

# THE EFFECT OF UREA SUPPLEMENTATION IN MATURATION MEDIA OF BOVINE OOCYTE IN VITRO TOWARDS EXPRESSION OF BAX, BCL-2 AND BAX/BCL-2 RATIO

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## ABSTRACT

This study aims to evaluate the expression of BAX, BCL-2, and BAX/BCL-2 ratio in maturation media of cow oocytes which supplemented with Urea in vitro because BAX and BCL-2 are the main regulators of apoptosis. A total of 263 oocytes from follicle aspirations originating from ovaries taken from slaughterhouses and were saturated with 3 addition of urea which was divided into three groups. The control group (P0) was control group without the addition of urea, P1 group was added with urea 20 mg/dL, while P2 group was added with urea 40 mg/dL. The results of in vitro oocyte maturation were continued with identification using immunocytochemical staining with the addition of BAX and BCL-2 antibodies. Positive results showed a brownish color on the oocyte and its cumulus. The results of this study indicated that there were significant differences ( $P < 0.05$ ) in BAX and BCL-2 expression, although both curves were equally increase. The increase in BCL-2 was more significant than BAX, while the BAX BCL-2 ratio did not show a significant difference ( $P > 0.05$ ) in which the curve of BAX/BCL-2 ratio was decreased. It can be concluded that the addition of urea does not affect the level of apoptosis.

Key words: apoptosis, BAX/BCL-2 ratio, oocyte, urea

## ABSTRAK

Penelitian ini bertujuan melihat ekspresi BAX, BCL-2, dan rasio BAX/BCL-2 dalam oosit yang ditambahkan urea dalam media maturasi in vitro karena BAX dan BCL-2 adalah regulator utama apoptosis. Sebanyak 263 oosit dari aspirasi folikel yang berasal dari ovarium yang diambil dari rumah potong dengan penambahan urea menjadi 3 kelompok. P0 adalah kelompok kontrol tanpa penambahan urea, P1 adalah penambahan urea 20 mg/dl dan P2 adalah penambahan urea 40 mg/dl. Hasil pematangan in vitro oosit akan dilanjutkan identifikasi dengan pewarnaan imunositokimia dengan penambahan antibodi BAX dan BCL-2. Hasil positif akan menunjukkan warna kecoklatan pada oosit dan kumulusnya. Hasil penelitian ini menunjukkan bahwa terdapat perbedaan yang signifikan ( $P < 0.05$ ) pada BAX dan BCL-2, meskipun kurva keduanya sama-sama naik, peningkatan BCL-2 lebih signifikan daripada BAX, sedangkan Rasio BAX/BCL-2 tidak menunjukkan perbedaan yang signifikan ( $P > 0.05$ ), kurva rasio BAX/BCL-2 mengalami penurunan. Berdasarkan hasil tersebut dapat disimpulkan bahwa penambahan urea tidak memengaruhi tingkat apoptosis.

Kata kunci: apoptosis, Rasio BAX/BCL-2, oosit, urea

## INTRODUCTION

Nutrition management is important in managing dairy farming. High protein food can stimulate milk production. Dairy farmers generally increase milk production by increasing protein intake in feeding, but in fact it can disrupt their reproductive system (Sinclair, 2008). Urea is a product of the metabolism of proteins of ruminants feed which present in the blood as Blood Urea Nitrogen (BUN). It spread throughout the body fluid including the follicles in the ovaries. Provision of protein in feed has an influence on the increase in urea in the blood and milk. The maximum limit of Blood Urea Nitrogen (BUN) is 15 mg/dL and the concentration of Milk Urea Nitrogen (MUN) for individual cow ranges from 8-25 mg/dL (Bazet *et al.*, 2010). High level of Blood Urea Nitrogen (BUN) can affect the process of oocyte maturation because the protein will be degraded and there will be an increase in ammonia content in the rumen which ultimately will increase the formation of urea in the liver, increase the level of urea in the blood, change the composition of fluid in the uterus, decrease uterine pH and reduce

conception rates (Dhali *et al.*, 2006). Previous studies suggested that the concentration of urea  $\geq 18$  mg/dL resulted in low Service per Conception (S/C) (Putri *et al.*, 2018).

In Vitro Maturation (IVM) of oocyte maturation level affects the fertilization process. Mature oocytes with good quality will result in high fertility rates. The rate of bovine oocytes development into blastocysts through In Vitro Fertilization (IVF) is only 30-40%. That number shows that the culture medium conditions greatly affect the potential development of the embryo and oocyte quality is an important development factor until the blastocyst stage. Immature oocytes can be obtained from cow ovaries in slaughterhouse by follicle aspiration or from live animals by laparoscopy or ultra sonography (Firmiati *et al.*, 2014).

Wattimena (2011) states that the success of in vitro maturation process is largely determined by several factors, including the quality of the maturation medium, the type of supplement in the maturation media, oocyte quality, risk of contamination, and culture media. Supplements used and good culture

conditions are supportive in increasing the ability of oocyte maturation *in vitro*. High blood urea concentration can induce protein denaturation that will lead to activation of damage/stress sensor which affects the growth of ovarian follicles in cow and oocyte development. Ultimately, it causes cell stress and cell damage (Galluzzi *et al.*, 2012). There are several causes of cell damage such as physical trauma, viral infection, toxin, spontaneous genetic mutations, and imbalances of metabolic performance, nutritional abnormalities and dysfunction of cell immunity. The mechanism of cell damage can be caused by ATP depletion (often due to hypoxia), cell membranes damage (due to myriad and also includes free radicals associated with oxygen), impaired cell metabolism, and genetic damage (McGavin and Zachary, 2007).

Chemicals, pharmaceuticals and toxin can affect cells through several mechanisms, namely blockade or stimulation of receptors on cell membranes, change of enzymes system, production of free radical toxins, change in cell permeability, chromosome destruction, modification of metabolic pathways, and destruction of cellular component (McGavin and Zachary, 2007). Apoptosis is a cellular destruction process which is very well regulated and is characterized by morphological and biochemical changes. Apoptosis can be stimulated by physiological and pathological conditions and plays an important role in maintaining normal homeostasis and the pathogenesis of several diseases. There are two causes of apoptosis, namely physiological causes, such as embryonic development during tissue formation and pathological causes which include viruses that trigger cell death, radiation, hypoxia and cell degeneration (Fink, 2005). The objective of this study was to prove that urea supplementation in *in vitro* maturation media of oocytes can reduce the percentage of maturation, increase the percentage of BAX, reduce the percentage of BCL-2 and increase the BAX/BCL-2 ratio.

## MATERIALS AND METHODS

### Population and Research Sample

The samples used in this study were cow ovaries which were acquired from slaughterhouse and taken to *In Vitro* Fertilization Sub Laboratory, Obstetrics Laboratory, Faculty of Veterinary Medicine, Airlangga University. Ovarian follicles fluid aspiration was performed to obtain oocytes that would be used as samples. The research sample was the immature cow oocytes which still had complex oocyte cumulus. The repetition was performed six times in accordance with Federer formula (1963) in the research by Kusrieningrum (2011).

### Ovarian Treatment

The first step in process of IVM process was separation of ovaries from surrounding tissue. The ovaries were washed using Phosphate Buffer Saline (PBS) 3 times. The next step was to perform oocyte

aspiration using an 18G needle and 10 mL syringe from follicle with diameter of 2-8 mm for oocyte collection. The aspirates were put into dissection media for 5 minutes in order to precipitate the oocytes. Washing and selection of oocytes were carried out in the dissection media.

### Maturation of Oocytes In Vitro (Maturation of Control)

For oocyte maturation process, Tissue Culture Medium (TCM) 199 was used with addition of Pregnant Mare Serum Gonadotropin (PMSG) 100 IU/ml, Human Chorionic Gonadotropin (HCG) 100 IU/mL, and Fetal Bovine Serum (FBS) 10%. In each petri dish, there are three drops of maturation media which contain 300  $\mu$ L/drop media that has been coated with mineral oil and then incubated in incubator with 5% CO<sub>2</sub>, 95% humidity, and temperature of 38.5° C for 22 hours (Widjiati, 2011).

### Maturation of Oocytes In Vitro (Maturation of Treatment)

For oocyte maturation process, the TCM-199 medium was added with 100 IU/mL PMSG, 100 IU/mL HCG, 10% FBS, and urea. The concentration of urea for supplementation in maturation media were 20 mg/dL and 40 mg/dL. Each oocyte replication obtained was divided into three groups, namely the control group (P0), the group with the addition of urea 20 mg/dL (P1), and the group with the addition of 40 mg/dL urea (P2). Each treatment group was divided into 3 parts, namely the percentage of maturation, BAX and BCL-2.

### Identification of BAX and BCL-2 Using Immunocytochemical Method

Oocyte which had been matured for 24 hours was placed on glass objects coated with poly-L lysine and dried at room temperature. Then, it was fixed in 120 mL cold methanol (-20° C) and then washed again with 10% PBS twice for 10 minutes. 3% H<sub>2</sub>O<sub>2</sub> was dropped and left for 10 minutes, the object glass was then rinsed with 10% PBS and incubated with anti-BAX/BCL-2 staining kit antibodies diluted with 5% Ab-diluents for 1 hour at room temperature. It was incubated with biotinylated secondary antibodies (1:600 in 10% PBS) at room temperature and then washed 10 times for 60 minutes in 10% PBS. It was incubated with 1:200 fluorophore-conjugated streptavidin in 10% PBS for 2-3 hours at 37° C and then washed 10 times for 60 minutes in PBS. The oocyte was stained with methyl green counterstaining and rinsed with sterile aquades. It was dried and viewed with a microscope with magnification of 100 times and 400 times. Positive results would be seen as brownish color (Hoffman *et al.*, 2008).

### Calculation of the Percentage of Mature Oocytes with Aceto Orcein Staining

Calculation of mature oocytes percentage based on the metaphase II stage could be done by aceto-

orcein staining. The oocyte samples of mature oocytes (MII) evaluation were taken from maturation medium according to the maturation period. Cumulus cells surrounding the oocyte were removed by repeated aspiration using a pipette tip with a diameter that matched the diameter of the oocyte. Oocytes that had been free of cumulus cells were fixed for 24 hours, stained with 1% aceto-orcein for 10 minutes and cleaned with aceto-glycerol. MII observation was carried out using an inverted microscope. Calculation of the percentage of oocyte maturation (oocytes which reached metaphase II stage was indicated by the presence of metaphase plates in aceto orcein staining and the presence of polar body I) performed by comparison of MII with the number of colored oocytes in percent (%).

#### Data Analysis

The data were tabulated and the data distribution was analyzed using analysis of variance.

### RESULTS AND DISCUSSION

#### In Vitro Maturation of Cow Oocytes

The number of oocytes which were selected from each ovary for in vitro maturation process ranged from 3-5 oocytes. After 24 hours maturation process, the data obtained in P0, P1, and P2 were 51.25%, 52.43%, and 46.88%, respectively. The average percentage of oocytes that reached metaphase II was 45.16% with total number of 263 oocytes (Figure 1).

#### Expression of BAX on In Vitro Matured Bovine Oocytes According to Staining Results

Immunocytochemical staining from BAX showed a significant effect of urea administration on BAX expression. Each treatment (P0, P1, P2) showed a significant difference ( $P < 0.05$ ). The number of BAX expressions increased from control group until treatment 2 group (Table 1). The results of

immunocytochemical staining from BAX can be seen in Figure 2.

The results of positive immunocytochemical examination of BAX in the control group was 22.3%, in group which was treated with urea 20 mg/dL was 42.05% and in group which was treated with urea 40 mg/dL was 56.6% (Table 1). Based from statistical analysis, it was found that there was a significant increase from control group to treatment 1 group and treatment 2 groups. Table 1 also showed a significant increase in BAX expression, therefore it can be concluded that administration of urea has a significant effect on BAX expression. BAX and BAK are genes that act as apoptotic regulators in cells (as pro-apoptotic signal). They are located within the mitochondria and endoplasmic reticulum and activate the caspase to regulate the apoptotic pathway (Allaire *et al.*, 2000; Ruiz-vela *et al.*, 2005).

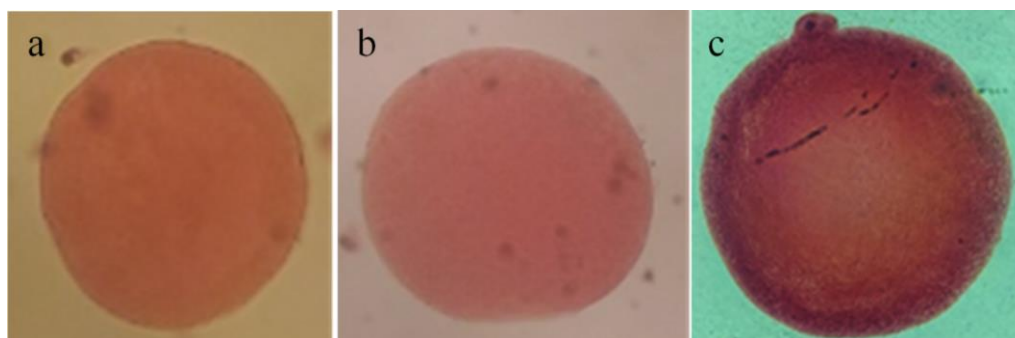
Inducible increased BAX or BAK expression results in changes in mitochondrial membrane potential, reactive oxygen species production and in certain circumstances cytochrome c release, which ultimately results in downstream caspase programs. Inducible p15 tBid expression in cells has a similar effect. In general, excessive expression of BCL-2 or BCL-XL will counteract these effects (Gross *et al.*, 1999). The complexity of this process does not prevent mitochondrial Tbid destruction and targeting in response to TNF/Fas but prevent cytochrome-c release (Gross *et al.*, 1999). However, other damage to mitochondria still remains and many cells will still die.

Apoptosis or programmed cell death occurs normally during development and aging process as a homeostatic mechanism for maintaining cell populations in tissue. Apoptosis also occurs as a defense mechanism, for example an immune reaction or when cells are damaged by disease or a destructive agent. Although there are various types of stimuli and conditions, both physiologic and pathologic, not all cells die in response to the same stimulus. It

**Table 1.** Expression of BAX, BCL-2, and BAX/BCL-2 ratio ( $\pm$ SD) in bovine oocytes which were matured in vitro using immunocytochemical staining

Group	N	BAX	BCL-2	Ratio of BAX/BCL-2
P0	6	22.31 $\pm$ 21.15 <sup>a</sup>	27.5 $\pm$ 32.21 <sup>a</sup>	2.3 $\pm$ 2.5 <sup>a</sup>
P1	6	42.31 $\pm$ 12.33 <sup>ab</sup>	52.85 $\pm$ 31.94 <sup>a</sup>	1.8 $\pm$ 2.6 <sup>a</sup>
P2	6	56.66 $\pm$ 25.17 <sup>b</sup>	89.58 $\pm$ 16.61 <sup>b</sup>	0.8 $\pm$ 0.2 <sup>a</sup>

<sup>a, b, ab</sup>Different superscripts within the same column indicate significant differences ( $P < 0.05$ )



**Figure 1.** The results of aceto orcein staining on oocytes. a= P0 (without urea treatment), b= P1 (with addition of urea 20 mg/dL), c= P2 (with addition of urea 40 mg/dL), there is formation of a polar body, 400x

is assumed that Urea activates changes in permeability of mitochondrial inner layer (inner mitochondrial permeability transition pores) which is regulated by  $Ca^{2+}$  matrix, pH and voltage (Ghobrial, 2005). BCL-2 family proteins can induce or inhibit the release of cytochrome-c into the cytosol which will activate Caspase 9 and Caspase 3, resulting in the process of apoptosis (Kumar *et al.*, 2005).

### Expression of BCL-2 on In Vitro Maturated Bovine Oocytes According to Staining Results

The results of immunocytochemical staining of BCL-2 showed a significant effect of urea administration with BCL-2 expression. The control group (P0) and treatment 1 (P1) did not show any significant difference ( $P>0.05$ ), meanwhile the control group (P0) and treatment 2 (P2) showed significant difference ( $P<0.05$ ) as well as treatment 1 (P1) and treatment 2 (P2). However, the number of BCL-2 expressions increased from control to treatment 2 (Table 1). The results of immunocytochemical staining of BCL-2 can be seen in Figure 3.

The results of positive immunocytochemical examination of BCL-2 in the control group was 27.5%, in group which was treated with urea 20 mg/dL was 52.85%, and in group treated with urea 40 mg/dL was 89.58 % (Table 1). From these results it could be concluded that there was a significant increase of BCL-2 expression from control group to treatment 1 group and treatment 2 groups. In this study, the increase of BCL-2 expression was higher than BAX. Its indicated that urea supplementation has a significant effect on BCL-2 expression.

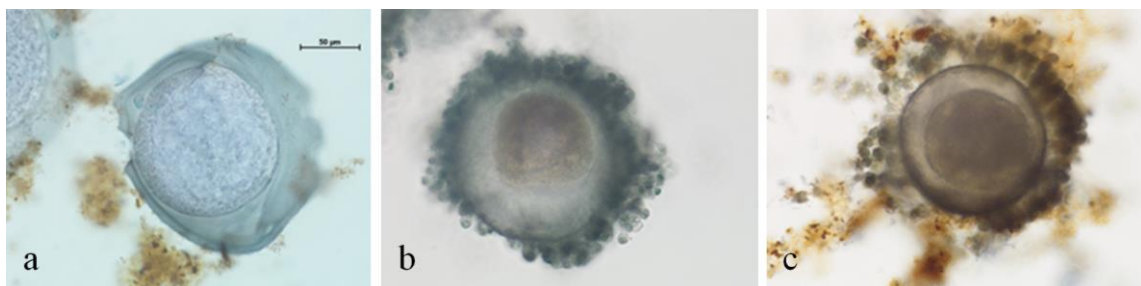
The BCL-2 family consists of pro-apoptotic protein (BAX, BAK, BAD, BCL-XS, BID, BIK, BIM, and HRK) and anti-apoptotic protein (BCL-2, BCL-XL, BCL-W, BFL-1, and MCL-1). Anti-apoptotic

members of BCL-2 family work by suppressing apoptosis through blockade of the cytochrome-c release, while pro-apoptotic members work as promoters. This effect depends more on the balance between BAX and BCL-2 than on BCL-2 itself (Reed *et al.*, 1994; Ghobrial *et al.*, 2005).

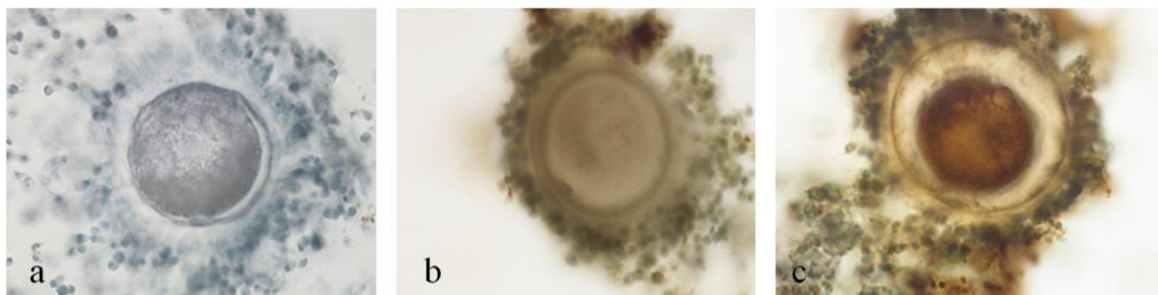
The BCL-2 family protein is the most important regulator of the intrinsic pathway and is also the main regulator in apoptosis (Shiozawa and Konishi, 2006). BCL-2 is included in a new category of oncogenes which could prolong cell survival without affecting cell proliferation. This protein is often over-expressed in various malignancies even in the absence of translocation of the t chromosome (Garuti *et al.*, 2002) which results in changes in the BCL-2 gene. Increased expression of BCL-2 can cause resistance to chemotherapy drugs and radiation therapy although a decrease in BCL-2 expression can stimulate apoptotic responses to anti-cancer drugs. Furthermore, excessive exposure of BCL-2 can result in accumulation of cell in the G0 phase of the cell cycle that will lead to a chemoresistant condition (Tamm *et al.*, 2001; Ghobrial *et al.*, 2005).

### Ratio of BAX/BCL-2 Expression on In Vitro Maturated Bovine Oocytes

There was a decrease of BAX/BCL-2 ratio on the control group (P0), treatment 1 (P1), and treatment 2 (P2) although the effect was not significant ( $P>0.05$ ) (Table 1). High levels of BCL-2 protein will prevent early cell death from apoptosis. BCL-2 protein will suppress apoptosis process by preventing caspase activation (Ghobrial *et al.*, 2005). Excessive amount of BCL-2 protein will decrease apoptosis and caused cancer through mitochondrial pathway. The selection of Vascular Endothelial Growth Factor Receptor-2 (VEGF/VEGFR2) pathway because VEGF/VEGFR2 is



**Figure 2.** Representative results of BAX immunocytochemical staining on oocytes in vitro maturation media. a= P0 (without urea treatment), b= P1 (with addition of urea 20 mg/dL). c= P2 (with addition of urea 40 mg/dL), 400x



**Figure 3.** Representative results of BCL-2 immunocytochemistry staining in oocytes in in vitro maturation media. a= P0 (without urea treatment), b= P1 (with an addition of urea 20 mg/dL). C= P2 (with addition of 40 mg/dL urea), 400x

considered as the most important proangiogenic pathway to increase all stages of angiogenesis including vascular permeability, endothelial cell survival, proliferation, migration or invasion of surrounding tissue, and formation of capillary blood vessels (Hoi *et al.*, 2014).

A high ratio of BCL-2/BAX will make the cells resistant to apoptotic stimuli while lower ratio will induce apoptosis. Increased expression of BCL-2 will affect the intrinsic pathway of apoptotic process because BCL-2 will increase Interleukin-Converting Enzyme (ICE)-like protease. BCL-2 will prevent Fas from carrying out apoptosis by inactivating ICE-like protease which will reduce the ability of Fas-FasL function as pro-apoptotic protein from Tumor Necrosis Factor (TNF) family members in the intrinsic apoptosis pathway. Transcription factor NF- $\kappa$ B can suppress the pro-apoptotic action of TNF- $\alpha$ . Currently, it is known that a key factor of apoptotic regulation is BCL-2/BAX ratio in which high ratio will make cells resistant to apoptotic stimuli and low ratio will stimulate apoptosis (Vaskivuo *et al.*, 2002).

## CONCLUSION

Supplementation of high-dose urea in in vitro maturation media of bovine oocytes can reduce the level of maturation due to oocyte damage as evidenced by immunocytochemical staining of BAX expression. On the other hand, supplementation of high-dose urea in in vitro maturation media of bovine oocytes can increase the expression of BCL-2. This proves that cell damage is not caused by apoptosis because BCL-2 is an anti-apoptotic protein.

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