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Apoptosis in the Bovine Blastocyst following Nuclear Transfer and *In Vitro* Fertilization

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핵치환과 체외수정에 유래된 소의 배반포에서의 Apoptosis

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ABSTRACT

The mechanisms underlying of the visual assessment and resulting in optimum embryonic development following *in vitro* maturation, fertilization, and culture are unclear. It was known that *in vitro* produced embryos show more frequent occurrence of fragmentation, which resulted in poor developmental potential and decreased implantation rate. The objective of this study was to investigate the apoptotic rates in bovine blastocyst derived from *in vitro* fertilization (IVF) and nuclear transfer (NT). In addition, the expression levels of *Bcl-2* and *Bax* gene were investigated in the blastocyst to confirm their potential roles in the regulation of apoptosis during preimplantation embryonic development. Analysis of apoptosis was carried out by using terminal deoxynucleotidyl transferase mediate dUTP nick end labeling (TUNEL) method. The levels of *Bcl-2* and *Bax* gene in the blastocyst derived from IVF and NT were determined by RT-PCR.

The proportion of TUNEL positive signal in blastocyst derived from NT was significantly higher than that in blastocyst derived from IVF ($p<0.001$). *Bcl-2* expression level of blastocyst derived from IVF was higher than that of blstocyst derived from NT. However, high expression level of *Bax* was observed in the blastocyst derived from NT. These results indicates that apoptosis is more responsible for fragmentation in bovine blastocyst derived from NT than IVF. These results suggested that the increase of developmental failure followed by NT could be caused by nuclear fragmentation as apoptosis.

(Key words: Apoptosis, Nuclear transfer, Blastocyst, *Bcl-2*, *Bax*)

Ⅰ. INTRODUCTION

In many species, the developmental competence of *in vitro* fertilized, nuclear transferred and cultured embryos remains low (Heyman, 2002). In addition, Nuclear transfer (NT) in mammalian species has been developed and advanced in the last 5 yr and many associated factors have been investigated (Kuhholzer, 2000). Successful cloning has now been achieved in several mammalian species as reported by the birth of offspring in sheep, cattle, goats, pigs

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and mice (Wilmut et al., 1997; Kato et al., 1998; Cibell et al., 1998; Polejaeva et al., 2000; Wakayama and Yanagimachi 1999). However, while mammalian nuclear transfer continue at a fast pace, there remains still significant problems associated with the low efficiency of the procedure resulting in the high rate of pregnancy loss throughout gestation.

The fundamental importance of apoptosis in normal organ development and differentiation has well been established. Using current *in vitro* procedures, less than half of inseminated bovine and human oocytes reached the blastocyst stage (Hardy and Handyside 1989; Keskintepe and Brackett 1996) and of these many do not implant or attach following embryo transfer. The explanation for the high rates of developmental failure remains unclear. But it is confirmed that arrested and developed embryos contain different proportions of cells with classic features of cellular death, including cytoplasmic, nuclear and DNA fragmentation (Hardy, 1997).

The loss of embryo viability can be due to suboptimal culture condition (Brison and Schultz 1997; O'Neil, 1998) or abnormality resulting from *in vitro* fertilization (Jurisicova et al., 1996; Levy et al., 1997), some embryos incubated *in vivo* are also highly apoptotic as judged by the fragmentation of blastomeres and terminal deoxynucleotidyl transferase mediate-dUTP nick end labelling (TUNEL) method (Warner et al., 1998). Yet very little is known how signalling mechanism triggers apoptosis in embryos and how they are controlled.

Two forms of cell death are known and can be distinguished on the basis of morphological and molecular criteria (Savill, 1994). Necrosis is characterised by nuclear disintegration, cellular swelling and rupture of internal and external membranes with the release of lytic enzymes and damaged to surrounding cells. In contrast, apoptosis characteristically affects single cells rather than groups of cells. Apoptosis-inducing events include various environmental stressors in addition to gross chromosome abnormalities. Morphological features of this form cell death include cytoplasm and chromatin condensation, DNA fragmentation, with the separation of the nucleus into discrete masses.

Every cell has cell death pathway as an apoptosis. It has been proposed that the tendency to apoptosis is continuously counterbalanced in the cell by genes stimulating cell survival and proliferation. Studies from extragonadal cell systems have shown that among the numerous proteins and genes involved members of the *Bcl-2* gene family play an key role in regulating apoptosis. At least 15 mammalian *Bcl-2* family members have been identified and categorized into two subgroup, anti-apoptosis group (*Bcl-2*, *Bcl-w*, *Bcl-xL*, AI) and pro-apoptosis group (*Bax, Bak, Bim, Bad, Bok*). Although anti-apoptotic homologues form with pro-apoptotic members, it is controversial whether dimerization is required for their activity. What is clear, however, that in most cases the ratio of pro-apoptotic to anti-apoptotic *Bcl-2* homologues within a cell determines whether the cell will live or die (Chao and Korsmeyer, 1998; Oltvai, 1993).

Therefore, because apoptosis levels are important parameters, they are emerging as a useful indicator of embryonic development and quality. The objective of this study was to investigate whether the differences in the quality of blastocyst derived from IVF and NT are partially caused by apoptosis.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturation (IVM) of Bovine Oocytes

Ovaries were collected from slaughterhouse and transported to the laboratory in saline at 37℃. Cumulus-oocyte complexes (COCs) were obtained by follicular aspiration and collected into TALP-HEPES (TL-HEPES) medium (Parrish et al., 1985) and

washed again three times with equilibrated in tissue culture Medium 199 (TCM-199) (Gibco BRL, GRAND Island, NY) supplemented with 2.2 g/L sodium bicarbonate, 10% fetal bovine serum (FBS; Gibco BRL), 0.22 μg/ml sodium pyruvate, 25 μg/ml gentamycin sulfate, 1 μg/ml porcine-Follicular Stimulating Hormone (p-FSH) (Schering Co., UK), and 1 μg/ml estradiol-17β (Sigma Chemical Co., St. Louise, MO). Culture were carried out at 39℃, 5% CO_2 in air for 22 hrs.

2. *In Vitro* Fertilization (IVF) of Bovine Oocytes

Matured COCs were washed with Sperm-TALP (Sp-TALP) medium and subsequently with Fertilization-TALP medium (Fert-TALP) (Rosenkrans et al., 1994). COCs transfered in a 44 μl drop of Fert-TALP droplet under paraffin oil. Frozen-thawed semen were separated on a discontinuous percoll gradient. Highly motile spermatozoa were added to Fert-TALP at a final concentration of 1×10^6 sperm /ml. Then 2 μl of PHE stock solution (2 mM Penicillamine, 20 μM Hypotaurine and 1 μM Epinephrine) were added to a Fert-TALP droplet to stimulate sperm motility. Culture was carried out at 39℃, 5% CO_2 in air for 22 hrs.

3. Preparation of Bovine Fetal Fibroblast

Primary bovine fetal fibroblasts (BFFs) were isolated from male fetuses of pregnant bovine female at 40 day in gestation. BFFs were cultured on 60 mm tissue culture plates (Falcon, Lincoln Park, NJ) in Dulbecco's Modified Eagles's medium (DMEM; Gibco BRL) supplemented with 10% FBS. After 7 days of culture, BFFs were trypsinized and washed three times with fresh changes of Ca^{2+} –, Mg^{2+} – free PBS (Gibco BRL). The cells were pelleted and resuspended in DMEM supplemented with 10% FBS. Thereafter, BFFs were routinely maintained on 50 ml tissue culture flasks (Falcon) upto 2～7 passage and used as donor cells for nuclear transfer.

4. Nuclear Transfer (NT) of Bovine Oocytes

The matured oocytes were derived in TL-HEPES supplemented with 0.1% hyaluronidase. The oocytes were enucleated by the aspiration of the first polar body and adjacent cytoplasm (approximately 30% of ooplasm) using a beveled pipette (30 μm in diameter) in CR1aa containing 0.3% bovine serum albumin (BSA) and 7.5 μM cytochalasin B (CB; Sigma). Enucleation was confirmed by staining aspirated portion of cytoplasm with 5 μg/ml Hoechst 33342 (Sigma), then the enucleated oocytes were incubated in CR_{1aa} containing 0.3% BSA until injection of donor cells. Single bFFs was inserted into the perivitelline space of enucleated oocyte by the enucleation pipette (30 μm in diameter).

Nuclear transfered oocytes were washed three times with fusion solution composed of 0.3 M mannitol, 0.1 mM $CaCl_2$ and 0.1 mM MgSO. Fusion was performed in a chamber with two platium electrodes at 1 mm apart overlaid with fusion solution. Membrane fusion was induced with double D.C. pulse of 2.5 kV/cm for 30 μsec delivered by BTX Electro Cell Manipulator 200 (Genetronics, San Diego, CA). This pulse was also utilized to simultaneously induce oocyte activation. Reconstructed oocytes were activated by treatment of ionomycine and 2.5 mM 6-Dimethylaminopurine (6-DMAP).

5. *In Vitro* Culture of Reconstructed Oocytes

Both reconstructed and *in vitro* fertilized oocytes were cultured in 50 μl of CR1aa containing 0.3% BSA for 24 hrs. Oocytes were co-cultured with bovine oviduct epithelial cells in 50 μl of CR1aa containing 10% FBS for 6 days. All oocytes were cultured at 39℃ in an atmosphere of 5% CO_2 in air.

6. Detection of Apoptotic Cells by Terminal Deoxynucleotidyl Transferase Mediate dUTP Nick End Labeling (TUNEL)

Zona pellucida of the blastocysts were removed by a brief exposure to 0.5% pronase and then washed in PPB (PBS containing 0.1% polyvinyl pyrrolidone and 1% BSA). Zona-free blastocysts were fixed in 4% paraformaldehyde in PBS for 1 hr at room temperature. Fixed embryos permeabilized with permeabilization buffer (TaKaRa) on ice for 5 min. The embryos were washed twice in PPB and incubated in labeling reaction mixture at 37℃ for 90 min (Labeling Safe Buffer 45 μl containing TdT Enzyme 5 μl; TaKaRa).

After washed twice in PPB, embryos were incubated in Anti-FITC HRP conjugate at 37℃ for 30 min (TaKaRa), and counter stained with propidium iodide (200 μl/ml) in PPB for 15 min. After washed twice in PPB, embryos were mounted on slide glass.

7. Assessment of Apoptosis in Bovine Blastocyst

FITC-labelled nuclei were counted as apototic cells and PI-labelled nuclei were counted as total cells. Apoptosis was analyzed with BIO-1D image analysis software (Andrew, 2000).

8. Analysis of Apoptosis Related Gene Expression by Reverse-Transcription Polymerase Chain Reaction(RT-PCR)

Single embryo was added to sampling buffer (PBS, 0.1% PVP, 1 U/μl RNAsin [Promega]), snap frozen in liquid nitrogen, and stored at −70℃ prior to use. The samples were transferred to ice and Oligo dT was added. The mixed samples were incubated at 70℃ for 5 min and then chilled on ice for 5 min. The incubated mixture were transfered to RT premix tube (Bioneer), and DEPC dH_2O added to fill up the reaction volume. RT reaction was performed at 42℃ for 60 min followed by denaturation at 94℃ for 5 min. After RT reaction, samples were stored at −20℃. PCR was carried out using a GeneAmp PCR system 2400 Thermal Cycler (Perkin- Elmer, CT). Each reaction material contained 10 μl of cDNA solution and PCR Premix (1 U Tag DNA polymerase, 250 μM dNTPs, 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$ [Bioneer]). Due to no information about exon and intron sequence on bovine *Bax* and *Bcl-2* gene. Primer for bovine *Bax* and *Bcl-2* were designed based on human *Bax* and *Bcl-2* gene structure to span intron.

Primer sequences for each cDNA are *Bax* forward primer 5'-CAGCTCTGAGCAGATCAT-3', anti-*Bax* primer 5'-CTGCAGCTCCATGTTACT-3' with and expected amplification product of 190 bp and *Bcl-2* forward primer 5'-GGTTACGATAA-CCGAGAG-3', anti-*Bcl-2* primer 5'-CAGACTGAG-CAGTGCCTT-3' with and expected amplification product of 500 bp. PCR conditions for *Bax* were 94 ℃ for 30 sec followed by 35 cycles of 94℃ for 30 sec, 57℃ for 30 sec, and 72℃ for 30 sec. PCR conditions for *Bcl-2* were 94℃ for 30 sec followed by 30 cycles of 94℃ for 30 sec, 60℃ for 30 sec, and 72℃ for 30 sec.

9. Statistical Analysis

The significant difference among treatment groups in each experiment was determined by the t-test and ANOVA analysis (Abacus, Berkeley, CA).

Ⅲ. RESULT

1. *In Vitro* Development of Reconstructed Oocytes by Nuclear Trasnfer

As shown in Table 1, the fusion and cleavage rates of oocytes derived from IVF and NT were 77% and 67%, respectively. Among cleaved embryos, 44% developed to the morula stage. The overall developmental rates of development to the blastocyst stage were 36% in nuclear transferred oocytes

Table 1. *In vitro* development of bovine oocytes following *in vitro* fertilization and nuclear transfer

	No. of oocytes	No. of embyos (%)				
		Fusion	2~cell	8~16 cell	Morula	Blastocyst
IVF	252	-	204 (81)	125 (61)	97 (48)	90 (44)[a]
NT	193	149 (77)	129 (67)	70 (47)	63 (42)	55 (36)[a]

[a] No significant difference was observed (P>0.05).

embryos and 44% in *in vitro* fertilization oocytes.

2. TUNEL Assay on Blastocysts derived from IVF and NT

The bovine blastocysts were analysed for the detection of DNA fragmentation using TUNEL labelling and morphological features of apoptosis (Fig. 1). The total number of cells were compared with dead cell index in day 7 blastocysts derived from NT. The apoptosis index in blastocysts were significantly higher than that in blastocyst derived from IVF (Fig. 2).

3. Expression of *Bax* and *Bcl-2* mRNA

Because this study showed that fragmented embryos were undergoing apoptosis, we expected that *Bax* and *Bcl-2* gene can be detected in bovine blastocyst by using the RT-PCR, an easy, inexpensive method for detecting gene transcripts in very small numbers of cell. RT- PCR analysis of the

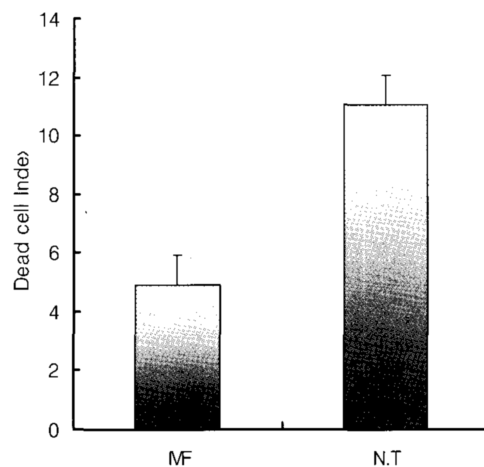

Fig. 2. Quantitative analysis of apoptosis by dead cell index in IVF and NT blastocyst. Values are mean±SEM (P<0.001).

Bax and *Bcl-2* gene in blastocyst derived from NT and IVF were shown in Fig. 3 and Fig. 4. *Bcl-2* expression level in blastocyst derived from IVF was higher than that in blastocyst derived from NT.

(A) **(B)**

Fig. 1. Detection of apoptosis and cell nuclei in bovine blastocysts derived from IVF (A) by and NT (B) TUNEL.

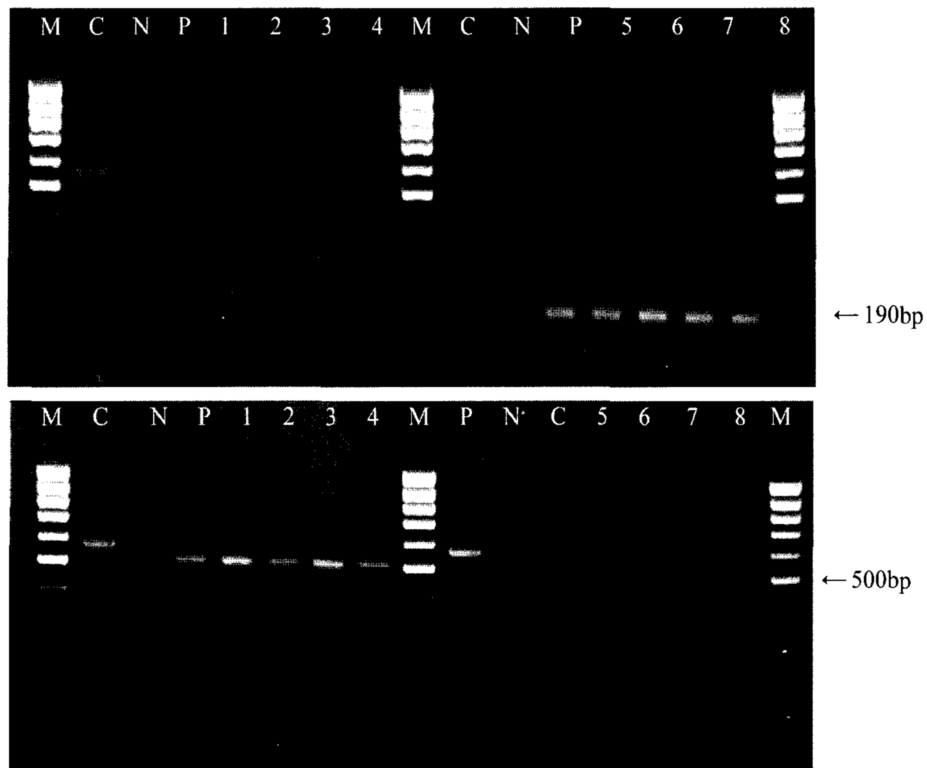

Fig. 3. RT-PCR analysis of *Bax* (A) and *Bcl-2* (B) in bovine blastocyst derived from IVF (Lane 1~4) and NT (Lane 5~8). M) size mark (100 bp ladder), P) positive control, N) negative control and C) GAPH control.

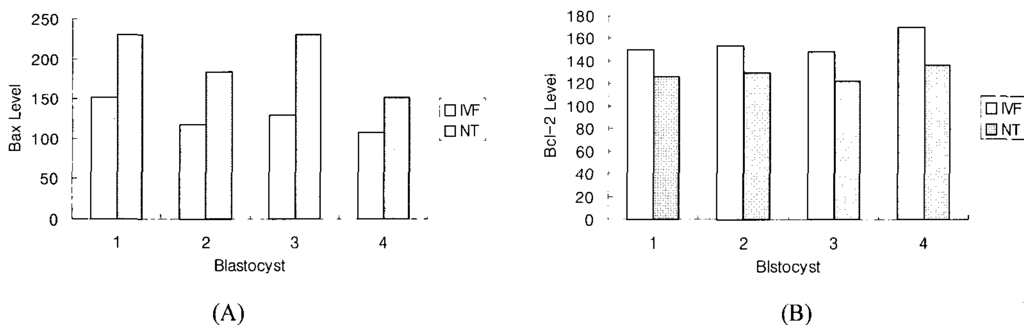

Fig. 4. Expression level of *Bax* (A) and *Bcl-2* (B) mRNA in blastocyst derived from IVF and NT ($P<0.05$). Values are mean ± SEM.

High expression of *Bax* gene was observed in blastocyst derived from NT.

Ⅳ. DISCUSSION

The results demonstrated the comparative index of apoptosis after bovine nuclear transfer and *in vitro* fertilization. Cleavage of DNA into oligonucleosome-sized fragments allows *in situ* labelling of

DNA strand breaks, combined with morphological characteristics, is commonly used to identify apoptosis (Savill, 1994; Negoescu et al., 1998). In this result, fragmented DNA detected by the TUNEL method was observed in bovine blastocyst derived from *in vitro* fertilization and nuclear transfer. The index of TUNEL staining was significantly higher in blastocysts derived from NT. Degenerated blastomeres are frequently observed in the late stage of embryos (Lindner and Wright, 1983; Plante and King, 1994), emphasizing that TUNEL staining in the present study reflects cell death. Futhermore, abundance of phagosomes has also been observed at the later stages, with all blastocysts displaying dead cells being phagocytosed by surrounding cell (Plante and King, 1994). Removal of degenerated cellular material by phagocytosis is considered as one of the most diagnostic features of apoptosis (MsConkey et al., 1996). Taken with the presence of other characteristic features including chromatin condensation, nuclear fragmentation, absence of neighboring cell destruction with TUNEL labelling, and extrusion of labelled cell into the blastocyst cavity, suggests that TUNEL labelling in this study is indication of apoptosis.

Apoptosis was first observed at the 8～16 cell stage of embryos with normal morphology. This was an unexpected observation since mouse embryos apoptosis does not occur at 8-cell stage or morulae derived from either *in vivo* or *vitro* (Handyside and Hunter, 1986; Brinson and Schultz, 1997). Similarly, Jurisicova et al. (1996) reported that human embryos from the 2-cell to the uncompacted morulae stage, was negative with regard to necrosis and apoptosis. It has been also reported that fragmentation was associated with apoptosis at the 1-cell stage during mouse embryonic development (Jurisicova et al., 1998). In this case, apoptosis may be related to the activation of embryonic genome. In mice and human, the major events take place at the 2 and 4-cell stage. However, in mice, activation of the embryonic genome is initiated at late stage of first cell cycle coincident with the first observation of apoptosis in this species. Apoptosis may ooccur due to damaged embryos that do not undergo appropriate activation of the embryonic genome. However, this does not explain the early expression of apototic genes in cow, as the embryos were neither fragmented nor abnormal. This proposal is supported by the high variation in the extent of apoptosis with transcription of early markers representing the activation of the embryonic genome in individual blastomeres (Byrne et al., 1999). Most embryos (90～100%) showed partial cell death from the early blastocyst to the hatched blastocyst stage compared with 2～16 stages (Byrne et al., 1999). Apoptotic index was inversely related to the number of cells at blastocyst stage.

There are two major protein families involved in the regulation of apoptosis: those that mediate the proteolytic breakdown of the cells, the caspase family; and those that regulate the activity of the caspases, the *Bcl-2* family (Thornberry and Lazebnik, 1998). Therefore, the *Bcl-2* family constitutes a cirtical intracellular checkpoint of apoptosis within a distal common cell death pathway (Oltvai et al., 1993; Cory and Adams, 1998). In this study, *Bax* and *Bcl-2* were detected in bovine blastocyst. The level of *Bcl-2* gene expression was much higher in blastocyst derived from IVF, whereas more *Bax* gene expression was observed in blastocyst derived from NT. These results suggest that blastocyst derived from NT are in a more advanced apoptotic process. Ginger et al. (1999) reported that maternal caspase are inherited during oogenesis and transcripts for some members of the *Bcl-2* family could be detected at every stage of preimplantation development. In addition, when fragmented blastocysts were compared to normal blastocysts, levels of *Bcl-2* gene expression tended to be lower in

fragmented blastocysts. Also, *Bcl-2* and *Bax* is related to their regulation of apoptosis during bovine oocyte maturation and early embryonic development (Ming Yuan and Rajadurai., 2002).

These results suggest that blastocyst derived from NT are more undergoing apoptotic process. However, no measurements of apoptosis were established in *in vivo* derived embryos and the results may not reflect the situation *in vivo*. Due to the complexity of NT procedures such as micromanipulation, NT procedures may cause embryonic damage, resulting in more increased apoptotic rate in cloned embryo. This finding indicates that measurements of apoptosis can be used as one of the markers for a useful tool to analyse the developmental potential in NT procedures. Also, this may be able to apply this knowledge to improving the overall efficiencies of NT.

Ⅴ. 요 약

포유동물의 체외수정란 최적발달조건을 수립하기 위하여 난자의 성숙, 수정 및 배양시 형태학적인 관찰만으로는 불충분하여 각 세포내 구성물 및 핵상 등의 변화를 조사하여 개별 수정란의 품질평가를 통하여 수정란 발달 능력을 향상시키는 방법의 개발이 필요하다. 최근 체외생산된 수정란이 발달율 및 착상율이 현저히 감소되는 원인을 조사한 결과 이러한 수정란에서 할구의 파편화가 보다 더 진행되는 것을 관찰하였다. 이에 본 실험의 목적은 체외수정과 핵치환 유래 소 배반포에서 세포사멸기전으로 알려진 apoptosis 조절 유전자로써 *Bcl-2*와 *Bax* 유전자의 전사체 발현량을 비교 조사함으로써 착상전 수정란 발달과정에서의 역할을 확립하고자 하였다.

Apoptosis의 분석은 TUNEL 방법으로 수행하였다. 체외수정과 핵치환유래 배반포에서 *Bcl-2*와 *Bax* 유전자의 발현량은 RT-PCR로 확인하였다. 핵치환유래 배반포에 있어 TUNEL 표식으로 확인된 비율은 체외수정된 배반포보다 유의하게 높다 (P<0.001). 체외수정된 배반포의 *Bcl-2*의 발현량은 핵치환유래 배반포보다 높다. 반대로 체외수정된 배반포의 *Bax* 발현량은 핵치환 유래 소 배반포보다 낮았다 (P<0.05). 이러한 결과는 체외수정보다 핵치환 유래 소의 배반포에서 좀더 많은 파편화를 초래함을 보여준다. 또한, 핵치환 유래 수정란의 발달 정지의 증가는 apoptosis와 같은 핵의 파편화로써 야기될 수 있다고 사료된다.

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