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The Apoptotic Effects of Alcohol Shock on Bovine Oocytes

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Abstract: Bovine oocytes collected from slaughterhouse should be subject to rigorous antiseptic procedures. In this lab, alcohol immersion was applied for preventing microbial infection instead of antibiotics. However, it remains to be investigated whether alcohol treatment would lead to unpredictable results. In the present study, the ovaries were fully washed with 75% alcohol for 1, 5 and 10 min, respectively. Only in the 10 min group, the oocytes apoptotic rates significantly increased ($p<0.05$) and the development rates significantly decreased ($p<0.01$) while detectable difference in apoptosis was not observed. Therefore, washing ovaries with 75% alcohol for <5 min constitutes no obvious influences on the development of oocytes and embryos and could be used as a convenient and rapid method to prevent microbial contamination.

Key words: Alcohol shock, apoptosis, bovine, oocyte, ovaries, China

INTRODUCTION

Collecting oocytes from slaughterhouse had become more and more popular since the culture system of embryos became a ready-to-use technique and the production had also been greatly improved. Normally, researchers obtain bovine oocytes from ovaries in the slaughterhouse or by transvaginal ovum pick-up (Merton *et al*., 2003).

In this process, microbial infections including bacteria, fungi and mycoplasmas are inevitably brought in due to direct or indirect contact. Therefore, ovaries isolated from livestock should be placed into normal saline or PBS containing thermos flask and washed several times before oocytes were pumped and all solution should be supplemented with antibiotics. Antibiotics are widely used in contamination prevention. However, many types of them could only inhibit rather than kill the microorganisms (Climent *et al*., 2002).

Although, it has not been reported that alcohol immersion could be applied for contamination prevention, this method has long been used in this lab and achieved ideal effects. However, it remains to be investigated whether the treatment would lead to unpredictable results such as apoptosis (Kotcha and Sulik, 1992; Ewald and Shao, 1993; Holownia *et al*., 1997; Zhang *et al*., 1998; Ikonomidou *et al*., 2000; Climent *et al*., 2002; Light *et al*., 2002).

Apoptosis is a form of cell death that can function to eliminate cells damaged by environmental stress which in early development is featured by some of morphological alterations, such as nuclear and cytoplasmic condensation, endoplasmic reticulum swelling and cytoplasmic blebbing (Feugang *et al*., 2002).

In this study, the effects of alcohol immersion on bovine oocytes will be detected in terms of DNA fragmentation and condensation, mature rates and embryonic development.

MATERIALS AND METHODS

Collection of oocytes: Newly isolated bovine ovaries were placed into normal saline in a thermos flask at 35°C and brought back to the lab within 3 h. Subsequently, they were fully washed with 75% alcohol for 1, 5 and 10 min, respectively. Then the Cumulus-Oocyte Complexes (COCs) were collected through mechanical operation.

The ovaries were then rinsed with normal saline twice, wiped up with sterile gauze and placed into 60 mm Petri dishes containing phosphate buffered saline (PBS, pH 7.4).

The ovarian follicles were cut up with a scalpel and the follicular fluid were collected into a 50 mL tube and kept still for 15 min. With the supernatant discarded, the remains were diluted with PBS from which COCs with >3 layers of cumulus cells were picked out.

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Culture and *in vitro* maturation of oocytes: The oocytes were matured *in vitro* as previously described (Koo *et al*., 2002). The COCs were covered with paraffin oil and incubated at 38.5°C in a humidified atmosphere with 5% CO_2 for 22 h.

Identification of mature oocytes: Mature COCs were washed in PBS to eliminate liquid paraffin and pipetted gently to remove cumulus cells. They were subsequently transferred into Petri dishes containing 400 μL 0.1% hyaluronidase for digestion and pipetted repeatedly. Granulosa cells could be completely disassociated 4 min later with oocytes covered with only zona pellucida left. Intact oocytes with homogenous cytoplasm and extruded first polar bodies were selected for further experiments.

Parthenogenesis of oocytes: The oocytes were induced parthenogenesis to evaluate their developmental potential. They were washed and then treated with 5 μmol L^{-1} ionomycin for 5 min. Afterwards, they were washed and activated in 2 mmol L^{-1} 6-DMAP for 4 h, washed with and cultured in preequilibrated SOFaa media supplemented with 5% FBS. The media were refreshed every 48 h. The cleavage rates and the blastocyst rates were determined at 48 h and 7 days after activation, respectively.

AnnexinV-FITC/PI assay: AnnexinV-FITC/PI assays were used to detect the apoptotic degree as previously described (Hao *et al*., 2003). The samples were mounted between a coverslip and a glass slide supported by 4 columns of a mixture of petroleum jelly and paraffin (9:1, vol/vol). Slides were scanned using a confocal laser-scanning microscope (Nikon TE-2000-E, Japan) with an argon-krypton laser at 488 and 543 nm and 2-channel scanning for the detection of FITC and PI, respectively. Exposure time of excitation lights was uniform for respective channels.

RESULTS AND DISCUSSION

Apoptotic rates of oocytes: The apoptotic rates were shown in Table 1, oocytes treated with alcohol for 1 and 5 min were nonsignificantly different from control ($p>0.05$) whereas those for 10 min significantly different ($p<0.05$). But the differences among groups treated with alcohol were nonsignificant compared with control (Fig. 1). Then researchers detected the single apoptotic oocytes, researchers deserved the difference among the normal, the apoptotic, the dead oocytes (Fig. 2).

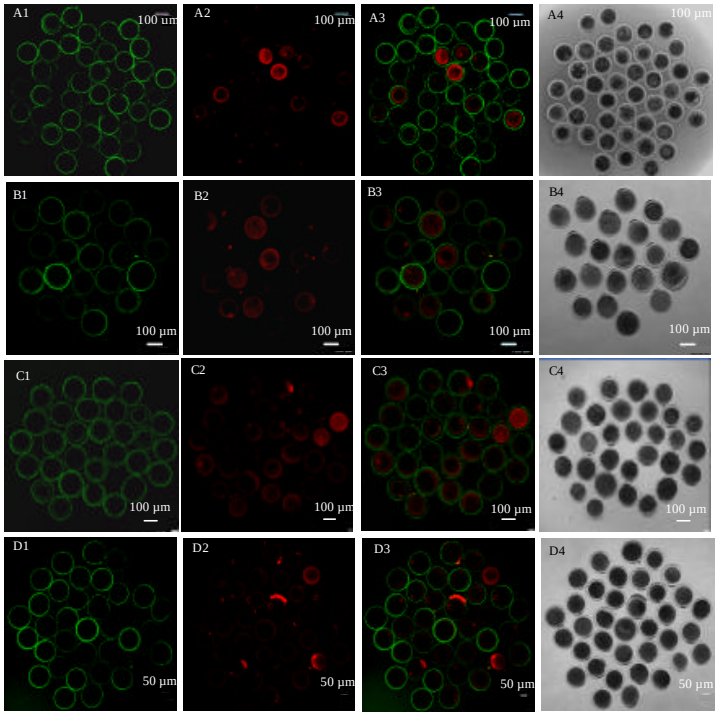

Fig. 1: Apoptotic detection of bovine oocytes upon alcohol exposure A1-A4: Normal oocytes. Acid sphingomyelinase was activated. Apoptotic oocyte from ovaries washed for B1-B4: 1 min.; C1-C4: 5 min. D1-D2 and 10 min. In D1-D4, cells showed shrinkage seriously and enlarged perivitelline space. Cells were immunostained with Annexin V-FITC (green) and counterstained with PI (red). Merge (green and red). Scale bar =100 μm

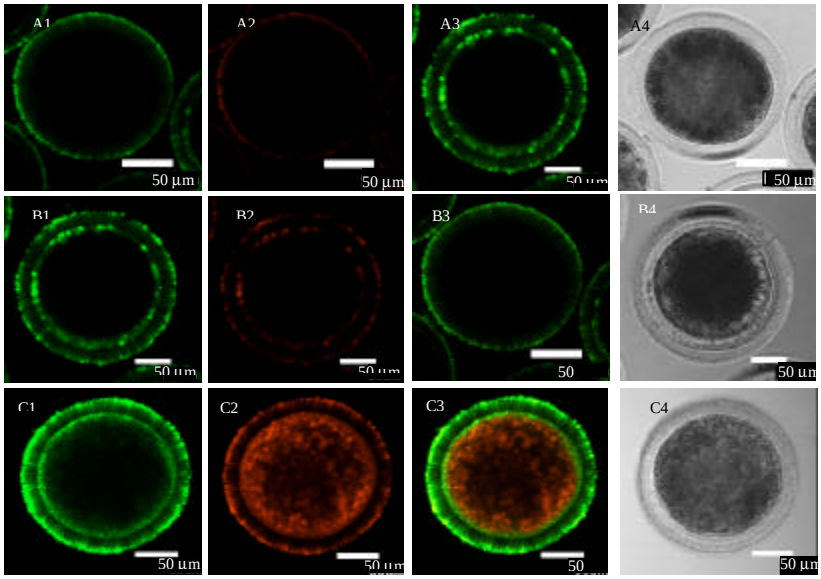

Fig. 2: Apoptotic degree of individual oocytes. A1-A4: Normal oocytes; B1-B4: Apoptotic oocyte. C1-C2: Dead oocytes, cells appeared seriously shrunk, with perivitelline space enlarged. Cells were immunostained with Annexin V-FITC (green) and counterstained with PI (red). Merge (green and red). Scale bar = 50 µm

Table 1: Cell death upon alcohol shock of bovine oocytes

Categories	0 min	1 min	5 min	10 min
The no. of oocytes	84	63	90	107
The no. of dead oocytes	11	9	7	8
The no. of apoptotic oocytes	9	9	15	26
Apoptotic rate (%)	12.33 (9/73)[a]	16.67 (9/54)[a]	18.07 (15/83)[a]	26.26 (26/99)[b]

[a,b]Values with different superscripts in the same row are significantly different ($p<0.05$)

Table 2: Effects of alcohol exposure on oocyte maturation and embryonic development

Categories	0 min	1 min	5 min	10 min
The number of COCs	54	31	46	34
Cleavage rate (%)	61.11 (33/54)[a]	58.06 (18/31)[a]	58.7 (27/46)[a]	35.29 (12/34)[c]
Blastula rate (%)	16.67 (9/54)[a]	16.13 (5/31)[a]	7.69 (2/46)[b]	5.88 (2/34)[b]

[a-c]Values with different superscripts in the same row are significantly different ($p<0.05$)

The cleavage and developmental rates: The cleavage and developmental rates of control were 61.11 and 12.96%, respectively (Table 2). The difference between the cleavage rates of control and that of oocytes treated with alcohol for 10 min was significant ($p<0.01$) while the oocytes treated with alcohol shock for 1 min exhibited nonsignificant differences with control in developmental rates.

The blastula rate: The cell number and blastula formation in embryos (Fig. 3), a lower number of cells within *in vitro* produced embryos can be attributed to the exposure to alcohol.

In the present study, apoptosis was observed in both control and the treated oocytes indicating constitutional apoptosis in normal culture and no differences were revealed among the treatments (Fig. 1). Several studies have described cell death during pre-implantation embryogenesis in a wide range of mammalian species, both *in vivo* and *in vitro*. Therefore, apoptosis may be involved in early embryonic arrest and the characteristic cytoplasmic fragments are the equivalents of apoptotic bodies, the end-product of apoptosis (Honda *et al*., 2005). Although, alcohol was not supplemented into the embryonic media directly, the results indicated that it did have some effects on oogenesis.

It was observed that naked ova increased and the cytoplasm darkened with increasing immersion time indicating that alcohol must have penetrated into them with certain mechanisms. COCs collected from follicular fluid were mainly those after secondary follicle stage whose surface structure were the folliculi and a layer of basement membrane. It was possible that alcohol immersion had altered the structure and permeability, leading to the following entry of alcohol and hence affected the COCs.

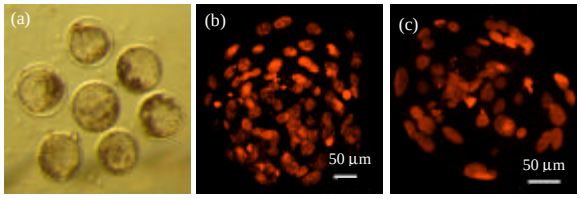

Fig. 3: Blastula and cell numbers within (PI staining): a) Normal blastulas; b) Cell number of normal blastula; c) Cell number of blastula from ovaries exposed to alcohol for 10 min. Bar = 50 µm

Apoptotic analysis suggested that some oocytes would enter apoptosis even in normal culture process and the treatment of alcohol had significant effects on the maturation and subsequent embryonic development of ovaries, aggravating their apoptosis with increasing time. It takes a long time for the toxicity of alcohol to become obvious for which reason only the newly isolated oocytes immersed for 10 min exhibited significant difference. However, the effects are distinctively obvious for the matured oocytes and those immersed for 10 min were significantly different which was aggravated with increased immersion time. Although, the oocytes did not exhibit significant difference in developmental rates, much fewer ones treated for 10 min survived at 8-cell phase and only numerable ones developed to blastula stage. Furthermore, alcohol immersed oocytes generated fewer cells when they entered blastula stage implying that the negative effects of alcohol are chronic, however the underlying mechanisms remain to be elucidated.

CONCLUSION

The results show treatment of newly isolated ovaries with 75% alcohol if the time is short enough, constitutes no obvious influences on the development of oocytes and embryos. Generally, it takes <5 min to wash them twice, shorter compared with the bovine oocytes treated for 5 min which is effective for contamination prevention and could be used as a convenient and rapid method.

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