3,4-Dihydroxyflavone Acts as an Antioxidant and Antiapoptotic Agent to Support Bovine Embryo Development In Vitro

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Abstract. The effects of two antioxidants, superoxide dismutase (SOD) and the flavonoid 3,4-dihydroxyflavone (DHF), on bovine embryo development in vitro were examined. Blastocyst development, total cell and inner cell mass (ICM) numbers, intracellular levels of reactive oxygen species (ROS), apoptotic indices and gene expression levels were examined before and after treatment of day 2 bovine embryos (22–4 cells) with various concentrations of 3,4-DHF or SOD for 6 days. Statistical analysis was performed using analysis of variance, with significance defined at the P<0.05 level. SOD had no significant effect on bovine embryo development at any tested concentration (control, 32.8%; 300 U/ml, 33.9%; 600 U/ml, 24.2%). In contrast, 10 μM 3,4-DHF promoted higher blastocyst development (39.3%) than any other concentration (control, 26.7%; 1 μM, 30.3%; 50 μM, 29.5%; 100 μM, 20.5%). Compared with 300 U/ml SOD, 10 μM 3,4-DHF resulted in significantly higher blastocyst development (44.2%) (control, 31.5%; SOD 300 U/ml, 33.6%). Treatment with 3,4-DHF increased the ICM cell number and reduced intracellular ROS production and apoptotic cell numbers. When O2 tension was decreased from 20% (high tension) to 5% (low tension), embryo development rates were doubled regardless of 3,4-DHF treatment. Under high O2 tension, 10 μM 3,4-DHF treatment may render bovine embryo development similar to a low O2 tension environment. The best blastocyst development was obtained under low O2 tension plus 10 μM 3,4-DHF treatment. The relative expression levels of antioxidant (MnSOD), antiapoptotic (Survivin, Bax inhibitor) and growth-related genes (IFN-τ, Glut-5) were significantly increased after 3,4-DHF treatment, while the expression levels of oxidant (Sox) and apoptotic genes (Caspase-3 and Bax) were reduced. These results suggest that 3,4-DHF may promote the in vitro development of bovine embryos through its antioxidant and antiapoptotic effects.

Key words: Antiapoptotic, Antioxidant, Bovine embryos, 3,4-Dihydroxyflavone, Superoxide dismutase (SOD)

In vitro bovine embryo production generally involves oocyte maturation, fertilization and zygote culture. For in vitro culture of mammalian embryos, an environment consisting of 5% CO2 and 95% air (~ 20% O2 total) is widely used [1]. However, a high O2 concentration during in vitro culture impedes embryonic development, perhaps due to the increased accumulation of reactive oxygen species (ROS) in the cytoplasm of developing embryos [2]. These ROS are highly reactive with intracellular macromolecules, including proteins, lipids and DNA, and may cause significant dysfunction such as enzyme inactivation, mitochondrial abnormalities or DNA fragmentation [2, 3]. Living organisms possess natural protective equivalents/ROS scavengers, which are intracellular antioxidants that counteract the negative effects of ROS [4]. However, antioxidant levels in vitro are lower than those in vivo; thus, antioxidant supplementation of the medium may support improved developmental capacity [5, 6]. Antioxidants include enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase, as well as cysteine and ascorbic acids and alpha-tocopherol. In the antioxidant system, SODs are the initial enzymes that promote the conversion of an anion superoxide to H2O2, which in turn is removed by catalase or glutathione peroxidase. SODs are characterized by their metal cofactor requirements and subcellular localizations.

The structures and functions of flavonoids have evoked considerable interest because of their antioxidant properties. The flavonoid structure is shared by the monomeric flavonols, flavones and flavanones. Some flavonoids exhibit potent antitumor properties and can modulate apoptosis, differentiation and the cell cycle, probably by virtue of their antioxidant functions [7]. Flavonoids may inhibit the generation of primary oxygen radicals and subsequent oxidation chains, since they are effective chelators of transition metal ions [8]. The number of hydroxyl substitutions in a flavonoid is thought to be a critical factor in its ROS-scaping ability [9].

The present study examined the use of a flavonoid as an antiiox-
dant agent in bovine embryo development in vitro. In particular, we compared the embryonic effects of treatment with 300 U SOD or with an optimal concentration of the flavonoid 3,4-dihydroxyflavone (DHF; Fig. 1A). The effects of 3,4-DHF treatment were also examined under high (20%) and low (5%) concentrations of oxygen tension. Antioxidant and antiapoptotic effects were examined by screening for intracellular ROS expression, DNA fragmentation and the gene expression level.

Materials and Methods

Chemicals and reagents
All chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated.

Production of bovine preimplantation embryos
The culture procedures used to produce preimplantation embryos from follicular oocytes were described previously [10]. Briefly, bovine ovaries were obtained from a slaughterhouse, and cumulus-oocyte complexes (COCs) were aspirated from 3- to 6-mm-sized visible follicles. The COCs were washed with HEPES-buffered Tyrode’s medium. They were then cultured for 22–24 h at 39 C in a 5% CO2 incubator in buffered Tyrode’s medium. They were then cultured for 22–24 h at 39 C in a 5% CO2 incubator in buffered Tyrode’s medium. COCs were transferred to fertilization (IVF) TL-STOCK medium [11] and inseminated with 2 µl of highly motile sperm (25×10⁶ spermatozoa/ml) recovered from frozen-thawed Hanwoo bull semen separated on a discontinuous 90% and 45% Percoll column (GE Healthcare, Bio-Sciences AB, Upsala, Sweden). Two microliters each of heparin (2 µg/ml) and PHE (18.2 M penicillamine, 9.1 M hypotaurine and 1.8 M epinephrine) were also added in a 44-µl IVF drop. The day of IVF was defined as day 0. Fertilization was assessed by the cleavage rate (≥2-cell) after 44 ± 2 h of incubation. For in vitro culture, cleaved embryos were incubated in CR1aa medium containing 3 mg/ml of fatty acid-free (FAF) BSA until day 4. Embryos were then transferred into CR1aa medium containing 10% FBS until day 8.

Blastocyst differential staining
The blastomere, inner cell mass (ICM) and trophectoderm (TE) cell numbers in blastocysts were counted by differential staining according to Thouas et al. [12]. Zona-intact blastocysts were incubated in 500 µl of Solution 1 (TL-HEPES–buffered medium with 1% Triton X-100 and 100 µg/ml propidium iodide, PI) for up to 30 sec or until the trophectoderm noticeably changed color to red and shrank slightly (monitored visually using a 25× dissecting microscope). Blastocysts were then immediately transferred into 500 µl of Solution 2 (fixative solution of 100% ethanol with 25 µg/ml bisbenzimide; Hoechst 33258) and stored at 4 C overnight. Fixed and stained blastocysts were transferred from Solution 2 directly into glycerol, taking care to avoid carry over of excessive amounts of Solution 2. Blastocysts were mounted onto a glass microscope slide in a drop of glycerol, gently flattened with a cover slip and visualized for cell counting. Labeled nuclei were observed by fluorescence microscopy with an ultraviolet filter (excitation, 330–385 nm; emission, 420 nm). The PI- and bisbenzimide-labeled TE nuclei appeared pink or red. Bisbenzimide-labeled ICM nuclei appeared blue.

Intracellular ROS levels
The H2O2 level in each embryo was determined by the dichlorodihydrofluorescein diacetate method (DCFDA; Molecular Probes, Eugene, OR, USA) described previously [13]. Blastocysts produced in vitro were transferred into culture medium containing 5 mM DCFDA, incubated for 20 min at 37 C, washed in PBS, placed on glass slides and covered with a cover glass. The fluorescence of DCF (excitation, 488 nm; emission, 520 nm) was measured using an inverted fluorescence microscope (Olympus, Tokyo, Japan). Recorded fluorescent images were analyzed using Image-Pro Plus 5.1 (Media Cybernetics, Bethesda, MD, USA) by counting the number of pixels after color inversion.

Terminal deoxynucleotid transferase (TDT)-mediated dUTP nick-end labeling (TUNEL) assay
Blastocysts were washed in PBS (pH 7.4) containing 1 mg/ml polyvinylpyrolidone (PBSD/PVP) and fixed in 3.7% paraformaldehyde in PBS for 1 h at room temperature (RT). After fixation, embryos were washed in PBS/PVP, permeabilized by incubation in 0.3% Triton X-100 for 1 h at RT, washed twice with PBS/PVP and incubated with fluorescein-conjugated dUTP and a TDT enzyme (Roche, Mannheim, Germany) in the dark for 1 h at 37 C. After counterstaining with a mixture of 40 µg/ml PI and 50 µg/ml RNase A for 1 h at 37 C to label nuclei, embryos were washed in PBS/PVP, mounted with slight coverslip compression and examined by fluorescence microscopy. Red, green and yellow (merged) indicate chromatin, fragmented DNA and fragmented DNA of an apoptotic blastomere, respectively. The apoptotic index was determined as the percentage of yellow blastomerex among the total number of red blastomeres.

Messenger RNA extraction
Messenger RNA for real-time RT-PCR was prepared from blastocysts using magnetic beads (Dynabeads mRNA Purification Kit; Dynal, Oslo, Norway) following the manufacturer’s instructions. Briefly, 15 oocytes or embryos were suspended in 100 µl of lysis/binding buffer [100 mM Tris-HCl (pH 7.5), 500 mM LiCl, 10 mM EDTA (pH 8.0), 1% lithium dodecyl sulfate (LiDS) and 5 mM DTT] and vortexed at RT for 5 min. A 50 µl aliquot of an oligo (dT) 25 magnetic-bead suspension was added to the samples and incubated at RT for 5 min. The hybridized mRNA and oligo (dT) beads were washed twice with washing buffer A [10 mM Tris-HCl (pH 7.5), 0.15 M LiCl, 1 mM EDTA and 1% LiDS] and once with washing buffer B [10 mM Tris-HCl (pH 7.5), 0.15 M LiCl and 1 mM EDTA]. The mRNA samples were eluted from beads in 15 µl of double-distilled DEPC-treated water.

Real-time RT-PCR quantification
After mRNA extraction, cDNA was synthesized using an oligo
(dT) 12–18 primer and Superscript reverse transcriptase (Invitrogen). Real-time RT-PCR (Bio-Rad, Chromo4) was performed using the primer sets shown in Table 1. In all experiments, histone H2A mRNA served as an internal standard. The threshold cycle (Ct) value represents the cycle number at which the sample fluorescence rose statistically above background noise. To monitor the reactions, the protocol provided with the DyNAmo SYBR green qPCR kit was used. This kit contains a modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM MgCl₂ and a dNTP mix that includes dUTP (Finnzyme Oy, Espoo, Finland). The PCR protocol used a denaturation step at 95°C for 15 min; amplification and quantification cycles that were repeated 40 times at 94°C for 30 sec, at 50 or 56°C for 1 min and at 72°C for 1 min using a single fluorescence measurement; and a melting curve program of 65–95°C with a heating rate of 0.2°C/sec and continuous fluorescence measurement. Samples were cooled to 12°C. SYBR Green fluorescence was measured after the extension step during the PCR reactions. PCR products were analyzed by generating a sequence-specific melting curve to distinguish non-specific from specific PCR products. The crossing point (CP), defined as the point at which fluorescence rises appreciably above background noise, was determined for each transcript. Gene expression was quantified by the 2-ddCt method [14].

**Experimental design**

Experiment 1. Effect of SOD treatment on bovine embryo development: *In vitro*-produced day 2 embryos (≥2–4 cells) were cultured in CR1aa medium alone (control) or containing 300 U/ml or 600 U/ml SOD. For *in vitro* culture, day 2 embryos were cultured in CR1aa containing 3 mg/ml FAF-BSA from days 2 to 4 and then in CR1aa containing 10% FBS until embryonic day 8. Experiments were replicated three times.

Experiment 2. Effect of 3,4-DHF treatment on bovine embryo development: To examine the effect of 3,4-DHF (INDOFINE Chemical, Hillsborough, NJ, USA) on bovine embryo development, day 2 embryos (≥2–4 cells) were cultured in CR1aa medium alone (control) or containing various concentrations (1, 10, 50 or 100 μM) of 3,4-DHF. The *in vitro* culture conditions were the same as in experiment 1. Experiments were performed four times.

Experiment 3. Comparison of the effect of optimal SOD or 3,4-DHF treatment on bovine embryo development: To compare the effect of optimal SOD or 3,4-DHF concentrations on bovine embryo development, day 2 embryos (≥2–4 cells) were cultured in CR1aa medium alone (control) or containing various concentrations (1, 10, 50 or 100 μM) of 3,4-DHF. The *in vitro* culture conditions were the same as in experiment 1. The embryo development rates and cell numbers were examined. Experiments were replicated four times.

Experiment 4. Analysis of the antioxidant and antiapoptotic effects of SOD or 3,4-DHF on bovine embryo development: The intracellular ROS level, apoptotic index and relative mRNA expression levels of eight candidate genes (Sox, MnSOD, Caspase-3, Bax, Survivin, Bax-1, IFN-τ and Glut-5) in *in vitro*-produced day 8 blastocysts treated with or without SOD or 3,4-DHF were examined.

Experiment 5. Development of bovine embryos treated with 3,4-DHF under various oxygen tensions: Embryo culture was performed in 5% CO₂ in air (high O₂ tension, ~20%) or in 5% O₂, 5% CO₂, and 90% N₂ (low O₂ tension) at 39°C. Day 2 embryos cultured under the high O₂ condition were equally distributed into high or low O₂ condition groups, and embryos were redistributed into groups with or without 3,4-DHF treatment. The base embryo culture was the same as in experiment 1. The embryo development rates and cell numbers were examined. Experiments were performed four times.

**Statistical analysis**

Differences in embryonic development, apoptotic index and relative gene expression levels between treatment and control groups...
were evaluated by analysis of variance (ANOVA) using the general linear model program PROC-GLM in the SAS software. P-values of < 0.05 were considered significant.

### Results

**Effect of SOD on bovine embryo development**

No significant differences in blastocyst rate were observed among the SOD treatment groups, although the result for the 600 U/ml SOD group was not statistically lower than that of the other groups (Table 2).

**Effect of 3,4-DHF on bovine embryo development**

The blastocyst development rate of the 10 μM 3,4-DHF group was significantly higher than that of the control or 100 μM 3,4-DHF group but not different from that of the 1 μM or 50 μM 3,4-DHF group (Table 3). The 3,4-DHF treatment effect was detected from the day 6 morula stage. The blastocyst development of embryos in the 10 μM 3,4-DHF group was higher than that of the other treatment groups. Embryos in the 100 μM 3,4-DHF group displayed the lowest embryo development rate among the treatment groups, with rates significantly lower than those of the 10 μM 3,4-DHF group.

**Comparison of the effect of optimal SOD or 3,4-DHF treatment on bovine embryo development**

Based on the results above, we compared the effects of treatment with 300 U/ml SOD or 10 μM 3,4-DHF on bovine embryo development in vitro. Figure 1B shows representative photomicrographs of day 8 blastocysts in each treatment group and their differential staining. The 10 μM 3,4-DHF group displayed the highest embryo development rate (Table 4). This difference was detected from the morula stage. The 10 μM 3,4-DHF group displayed a significantly increased ICM cell number, but this treatment did not affect the increase in the total cell number.

#### Antioxidant and antiapoptotic effects of SOD and 3,4-DHF on bovine embryo development

Figure 1C shows representative phase contrast (upper panels) and fluorescence photomicrographs (lower panels) of day 8 blastocysts developed in vitro under different culture conditions (control, 300 U/ml SOD, or 10 μM 3,4-DHF). Compared with the antioxidant treatment groups, the fluorescence level of the control group embryos was very high, and the level of the 3,4-DHF treatment group was the lowest. When this result was quantified using image analysis, the mean fluorescent densities of embryos in each treatment group were significantly different (control, 106 ± 16.6; SOD, 59 ± 20.5; 3,4-DHF, 33 ± 10.6). Adding antioxidants reduced the intracellular ROS production. Signal intensity levels were significantly decreased in the 3,4-DHF treatment group (Fig. 1D).

Approximate values for the apoptotic index per blastocyst were determined using fluorescence microscopy with the TUNEL assay (Fig. 1E–F). The apoptotic index of the 10 μM 3,4-DHF treatment group (2.96 ± 2.4%) was significantly lower than that of the control (6.87 ± 4.3%). However, the apoptotic index was not significantly different between the antioxidant treatment groups.

Expression levels of candidate antioxidant (MnSOD), antiapoptotic (Survivin and Bax inhibitor-I) and implantation genes (interferon tau, IFN-τ) were significantly increased after 3,4-DHF or SOD treatment. In contrast, expression levels of candidate oxidant (SOX) and apoptotic genes (Caspase-3 and Bax) were similar. Expression of the metabolic gene Glut-5 was significantly increased after 3,4-DHF treatment compared with the other groups (Fig. 2).

### Tables

#### Table 2. Effect of SOD on bovine embryo development in vitro (r=3)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2 ≥2–4 cell</th>
<th>No. (%) of embryos developed to ≥8 cell</th>
<th>Day 4 ≥ morula</th>
<th>Day 8 ≥ blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>122</td>
<td>100 (82.0)</td>
<td>64 (52.5)</td>
<td>40 (32.8)</td>
</tr>
<tr>
<td>300 U/ml SOD</td>
<td>124</td>
<td>101 (81.5)</td>
<td>68 (54.8)</td>
<td>42 (33.9)</td>
</tr>
<tr>
<td>600 U/ml SOD</td>
<td>124</td>
<td>91 (73.4)</td>
<td>56 (45.2)</td>
<td>30 (24.2)</td>
</tr>
</tbody>
</table>

#### Table 3. Effect of 3,4-DHF on bovine embryo development in vitro (r=4)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2 ≥2–4 cell</th>
<th>No. (%)* of embryos developed to ≥8 cell</th>
<th>Day 4 ≥ morula</th>
<th>Day 8 ≥ blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>120</td>
<td>95 (79.2)</td>
<td>52 (43.3)*</td>
<td>32 (26.7)*</td>
</tr>
<tr>
<td>1 μM 3,4-DHF</td>
<td>122</td>
<td>95 (77.9)</td>
<td>57 (46.7)*</td>
<td>37 (30.3)*</td>
</tr>
<tr>
<td>10 μM 3,4-DHF</td>
<td>122</td>
<td>99 (81.1)</td>
<td>64 (52.5)*</td>
<td>48 (39.3)*</td>
</tr>
<tr>
<td>50 μM 3,4-DHF</td>
<td>122</td>
<td>89 (73.0)</td>
<td>53 (43.4)*</td>
<td>36 (29.5)*</td>
</tr>
<tr>
<td>100 μM 3,4-DHF</td>
<td>122</td>
<td>90 (73.8)</td>
<td>50 (41.0)*</td>
<td>25 (20.5)*</td>
</tr>
</tbody>
</table>

* Means in the column without common superscripts are significantly different (P<0.05).
Development of bovine embryos treated with 3,4-DHF under various oxygen tensions

The bovine embryo development rates were significantly higher under low O₂ tension than under high O₂ tension when 3,4-DHF was not added. Under high O₂ tension, the blastocyst rate was significantly higher in the 3,4-DHF group than in the no-treatment group. Under low O₂ tension, no difference in blastocyst rate was seen between the 3,4-DHF and no-treatment groups. The total cell numbers were significantly different among the low O₂ tension groups but not among the high O₂ tension groups. The ICM number was not different between the treated and untreated groups under low or high O₂ tension. The best result was produced under low O₂ tension with 10 μM 3,4-DHF treatment (Table 5).

Discussion

The results of the present study demonstrate that the antioxidant flavonoid 3,4-DHF has positive effects on in vitro bovine embryo development and improves the ICM numbers while significantly decreasing intracellular ROS production and the number of apop-
totic cells in the blastocyst. Treatment with 3,4-DHF significantly increased the expression levels of antioxidant (MnSOD), antiapoptotic (Survivin, Bax inhibitor) and growth-related genes (Glut-5, IFN-τ), but did not alter the expression of candidate oxidant (Sox) or apoptotic genes (Caspase-3, Bax). It had a stronger effect than treatment with SOD on bovine embryo development due to its highly potent antioxidant and antiapoptotic characteristics.

The arrest of embryo development in vitro is caused by a high abundance of ROS resulting from the relatively higher ambient oxygen concentration and lower levels of free radical scavengers than under in vivo conditions [15]. Addition of free radical scavengers and metal chelators, such as SOD, EDTA and thioredoxin, to the culture media under normal ambient oxygen conditions may enhance embryo development [16]. In this study, we compared the treatment effect of two reagents, SOD and 3,4-DHF, as antioxidants on bovine embryo development. SOD catalyzes the dismutation of superoxide into oxygen and H₂O₂, presenting an important antioxidant defense in nearly all cells exposed to oxygen. As an antioxidant agent and an effective chelator of transition metal ions, 3,4-DHF reduces oxygen and nitrogen free radicals and inhibits the generation of primary oxygen radicals and subsequent chain oxidation [17].

Unexpectedly, SOD treatment did not significantly affect bovine embryo development compared with the untreated control. Embryo development following treatment with 600 U/ml SOD was lower than that of the control or the 300 U/ml SOD treatment group. Li et al. [18] reported increasing positive effects on rabbit embryo development when 100 or 300 U/ml SOD was used, with no further benefits at concentrations >600 U/ml. The 3,4-DHF treatment at 10 μM resulted in significantly higher embryo development rates compared with the untreated control. In the present study, the highest concentration (100 μM) of 3,4-DHF also resulted in significantly higher embryo development rates compared with the untreated control. In the present study, the highest concentration (100 μM) of 3,4-DHF also resulted in significantly higher embryo development rates compared with the untreated control.

### Table 4. Effect of SOD or 3,4-DHF on bovine embryo development in vitro (r=4)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2 ≥2–4 cell</th>
<th>Day 4 ≥8 cell</th>
<th>Day 6 ≥morula</th>
<th>Day 8 ≥blastocyst</th>
<th>No. of total cells (ICM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>130</td>
<td>96 (73.8)</td>
<td>54 (41.5)</td>
<td>41 (31.5)</td>
<td>90.0 ± 17.7 (17.5 ± 4.7)</td>
</tr>
<tr>
<td>300 U SOD</td>
<td>128</td>
<td>95 (74.2)</td>
<td>51 (39.8)</td>
<td>43 (33.6)</td>
<td>94.6 ± 10.3 (21.1 ± 5.0)</td>
</tr>
<tr>
<td>10 μM 3,4-DHF</td>
<td>129</td>
<td>103 (79.8)</td>
<td>83 (64.3)</td>
<td>57 (44.2)</td>
<td>104.2 ± 13.9 (33.5 ± 4.3)</td>
</tr>
</tbody>
</table>

* Means in the column without common superscripts are significantly different (P<0.05).

### Table 5. Effect of 3,4-DHF treatment under conditions of high or low oxygen tension on bovine embryo development in vitro (r = 4)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>O₂</th>
<th>3,4-DHF</th>
<th>Day 2 ≥2–4 cell</th>
<th>Day 4 ≥8 cell</th>
<th>Day 6 ≥morula</th>
<th>Day 8 ≥blastocyst</th>
<th>No. of total cells (ICM)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>−</td>
<td>3,4−DHF</td>
<td>126 (94.6)</td>
<td>56 (44.4)</td>
<td>36 (28.6)</td>
<td>92.7 ± 17.3 (20.9 ± 7.7)</td>
<td></td>
</tr>
<tr>
<td>(~20%)</td>
<td>+</td>
<td></td>
<td>126 (98 (77.7)</td>
<td>66 (52.4)</td>
<td>54 (42.9)</td>
<td>107.6 ± 13.4 (35.5 ± 10.3)</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>−</td>
<td></td>
<td>123 (98 (79.7)</td>
<td>73 (59.3)</td>
<td>60 (48.8)</td>
<td>129.3 ± 13.1 (39.5 ± 13.5)</td>
<td></td>
</tr>
<tr>
<td>(~5%)</td>
<td>+</td>
<td></td>
<td>123 (102 (82.9)</td>
<td>78 (63.4)</td>
<td>66 (53.7)</td>
<td>159.2 ± 15.5 (42.8 ± 12.0)</td>
<td></td>
</tr>
</tbody>
</table>

* 3,4-DHF, 10 μM. ** Means in the column without common superscripts are significantly different (P<0.05). No: Mean ± SD

### Fig. 2. Relative mRNA expression levels of selected genes in bovine day 8 blastocysts.

*P<0.05 among the three groups.

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Table 4. Effect of SOD or 3,4-DHF on bovine embryo development in vitro (r=4)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 2 ≥2–4 cell</th>
<th>Day 4 ≥8 cell</th>
<th>Day 6 ≥morula</th>
<th>Day 8 ≥blastocyst</th>
<th>Cell number of blastocyst (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>130</td>
<td>96 (73.8)</td>
<td>54 (41.5)</td>
<td>41 (31.5)</td>
<td>90.0 ± 17.7 (17.5 ± 4.7)</td>
</tr>
<tr>
<td>300 U SOD</td>
<td>128</td>
<td>95 (74.2)</td>
<td>51 (39.8)</td>
<td>43 (33.6)</td>
<td>94.6 ± 10.3 (21.1 ± 5.0)</td>
</tr>
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<td>10 μM 3,4-DHF</td>
<td>129</td>
<td>103 (79.8)</td>
<td>83 (64.3)</td>
<td>57 (44.2)</td>
<td>104.2 ± 13.9 (33.5 ± 4.3)</td>
</tr>
</tbody>
</table>

* Means in the column without common superscripts are significantly different (P<0.05).
in lower embryo development compared with the control. This result suggests that 3,4-DHF has two different actions, similar to
green tea polyphenol [4], acting as an antioxidant at lower concen-
trations and as a prooxidant at higher concentrations. Based on the
results above, the effects of SOD (300 U/ml) and 3,4-DHF (10 μM)
on bovine embryo development in vitro were compared. Treatment
with 3,4-DHF improved embryo development compared with the
other groups, whereas 300 U/ml SOD had no effect. It also
improved cell numbers by increasing the number of ICM cells in
blastocysts, whereas SOD treatment did not affect cell numbers in
embryos.

The O2 tension in the oviducts or uterus in several species is only
30–40% of that in air [19]. In vitro embryonic culture under high
O2 tension may produce free radicals, which are detrimental to
development [20]. Reduction of O2 from 20% to 5% reportedly
enhances cattle embryonic development [21–23]. All of the
embryos mentioned above (Tables 2–4) were cultured at 39 C in a
5% CO2 incubator. This condition is a high (approximately 20%)
O2 atmosphere, in which ROS levels are also expected to be high
[15]. When embryo development was compared under high and
low (5%) O2 atmospheres, reducing O2 tension from 20% to 5%
doubled the blastocyst rates regardless of 3,4-DHF treatment.
Under high O2 tension, the addition of 10 μM 3,4-DHF had a fur-
ther positive effect on bovine embryo development by increasing
the cell numbers in blastocysts. Thus, the best result for embryo
development was obtained under low O2 tension in the presence of
3,4-DHF.

The ROS levels in control group embryos were significantly
higher than those in the antioxidant treatment groups. There were
significant differences in the fluorescent densities (Fig. 1D), with
both antioxidant treatments (SOD, 3,4-DHF) presenting remark-
ably reduced ROS production. The effect of 3,4-DHF was
significantly higher than that of SOD. When embryos were cul-
tured in the presence of 10 μM 3,4-DHF, the apoptotic index was
significantly lower than that of the untreated control group. Oxida-
tive stress such as that caused by OH- induces DNA fragmentation
in cells [2], probably due to an increase in the number of DNA dou-
ble-strand breaks. Double-strand DNA breaks would be induced
by a high frequency in embryos cultured under the 20% O2 condi-
tion [1].

In this study, real-time RT-PCR was used to evaluate the relative
expression levels of oxidation-, apoptosis- and growth-related
genes. Antioxidant MnSOD gene expression levels were increased
after 3,4-DHF or SOD treatment (Fig. 2). MnSOD helps to main-
tain intracellular ROS levels and the redox balance; increased
MnSOD levels protect normal tissue against oxidative stress [24].
MnSOD modulates the activation of several redox-sensitive tran-
scriptional factors, such as NF-κB and AP-1, and alters gene
expression associated with apoptosis [25]. Higher MnSOD expres-
sion has been reported in embryos exposed to in vivo conditions or
reduced O2 tensions compared with those cultured under high O2
tension [26]. In the present study, Survivin, Bax-inhibitor, Glut-5
and IFN-τ transcripts showed higher expression levels after 3,4-DHF
therapy than the untreated or SOD-treated groups.

Embryo culture under low oxygen conditions is reportedly asso-
ciated with fewer perturbations in the global pattern of gene
expression and more closely resembles that of in vivo control
embryos [27]. Rizos et al. [28] showed that differences in mRNA
expression patterns are related to the quality of the bovine blasto-
cysts produced under different culture conditions. From this
viewpoint, high expression levels of the MnSOD, Survivin, Bax-Ι,
Glut-5 and IFN-τ transcripts appear to be indicative of high-quality
embryos, while high relative levels of Sox, Caspase-3 and Bax
transcripts are indicative of low-quality embryos. Supplementation
of the two types of antioxidants appeared to increase the antioxid-
ant and antiapoptotic gene transcript levels. The expression levels
of antiapoptotic and growth-related genes appeared to be higher in
the 3,4-DHF treatment group than in the SOD treatment group.
Together, these results suggest that 3,4-DHF supplementation
improves the embryo culture environment by significantly reduc-
ing the ROS content and apoptotic cell number and enhancing
antioxidant and antiapoptotic gene expression. The flavonoid 3,4-
DHF is therefore a highly potent antioxidant that promotes the in
vitro developmental capacity of bovine embryos by antioxidant and
antiapoptotic effects.

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References

1. Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effects of oxygen concentration and
antioxidants on the in vitro developmental ability, production of reactive oxygen spe-
cies (ROS), and DNA fragmentation in porcine embryos. Theriogenology 2004; 62: 1136–
1197.

2. Guerin PEI, Mouatassim S, Menezo Y. Oxidative stress and protection against reactive
oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod

3. Halliwell R, Gutteridge JMC. The chemistry of oxygen radicals and other derived
1989; 22–85.

4. Wang ZG, Yu SD, Xu ZR. Improvement in bovine embryo production in vitro by treat-
ment with green tea polyphenols during in vitro maturation of oocytes. Anim Reprod Sci

5. Livingston T, Eberhardt D, Edwards JL, Godkin J. Retinol improves bovine embry-

6. Ali AA, Biloadeus JF, Sizard MA. Antioxidant requirements for bovine oocytes varies
during in vitro maturation, fertilization and development. Theriogenology 2003; 59: 939–
949.

7. Lee EE, Kim JH, Kang YJ, Cho SG. The anti-apoptotic and anti-oxidant effect of eri-

8. Afanas’ev IB, Dorozhko AI, Brodskii AV, Kostyk VA, Potapovitch AI. Chelating
and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in

ascorbate/F2- induced oxidative stress in cultured retinal cells. Biochem Pharmacol

10. Park SP, Kim EY, Kim DI, Park NH, Won YS, Yoon SH, Chung KS, Lim JH. Systems for
production of calves from Hanwoo (Korean native cattle) IVM/IVF/IVC blasto-

11. Barioler BD, Leibfried ML, Lieberman G. Development of preimplantation embryos

12. Thouas GA, Kontiatis NA, French AJ, Jones GM, Trounson AO. Simplified tech-
nique for differential staining of inner cell mass and trophectoderm cells of mouse and


