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Effect of oocyte vitrification before and after in vitro maturation towards Bcl-2, Bax and Bcl-2/Bax ratio expression

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ABSTRAK

Tujuan: Melihat ekspresi Bcl-2, Bax dan rasio Bcl-2/Bax pada oosit dan kumulus yang divitrifikasi sebelum dan sesudah maturasi in vitro.
Bahan dan Metode: Maturasi dilakukan dalam medium TC 100 µl selama 24 jam. Vitrifikasi diawali dengan pencucian oosit dalam medium PBS yang disuplementasi serum 20% selama 1-2 menit, dilanjutkan dalam medium PBS + serum 20% + etilen glikol 10% selama 10-14 menit. Oosit kemudian dipindahkan dalam medium vitrifikasi PBS + serum 20% + sukrosa 0,5M + etilen glikol 15% + PROH 15% selama 25-30 detik. Thawing dilakukan dengan cara oosit direndam secara berturut dalam media : 1). PBS + 20% serum + sukrosa 0,5M, 2). PBS + 20% serum + sukrosa 0,25M, dan 3). PBS + 20% serum + sukrosa 0,1M. Dilakukan pemeriksaan imunositokimia untuk melihat ekspresi Bcl-2, Bax dan ratio Bcl-2/Bax.
Hasil: Ekspresi Bcl-2 pada oosit kelompok kontrol berbeda signifikan dibanding kelompok perlakuan, sementara pada kumulus berbeda signifikan dengan kelompok K1. Ekspresi Bax pada oosit dan kumulus kelompok kontrol berbeda signifikan dengan kelompok perlakuan. Ekspresi rasio Bcl2/Bax pada oosit dan kumulus tidak berbeda signifikan pada semua kelompok.
Simpulan: Rasio ekspresi Bcl2/Bax tidak berbeda signifikan pada semua kelompok

Kata kunci : Bcl-2, Bax, vitrifikasi, maturasi in vitro

ABSTRACT

Objectives: to compare the expression of Bcl-2, Bax and Bcl-2/Bax ratio in cumulus cell and oocyte between vitrified oocyte pre and post in vitro maturation.
Materials and Methods: Maturation was operated in medium TC 100 µl for 24 hours. Vitrification begins with washing oocyte in PBS basic medium supplemented of 20% serum for 1-2 minutes, followed by equilibration medium PBS + 20% serum + 10% ethylene glycol for 10-14 minutes, then transferred to 20% serum + PBS + 0.5 M sucrose + 15% ethylene glycol + PROH 15% for 25-30 seconds. Thawing is processed by submerging the oocytes in the media: 1). PBS + 20% serum + 0.5 M sucrose, 2). PBS + 20% serum + 0.25 M sucrose, and 3). PBS + 20% serum + 0.1 M sucrose. Imunocytochemistry observed the expression of Bcl-2, bax and Bcl-2/bax ratio.
Results: Bcl-2 expression on oocyte in control group differed significantly with treatment group, Bcl-2 expression on cumulus in control group differed significantly with treatment 1 group. Bax expression on oocyte in control group differed significantly with treatment group. Bax expression on cumulus in control group differed significantly with treatment group. Bcl-2/Bax expression ratio on oocyte and cumulus did not differ significantly in all group
Conclusion: No difference Bcl-2/Bax expression ratio on oocyte and cumulus between vitrified oocyte pre and post in vitro maturation.

Keywords : Bcl-2, Bax, vitrification, in vitro maturation

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INTRODUCTION

Oocyte vitrification has become a growing and promising technology in assisted reproductive technologies. Vitrification is promising because it can prevent the formation of ice crystals, with the use of high concentrations of cryoprotectants and which can allow transformation of liquid to solid form without the formation of crystals [6]. The temperature commonly used is -196^{o}C, which is the temperature of liquid nitrogen. At low temperatures, water's form would be solid and there will be no biological reactions happen[15]. Oocyte vitrification is preferred, especially in women with no partner, and is ethically more safe[20], but until now the viability of oocytes after vitrification is still low.

Vitrification can be performed on immature and mature oocytes. Immature oocytes will stop at prophase I stage. The cytoplasm and nucleus will undergo a series of changes to reach metaphase II stage where oocytes are mature, ready for fertilization, and have the ability to support embrional development[10]. Frozen oocytes deposits usually take longer time to develop because of the typical physical properties of the oocytes.

Firstly, human oocytes have the critical specific size where the ratio of volume and surface is small and cause limited penetration of water and cryoprotectants through the plasma membrane. This results difficulties for oocytes to be protected from the formation of intracellular ice crystals[2]. Secondly, the mature oocyte contains spindle thread which is highly vulnerable to any temperature downturn[3].

Decrease in temperature affects the integrity of some of the structures that are important for fertilization and embryo development, such as the pellucide zone, cortical granules, cytoskeleton, spindle thread and

condensed chromosomes [16]. Frozen oocytes deposit may result in degeneration. Observations on degenerating oocytes post vitrification show signs of apoptosis such as cytoplasm condensation, cytoplasm fragmentation, formation of apoptotic bodies, biochemical markers, DNA fragmentation, and caspase activation. These biochemical markers are detected not only in morphologically apoptotic oocytes but also in morphologically not apoptotic oocytes[17]. Apoptotic marker genes such as C-myc, p53, Bcl-2, Bax and caspase[9] are expressed in oocytes after vitrification[7].

There are two important components that regulate the process of apoptosis, the Bcl-2 and caspase. Bcl-2 is composed of anti-apoptotic genes (Bcl-2 and Bcl-XL) and pro-apoptotic genes (Bax). Bax is antagonistic to Bcl-2, and results damage to the cytoplasm and induces apoptosis. High apoptotic activity is always associated with the low ratio of Bcl-2/Bax. Until now, research towards the effect of vitrification on apoptosis of cumulus cells and oocytes has not been massively done.

MATERIALS AND METHODS

Samples were collected from sheep oocyte, derived from follicles measuring 3 to 5 mm. This research was a laboratory explorative comparison with *post test only control group* design. Oocytes obtained were divided into three groups. Only IVM was performed to the control group. IVM continued by vitrification was performed in group 1 and vitrification continued by IVM was done to group 2. *In vitro* maturation was performed using an incubator with temperature of 38^0C, 5% CO2 at 95% humidity for 24 hours in TC medium. Vitrification began with washing oocytes in PBS supplemented with basic medium 20% serum for 1-2 minutes, followed by oocytes equilibration in PBS + 20% serum + 10% ethylene glycol medium for 10-14 minutes. Oocytes were transferred to vitrification medium containing PBS + 20% serum + 0.5M sucrose + 15% ethylene glycol + 15% PROH for 25-30 seconds. *Thawing* was carried out by dipping the *hemistraw* into the thawing media. Oocytes were soaked into some media, consecutively : 1) PBS + 20% serum + 0.5M sucrose, 2). PBS + 20% serum + 0,25M sucrose, and 3). PBS + 20% serum + 0.1 M sucrose. Immunocytochemistry examination was done by washing preparations with PBS for 2x5 minutes, proteolytic digestion with 0.025% trypsin in an incubator at a temperature of 37^0C for 15 minutes, followed by H2O2 for 10 minutes, primary antibody for 60 minutes, and next was the addition of Biotinylated link for 30 minutes, followed by administration of streptavidin for 30 minutes, and DAB (diamonibenziddine) chromagen which was diluted with DAB plus 2% subtrate for 6-10 minutes. Washing with PBS was done for 2x5 min for each stages turn to clean up residual materials. Counterstain using methyl green 3 was done for 5-10 minutes at room temperature and hereafter the preparations were ready to be examined under a microscope to read the results of staining index remmele scale (IRS) which is the result of multiplying the immunoreactive cells percentage score and color intensity score of immunoreactive cells. The data were analyzed using one-way ANOVA.

RESULTS AND DISCUSSION

This study observed the expression of Bcl-2 and Bax in vitrified oocytes and cumulus cells before and after *in vitro maturation*. Expression of Bcl-2 and Bax could be seen in immunocytochemistry staining. Staining results were assessed semiquantitatively according to the modified Remmele method. Positive expression was cha-racterized by a brownish color in oocytes and cumulus cells

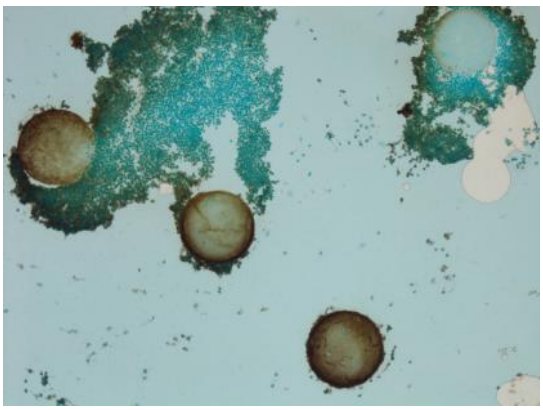

Figure 1. Expression of Bcl-2 by immunocytochemistry examination

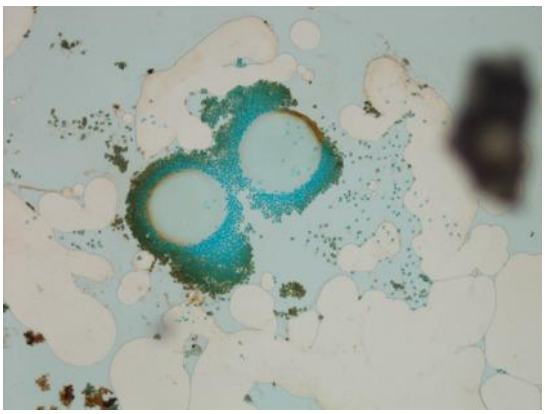

Figure 2. Expression of Bax by immunocytochemistry examination.

Table 1 One-way ANOVA analysis to determine differences in the expression of Bcl-2 in control oocytes group compared with treatment groups

Groups	Bcl-2 Expression ± SD
IVM (K0)	1.4 ± 1.8[a]
IVM+Vitrificaion (K1)	6.62 ± 3.24[b]
Vitrification+IVM (K2)	8.38 ± 2.56[b]

Different superscripts in the same column indicate significant differences at p <0.05

Table 2. One-way ANOVA analysis to determine differences in the expression of Bax in control oocytes group compared with treatment groups

Groups	Bax Expression ± SD
IVM (K0)	2.46 ± 2.4[a]
IVM+Vitrification (K1)	7.07 ± 3.76[b]
Vitrification+IVM (K2)	6.37 ± 2.69[b]

Different superscripts in the same column indicate significant differences at p <0.05

Table 3. One-way ANOVA analysis to determine differences in the ratio of BCl2/Bax expression in control oocytes group compared with treatment groups

Groups	Bcl-2/Bax expression ratio ± SD
IVM (K0)	0.95 ± 1.6[a]
IVM+Vitrification (K1)	2.36 ± 3.50[a]
Vitrification+IVM (K2)	1.54 ± 0.92[a]

Different superscripts in the same column indicate significant differences at p <0.05

Table 4. One-way ANOVA analysis to determine differences in the expression of Bcl-2 in control cumulus cells group compared with treatment groups

Groups	Bcl-2 Expression ± SD
IVM (K0)	2.27 ± 2.91[a]
IVM+Vitrification (K1)	7.54 ± 3.96[b]
Vitrification+IVM (K2)	4.40 ± 1.68[b]

Different superscripts in the same column indicate significant differences at p <0.05

Table 5. One-way ANOVA analysis to determine differences in the expression of Bax in control cumulus cells group compared with treatment groups

Groups	Bax Expression ± SD
IVM (K0)	4.69 ± 2.65[a]
IVM+Vitrification (K1)	8.17 ± 2.45[b]
Vitrification+IVM (K2)	4.6 ± 1.67[b]

Different superscripts in the same column indicate significant differences at p <0.05

Table 6. One-way ANOVA analysis to determine differences in the ratio of BCl2/Bax expression in control cumulus cells group compared with treatment groups

Groups	Bcl-2/Bax expression ratio ± SD
IVM (K0)	1.22 ± 1.88[a]
IVM+Vitrification (K1)	1.00 ± 0.50[a]
Vitrification+IVM (K2)	0.10 ± 0.35[a]

Different superscripts in the same column indicate significant differences at p <0.05

Frozen-stored immature oocytes remains a major challenge in assisted reproductive technology. Frozen-stored immature oocytes gave great hope to store oocytes in large quantities. In patients with reproductive organs cancer, frozen-stored oocytes immature gave hope to store oocytes without stimulation.

This study was aimed to compare the effect of vitrification towards the apoptotic degree in vitrified oocytes before and after in vitro maturation. Markers of apoptosis were seen from the expression of Bcl-2, Bax and the ratio of Bcl-2/Bax. Apoptosis' markers were seen also in cumulus cells, since the presence of cumulus cells is still needed by oocytes to achieve their maturation stage.

Bcl-2 has anti-apoptotic function as to improve cell's survival, while Bax has proapoptotic function that increases cell's death[25]. Bcl-2 expression in oocytes and cumulus cells was not significantly different between groups that vitrified before and after *in vitro* maturation although the result was significantly different with the control group. This showed that the vitrification done before and after *in vitro* maturation had no effect on cell survival. Bcl-2 is expressed in the immature oocyte both in oocytes and cumulus cells which are exposed only to the vitrification medium and those which are frozen and thawed.[23] Immature oocytes' vitrification using some tools showed that compared to other tools, the highest expression of Bcl-2 is found in the use of hemistraw and cryotop[21], and these results are similar with this research that uses hemistraw for vitrification.

Bax' expression in oocytes and cumulus cells was not significantly different between in groups that were vitrified before and after in vitro maturation, although it is significantly different from the control group. Research done by Rao et al give same result where the expression of Bax in the vitrified group with hemistraw was higher than in the control group. In this study, the expression of Bcl-2 in the K2 group's oocytes was higher than K1, therefore at the cumulus group, K2 expression of Bax was lower than the K1 since the presence of oocyte plays an important role in preventing cumulus cells' death by increasing anti-apoptotic

protein, Bcl-2, and suppressing pro-apoptotic protein, Bax. In COCs culture, apoptosis occured in the outermost layer of cumulus, whereas in cumulus' and oocytes' culture apoptosis was found in a layer of innermostt cumulus[13].

Although the expression of Bcl-2 and Bax were high in both treatment groups compared to the control groups, the ratio of Bcl-2/Bax in treatment group did not significantly differ from the control group. This effect depended on the balance between Bcl-2 and Bax compared to Bcl-2 only[9]. The incidence of apoptosis has no effect on the oocyte- cumulus complexes' morphology. Observation by light microscope showed normal morphology in both on vitrification that were done before and after *in vitro* maturation and there were no differences with the control group[8].

The ratio of Bcl-2/Bax in the K2 group was the lowest compared to the other two groups even though the difference was not significant. This indicates that the oocyte vitrification can be performed in either before or after *in vitro* maturation. Frozen-deposit oocytes in the GV phase is more sensitive than MII phase because it will damage the junction between oocytes with cumulus cells surrounding it and because there were two different cell types that should be frozen[11].

Different result was shown by Men who found out that oocyte vitrification in GV phase was more acceptable than MII phase, because in MII phase, oocytes have had cortical granules and spindle thread that is sensitive to the freezing process. Moreover, oocytes in phase GV still have limited genetic material[18,21]

Immature oocytes and cumulus cells vitrification is a major challenge. Cumulus cells on immature oocyte have major role in the process of *in vitro* maturation and oocyte's development[22]. Besides, the cumulus cells surrounding the oocyte, while being frozen, can control the penetration of cryoprotectants that are toxic, prevent cell swelling at the time of cryoprotectants replacement and finally produce productive vitrification[19]. Nonetheless, the presence of cumulus cells layer and glycolproteins that surround oocytes can reduce the rate of penetration of cryoprotectant[24,4], resulting in uneven spread of cryoprotectants within the cell that results frozen oocyte's survival[14].

Communication between cumulus cells and oocytes occurs via paracrine signaling and gap junction as a mediator that helps the work of FSH. It takes at least three layers of compact cumulus cells to protect the oocyte and required for *in vitro* maturation[7]. Cumulus complexes and cryoprotectants do not damage the integrity of the membrane of cumulus cells which shows that communication between oocytes and cumulus still occurs. Nevertheless, once it is exposed to N2, membrane of cumulus cells experience damage that also damage communication between oocytes with cumulus cells[1].

In this study, the ratio of Bcl-2/Bax in cumulus cells is highest on the control group but this is not followed by a good condition of oocytes. Oocyte in the control group in fact showed the lowest ratio of Bcl-2/Bax compared to other groups. Cumulus in group 2 showed lowest ratio of Bcl-2/Bax but not the oocytes' ratio of Bcl-2/Bax. This indicates that after vitrification, the interdependent relation between oocytes and cumulus doesn't occur any longer. Frozen-deposit causes changes in the function of cumulus[12]. The exposure of cryoprotectants causes irregularity of actin filaments in the transzonal process which showed contact between the cumulus cells and ocytes[16]. Oocyte and cumulus complex included in N2 will lose their ability to communicate with each other, most of cumulus' cytoplasms are vacuolated, cumulus cells' projections appears to be damaged and discontinued[1].

Different things happen in the cumulus cells of mature oocyte. Cumulus cells are no longer required to protect the oocyte in the process of vitrification. Vitrification in mature oocytes without cumulus indicates survival rate and development of the embryo until 8 cells higher than cumulus[5]. However, the presence of cumulus is still required for embryonic development until blastosis stage[27].

CONCLUSION

There is no difference in the ratio of Bcl2/Bax expression in oocytes and cumulus cells of oocytes which are vitrified before and after *in vitro* vitrification.

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