

9-1-1995

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Harvey, M B; Arcellana-Panlilio, M Y; Zhang, X; Schultz, G A; and Watson, A J, "Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary bovine oviduct cultures employed for embryo coculture." (1995). *Obstetrics & Gynaecology Publications*. 84.

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## Expression of Genes Encoding Antioxidant Enzymes in Preimplantation Mouse and Cow Embryos and Primary Bovine Oviduct Cultures Employed for Embryo Coculture<sup>1</sup>

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### ABSTRACT

Preimplantation embryos from a variety of mammalian species contrast markedly in their response to culture in vitro. Murine preimplantation embryos display a wider tolerance than other mammalian species to culture environments, and this has contributed to the development of several effective defined culture media. Embryo coculture on somatic cells remains the most effective method of supporting reasonable rates of bovine preimplantation development in vitro. The patterns of gene expression for several antioxidant enzymes during preimplantation murine and bovine development were examined by use of the reverse transcription-polymerase chain reaction technique to determine whether the differential developmental capacity of mammalian preimplantation embryos in culture may reflect variations in the patterns of expression for a series of antioxidant enzymes. Transcripts for catalase, CuZn-containing superoxide dismutase (CuZn-SOD), Mn-SOD, glutathione peroxidase (GPX), and glutamylcysteine synthetase (GCS) were detected in mouse embryos at all stages of development regardless of in vivo or in vitro development. Preimplantation cow embryos produced by in vitro procedures expressed mRNAs for catalase, CuZn-SOD and GPX, whereas transcripts for Mn-SOD were not detected at any stage. GCS transcripts, although present in stages up to the morula, were not detected in cow blastocysts. Analysis of antioxidant gene expression in both bovine primary oviductal cell monolayer cultures and nonattached, ciliated oviductal cell vesicle cultures revealed a constitutive pattern of expression of all five enzymes for the 8-day culture interval. These experiments suggest that differences in gene expression may contribute to the variation in the ability of embryos to develop in vitro with respect to levels of oxygen and dependence on coculture.

### INTRODUCTION

Preimplantation embryos from several mammalian species can be removed from the female reproductive tract and successfully cultured in vitro. However, the efficiency with which this is achieved varies markedly among species [1–15]. Mouse embryos develop in simple defined media supplemented with protein and carbohydrate substrates [14, 15], whereas the majority of sheep and cow embryo culture media include serum and utilize embryo coculture with somatic cells to support development to the blastocyst stage [1–10]. This role of coculture may be influenced by the culture atmosphere (O<sub>2</sub> level) since high rates of ovine preimplantation embryo development occur when ovine embryos are cultured in medium alone under a reduced O<sub>2</sub> (5% CO<sub>2</sub>:5% O<sub>2</sub>:90% N<sub>2</sub>) culture atmosphere [6–8].

Recently, free oxygen radicals (FORs) have been implicated as major causes of embryonic arrest and cell death [16, 17]. Biological systems have evolved several mecha-

nisms to offset the potential deleterious effects of FORs, including iron-binding proteins, free radical scavengers, and enzyme systems that quickly convert FORs into innocuous intermediate compounds [16–23]. Mammalian preimplantation embryos could vary in their capacity to reduce these deleterious effects of exposure to FORs, and this may contribute, in part, to their differential capacities to sustain development in vitro. The aim of this study was to define the temporal patterns of expression of genes encoding the principal antioxidant enzymes that function intracellularly to neutralize the damaging effects of FORs. The quantity of research material that can be obtained from preimplantation embryos is quite limited [24]. For this reason, mRNA phenotyping by reverse transcription-polymerase chain reaction (RT-PCR) was employed because it is sufficiently sensitive to allow characterization of the patterns of expression of low-abundance transcripts in small amounts of starting material [25–27]. Our analysis was extended to include both bovine primary oviductal cell monolayer cultures and non-attached oviductal cell vesicle cultures. These primary oviduct cultures display different capacities for facilitating bovine preimplantation development in vitro [1, 7, 28–32]. Bovine zygotes cultured on oviductal monolayers must be moved to fresh cultures every 72 h to sustain their development [1], whereas microdrop cultures containing the non-attached oviductal vesicles support development through the first week without transfer to fresh cultures [32]. The present study, therefore, also examined whether these two

Accepted May 3, 1995.

Received March 1, 1995.

<sup>1</sup>This research was supported by grants MT-4854 to G.A.S. and MT-12107 to X.Z. from the Medical Research Council (MRC) of Canada, and by NSERC Canada Operating grants to X.Z. and A.J.W. M.B.H. is the recipient of a postdoctoral fellowship from the Alberta Heritage Foundation for Medical Research (AHFMR). X.Z. and A.J.W. are Scholars of the MRC of Canada.

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primary bovine oviduct cultures vary in their expression of antioxidant enzyme genes during an 8-day culture interval. The results suggest that differences in gene expression may contribute to variation in the ability of mammalian embryos to develop in vitro with respect to ambient levels of oxygen and dependence on coculture.

## MATERIALS AND METHODS

### *Mouse Embryo Recovery and Culture*

Fertilized eggs, 2-cell embryos, morulae, and blastocysts were collected at 24 h, 48 h, 72 h, and 96 h after 7.5 IU hCG administration from mated, superovulated CD-1 mice (Charles River Breeding Laboratories, Lachine, PQ, Canada). To obtain cultured embryos, fertilized eggs were collected (24 h post-hCG) and cultured in M1G medium [33] containing 10 mM EDTA for 24 h, 48 h, and 72 h to reach 2-cell embryo, morula, and blastocyst stages, respectively.

### *Bovine Embryo and Oviductal Cell Culture*

Bovine embryos were produced by in vitro fertilization of in vitro-matured oocytes collected from slaughterhouse ovaries as previously described [1, 7, 32]. Embryos representing several stages of preimplantation development were obtained after coculture of fertilized eggs with bovine nonattached oviductal vesicles. Cumulus-oocyte-complexes (COCs) were collected from slaughterhouse ovaries by a razor blade slashing technique that perforates the follicular wall and releases its contents. The follicular contents were pooled, and the COCs were collected under a dissecting microscope prior to four washes with oocyte collection medium (Hepes-buffered TCM-199 [Gibco, Grand Island, NY] + 2% serum). The washed COCs were placed into maturation medium (TCM-199 + 10% [v/v] fetal bovine serum [FBS; Gibco] supplemented with sodium pyruvate [35 µg/ml; Sigma Chemical Company, St. Louis, MO], 5 µg/ml FSH [Follitropin; Vetrapharm, London, ON], 5 µg/ml LH [Vetrapharm], and 1 µg/ml estradiol-17β [Sigma]) for 24 h at 38.6°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. The matured oocytes were fertilized in vitro employing frozen-thawed bovine semen (Semex Canada Inc., Guelph, ON, Canada) using standard "swim-up" procedures [1, 25, 34]. The COCs were removed from maturation medium and washed four times in Hepes-buffered modified Tyrode's solution prior to their placement into pre-equilibrated fertilization drops (50 COCs/300 µl drop) consisting of bicarbonate-buffered modified Tyrode's solution [34] under light paraffin oil (BDH Inc., Toronto, ON, Canada). After "swim-up," approximately 90 µl of concentrated sperm solution (or  $2.25 \times 10^5$  motile spermatozoa) was added to each fertilization drop. The sperm/COC droplets were incubated for 18 h at 38.6°C in a humidified 5% CO<sub>2</sub> in air atmosphere before removal of the remaining cumulus cell

investment. The ensuing zygotes were placed into 50-µl culture drops of TCM-199 medium + 10% FBS under oil, into which up to 40 nonattached ciliated primary oviduct epithelial cell vesicles were added [32]. The oviductal vesicle cocultures supported bovine preimplantation development to the hatched blastocyst stage without requiring embryo transfer to fresh cultures. The cultures were maintained by the addition of a further 50 µl of TCM-199 + 10% FBS medium to each embryo coculture drop after 48 h [32]. All of the collected COCs were used in this study, and no oocyte selection strategy was employed. These culture conditions routinely supported an overall cleavage rate of 70% of matured oocytes, with up to 25% of the matured oocytes progressing to the blastocyst stage after an 8-day culture interval.

### *Establishment of Primary Oviductal Epithelial Cell Cultures*

The bovine oviduct primary cell cultures were established as outlined [1, 32]. The oviducts were trimmed of all attached fat and connective tissue prior to two washes in Hanks' Balanced Salt Solution (HBSS, Gibco). For establishment of monolayer cultures, the epithelial cells were isolated by filling the oviduct lumen with 0.05% trypsin in Ca<sup>2+</sup>-, Mg<sup>2+</sup>-free HBSS (Gibco) and incubating at 38.5°C for 20 min. The oviduct contents were squeezed into a 35-mm Petri dish. The cells were dispersed by forcing them through an 18-gauge needle attached to a 5-ml syringe three times before the samples were transferred into 15-ml conical tubes containing 10 ml of HBSS for washing by centrifugation. The cells were resuspended in fresh HBSS and washed three more times before final resuspension in the appropriate volume of TCM-199 medium + 10% FBS. The cultures were established by addition of  $1 \times 10^6$  cells per well of a 24-well plate containing 1 ml of TCM-199 + 10% FBS medium per well. By 48 h, approximately 50% of the surface of each well was covered by attached cells. By 72 h, the monolayers were confluent. The cultures were maintained for up to eight days by removing the old medium and adding 1 ml of fresh culture medium every 48 h.

The nonattached epithelial cell vesicles were isolated by simply squeezing the epithelial cell sheets from the oviduct into 15-ml plastic centrifuge tubes containing 10 ml of HBSS. Trypsin digestion was not employed in the establishment of these cultures. The isolated epithelial cell sheets were washed four times in HBSS prior to the addition of up to 70 µl of concentrated epithelial cell sheet suspension per 35-mm Petri dish containing 3 ml of TCM-199 + 10% FBS medium. The cell sheets were cultured for 24 h under an atmosphere of 5% CO<sub>2</sub> in air at 38.6°C. During this interval, the epithelial sheets formed ciliated tubular vesicles that actively moved throughout the culture dish. Up to 40 active epithelial vesicles were selected and transferred into the embryo culture drops to support development to the blas-

TABLE 1. Antioxidant enzyme PCR primer sequences.

Gene	Primer sequence	Reference	Fragment size (bp)	Restriction diagnosis	
				Enzyme	Size of fragments (bp)
Catalase	5' Primer = 5'GCAGATACCTGTGAAGTCTC3' 3' Primer = 5'GTAGAATGTCCGCACCTGAG3'	40	229	<i>Sph</i> 1	66, 163
Mn-SOD	5' Primer = 5'GCACATTAACGCGCAGATCA3' 3' Primer = 5'AGCCTCCAGCAACTCTCCTT3'	38	241	<i>Rsa</i> 1	85, 156
CuZn-SOD	5' Primer = 5'AAGGCCGTGTGCGTGCTGAA3' 3' Primer = 5'CAGGTCTCCAACATGCCTCT3'	38	246	<i>Pst</i> 1	170, 76
GPX	5' Primer = 5'CCTCAAGTACGTCGACCTG3' 3' Primer = 5'CAATGTCGTTGCGGCACACC3'	37	197	<i>Taq</i> 1	30, 167
GCS	5' Primer = 5'CCTTCTGGCACAGCACGTTG3' 3' Primer = 5'TAAGACGGCATCTCGCTCTC3'	39	346	<i>Hinf</i> 1	82, 260

tocyst stage. Cultures were maintained by adding an additional 50  $\mu$ l of fresh culture medium to each embryo culture drop after 48 h, and the cultures were maintained for an 8-day interval.

#### RNA Extraction

Total RNA was prepared from pools of 50–150 preimplantation mouse embryos collected in vivo and from similar numbers of in vitro-produced mouse and cow embryos in the presence of 10  $\mu$ g of carrier *Escherichia coli* ribosomal RNA (Boehringer-Mannheim, Laval, PQ, Canada) by phenol-chloroform extraction and ethanol precipitation as previously described [25–27, 35]. Bovine zygotes were pooled into the following groups: 1) 1-cell (zygotes); 2) 6–8-cell stage; 3) Day 6 morulae; and 4) Day 8 blastocysts. Mouse embryos represented developmental groups of 1) in vivo eggs; 2) in vivo 2-cell stage; 3) in vivo morulae; 4) in vivo blastocysts; 5) in vitro 2-cell stage; 6) in vitro morulae; and 7) in vitro blastocysts. Embryos were placed into 100  $\mu$ l of extraction buffer (0.2 M NaCl, 1 mM EDTA, 25 mM Tris HCl, pH 7.4) and were vortexed with equal volumes of phenol and chloroform:isoamyl alcohol (24:1), centrifuged, re-extracted with 100  $\mu$ l of chloroform:isoamyl alcohol, and then precipitated with 95% ethanol at  $-20^{\circ}\text{C}$  overnight. The samples were then centrifuged, washed with 70% ethanol ( $-20^{\circ}\text{C}$ ), recentrifuged, and air-dried before being dissolved in sterile distilled water. Total RNA was also extracted from bovine oviductal cell culture preparations by lysis in guanidinium isothiocyanate (Pharmacia, Quebec, PQ, Canada) followed by ultracentrifugation through CsTFA [36]. Aliquots of 1  $\mu$ g of total RNA were used for reverse transcription.

#### RT-PCR

RNA was reverse-transcribed by oligo(dT) priming and MMLV reverse transcriptase (Gibco BRL, Burlington, ON), and the cDNA derived from the equivalent of total RNA from

10 embryos was used in PCR to specifically amplify cDNAs of interest as previously described [25–27, 35]. The PCR products were resolved on 2% agarose gels containing 0.5  $\mu$ g/ml ethidium bromide.

#### PCR Primers

Primer pairs were obtained from the Regional DNA Synthesis Laboratory, University of Calgary. Complementary DNA samples were first tested and discarded if found to be contaminated with genomic DNA. Genomic contamination was assayed by PCR with a primer pair for mouse  $\beta$ -actin, which produces a predicted 243-bp fragment for the cDNA and a 330-bp fragment (due to presence of an intron) if contaminating genomic DNA is present [37–42]. At no time in any of these experiments was the larger genomic actin DNA fragment detected. Primer pairs used in the PCR reaction were derived from published mouse sequences, and the sizes of the expected PCR fragments are shown in Table 1. To confirm identity, PCR products were subjected to cleavage with an appropriate diagnostic restriction enzyme (Table 1). The bovine embryo and oviduct cell PCR products for glutamylcysteine synthetase (GCS) were further subjected to Southern blot analysis to ensure recognition of the appropriately sized PCR product in these samples.

## RESULTS

To develop an mRNA phenotypic map for the expression of various antioxidant genes in early mouse and cow embryos and primary oviduct cell cultures, RT-PCR studies were carried out for catalase, CuZn-containing superoxide dismutase (CuZn-SOD), Mn-SOD, glutathione peroxidase (GPX), and GCS [37–42]. In all instances, the assays were repeated at least twice with different embryo batches, and the identity of these PCR products was confirmed by restriction digestion or Southern blot analysis (Table 1). Examples of PCR results are presented in Figures 1, 2, and 3. Figure 1 displays the typical pattern of expression for these antiox-

## MOUSE

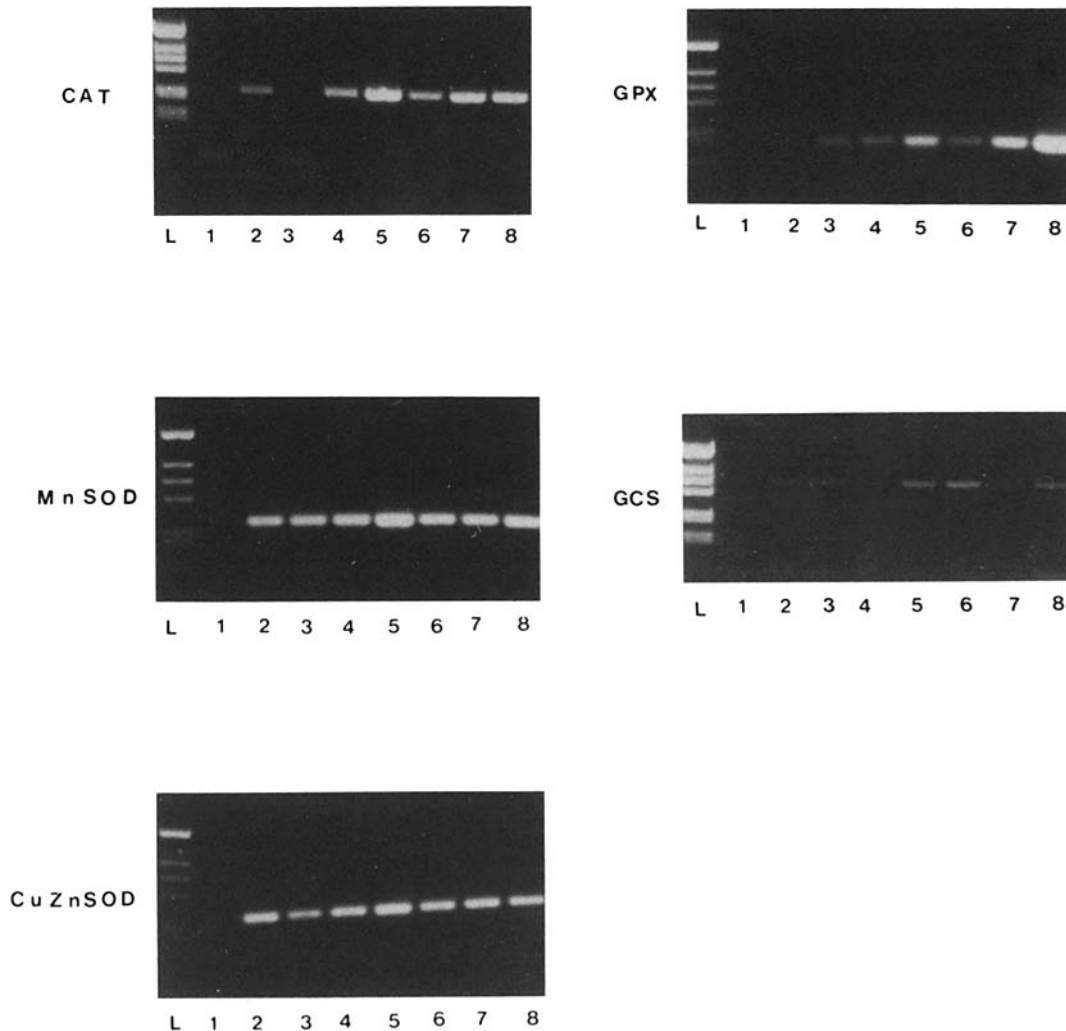


FIG. 1. Gene expression of antioxidant enzymes: mouse embryos. Each embryo lane represents cDNA aliquot derived from total RNA from equivalent of 10 embryos. RNA preparations were reverse-transcribed and amplified by 40 cycles of PCR. Transcripts encoding catalase (CAT), Mn SOD, CuZn SOD, GPX, and GCS were investigated. Lane L, DNA ladder (bands from top to bottom: 1018 bp, 516/506 bp, 394 bp, 344 bp, 298 bp, 220/200 bp, 154/142 bp). For mouse embryos: Lanes 1, negative control (no cDNA); 2, fertilized eggs; 3, in vivo 2-cell embryos; 4, in vivo morulae; 5, in vivo blastocysts; 6, in vitro 2-cell embryos; 7, in vitro morulae; 8, in vitro blastocysts. All five antioxidant PCR products were constitutively expressed within both in vivo and in vitro mouse embryo samples.

oxidant enzymes in cultured and noncultured murine preimplantation embryos. Figure 2 summarizes the expression patterns for these mRNAs within a bovine preimplantation developmental series, and Figure 3 displays the antioxidant expression patterns within the two oviductal primary cell cultures. Transcripts for  $\beta$ -actin were detected in all of these samples (data not shown).

Transcripts encoding catalase, CuZn-SOD, Mn-SOD, GPX, and GCS were detectable in all stages of in vivo preim-

plantation mouse embryos (from 1-cell to blastocyst stages; Fig. 1, lanes 2–5). In the case of catalase, there was decreased abundance at the 2-cell stage (lane 3), which was probably due to degradation of maternal mRNA. This pattern of expression is typical of many genes that are constitutively expressed during early development; namely, there is strong expression in the oocyte and 1-cell embryo, decreased abundance at the 2-cell stage due to degradation of maternal mRNA, and reaccumulation in morulae and blas-

## COW

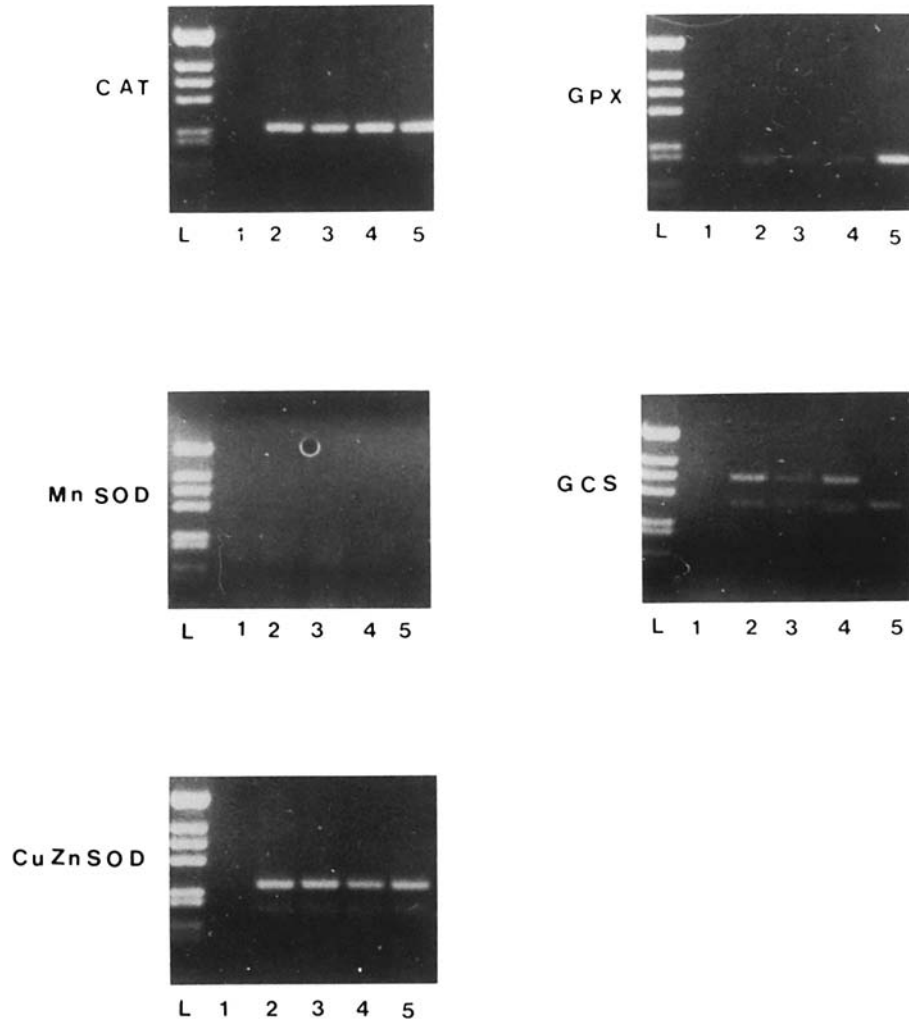


FIG. 2. Gene expression of antioxidant enzymes: cow embryos. Each embryo lane represents cDNA aliquot derived from total RNA from equivalent of 10 embryos. RNA preparations were reverse-transcribed and amplified by 40 cycles of PCR. Transcripts encoding catalase (CAT), Mn SOD, CuZn SOD, GPX, and GCS were investigated. Lane L, DNA ladder (bands from top to bottom: 1018 bp, 516/506 bp, 394 bp, 344 bp, 298 bp, 220/200 bp, 154/142 bp). For cow embryos: Lanes 1, negative control (no cDNA); 2, fertilized eggs; 3, 6-8-cell embryos; 4, morulae; 5, blastocysts (Day 8). Transcripts encoding catalase, CuZn SOD, and GPX were constitutively expressed. No PCR products for Mn SOD were detected in any embryo stage whereas GCS transcripts were expressed up to morula stage.

tocysts due to new transcription from the zygotic genome [43]. Analysis of *in vitro*-cultured 2-cell embryos, morulae, and blastocysts (Fig. 1, lanes 6, 7, and 8, respectively) showed a pattern of gene expression for antioxidant enzymes identical to that observed for their *in vivo* counterparts (Fig. 1). The identity of the murine preimplantation embryo antioxidant PCR products was confirmed by conducting diagnostic restriction enzyme digestions. For example, restriction enzyme digestion of the catalase PCR product with *Sph* 1 resulted in the predicted product sizes

of 66 and 163 bp. Likewise, restriction enzyme digestions of the remaining murine antioxidant PCR products resulted in the production of DNA fragments of predicted size for each product (see Table 1 for fragment sizes).

In early bovine embryos, transcripts for catalase, CuZn-SOD, and GPX were expressed in all preimplantation embryo stages (Figure 2). The signal obtained for GPX was barely detectable by ethidium bromide staining prior to the blastocyst stage, but a strong signal was observed from the RT-PCR analysis of blastocyst RNA. No PCR products for

## OVIDUCT

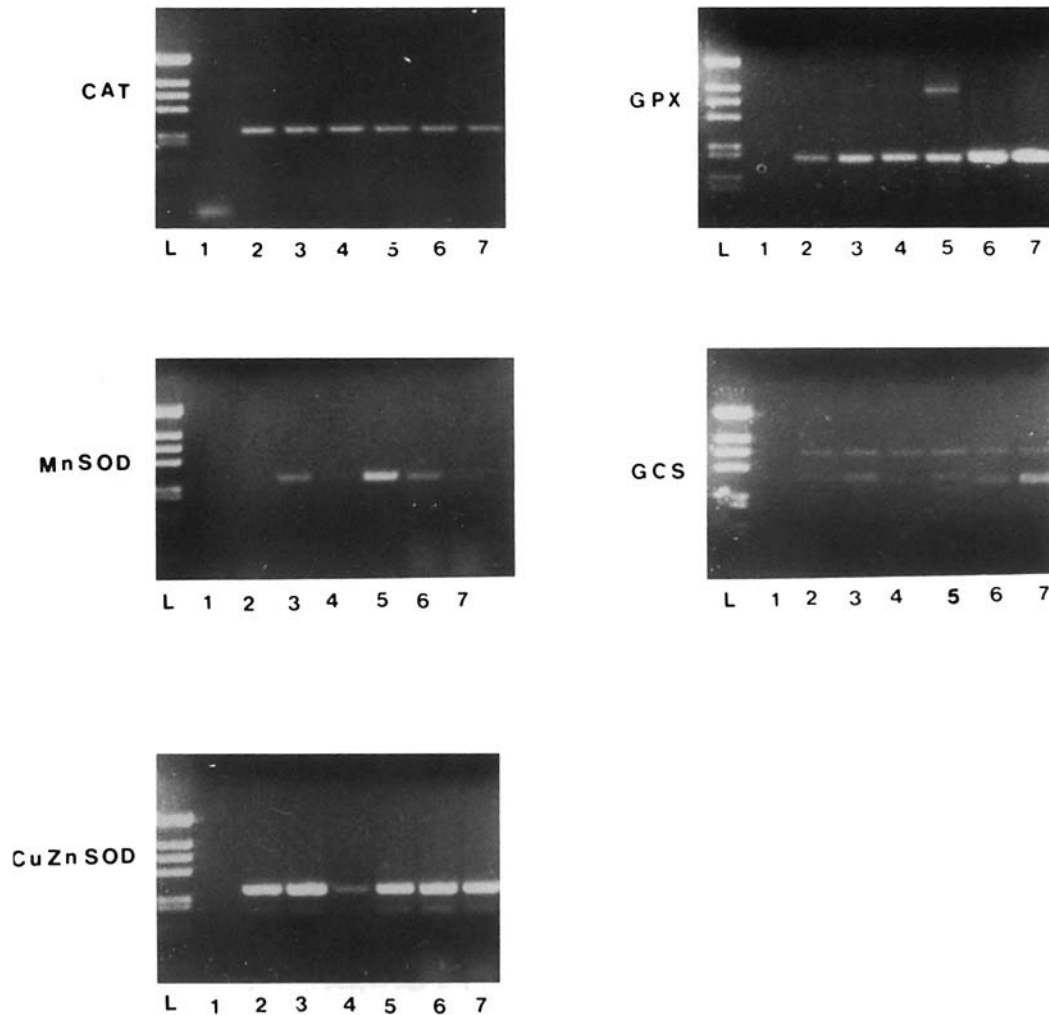


FIG. 3. Gene expression of antioxidant enzymes: bovine oviductal coculture cells. Each oviduct cell lane was produced with cDNA derived from approximately 20 ng total RNA. RNA preparations were reverse-transcribed and amplified by 40 cycles of PCR. Transcripts encoding catalase (CAT); Mn SOD, CuZn SOD, GPX, and GCS were investigated. Lane L, DNA ladder (bands from top to bottom: 1018 bp, 516/506 bp, 394 bp, 344 bp, 298 bp, 220/200 bp, 154/142 bp). For oviduct cells: Lanes 1, negative control (no cDNA); 2, fresh oviduct cells; 3, monolayers after 48-h culture; 4, monolayers after 96-h culture; 5, monolayers after 8-day culture; 6, vesicles after 24-h culture; 7, vesicles after 8-day culture. Both primary oviduct cultures displayed identical antioxidant expression patterns as all five antioxidant genes were constitutively expressed throughout 8-day culture interval.

Mn-SOD mRNA were detected by ethidium bromide staining at any stage of bovine preimplantation development, whereas all mouse embryo samples yielded very strong signals. The intensity of signals for GCS, like those for mouse embryos, varied from one stage to the next, but traces were detectable at all bovine embryo stages except the blastocyst stage (Fig. 2). This result was repeated several times on different proven bovine blastocyst cDNA samples, and at no time was a GCS PCR product of appropriate size detected in bovine blastocysts. The GCS primers also amplified a second smaller (than predicted size) PCR product in all bovine

embryo samples (Fig. 2). This second PCR product remained despite extensive efforts to optimize the PCR conditions. The identity of this second product remains obscure, but the identity of the appropriately sized GCS bovine embryo amplicon was confirmed by conducting a Southern blot analysis with a labeled GCS cDNA probe (Fig. 4). All other bovine embryo antioxidant PCR products were identified by conducting diagnostic restriction enzyme digests, and in all cases the predicted fragment sizes (see Table 1) were observed.

Analysis of antioxidant gene expression in both oviductal

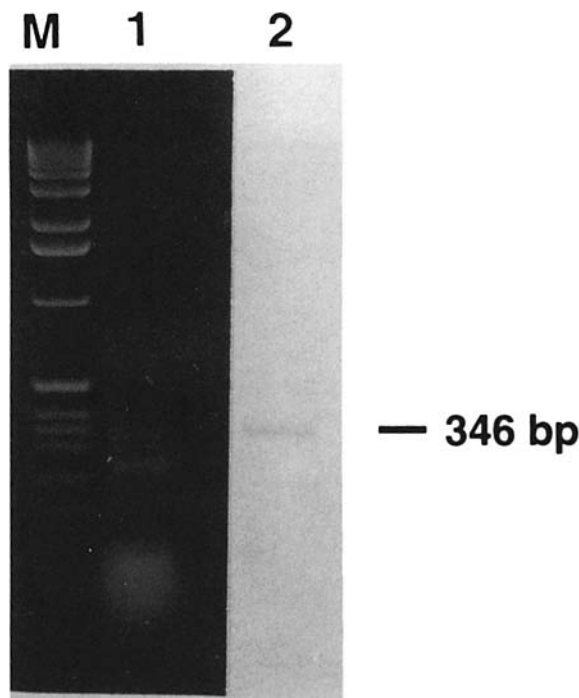


FIG. 4. Southern hybridization to verify identity of GCS amplified product. Total RNA from 48-h oviduct cultures was reverse-transcribed and then amplified by use of the GCS primers as described. Aliquot of PCR product was run on 2% agarose gel alongside 1-kb ladder molecular weight markers (Gibco-BRL). Gel was photographed with ruler beside it in order to facilitate identification of hybridization bands later on. Gel was then transferred onto Hybond + nylon membrane, baked, prehybridized, and then hybridized with 3' end  $^{32}\text{S}$ -dATP tailed GCS 30-mer (CTA GAC TTC CTC ATT CCA CTG TCC AAG GTT) located internal to expected PCR product sequence. After overnight incubation at 65°C, membrane was washed to a stringency of 0.5-strength saline sodium citrate at 65°C, placed against autoradiographic film, and exposed overnight. Lane M, molecular weight ladder; lane 1, ethidium bromide-stained PCR product; lane 2, autoradiograph of same PCR product lane after hybridization with GCS probe. Only upper GCS product (predicted size) hybridizes with  $^{32}\text{S}$ -labeled oligonucleotide.

primary cell monolayer cultures and nonattached ciliated vesicle cultures displayed constitutive expression for catalase CuZn-SOD, GPX, and GCS for an eight-day interval (Fig. 3). The mRNAs encoding Mn-SOD yielded RT-PCR products that varied in intensity over the complete culture interval but were still present at all stages examined. The absence of Mn-SOD PCR products in cow embryo experiments was not simply due to failure of mouse primers to interact with homologous bovine sequences, because positive signals were obtained with RNA samples derived from bovine oviduct cells (Fig. 3). As for bovine preimplantation embryos, the GCS primer set amplified two PCR products in all oviduct samples. The specific GCS PCR product was once again identified by conducting a Southern blot analysis with a labeled GCS cDNA probe (Fig. 4). All other antioxidant oviductal cell PCR products were identified by conducting the appropriate diagnostic restriction enzyme digests, resulting in all cases in the production of cleavage products of expected size (Table 1).

## DISCUSSION

Preimplantation embryos of all species studied thus far display characteristic culture blocks associated with the timing of embryonic genome activation [16]. Thus, it is at the stage when the control of development is changing from an exclusively posttranscriptional level to a transcriptional level [44] that the embryo appears to be most vulnerable to environmental insults. Recent attention has now focused on free oxygen radicals (FORs) as major causal agents for in vitro embryonic arrest (see [16] for review).

Formation of FORs is a normal process occurring within cells when there is leakage of electrons to oxygen during electron transfer reactions [16, 17, 45]. This occurs either in the mitochondrial respiratory chain cytochrome P450 oxidases, in the endoplasmic reticulum, or by the activity of oxidase systems [16, 17, 45]. FORs exert a powerful oxidizing potential and have a pronounced effect on DNA, RNA, and protein synthetic activities [16–23]. The extent of their damage to cell systems is dependent on the balance between their rates of production and removal [45]. The deleterious effects of FORs are counterbalanced by antioxidants that neutralize the activated oxygen species. While there are numerous nonenzymatic antioxidant agents including vitamins E and C,  $\beta$ -carotene, urate, and taurine, specific antioxidant enzymes are produced by cells and are able to detoxify  $\text{O}_2$ ,  $\text{H}_2\text{O}_2$ , and organic peroxides [16, 17, 45]. These enzymes include Mn-SOD and CuZn-SOD, which catalyze the dismutation reaction, removing  $\text{O}_2$  species; catalase, which catalyses the decomposition of hydrogen peroxide to oxygen and water; and GPX, which utilizes glutathione as a reducing source to catalyze the removal of both hydrogen peroxide and lipid peroxides [16, 17, 45]. The aim of the present study was to examine and contrast the expression of antioxidant enzymes in mouse embryos, which develop efficiently in vitro in simple culture media, and cow embryos, for which in vitro development is more heavily dependent on the positive influences of embryo coculture with somatic cells. Our analysis was extended to include these coculture cells since their ability to maintain bovine embryo development in vitro may, in part, reflect a capacity to express the genes encoding these antioxidant enzymes.

Transcripts for catalase, Mn-SOD, CuZn-SOD, GPX, and GCS were detected in both in vivo- and in vitro-produced mouse embryos at all stages of development. It should be stressed that no attempts were made to determine whether the translated products from these mRNAs were present within early embryos or oviductal cultures. This analysis awaits future studies. The expression of GCS, which is the rate-limiting enzyme in the biosynthesis of glutathione [46], is consistent with the detection of glutathione in mouse oocytes and embryos [23]. It should be noted that EDTA is necessary to overcome the 2-cell block for the CD1 mouse embryos used in this study [16]. EDTA, transferrin, and other



iron-binding agents all promote the development in vitro of embryos from arresting-strain mice to the blastocyst stage by chelating ferrous irons [16]. Most laboratory chemicals contain traces of iron sufficient to catalyze formation of highly toxic hydroxy radicals [47].

In bovine embryos, the mRNAs encoding catalase, CuZn-SOD, GPX, and GCS were detected throughout preimplantation development, whereas no Mn-SOD mRNAs were detected. It is clearly recognized that numerous additional enzymatic and nonenzymatic antioxidant defenses exist within cells other than the enzymes investigated in this study. Nonetheless, our results have certainly indicated that mouse embryos synthesize a more complete array of antioxidant defenses than do cultured bovine embryos, and this result may have unmasked a component of the mechanism that underlies the variation in developmental capacity displayed by the preimplantation embryos of these two species in vitro. Furthermore, the beneficial influence of coculture on bovine preimplantation development under a 5% CO<sub>2</sub> in air atmosphere may reflect the fact that these embryos do not possess the full array of antioxidant enzymes required to cope with FOR production.

It is possible that coculture cells support bovine embryo development in vitro in part by offsetting the potential deleterious effects of exposure to high O<sub>2</sub> by metabolizing FORs present in the culture medium. Our results support this possibility since RT-PCR of antioxidant transcripts showed that all five antioxidant enzymes were constitutively expressed in both bovine oviductal cell monolayers and nonattached epithelial cell vesicle cultures during an 8-day culture period. This proposed role for the oviduct cell cultures is supported by studies in which embryo development has been enhanced by the addition of SOD to defined culture media [48, 49]. However, the results stemming from the addition of exogenous SOD to media are variable and in dispute because it is not clear how exogenous SOD could remove intracellular superoxides that do not readily cross cell membranes [16]. It is also possible, and perhaps likely, that the coculture cells support embryo development by producing additional antioxidant agents (other than those examined in this study) that are able to enter the embryo and neutralize FORs.

In conclusion, our results show that preimplantation mouse embryos express transcripts for catalase, SODs, and enzymes important in FOR neutralization by glutathione. Cow embryos do not express mRNAs encoding Mn-SOD. Could different capacities to offset the deleterious effects of exposure to high O<sub>2</sub>, in part, reflect the varying developmental potentials of mammalian preimplantation embryos in vitro? An understanding of the programming of early embryos will ultimately indicate what antioxidant defenses are missing and thus allow us to specifically address these inadequacies by adding exogenous antioxidant agents to neutralize FOR action at the site of production. Candidates for

such agents presently include taurine, pyruvate, and  $\alpha$ -tocopherol [16]. The ability to limit FOR damage of embryos should promote increased preimplantation embryo production efficiency, which would improve the success outcomes of both human and agricultural in vitro fertilization and embryo transfer programs.

## ACKNOWLEDGMENTS

Appreciation is extended to Ms. Barbara Lowery for the preparation of the final manuscript. We are grateful to Dr. Xia Ping, Mr. John Looye, Mr. Jeff Smith, Mr. Shawn Gordon, and the members of the ABEL Laboratories, under Dr. Keith Betteridge's direction, University of Guelph, for assistance with the bovine ovary and oviduct collections. We also thank Dr. Bob Stubbings, Semex Canada, for supplying the bovine semen employed in this study.

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