitro* [21]. Studies achieved by Downs and his co-workers showed that the inclusion of FSH in culture medium results in an increased concentration of cAMP, which augments meiotic arrest [22]. Once the amount of cAMP generated reaches a certain threshold a positive maturation signal is transmitted. After 2 h of the addition of LH was found to exert an additive stimulatory effect on oocyte

maturation [23]. LH, as supplied to SMART medium in the current study, has been reported to modulate activity of the somatic cells of the follicle by 'uncoupling' the communication between the cumulus cells and the oocyte, leaving it free to resume meiosis [24]. LH also found to supports follicular growth by providing androgen substrate for the granulosa cell aromatase and triggers the resumption of oocyte maturation [25].

A significant differences ($P<0.05$) were appeared in IVM percentages for G4 group when comparing with G1 and G2 groups in vitrification technique group. Follicular fluid (FF) is an environmental factor in which oocytes are nourished and undergo maturation *in vivo*. FF contains a variety of peptide growth factors [26], some of which have been suggested to play a key role in the ability of oocytes to undergo nuclear and cytoplasmic maturation [27]. Moreover, FF is reported to consist of various growth factors, follicle-stimulating hormone (FSH), leutinizing hormone (LH) electrolytes, amino acids, and several other nutrients although it may also contains oocyte maturation inhibitory factor [28]. The addition of gonadotrophins reverts this inhibitory effect, increasing the nuclear maturation rate *in vitro* [29].

In the present study, the addition of follicular fluid (FF) enhance the rates of oocytes maturation specially for addition 10% comparing with 5% FF in all groups of this study. Also, the results of IVM for G4 that supplemented with (sucrose 4%, FF 10% and Gn) recorded the highest IVM percentage noticed in control group (65.52%), followed by IVM percentage of vitrification group (50%). The results of the present study are in agreement with results obtained in previous studies on the effects of FF supplementation of IVM medium in other species. In pigs, FF induced oocyte maturation *in vitro* and improved the rate of male and female pronuclei formation [30] and subsequent developmental capacity [31]. Kim *et al*., and Romero-Arredondo *et al*., also reported beneficial effects of the addition of FF to the maturation medium on the maturation and developmental ability of bovine oocytes [32]. In humans, the addition of mature FF to IVM medium of immature oocytes from unstimulated ovaries was shown to increase both the fertilization rates and embryo quality [33]. A recent study revealed that FF promotes oocyte cytoplasmic maturation during IVM and suggested that the major role of FF is to provide protection against oxidative stress [34].

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