Protective Effect of BOEC Co-Culture System against Nitric Oxide on Development of Bovine IVM/IVF Embryos

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ABSTRACT

Somatic cells such as oviduct epithelial cell, uterine epithelial cell, cumulus-granulosa cell and buffalo rat river cell has been used to establish an effective culture system for bovine embryos produced in *in vitro*. But nitric oxide (NO) metabolites secreted from somatic cells were largely arrested the development of bovine *in vitro* matured/ *in vitro* fertilized (IVM/IVF) embryos, suggesting that NO was induced the embryonic toxic substance into culture medium. The objective of this study was to investigate whether BOEC co-culture system can ameliorate the NO-mediated oxidative stress in the culture of bovine IVM/IVF embryos. Therefore, we evaluated the developmental rate of bovine IVM/IVF embryos under BOEC co-culture system in the presence or absence of sodium nitroprusside (SNP), as a NO donor, and also detected the expression of growth factor (*TGF-β*, *EGF* and *IGFBP*) and apoptosis (*Caspase-3*, *Bax* and *Bcl-2*) genes. The supplement of SNP over 5 uM was strongly inhibited blastocyst development of bovine IVM/IVF embryos than in control and 1 uM SNP group (Table 2). The developmental rates beyond morulae stages of bovine IVM/IVF embryos co-cultured with BOEC regardless of SNP supplement (40.4% in 5 uM SNP+ BOEC group and 65.1% in BOEC group) were significantly increased than those of control (35.0%) and SNP single treatment group (23.3%, $p<0.05$: Table 3). The transcripts of *Bax* and *Caspase-3* genes were detected in all experiment groups (1:Isolated fresh cell (IFC), 2:Primary culture cell (PCC), 3:PCC after using the embryo culture, 4: PCC containing 5 uM SNP and 5: PCC containing 5 uM SNP after using the embryo culture), but *Bcl-2* gene was not detected in IFC and PCC (Fig. 1). In the expression of growth factor genes, *TGF-β* gene was found in all experimental groups, and *EGF* and *IGFBP* genes were not found in IFC and PCC (Fig. 2). These results indicate that BOEC co-culture system can increase the development beyond morula stages of bovine IVM/IVF embryos, possibly suggesting the alleviation of embryonic toxic substance like nitric oxide.

(Key words : Bovine oviduct epithelial cell, Bovine IVM/IVF embryo, SNP, Nitric oxide, Growth factor, Apoptosis gene)

INTRODUCTION

Co-culture system that used for culturing the embryo in *in vitro* is a potentially simple model system mimicking the *in vivo* environment to overcome the *in vitro* cell block events and to obtain a large number of good qualities embryos derived from IVM/IVF oocytes. Co-culture system may exert a positive influence on early development by the secretion of embryotrophic factors into culture medium or by reduction of the negative effects of toxic components of the culture environment. It seems likely that embryo co-culture is effective by both of these routes(Winger *et al.*, 1997). The feeder cells are produced the some kinds of growth factors and other specific proteins to safely develop the embryos produced by *in vitro*. Growth factors, cytokines and other proteins are representative embryotrophic factors secreted by feeder cells. The major embryotoxic factors in *in vitro* culture system may be the reactive oxygen species (ROS) and deleterious heavy metal ions into the culture medium. ROS is the greatest factor of damaging and/or retarding the embryo development *in vitro* (Ashok *et al.*, 2003; de Lamirande and Gagnon, 1992). At physiological concentrations, hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical (OH^-) and nitric oxide (NO^-) are considered the major ROS that participate in redox reactions in diverse biological processes (Hancok *et al.*, 2001).

Nitric oxide (NO) plays an important role in the regulation of various metabolic pathways and reproductive phenomenon in mammals (Brune *et al*, 1995). NO

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is a gaseous, free-radical molecule that act as an intracellular messenger and regulates various reproductive process, such as steroidogenesis, pregnancy, folliculogenesis, and tissue remodeling. NO is produced in human placenta, decidua and the endometrium during pregnancy (Norman and Cameron, 1996) and regulate the preimplantation embryo development through regulation of mitotic division (Tranguch *et al*, 2003). NO is not only required for normal preimplantion embryo development but must be produced within a limited range of concentrations. Excess NO halts embryo development, possibly through the production of free radicals and lead to development arrest and/or apoptosis of embryos (Chen *et al.*, 2001). Thus NO prevents embryos from developing beyond the two cell stage in mouse (Nathan and Xie, 1994). Early embryonic loss is associated with local production of NO by the decidual cell in the mouse uterus (Gouge *et al.*, 1998).

Therefore, the objectives of the present studies were to examine the protective effects of the BOEC co-culture system against nitric oxide in *in vitro* culture condition of bovine IVM/IVF embryos, and to detect the temporal expression patterns of growth factor genes (*TGF-β*, *EGF* and *IGFBP*) and apoptosis genes (*Bax*, *Caspase-3* and *Bcl-2*) in BOEC used or not used in *in vitro* culture of bovine IVM/IVF embryos.

MATERIAL AND METHODS

In Vitro Production of Bovine IVM/IVF Embryos

The cumulus-oocyte complexes (COCs) aspirated from the ovaries obtained from an abattoir were matured in TCM 199 (Gibco, USA) that were supplemented with 10% fetal bovine serum (FBS, Gibco), 1 ug/ml estradiol-17β, 5ug/ml luteinizing hormone, 0.5 ug/ml follicle stimulating hormone at 38.5℃ in a humidified 5% CO_2 in air. Following the maturation, 10~15 matured oocytes were transferred to 50 ul of fertilization drops (BO medium; Brackett and Oliphant, 1975). Frozen/thawed Hanwoo semen were washed in BO medium containing 10 mM caffeine and 50 ul sperm suspension was introduced into each fertilization drops. Presumptive zygotes produced at 6~8 hr after insemination were cultured for 34~36 hr in CR1aa medium and 2- and 8-cell embryos were randomly allocated into each experimental groups. The development effects of the NO donor were examined by determining the appearance and developmental stages on day 7~8 of culture in the following concentration groups : 0, 1, 5, 10 and 25 uM SNP. The total cell numbers of blastocysts were evaluated by Hoechst 33342 staining. All chemicals used in this study were purchased from Sigma-Aldrich (USA) unless otherwise stated.

Isolation and Primary Culture of Bovine Oviduct Epithelial Cell (BOEC)

Bovine oviducts were transported on ice from the slaughterhouse to the laboratory within 1~2 hrs. The oviducts were carefully trimmed on an ice and rinsed with phosphate- buffered saline containing 1% antibiotics and antimycotic solution (Gibco). The epithelial cells were dispersed by forcing medium through an 18-gauge needle attached to a 10 ml syringe and then the samples were transferred into 50 ml conical tubes containing 25 ml of Dulbecco's modified eagle medium (DMEM-low-glucose, Gibco) with 10% FBS and washed twice by centrifugation (200 ×g) at 4℃. Cell was adjusted at 3×10^6 cell/ml with DMEM with 10% FBS. The cell pellet was then equally dispensed as 1 ml volumes into 4-well tissue culture dish (Nunc, Denmark) and cultured at 38.5℃ in 5% CO_2 in humidified air. The medium of the culture was changed by fresh medium in every two days. Confluent monolayers with some ciliary activity formed within 2~3 days were affirmed and cultured with or without embryos for 6~7 days, and the other one were used for gene detection.

RNA Isolation of BOEC

Total RNA was extracted from isolated of BOEC or BOEC monolayer used or not used the embryo culture by guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Oviductal cells were placed into extraction solution (0.025 M sodium citrate, 0.5% sarcosyl, 4 M guanidiumthiocyanate, pH 7.0) and were vortexed with phenol, chloroform : isoamyl alcohol (24:1) and 2M NaOAC, centrifuged with 20,000 ×g at 4℃ for 20 min and transferred the supernatant to the new tube. After adding isopropanol, the tube was shaked gently and then precipitated with isopropanol at −20℃ for overnight. The samples were then centrifuged, washed with 70% ethanol, recentrifuged and air-dried before being dissolved in diethylprocarbonate (DEPC) treated distilled water. The yield of extracted total RNA for each sample was determined by UV spectrophotometer at 260 nm.

Reverse-transcription Polymerase Chain Reaction (RT-PCR) Analysis

The mRNA collected from the total RNA sample was analyzed to detect the genes by the RT-PCR. Reverse-transcriptase generation of cDNA was performed on 2 ug of total RNA, a final volume of 2 ul, the reverse transcription reagents (RT mixture), RT buffer, 0.5 mM of each dNTP, 0.5 ug oligo(dT), 10IU RNase-inhibitor (Gibco) and 500IU reverse transcriptase (Ambion) carried out at 42℃ for 60min followed by denaturation for 10min at 98℃ and the remaining of RT products were storage at 4℃. Subsequent PCR analy-

Table 1. Oligonucleotide primer sequence for growth factors and apoptosis genes

mRNA	Type of primer	Primer sequence (5' 3')	Annealing temperature (℃)	Product size (bp)	Genebank accession number
TGF-β	Forward	GCGAGCGCAGCGAGGAATACT	66	687	L08375
	Reverse	GGCACGCCCCGGCACAGAAG			
EGF	Forward	GCACCCATGGCAGAAGGAGA	55	718	X81380
	Reverse	TTTTTGCAGGAACATTTACACG			
IGFBP	Forward	CCGAGGAGGACCGCAGTGTAG	58	455	AF085482
	Reverse	ATCGTGTCCTTGGCAGTCTTTTGT			
Bax	Forward	GCCCCTGTCGTCCTTTGTCC	63	678	AF098067
	Reverse	TGGCGAGGAGCTGGTGCTGG			
Bcl-2	Forward	CGACTTTGCAGAGATGTCCA	63	274	L14680
	Reverse	TAGTTCCACAAAGGCATCCC			
Caspase-3	Forward	GAAGCAAATCAATGGACTCTGGA	63	509	AB029345
	Reverse	GTCTGCCTCAACTGGTATTTTCTG			
B-actin	Forward	ATCACCATCTTCCAGGAGCG	58	280	AF261085
	Reverse	GATGGCATGGACTGTGGTCA			

sis was performed on 2 ul of the cDNA in final volume of 20 ul, the PCR mixtures consisted of PCR buffer, 0.2 mM dNTP, 10 uM primer and 2 IU Taq polymerase. PCR was then carried out for 3 min at 95 ℃, 50 sec at 60℃, 1 min at 72℃. Primer sequences used in this study are indicated in Table 1. For each sample, PCR amplification products was analyzed on 2.0% agarose gel stained with 0.5ug/ml ethidium bromide, visualized under UV light and photographed. Standard DNA markers(1kb DNA ladder) were also used to determine the size of amplified products.

Statistical Analysis

The SAS mixed linear model program was used to analyze the data. Percentage of developmental stages and cell numbers of blastocyst in each treatment were compared for differences through use of Duncan's modified multiple range tests. Values at 5% level or less ($p<0.05$) were considered statistically significant.

RESULTS

The effects of sodium nitroprusside (SNP), a nitric oxide donor, on the development of bovine IVM/IVF embryos were summarized in Table 2.

No effects of SNP were observed on development to the morula stage. Treatment of 5 uM SNP group or more (5 uM, 19.4%; 10 uM, 13.9%; 25 uM. 0%) in the developmental rates of blastocyst were significantly dropped when compare to control(36.1%) and 1 uM SNP groups (33.3%, $p<0.05$). Out of 36 zygotes, there was not developed to the blastocyst in 25 uM SNP group. The total cell numbers of blastocysts were decreased according to the increasing the dose of SNP.

The protective effects of BOEC co-culture system against SNP on development of bovine embryos was shown in Table 3.

Protective effects of BOEC co-culture system against SNP on formation of blastocyst was significantly higher in treatment of 5 uM SNP with BOEC monolayer groups (19.4%) than in control (10.0%) and in 5 uM SNP single groups (3.3%, $p<0.05$). But, 5 uM SNP with BOEC monolayer groups (40.4%) on the developmental rates beyond morula stage was not differ to control groups (35.0%). In the cell numbers of blastocyst, BOEC co-culture groups irrespective of SNP supplemention (85.4±4.8 in BOEC co-culture group and 83.8±4.3 in BOEC plus 5 uM SNP group) was slightly larger than those of BOEC-free groups (80.7±3.1 in control and 75.8±5.5 in 5 uM group).

To establish an mRNA phenotypic map for the expression of various growth factors and apoptosis genes, BOEC in the presence or absence of SNP used or not used for embryo culture were analyzed to detect the genes by RT-PCR.

Fig. 1 displayed the expression patterns for apop-

Table 2. Effects of SNP on *in vitro* development of bovine IVM/IVF embryos

SNP (μM)	No. of IVM/IVF embryos	No. of embryos developed to (%, Mean±SD)			Cell no. of blastocysts
		Pre-morulae	Morulae	Blastocysts	
0	36	13(36.1±12.0^{c})	10(27.8±2.9^{a})	13(36.1±9.6^{a})	84.4±4.6^{a}
1	36	15(41.7±8.3^{bc})	9(25.0±0.0^{a})	12(33.3±8.3^{a})	82.8±3.8^{ab}
5	36	19(52.8±6.1^{bc})	10(27.8±12.7^{a})	7(19.4±9.7^{b})	78.4±5.9^{bc}
10	36	22(61.1±2.7^{ab})	9(25.0±9.1^{a})	5(13.9±8.3^{b})	74.1±8.0^{c}
25	36	29(80.6±11.0^{a})	7(19.4±11.0^{a})	0(0±0.0^{c})	0.0±0.0^{d}

$^{a\sim d}$ Values with different superscripts within columns are significantly differ, $p<0.05$.

Table 3. Effects of SNP and BOEC co-culture system on the development of bovine IVM/IVF embryos

SNP (μM)	BOEC	No. of IVM/IVF embryos	No. of embryos developed to (%, Mean±SD)			Cell no. of blastocysts
			Pre-morulae	Morulae	Blastocysts	
0	-	60	39(65.0±1.8^{b})	15(25.0±6.3^{b})	6(10.0±4.5^{c})	80.7±3.1^{b}
0	+	63	22(35.0±6.2^{c})	23(36.5±8.3^{a})	18(28.6±3.4^{a})	85.7±4.8^{a}
5	-	60	47(78.3±6.9^{a})	11(18.3±9.1^{c})	2(3.3±2.7^{c})	75.8±5.5^{c}
5	+	62	37(59.7±2.4^{b})	13(21.0±7.2^{bc})	12(19.4±4.8^{b})	83.8±4.3^{ab}
Overall mean						
BOEC	-	120	86(71.5±8.5^{A})	26(22.0±7.8^{A})	8(6.5±5.0^{B})	
	+	125	59(47.3±14.2^{B})	36(28.8±11.1^{A})	30(23.9±6.2^{A})	
SNP	0	123	61(19.9±17.0^{B})	38(31.0±9.1^{A})	24(19.2±10.8^{A})	
	5	122	84(68.9±11.1^{A})	24(19.9±7.4^{B})	14(11.3±9.5^{A})	

^{a,b,c,A,B} Value with different superscripts within columns are significantly differ, $p<0.05$.

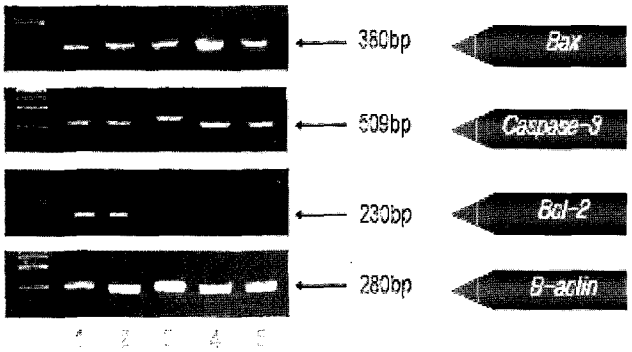

Fig. 1. mRNA expression of apoptosis genes on BOEC with or without SNP used or not used for *in vitro* culture of bovine embryos. 1: IFC, 2: PCC, 3: PCC after using the embryo culture, 4: PCC containing 5 uM SNP, 5: PCC containing 5 uM SNP after using the embryo culture.

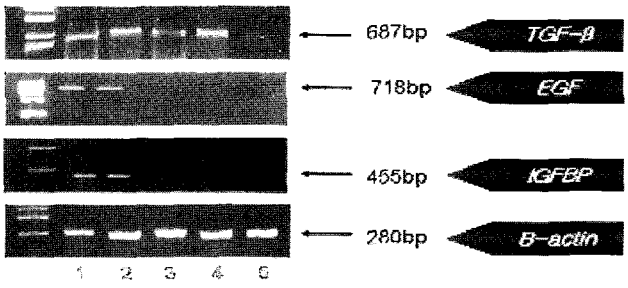

Fig. 2. mRNA expression of growth factor genes on BOEC with or without SNP used or not used for *in vitro* culture of bovine embryos. 1: IFC, 2: PCC, 3: PCC after using the embryo culture, 4: PCC containing 5 uM SNP, 5: PCC containing 5 uM SNP after using the embryo culture.

tosis genes in BOEC treated with or without SNP used or not used for embryo culture. Expression of *Bcl-2* was detected in IFC and PCC groups, the other side, *Bax* and *Caspase-3* gene were expressed in all treatment groups.

Fig. 2 was shown the expression patterns for gr-

owth factor genes in BOEC treated with or without SNP used or not used for embryo culture. Transcripts for *EGF* and *IGFBP* were expressed in IFC and PCC groups. And, *TGF-β* gene was detected in all treatment groups.

DISCUSSION

Recently, developmental rates to blastocysts in simple culture media in *in vitro* remain low and range between 20~30 percent in bovine embryo culture (Carolan *et al.*, 1996; Krisher *et al.*, 1999). Various factors including the oocyte itself, protein source, somatic cells, culture media, oxygen tension and energy substrates may affect preimplantation embryo development *in vitro* (Bavister, 1995). ROS have been implicated as major cause of embryonic arrest and cell death (Ashok *et al.*, 2003). ROS are highly reactive with complex cellular molecules such as proteins, lipids and DNA, and cause serious dysfunction such as enzyme inactivation, mitochondrial abnormality or DNA fragmentation (Guerin *et al.*, 2001). ROS are derived from the metabolism of molecular oxygen and included superoxide anion radical, singlet oxygen, hydrogen peroxide, hydroxyl radical and nitric oxide radical. The super oxide anion, hydrogen peroxide and nitric oxide are considered the major reactive species that participate in redox reactions in diverse biological processes (Hancok *et al.*, 2001). In diverse cell types, the intracellular redox state is important in regulation of enzymes presumably involved in single transduction mechanism (Hancok *et al.*, 2001).

NO is a free radical molecule that has been demonstrated to be an intracellular messenger and regulates smooth muscle cell tone, platelet aggregation and adhesion, cell growth, apoptosis, neurotransmission and injury as well as infection-induced immune reactions (Rosselli *et al.*, 1998). NO acts through the cGMP pathway. NO mediates cGMP signal transduction system and also mediates its effects through stimulation of cyclooxygenase enzymes in order to ensure the normal preimplantation embryo development and pregnancy. The wide distribution of NOS in diverse cells of the female and male reproductive organs (Rosselli, 1997) suggests a major role for NO in the physiological regulation of reproduction. The critical concentration of NO and cGMP is required for normal embryo development and enhance the developmental rates through regulation of mitotic division in these embryos. However, excess NO halts or retards embryo development and/or apoptosis of the mouse embryo (Tranguch *et al*, 2003), possibly through the production of free radicals.

Our study were evaluated the effects of SNP on developments of Hanwoo IVM/IVF embryos. In different concentration of SNP (1~25 uM), a nitric oxide donor, treatment of 5 uM SNP group were significantly dropped when compare to control and 1 uM SNP groups in the developmental rates of blastocyst ($p<0.05$). The development rate to blastocysts in SNP groups supplemented more than 5 uM were significantly decreased in control and 1 uM SNP groups ($p<0.05$), and cell numbers of blastocyst was similar trend to the blastocyst development results. This results is in agreements with Lim and Hansel (1998), and Orsi (2006) suggesting that preimplantation *in vitro* embryo developments were inhibited by NO supplementation (more than 10 uM SNP). This finding of an inhibitory role of NO in early development is confirmed by our results showing that the presence of a NO donor (SNP) in the culture system greatly inhibits embryo development beyond morula stage.

Many researcher has been endeavored to develope the culture methods to remove and/or ameliorate the embryotoxic factor such as a free radical. Antioxidative enzymes play a role in protecting cell from oxidative stress-induced cell death (Chandra *et al.*, 2000). Co-culture system using somatic cell can improved the development of bovine IVM/IVF embryos, possibly through the releasing of the embryotropic factors and the removing or alleviating of the embryotoxic substance into culture medium (Liu *et al.*, 1998). NO produced from somatic cell has an inhibitory role in preimplantation development of IVF-derived embryos. It is necessary to develop an effective method for removal of embryotoxic NO during embryo culture. We hypothesized that BOEC co-culture system can improve the developments of bovine embryos during *in vitro* culture, indicating the removing and/ or ameliorating of the embryotoxic factors.

Our study were examined the protective effects of BOEC co-culture system against nitric oxide on the development of bovine IVM/IVF embryos. The protective effects of BOEC co-culture system against nitric oxide on development of embryos was significantly higher in treatment of 5 uM SNP with BOEC monolayer groups than in control and in 5 uM SNP single groups ($p<0.05$). This study agrees with the finding of some researchers (Joo *et al.*, 1999; Lim and Hansel, 1998) that SNP was inhibited the development of bovine or mouse embryos *in vitro*. This study agreed with the findings of Gandolfi *et al.* (1989) reported that the beneficial effects of blastocyst development are thought to be due to embryotrophic factors provided by the epithelial cells. The results of this study suggest that co-culture system most likely affects embryo development by scavenging NO, but specific mechanism is still unclear.

Our efforts have focused on defining possible embryotrophic and/or detoxifying role of oviduct by characterizing the expression of mRNAs encoding growth

factors transcripts and apoptosis-related genes in primary bovine oviductal cultures.

In co-culture system, somatic cells derived from female reproductive organ may secrete embryotrophic factors, which may include growth factors such as *IGF, TGF* and *EGF* (Watson *et al.*, 1994), and antioxidant enzymes (Harvey *et al.*, 1995) and apoptosis-related substances. But extensive biochemical and morphological studies have revealed several different mechanisms involved in the induction of embryonic developmental factors, the key events underlying the development of embryos remain largely unknown.

Thus, we examined whether growth factor and apoptosis-related genes were expressed in BOEC treated with or without SNP, which was used or not used in *in vitro* culture of bovine IVM/IVF embryos. In the expression of growth factors, *TGF-β* gene was detected in all experimental groups. Also, transcripts for *EGF* and *IGFBP* were expressed in IFC and PCC groups. This results of our study agreed with results of Watson *et al.* (1994) and Winger *et al.* (1997) reported that primary bovine oviductal epithelial cells express the *EGF, TGF-β* and *IGFBP*. Our study was detected different patterns of mRNA expression for apoptosis genes in BOEC in the presence or absence of SNP, which was used or not used for embryo cultures. Apoptosis gene such as *Bax* and *Caspase-3* were observed in all experimental groups. On the other hand, transcription for *Bcl-2* was detected in IFC and PCC groups.

In conclusion the present study demonstrates that the co-culture system of bovine oviduct epithelial cell can improve the developmental rates of bovine IVM/IVF embryos, indicating that the alleviation of embryotoxic factor such as nitric oxide.

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REFERENCES

1. Ashok BT, David L, Chen YG, Garikapaty VP, Chander B, Kanduc D, Mittelman A, Tiwari RK (2003): Peptide mitmotopes of on oncoproteins as therapeutic agents in breast cancer. Int J Mol Med 11: 465-471.
2. Bavister BD (1995): Culture of preimplantation embryos: facts and artifacts. Hum Reprod Update 1: 91-148.
3. Brackett BG, Oliphant G (1975): Capacitation of rabbit spermatozoa *in vitro*. Biol Reprod 12:260-274.
4. Brune B, Mebmer UK, Sandau K (1995) The role of nitric oxide in cell injury. Toxico Letter 83:233-237.
5. Carolan C, Lonergan P, Khatir H, Mermillod P (1996): *In vitro* production of bovine embryos using individual oocytes. Mol Reprod Dev 45:145-150.
6. Chandra J, Samali A, Orrenius S (2000): Triggering and modulation of apoptosis by oxidative stress. Free Rad Biol Med 29:323-333.
7. Chen HW, Jiang WS, Tzeng CR (2001): Nitric oxide as a regulator in pre-implantation embryo development and apoptosis. Fertil Steril 75:1163-1171.
8. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159.
9. de Lamirande E, Gagnon C (1992) Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. J Androl 13:368-378.
10. Gandolfi F, Brevini TA, Richardson L, Brown CR, Moor RM (1989) Characterization of proteins secreted by sheep oviduct epithelial cells and their function in embryonic development. Development 106:303-312.
11. Gouge RC, Marshburn P, Gordon BE, Nunley W, Huet-Hudson YM (1998): Nitric oxide as a regulator of embryonic development. Biol Reprod 58: 875-879.
12. Guerin P, El Mouatassim S, Menezo Y (2001): Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 7:175-189.
13. Hancock J, Desikan R, Neill S (2001) Role of oxygen species in cell signaling pathways. Biochem Soc Trans 29:345-350.
14. Harvey MB, Arcellana-Panlilio MY, Zhang X, Schultz GA, Watson AJ (1995): Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary bovine oviduct cultures employed for embryos coculture. Biol Reprod 53:530-538.
15. Joo BS, Park SH, Park SJ, Kang HS, Moon HS, Kim HD (1999) The effect of nitric oxide on sperm cell function and embryo development. J Reprod Immunol 42:327-334.
16. Krisher RL, Lane M, Bavister BD (1999): Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. Biol Reprod 60:1345-1352.
17. Lim JM, Hansel W (1998): Improved development of *in vitro*-derived bovine embryos by use of a nitric oxide scavenger in a cumulus-granulosa cell coculture system. Mol Reprod Dev 50:45-53.

18. Liu LPS, Chan STH, Ho PC, Yeung WSB (1998) Partial purification of embryotrophic factors from human oviductal cells. Hum Reprod 13:1613-1619.
19. Nathan C, Xie QW (1994): Regulation of biosynthesis of nitric oxide. J Biol Chem 269:13725-13728.
20. Norman JE, Cameron IT (1996): Nitric oxide in the human uterus. Rev Reprod 1:61-68.
21. Orsi NM (2006): Embryotoxicity of the nitric oxide donor sodium nitroprusside in preimplantation bovine embryos *in vitro*. Anim Reprod Sci 91(3-4):225-236.
22. Rosselli M (1997): Nitric oxide and reproduction. Mol Hum Reprod 3:639-642.
23. Rosselli M, Keller PJ, Dubey RK (1998): Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. Hum Reprod Update 4:3-24.
24. Tranguch S, Steuerwald N, Huet-Hudson YM (2003): Nitric oxide synthase production and nitric oxide regulation of preimplantation embryo development. Biol Reprod 68:1538-1544.
25. Watson AJ, Watson PH, Arcellana-Panlilio M, Wares D, Walker SK, Schultz GA, Armstrong DT, Seamamark RF (1994): A growth factor phenotype map for ovine preimplantation development. Biol Reprod 50:725-733.
26. Winger QA, Rior P, Han VKM, Armstrong DT, Hill DJ, Watson AJ (1997): Bovine oviductal and embryonic insulin-like growth factor binding proteins: Possible regulators of embryotrophic insulin-like growth factor circuits. Biol Reprod 56:1414-1423.

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