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Expression of Cu/Zn and Mn Superoxide Dismutases During Bovine Embryo Development: Influence of In Vitro Culture

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ABSTRACT Temporal pattern of expression of Cu/Zn and Mn superoxide dismutases (SODs) was investigated in bovine oocytes and embryos produced in vitro in two different culture conditions and in vivo after superovulation. SODs were examined at a transcriptional level in single oocytes and embryos by reverse transcriptase–polymerase chain reaction (RT-PCR) and, at a protein level, by Western blotting on pools of embryos. mRNA encoding Cu/Zn SOD were detected in in vitro bovine embryos throughout preattachment development as well as in in vivo derived morulae and blastocysts. Transcripts for Mn SOD gene were detected in most immature and in vitro matured oocytes as well as in some zygotes and 5- to 8-cell embryos while no transcript was found at the 9- to 16-cell stage in both culture conditions. In vitro embryonic expression of Mn SOD was detected earlier in the presence of serum. Half of the morulae showed the transcript if cultured with 5% serum while none without serum. At the blastocyst stage Mn SOD could be detected independently of culture conditions. For in vivo–derived embryos Mn SOD transcripts were detected both in morulae and blastocysts. Immunoblotting analyses revealed that Cu/Zn SOD and Mn SOD were also present at a protein level in in vitro-derived zygotes and blastocysts. Together these data demonstrate, for the first time, that Mn SOD is transcribed and that Cu/Zn and Mn SOD proteins are expressed in preimplantation bovine embryos. Finally, they suggest that Mn SOD transcription is altered by in vitro culture conditions. *Mol. Reprod. Dev. 58:45–53, 2001.* © 2001 Wiley-Liss, Inc.

Key Words: RT-PCR; bovine oocyte; preimplantation development; antioxidant enzymes; in vitro/in vivo

INTRODUCTION

Oxidative stress appears to be one of the causes of impaired in vitro embryo development. An increased production of peroxides was measured in mouse embryos cultured in vitro (Nasr-Esfahani and Johnson, 1990; Goto et al., 1993) suggesting that during embryo culture the equilibrium between reactive oxygen species (ROS) production and scavenging is disrupted. Higher ROS generation has been ascribed to environmental factors such as light exposure, high oxygen tension, presence of heavy metals in culture media (reviewed by Johnson and Nasr-Esfahani, 1994) as well as to disorders in embryo metabolism (Rieger, 1992).

Supplementation of culture media with ROS scavengers and metal chelators, which are normally present in the genital tract, has been shown to promote bovine embryo development (Johnson and Nasr-Esfahani, 1994). Little information is available concerning antioxidative defense in preimplantation embryos. Some studies suggest that cellular enzymatic systems against oxidative injury are not fully developed in in vitro produced bovine embryos (Harvey et al., 1995) and preimplantation mouse embryos (El-Hage and Singh, 1990). In the antioxidant system the superoxide dismutases (SOD) are the initial enzymes, they allow the conversion of the anion superoxide (O^{2-}) to H_2O_2, which in turn is removed by catalase or glutathione peroxidase. SODs are characterized by their metal requirements and by their subcellular localization. The manganese-containing SOD (Mn SOD) is found in the mitochondria (Weisiger and Fridovich, 1973) while the copper-zinc–containing SOD (Cu/Zn SOD) is cytoplasmic (McCord and Fridovich, 1969). Both transcripts have been found in mouse oocytes and during embryonic development (Harvey, 1995; El Mouatassim et al., 1999). In human oocytes Mn SOD mRNA seems to be unadenylated before maturation (El Mouatassim et al., 1999). In the bovine transcripts encoding Mn SOD were not found in in vitro derived morulae, blastocysts, and early cleavage stages embryos whereas transcripts for Cu/Zn SOD were detected throughout bovine embryo development (Harvey et al., 1995).

The present study aimed at testing the influence of in vitro culture conditions on SODs expression in bovine

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embryos. For this purpose, temporal patterns of expression of Mn SOD and Cu/Zn SOD were compared between in vivo flushed embryos and in vitro produced embryos cultured with and without 5% serum. Presence of Mn SOD and Cu/Zn SOD was examined both at a transcriptional level by reverse transcriptase–polymerase chain reaction (RT-PCR) and at a posttranscriptional level by immunoblotting.

MATERIALS AND METHODS

In Vitro Bovine Embryo Production

Cumulus–oocyte complexes (COC's) collected by puncturing slaughtered cow ovarian follicles were matured and fertilized in vitro as previously described (Kaidi et al., 1998). Briefly, COCs were matured during 24 hr in tissue culture medium 199 (TCM 199; Gibco, Paisley, Scotland) with 10% fetal calf serum (FCS) and 10 ng/ml Epidermal Growth Factor (Sigma, St Louis, Missouri) then fertilized 18 hr in TALP medium (Tyrode albumin lactate pyruvate) with 2×10^6 spermatozoa/ml separated on a discontinuous density gradient of Percoll (Pharmacia, Uppsala, Sweden). Presumed zygotes were denuded 18 hr after insemination (HPI) and cultured in a modified Synthetic Oviduct Fluid medium (mSOF) (Holm et al., 1999) with or without 5% FCS and under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. When cultured under those conditions, about 75% of the zygotes were cleaved at day-3 postinsemination (pi) and 30% had reached the blastocyst stage at day-8 pi.

In Vivo Embryos Collection

All experimentations on animals were performed under the agreement LA1220030 issued from the Ministery of Agriculture of Belgium. Protocols were reviewed and accepted by the Ethical Committee of our University (n°98-30-01). Ten days post-oestrus cows were stimulated with a total treatment of 500 μg of porcine FSH combined with 100 μg of pLH (Stimufol; Rhône Mérieux, Brussels, Belgium) injected in eight decreasing doses over 4 days. On day 3 of this treatment they received 500 μg of cloprostenol (Estrumate, Schering-Plough Animal Health). Oestrus was expected on day 5 and cows were inseminated twice on that day. In vivo embryos were collected on day-7 pi by flushing the uterus with 800 ml of phosphate-buffered (PBS).

Bovine Oviduct Epithelial Cells Culture Used as Positive Control

Oviducts were recovered from slaughtered cows. Epithelial cells were cultured as previously described (Van Langendonckt et al., 1995). After isolation, oviduct cells were washed in TCM 199 supplemented with 10% FCS and further cultured in this medium until confluency. Confluent cell monolayers were washed with Dulbecco's PBS and cells designed for protein analysis were detached and lysed in a buffer containing 0.5 g of urea per ml, 40 μl of Triton X-100 per ml (Boehringer, Mannheim, Germany), 20 μl of β-mercaptoethanol per ml and those used for RNA analysis were lysed by adding Tripure (Boeringher).

Preparation of Embryo Extracts

Immature oocytes were isolated just after the follicles aspiration while mature oocytes were recovered after 24 hr of maturation. In vitro produced (IVP) embryos were collected at 18 hr pi (HPI) for the 1-cell stage, at 70 HPI for the 5- to 8-cell stage, at 98 HPI for the 9- to 16-cell stage, at 120 HPI for the morula stage with serum and 140 HPI without serum and 165 HPI for the blastocyst stage for both conditions. This timing corresponds to the peak of appearance of the different embryonic stages in our culture conditions (Van Langendonckt et al., 1997).

Oocytes and embryos were incubated 5 min at 37°C in Dulbecco's PBS containing 0.25% trypsin and vortexed for 2 min to remove adherent cumulus cells. This procedure allows elimination of all the adherent cumulus cells (Lequarré et al., 1997). After two washes in PBS for already denuded embryos and five washes for immature and mature oocytes, the embryos were transferred in a minimal volume of medium to a 0.5 ml Eppendorf tube and stored at −70°C. Samples of the last wash were collected as a negative control checking for cumulus cells contamination. Samples designed for PCR analysis were stored individually and lysed by adding tripure and those used for immunodetection were stored in one group of 50 and lysed by repeated freezing and thawing just prior to analysis.

Detection of Transcripts Encoding Beta-Globin, Beta-Actin, Cu/Zn, and Mn Superoxide Dismutases

RNA from individual embryos was extracted, reverse transcribed, and amplified as already described (Lequarre et al., 1997). RNA from bovine oviduct cells was extracted using the same procedure and treated in parallel as positive control. Prior to RNA extraction 1 pg of rabbit globin mRNA (Gibco-BRL, Paisley, Scotland) was added as an internal standard and 20 μg of glycogen (Boehringer, Mannheim, Germany) was added as a carrier. Briefly, total RNA was extracted with Tripure (Boehringer) according to the manufacturer's specifications. After precipitation total RNA was resuspended in 5 μl of DEPC-treated water, reverse-transcribed for 1 hr at 37°C in 10 μl of Tris–HCl buffer containing 75 mM KCl, 3 mM $MgCl_2$, 200 μg of oligo $dT_{(12–18)}$ (Pharmacia, Uppsala, Sweden), 1 mM of each dNTP (Boehringer), 15 U of RNA guard (Pharmacia), and 100 U MMLV reverse transcriptase (Gibco-BRL). The sample volume was then extended to 30 μl and aliquoted in 5 μl portions.

Twenty base–long primer pairs were selected in interspecies conserved regions, according to the criteria given in Oligo 4.0-s software. Their sequences are presented in Table 1 with corresponding references; they were obtained from Gibco-BRL. To ensure that the detected amplification product did not result from cont-

TABLE 1. Primers Used for PCR

Gene	Primer sequence	Reference	RNA size (bp)
β-Globin			
Sense	5′ GGCAAGGTGAATGTGGAAGA 3′	Rabbit	201
Antisense	5′ CTTTGAGGTTGTCCAGGTGA 3′	(Hardison et al., 1979)	
β-Actin			
Sense	5′ GCGTGACATCAAGGAGAAG 3′	Bovine	432
Antisense	5′ TGGAAGGTGGACAGGGAGG 3′	(Degen et al., 1983)	
Cu/Zn SOD			
Sense	5′ GAAGAGAGGCATGTTGGAGA 3′	Bovine	220
Antisense	5′ CCAATTACACCACGAGCCAA 3′	(Hallewell et al., 1991)	
Mn SOD			
Sense	5′ CACCACAGCAAGCACCACGC 3′		
Antisense	5′ TCCCACACGTCAATCCCCAG 3′	Bovine	409
Internal probe	5′ GGAATTGCTGGAAGCCATCA 3′	(Meyrick et al., 1994)	

aminating genomic DNA, primer pairs always spanned at least one intron.

The mRNAs encoding β-globin, Mn SOD, Cu/Zn SOD, and β-actin were amplified separately on each oocyte or embryos on $\frac{1}{5}$th of the reverse transcribed RNA. The PCR reaction mixture consisted of 50 μl of 10 mM Tris–HCl, pH 8.3 containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 mM of primers, 0.05 mM of each dNTPs, and 1 U of thermostable DNA polymerase, Dynazyme (Finnzymes, Espoo, Finland). After a hot start of 5 min at 95°C, 35 or 40 cycles of amplification (depending on the primer pair used) were run as follows: DNA was denatured at 95°C for 1°min, reannealing was achieved at 55 or 59°C for 50 sec, an extension at 72°C for 1 min, and a final extension step was performed for 5 min at 72°C. Products of amplification were separated on agarose gel and stained with ethidium bromide. The electrophoresis standards used were 100 bp Molecular Rulers (Bio-Rad, Richmond, CA). Identity of the amplification products was checked by sequencing (Eurogentec, Liège, Belgium). Under these conditions Mn SOD and actin PCR products were detected from 10 ng down to 0,01 ng of total oviduct cells RNA and down to 0.05 ng for Cu/Zn SOD (data not shown).

Southern Blot Analysis of Mn SOD RT-PCR Products

Following electrophoresis, PCR products were blotted overnight onto a Hybond-N nylon membrane (Amersham, Life Science, Buckinghamshire, England) by capillary action in 10 × SSC. After transfer and DNA fixation by UV irradiation, hybridization was performed at 50°C in 6 × SSC with a 20 bp probe corresponding to an internal sequence of the PCR product (Table 1) and end-labeled by kination with 1 μl of $\gamma AT^{32}P$ (3000 Ci/mmol, 10 mCi/ml, Amersham, Pharmacia, Biotech.) as described by Sambrook et al. (1989).

Immunodetection of Cu/Zn and Mn Superoxide Dismutases

Superoxide dismutases were detected in oviduct cells and embryos by Western blot as already described (Vansteenbrugge et al., 1997). Proteins from oviduct and embryo lysates were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970). Fifty microliter of sample buffer was added to each cellular extract and the samples were loaded on a 10–20% polyacrylamide gel (Ready gel, Bio-Rad, CA). Prestained SDS-PAGE standards (low molecular weight range, Bio-Rad) were used as a reference. After electrophoresis, proteins were transferred onto a prewetted nitrocellulose membrane (Hybond-C, 0.45 mm thick; Amersham) using a semidry transfer apparatus (Trans-Blot SD, semi-dry transfer cell, Bio-Rad). After blotting, the membrane was soaked in Tris-buffered saline (TBS) containing 3% nonfat powdered milk as blocking agent. Further washing and incubation steps were performed at room temperature in TBS containing 1.5% milk.

For the detection of Cu/Zn SOD, an anti-r-bovine SOD antiserum was used. The antiserum was obtained by immunization of a rabbit with Cu/Zn SOD from bovine erythrocytes (Calbiochem, San Diego, CA). Immunization was performed by injection of 100 μg of Cu/Zn SOD followed by three booster injections at 1 month interval. After blocking, the membrane was incubated for 1 hr with the primary antiserum (1:400), cross-reactivity was visualized by means of anti-rabbit Ig G biotin-conjugated second antibody (1:20,000; 1 hr, Sigma Chemical, St Louis, MO) followed by a streptavidin–alkaline phosphatase biotinylated complex (1:200, 1 hr; Dako, Prosan, Ghent, Belgium) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) as color development reagent. The membrane was washed four times for 10 min each in TBS supplemented with 1.5% milk between each step [20].

Mn SOD was evidenced with an anti-human liver Mn SOD antiserum raised in the sheep (Calbiochem). Incubation with the primary antibodies was performed for 1 hr at a 1:400 dilution. Anti-sheep peroxidase-conjugated antibodies (1:100,000, 1 hr, Sigma) were used as secondary antibodies and visualized by a highly sensitive enhanced chemiluminescent detection procedure (SuperSignal ultra luminol enhancer solution and ultra

stable peroxide solution, Pierce, Rockford, IL). After addition of the luminol/peroxidase reagent, the light signal was detected after exposure of the membrane for 5 min on an ECL hyper-film (Amersham). At least six washes of 10 min each were performed between each step.

RESULTS

Identification of Amplified cDNAs

cDNA was prepared from individual bovine oocytes and embryos and from oviduct cells. As shown in Fig. 1, PCR products showed the expected size (reported in Table 1) and their identity was confirmed by sequencing. The sequence of the fragments was compared with published bovine mRNA sequence and showed 98% and 99% identity, respectively, for Cu/Zn SOD (Hallewell et al., 1991) and Mn SOD (Meyrick and Magnuson, 1994) and 91% identity with the murine sequence for β-actin (Tokunaga et al., 1986). Mn SOD amplified product was confirmed for specificity by Southern blot analysis using an oligonucleotide probe corresponding to the bovine sequence (Table 1).

On each sample cDNA derived from one exogenously supplied mRNA (β-globin) and three endogenous mRNA (β-actin, Mn SOD, and Cu/Zn SOD) were amplified by separate PCR analyses. β-globin mRNA was detected after RT-PCR in 97% (n = 228) of the analyzed samples and the intensity of the ethidium bromide stained band was similar in all samples. When no signal was detected for this reporter gene, either total RNA of the sample was lost or degraded, or β-globin amplication failed. As no amplification could be obtained with any other genes, RNA must have been lost during the extraction or RT procedures. Transcripts encoding for β-actin were detected in 82% of the embryos regardless of the developmental stage. Figure 1 shows representative results obtained on individual immature and mature oocytes. Only samples showing both β-actin and β-globin amplified products were further considered for Cu/Zn and Mn SOD analyses.

Transcription of Cu/Zn Superoxide Dismutase During Bovine Embryo Development

After RT-PCR transcripts encoding Cu/Zn SOD were found in 67% (6/9) of the in vivo morulae and in 80% (12/15) of the in vivo blastocysts (Fig. 2). Under in vitro conditions, transcripts were detected throughout embryonic development performed without serum. The same results were obtained in culture with serum (data not shown). Cu/Zn SOD transcripts were detected in 81% of the embryos showing both β-globin and β-actin transcription regardless of the culture conditions and developmental stages.

Transcription of Mn Superoxide Dismutase During Bovine Embryo Development

Seventy-three percent (11/15) of the in vivo derived blastocysts as well as 67% (6/9) of morulae showed transcription Mn SOD (Fig. 3). Under in vitro condi-

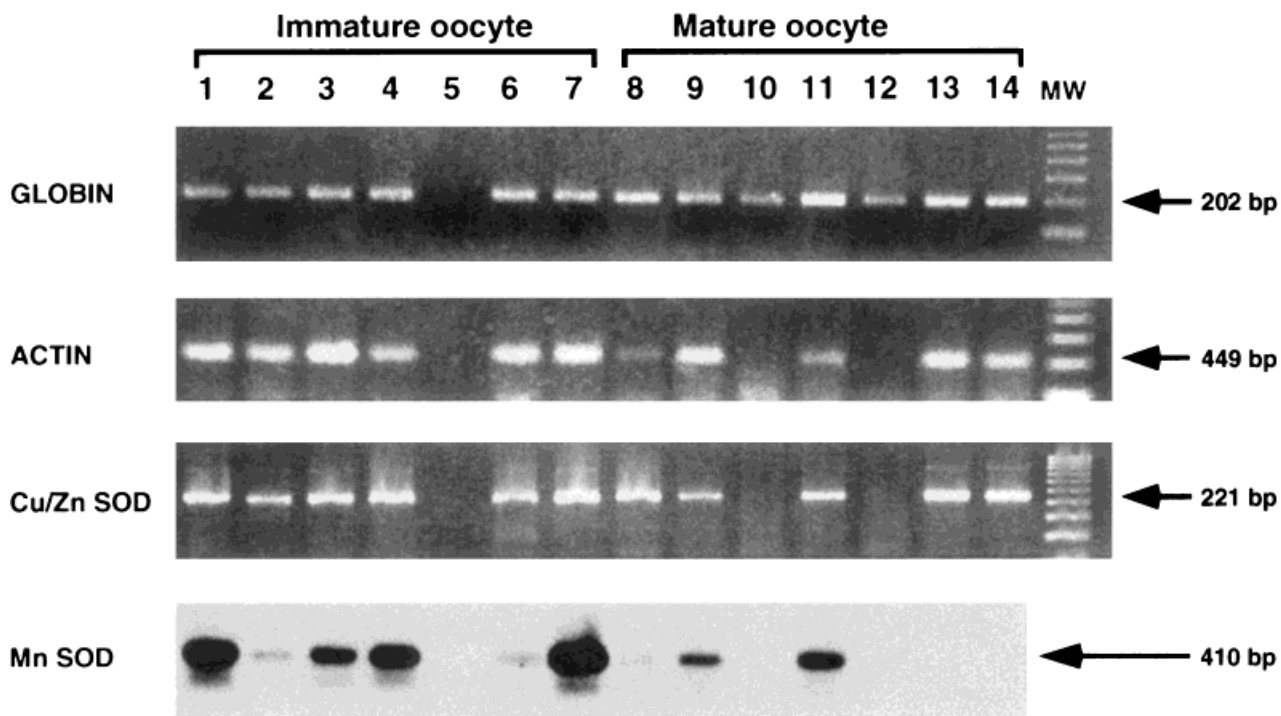

Fig. 1. mRNA expression on single immature and in vitro matured bovine oocytes. RT-PCR products were separated on agarose gels stained with ethidium bromide. For Mn SOD, the gel was Southern blotted and hybridized with a radiolabeled oligonucleotide internal to the amplicon. Sample 5: no signal with any amplification. Samples 10 and 12: signal for β-globin but not for any other genes, RNA degraded inside the oocyte before extraction, samples 13 and 14: actin and Cu/Zn SOD signal but no signal for Mn SOD, oocytes with heterogeneous mRNA content.

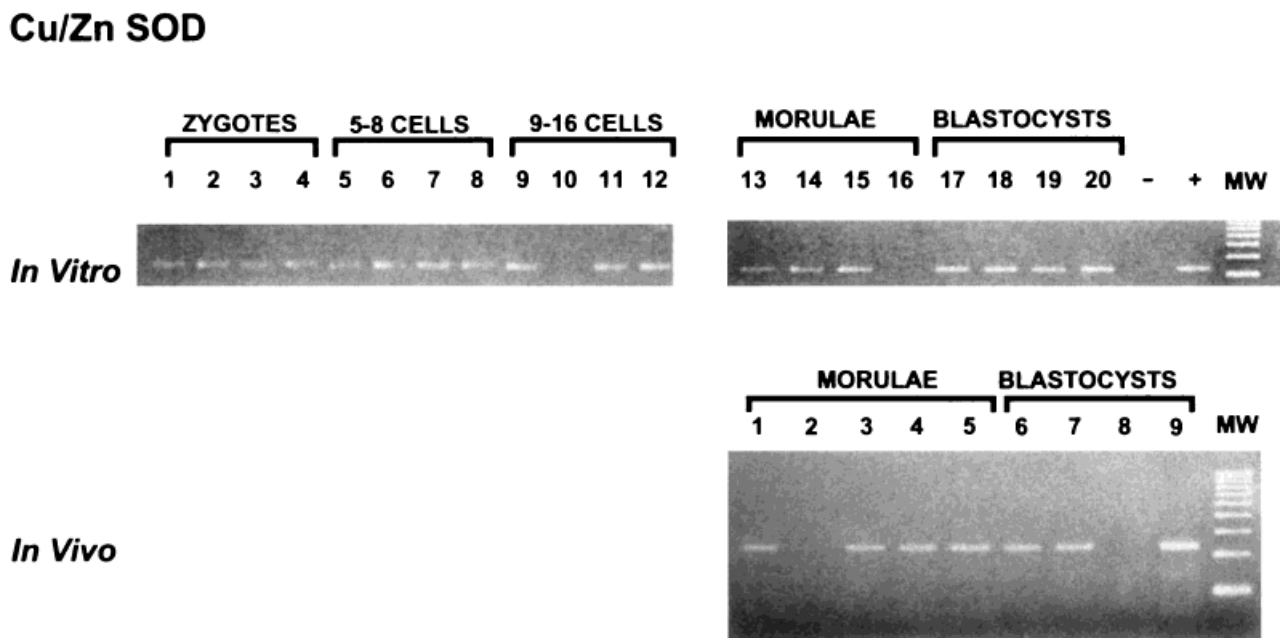

Fig. 2. Cu/Zn SOD RT-PCR on single in vitro and in vivo produced embryos collected at successive developmental stages. + positive PCR control (cDNA from oviduct cells) and − negative RT-PCR control (no RNA); MW, 100 bp Molecular Rulers. The experiment was repeated four times in vitro and three times in vivo, a representative result is shown.

tions, Mn SOD transcripts were determined by ethidium bromide staining of the PCR products in 72% (18/25) of the immature oocytes and 60% (12/20) of mature oocytes but only 43% (6/14) of the zygotes. Statistical association between Mn SOD expression and embryo stages was compared using the χ^2-test. The decrease in ratio for immature oocytes, mature oocytes, and zygotes expressing the gene was not significant. Nevertheless, RT-PCR signal was no more detectable with ethidium bromide at the 5- to 8-cell, 9- to 16-cell, and morula stages while it was detected in 79% (11/14) of the in vitro produced (IVP) blastocysts. After Southern blot analysis of the gel a signal was also detected in 58% of the 5- to 8-cell stage (7/12) but no Mn SOD mRNA was found in 9- to 16-cells (0/15) and morulae (0/14) that were produced without serum even when pools of 10 morulae were examined (data not shown). Under culture conditions including 5% serum, Mn SOD was detected in 58% of the morulae (14/24) and in 74% of the blastocysts (14/19) (Fig. 2).

Immunodetection of Cu/Zn and Mn SOD in IVP Zygotes and Blastocysts

As shown in Fig. 4, the Cu/Zn SOD protein was detected in bovine zygotes and day-7 blastocysts produced in vitro: the anti-Cu/Zn SOD antiserum recognized one band at 16 kDa, corresponding to the molecular weight of the bovine protein. Mn SOD was also evidenced in in vitro bovine zygotes and blastocyst extracts: one band showing the expected molecular weight (20 kDa) was detected in both extracts by anti-Mn SOD antibodies using a highly sensitive chemiluminescence detection procedure. Embryos were all derived from in vitro culture without serum. No bands were found when the first antibody was replaced with the preimmune serum or sheep serum.

DISCUSSION

The present study shows that the ubiquitous enzyme Cu/Zn SOD catalyzing superoxide dismutation in the cytosol (Fridovich, 1995) is expressed in bovine embryos as it is in most eucaryotic cells. Transcripts encoding for Cu/Zn SOD were detected in immature as well as in in vitro matured bovine oocytes indicating their inheritance from the maternal pool of mRNAs. Expression of Cu/Zn SOD was found throughout in vitro bovine embryo development confirming the results of Harvey et al. (1995). Most individual zygotes, 5- to 8-cell stage embryos, 9- to 16-cell stage embryos, morulae, and blastocysts showing actin mRNA also contained Cu/Zn SOD transcripts. Interestingly, transcripts for Cu/Zn SOD were also present in in vivo derived bovine morulae and blastocysts demonstrating that its transcription is not peculiar to in vitro cultured embryos. The protein was detected by immunoblotting in in vitro produced bovine zygotes as well as in blastocysts. Unfortunately, its activity could not be measured even when applying a highly sensitive chemiluminescence method (data not shown). The fact that Cu/Zn SOD was detected throughout development was not surprising. This enzyme seems to be the first line of defense against oxygen-derived free radicals (Michiels et al., 1994) and is likely to be essential together with peroxide scavengers for protecting embryos against oxidative insult (Johnson and Nasr-Esfahani, 1994). However, additional extracellular protection against superoxide anion toxicity seems also to be required in vivo as well as in vitro since Cu/Zn SOD has been found in oviduct and

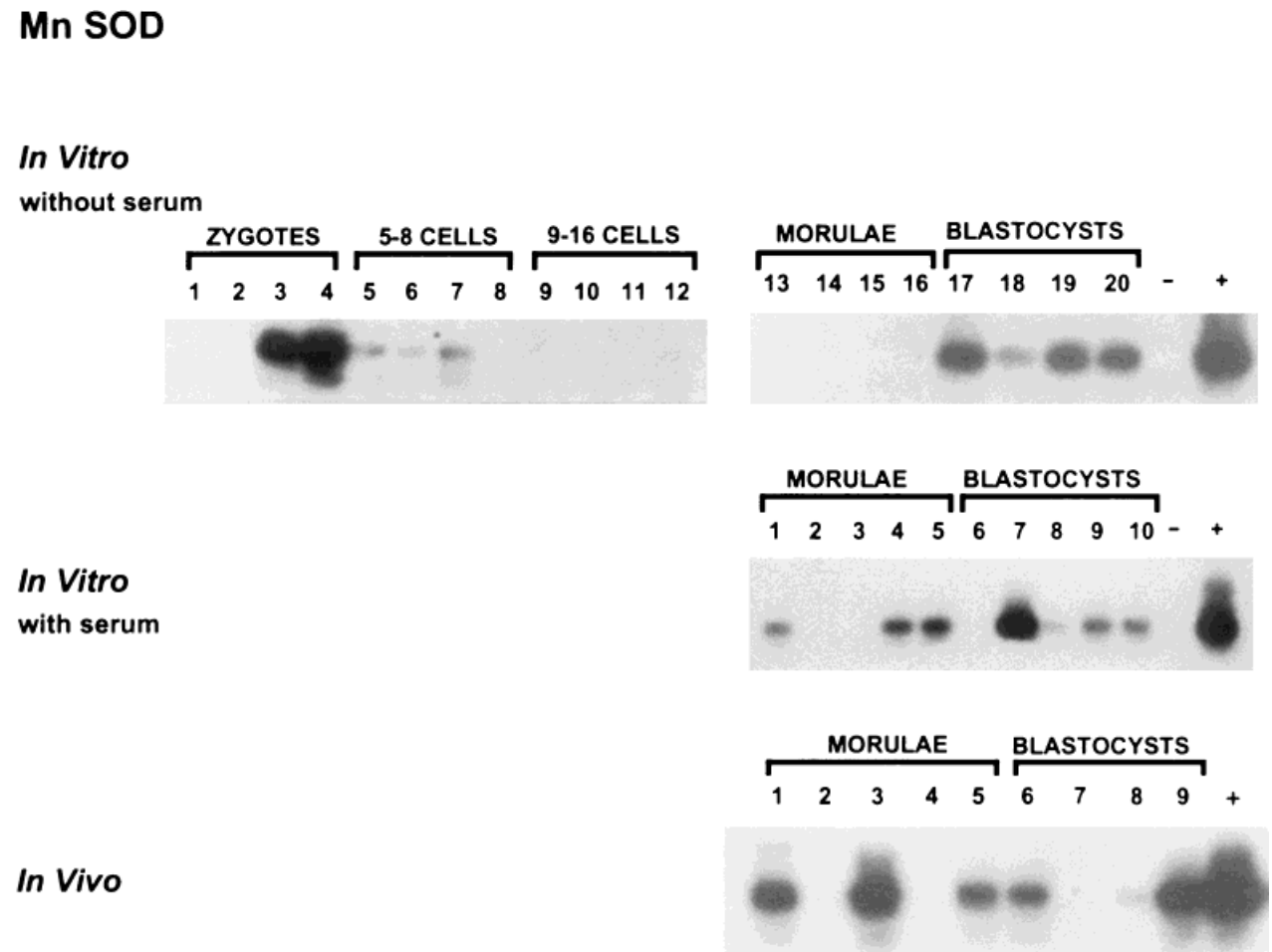

Fig. 3. Mn SOD RT-PCR on single in vitro and in vivo produced embryos. Different developmental stages are illustrated. + positive PCR control (cDNA from oviduct cells) and − negative RT-PCR control (no RNA); MW, 100 bp Molecular Rulers. The experiment was repeated four times in vitro and three times in vivo, a representative result is shown.

uterine secretion in the mouse (Chun et al., 1994) and rabbit (Noda et al., 1991). Several studies have shown that supplementation of culture media with Cu/Zn SOD had a favorable effect on embryo development in bovine (Lauria et al., 1994; Fujitani et al., 1997), mouse (Umaoka et al., 1991; Nonogaki et al., 1992) and rabbit (Li et al., 1993).

Transcripts for the mitochondrial Mn SOD were not detected previously by Harvey et al. (1995) during bovine embryonic development. Here, with another set

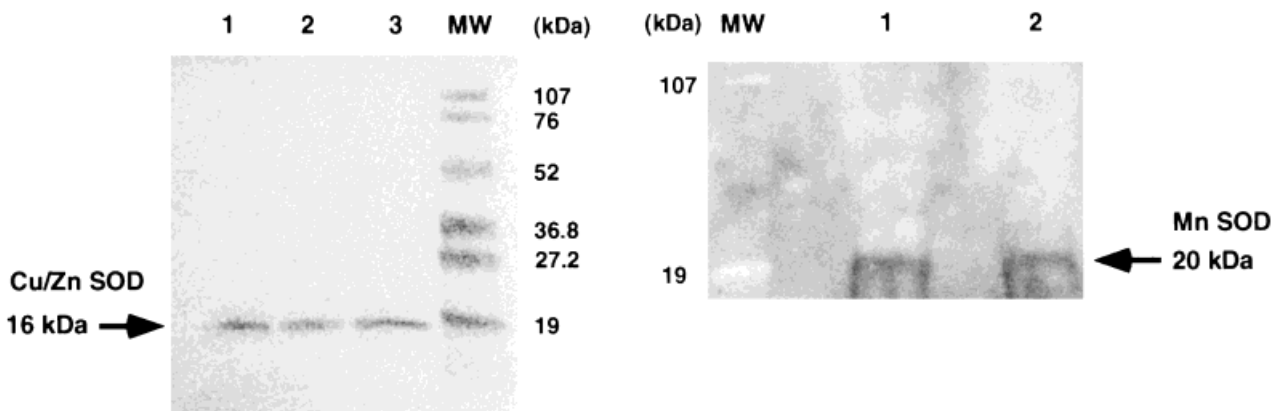

Fig. 4. immunoblotting: Lane 1: 50 zygotes; Lane 2: 50 blastocysts in vitro produced; Lane 3: oviduct cells; MW, low range molecular weight. One band detected at 16 kDa for Cu/Zn SOD and one band at 20 kDa for Mn SOD.

of primers and under different in vitro culture conditions, we report Mn SOD expression at the mRNA level from immature oocyte up to the 5- to 8-cell stage followed by embryonic expression at the morula or at the blastocyst stage depending on culture conditions. Absence of Mn SOD mRNA, previously reported for IVP bovine embryos, could result from their coculture conditions. In coculture, cells contribute to embryo development both by secreting embryotrophic factors and by removing embryotoxic components (Bavister, 1992). Consequently it could change the oxidative stress and delay the timing of induction of Mn SOD expression. On the other side, one of the primers used in that study was spanning a region not well conserved between species, it has four mismatches with the bovine sequence established in our study so it is possible that their amplification was not sensitive enough to detect low abundance of mRNA.

Here, unlike the human oocyte at the germinal vesicle stage, Mn SOD messengers were already adenylated at the immature stage as it is described for the mouse oocyte (El Mouatassim et al., 1999). Most immature oocytes had a strong Mn SOD PCR signal but the signal was detected in fewer samples as development proceeded (72% of immature oocytes vs. 63% of mature oocyte and 43% of the zygotes). Although this decrease was not significant, an enhanced sensitivity of the method was necessary to detect the signal in 5- to 8-cell embryos. So maternal Mn SOD mRNA, accumulated in the oocyte, is progressively degraded during maturation and early cleavage as described for many genes (Bachvarova et al., 1989; Bilodeau-Goeseels and Schultz, 1997; Wrenzycki et al., 1998).

After degradation of maternal mRNA, embryonic expression of Mn SOD was found to be dependent on culture conditions. No expression could be detected before the blastocyst stage when culture was performed in the absence of serum while for in vivo embryos and for in vitro embryos cultured with 5% serum, Mn SOD mRNA was already expressed in half of the analyzed morulae. In vitro culture can alter gene expression, Stojanov and co-workers (Stojanov and O'Neill, 1999; Stojanov et al., 1999) have described a delay in zygotic expression in in vitro cultured mouse embryo vs. fresh in vivo embryo for PAF receptor and for IGF-II. In bovine embryo, different patterns of expression have been reported in blastocysts produced in vitro compared with the in vivo ones (Wrenzycki et al., 1996; Eckert and Niemann, 1998). Different in vitro culture conditions can also modulate the expression system. In a recent study, Wrenzycki et al. (1999) have compared the expression level for eight genes in bovine embryos produced in two in vitro culture systems: one containing 0.1% polyvinyl alcohol (PVA) and the other containing 10% oestrus cow serum. A lower expression was observed in the presence of serum except for the heat shock protein gene (*HSP70*) with an expression significantly increased with serum as we report here for Mn SOD at the morula stage. Serum contains numerous defined and undefined factors that may interact with the expression system as, for example, growth factors such as TNFα known as an inductor of Mn SOD transcription (Wong and Goeddel, 1988). The role of serum in in vitro embryo culture should be very carefully considered.

We have also to keep in mind that kinetics of development can depend on culture conditions. Van Soom et al. (1997) have reported a slower transition from morula to blastocyst stage in vivo compared to their in vitro counterparts. Compaction in cattle takes place at the 32-cell stage but some in vivo compacted morulae could be found with as many as 95 cells, implying that the compacted stage remains unchanged during more than one cleavage division in in vivo embryos. According to these data an in vivo morula can have more than twice the number of cells than an in vitro morula. This higher cell number could explain why some in vivo morulae expressed Mn SOD but none in vitro without serum. Unfortunately our actin PCR results could not be used efficiently to quantify the cell numbers of each embryo. On the other side, in vitro, transition from the 16-cell stage up to the morula stage occurs nearly one day earlier in the presence of serum (Van Langendonckt et al., 1997) but morulae with serum are not characterized by a higher number of cells. So developmental acceleration with serum is maybe associated with a different temporal pattern of gene expression.

The activity of Mn SOD is restricted to the mitochondria and plays a key role in protecting them from oxidative damage (Fridovich, 1995). As oxidative metabolism increases sharply at the morula stage (Thompson et al., 1996) it could explain why transcription onset of Mn SOD started at the earliest at that stage. Moreover, Mn SOD expression was found to be associated with cellular differentiation (St Clair et al., 1994) and compaction at the morula stage correlate with the first differentiation event (Shehu et al., 1996).

Independently of Mn SOD mRNA variations during preimplantation development, the protein was detectable at the zygote and at the blastocyst stage in embryos cultured in vitro. At that blastocyst stage either the protein was newly synthesized or it was stable enough to persist and protect the embryo during the in vitro culture period. A temporal pattern of protein expression is difficult to demonstrate as it would need a large number of bovine embryos.

Finally, whatever was the stage of embryonic development, around 20% of the samples analyzed did not show a signal after actin amplification as we had already reported (Lequarré et al., 1997). Actin is probably not the best reporter messenger since its expression level can vary according to culture conditions (Church et al., 1992) and even according to each blastomere of an embryo (Krüssel et al., 1998). A recent study (Steuerwald et al., 1999), using highly sensitive fluorescent RT-PCR, also demonstrate variability in actin mRNA content on single human oocyte. Absence of actin PCR signal for some samples in our study could be indicative of a low quality oocyte or embryo. Moreover, samples positive for both actin and globin were

not necessarily positive for the two SODs, Mn SOD disappearing faster usually. The stability of a mRNA is a function of its primary and higher-order structure, as well as its interactions with a variety of trans-acting RNA-binding proteins (Liebhaber, 1997) so it will be different for each messenger analyzed and it could vary according to the quality of the sample analyzed.

In conclusion, we have showed here the expression of the cytosolic Cu/Zn SOD and the mitochondrial Mn SOD in bovine oocytes and embryos at both mRNA and protein levels. Embryonic mRNA detection of Mn SOD is dependent on culture conditions and is induced earlier in the presence of serum. The mRNA content of individual oocyte and embryo is often heterogeneous which may reflect different potential of development.

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