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Native plants (*Phellodendron amurense* and *Humulus japonicus*) extracts act as antioxidants to support developmental competence of bovine blastocysts

Geon-Yeop Do[1,2,a], Jin-Woo Kim[1,a], Hyo-Jin Park[1], Seung-Bin Yoon[3,4], Jae-Young Park[1], Seul-Gi Yang[1], Bae Dong Jung[5], Yong-Soo Kwon[6], Man-Jong Kang[7], Bong-Seok Song[3,4], Sun-Uk Kim[3,4], Kyu-Tae Chang[3,4], and Deog-Bon Koo[1,*]

* **Corresponding Author:** Deog-Bon Koo
Tel: +82-53-850-6557, **Fax:** +82-53-850-6559,
E-mail: dbkoo@daegu.ac.kr

[1] Department of Biotechnology, College of Engineering, Daegu University, Gyeongsan 38453, Korea
[2] Maria Fertility Hospital, Daegu 41242, Korea
[3] Futuristic Animal Resource and Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju 28116, Korea
[4] National Primate Research Center, Korea Research Institute of Bioscience and Biotechnology, Cheongju 28116, Korea
[5] College of Veterinary Medicine & Institute of Veterinary Science, Kangwon National University, Chuncheon 24341, Korea
[6] College of Pharmacy, Kangwon National University, Chuncheon 24341, Korea
[7] Department of Animal Science, College of Agriculture and Life Sciences, Chonnam National University, Gwangju 61186, Korea

[a] The first two authors contributed equally to this work.
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Objective: *Phellodendron amurense* (*P. amurense*) and *Humulus japonicus* (*H. japonicus*) are closely involved in anti-oxidative response and increasing antioxidant enzymes activities. However, the effects of their extracts on development of preimplantation bovine embryos have not been investigated. Therefore, we investigated the effects of *P. amurense* and *H. japonicus* extracts on developmental competence and quality of preimplantation bovine embryos.
Methods: After *in vitro* fertilization, bovine embryos were cultured for 7 days in Charles Rosenkrans amino acid medium supplemented with *P. amurense* (0.01 µg/mL) and *H. japonicus* (0.01 µg/mL). The effect of this supplementation during *in vitro* culture on development competence and antioxidant was investigated.
Results: We observed that the blastocysts rate was significantly increased (p<0.05) in *P. amurense* (28.9%±2.9%), *H. japonicus* (30.9%±1.5%), and a mixture of *P. amurense* and *H. japonicus* (34.8%±2.1%) treated groups compared with the control group (25.4%±1.6%). We next confirmed that the intracellular levels of reactive oxygen species (ROS) were significantly decreased (p<0.01) in *P. amurense* and/or *H. japonicus* extract treated groups when compared with the control group. Our results also showed that expression of cleaved caspase-3 and apoptotic cells of blastocysts were significantly decreased (p<0.05) in bovine blastocysts derived from both *P. amurense* and *H. japonicus* extract treated embryos.
Conclusion: These results suggest that proper treatment with *P. amurense* and *H. japonicus* extracts in the development of preimplantation bovine embryos improves the quality of blastocysts, which may be related to the reduction of ROS level and apoptosis.

Keywords: Bovine Embryo; *Phellodendron amurense*; *Humulus japonicas*; Reactive Oxygen Species (ROS); Antioxidants

INTRODUCTION

In vitro production of mammalian embryos is essential for breeding, infertility therapy and fertility management in domestic animals [1]. However, *in vitro* produced (IVP) embryos cultivated under suboptimal culture conditions show decreased developmental competence and quality compared with *in vivo* produced embryos, [2-4]. Thus, many studies were conducted to improve the quality of IVP embryos via improvement of culture medium with growth factors and anti-oxidants.

In vitro production of mammalian embryos is commonly conducted under 5% CO_2 in air. However, IVP embryos grown under high O_2 concentrations have poor blastocysts qualities because of the increased accumulation of reactive oxygen species (ROS) in mammalian embryos

[5]. ROS, such as superoxide anions ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$) and induced oxidative stress, are known to damage the lipids, proteins and nucleic acids [6]. Therefore, the addition of antioxidants into culture medium improves developmental competence and qualities of IVP embryos.

Apoptosis is a programmed type of cell death that plays important roles in homeostasis and elimination of damaged cells [7]. In general, apoptosis is related to embryonic development and growth. Additionally, it is an important indicator of inadequate conditions for mammalian embryo development [8]. In addition, apoptosis is greater in IVP embryos than *in vivo* produced embryos. Therefore, decreasing apoptotic cells in blastocysts indicate improved culture conditions.

Phellodendron amurense (*P. amurense*) and *Humulus japonicus* (*H. japonicus*), which are usually found in forests of China, Korea, and Japan, are widely used in traditional medicine to treat pneumonia, diarrhea, pulmonary, tuberculosis and dysentery, as well as to promote blood circulation [9,10]. Extracts of *P. amurense* and *H. japonicus* have recently been reported to contain isoquinoline, alkaloids, phenolic compounds, flavone glycosides, terpenes, lupulones, phenolics and flavonoids [11-13]. Moreover, recent studies have shown that *P. amurense* and *H. japonica* extracts possess anti-oxidant functions [11,13]. However, the antioxidant effects of *P. amurense* and *H. japonica* extracts during preimplantation development of bovine embryos has not been thoroughly investigated.

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized by the pineal gland in the brain [14], and its secretion is dependent on the sleep-wake cycle, with the highest levels occurring at night [15]. Melatonin acts as a direct scavenger of toxic ROS, and also has the ability to decrease the formation of ROS. This compound also induces the activity of antioxidant enzymes and protects against the damage that may occur in response to oxidative stress. In particular, it plays an important role in decreasing and protecting against mitochondrial oxidative stress, as well as reducing apoptosis [16]. In addition, many studies have reported that melatonin enhances mouse, porcine, bovine and human embryo developmental competence [17-19]. Thus, we used melatonin as a positive control to confirm the effects of *P. amurense* and/or *H. japonicus* extracts on developmental competence through their roles as anti-oxidants in bovine embryos.

The present study was conducted to investigate the effects of addition of *P. amurense* and *H. japonicus* into culture medium on the developmental competence of preimplantation bovine embryos. We also assessed the effects of *P. amurense* and *H. japonicus* on elimination of ROS. Finally, the expression of apoptotic factor (cleaved caspase-3) and the index of apoptotic cells were investigated in bovine blastocysts after treatment with *P. amurense* and *H. japonicus* extracts.

METERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA)

Preparation of *P. amurense* and *H. japonicus*

P. amurense and *H. japonicus* extracts were obtained from Kangwon National University. Approximately 300 g of the dried *P. amurense* and *H. japonicas* were boiled in 3 L of methanol for 4 h. The extracts were then filtered using absorbent cotton, after which the solvent was removed from the filtered extracts by rotary vacuum evaporation at 40°C. Dried extracts were subsequently stored at room temperature until analysis. The extracts were dissolved in dimethyl sulfoxide before use.

In vitro production of bovine embryos

Bovine ovaries were collected from a local slaughterhouse (Gyeongsan, Gyeongbuk, Korea) and then transported to the laboratory in 0.9% saline supplemented with 75 μg/mL potassium penicillin G while maintained at 30°C to 35°C. Cumulus-oocyte complexes (COCs) were aspirated from 3 to 6 mm follicles using a disposable 10 mL syringe with an 18-gauge needle, after which COCs with surrounding cumulus cells and homogeneous cytoplasm were selected. The COCs were then washed three times in the Tyrode's lactate-N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (TL-HEPES) and twice in *in vitro* maturation (IVM) medium, after which 50 immature COCs were matured in 500 μL of IVM medium in a four-well multi-dish (Nunc, Roskilde, Denmark) for 20 to 22 h at 38.5°C under 5% CO_2 in air. The medium used for oocyte maturation was TCM-199 (Gibco Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco Life Technologies, Inc., USA), 10 IU/mL pregnant mare serum gonadotropin, 10 IU/mL human chorionic gonadotropin, 0.6 mM cysteine, 0.2 mM sodium pyruvate, 10 ng/mL epidermal growth factor, 25 μM β-mercaptoethanol, 25 μg/mL gentamycin and 1 μg/mL 17 β-estradiol. After culturing for IVM, 15 oocytes were fertilized with sperm that had been frozen and thawed at 2×10^6 cells/mL in 50 μL fertilization medium (Fert-TALP). When the sperm were added to the fertilization drops, 2 μg/mL heparin, 20 μM penicillamine, 10 μM hypotaurine, and 1 μM epinephrine were also added. At 22 h after insemination, the cumulus-enclosed oocytes were detached using gentle pipetting and then transferred to Charles Rosenkrans amino acid (CR1aa) medium containing 0.3% bovine serum albumin (BSA), 1× basal medium Eagle amino acids, and 1× minimum essential medium nonessential amino acids for *in vitro* culture (IVC). After culture for 3 days, we further cultured the cleaved embryos in a medium consisting of 50 μL of CR1aa (with 10% FBS) for 4 days at 38.5°C under 5% CO_2 in air. During the culture periods, presumptive embryos were treated with 0.01 μg/mL *P. amurense* and/or 0.01 μg/mL *H. japonicus* [20] and 0.1 μM melatonin [21] by the addition to the culture medium. The rates of cleavage and blastocyst stage embryos were determined on day 3 and 7, respectively.

Measurement of ROS levels

The ROS levels in blastocysts were measured using the dichlorodihydrofluorescein diacetate method (H_2DCFDA; Invitrogen Molecular Probes, Eugene, OR, USA) as previously described [5]. Blastocysts were transferred into CR-1aa (with 10% FBS) medium containing 5 μM H_2DCFDA for 20 min at 38.5°C under 5% CO_2 in air. The permeabilized blastocysts in H_2DCFDA were washed three times with 0.1% polyvinyl alcohol (PVA) in phosphate buffer solution (PBS). The fluorescent images were detected by epifluorescence microscopy (IX 51; Olympus, Tokyo, Japan) and then analyzed using the Image J software 1.38 (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining

Blastocysts were washed with 0.1% PVA in PBS, then fixed in 4% paraformaldehyde (PFA) in PBS for 1 h at room temperature, after which they were made permeable by incubation with 0.2% Triton X-100 at room temperature and then incubated in 0.1% PVA in PBS containing 1% BSA overnight at 4°C. Samples were then incubated in 10% FBS in PBS (v/v) for 45 min at 37°C, then incubated with cleaved caspase-3 antibodies (cat. no. 9664; Cell Signaling Technology, Danvers, MA, USA) diluted 1:2,500 overnight at 4°C and washed with 0.1% PVA in PBS at least three times. The sample was subsequently reacted with the secondary antibodies, rhodamine-conjugated goat anti-rabbit immunoglobulin (Ig) G (cat. no. 31463; Thermo Scientific, Rockford, IL, USA), then diluted 1:50 for 2 h at room temperature. After washing, the samples were incubated in Hoechst 33342 (w/v) for 20 min at room temperature. Finally, the samples were mounted on glass slides and examined with a confocal laser-scanning microscope (Zeiss LSM 700 META; Carl Zeiss, Jena, Germany).

Assessment of apoptosis in blastocysts

Apoptotic cells among blastocysts were detected using an *In Situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. At Day 7, the IVP blastocysts were washed with 0.1% PVA in PBS, then fixed in 4% PFA (v/v) in PBS for 1 h at room temperature. Blastocysts were then permeabilized using 0.1% (v/v) Triton X-100 for 30 min at 4°C. The fixed embryos were subsequently incubated in terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction medium for 1 h at 38.5°C, then washed and mounted on slides. Whole-mount embryos were examined under an epifluorescence microscope (Olympus, Japan) following a TUNEL assay and DAPI staining to determine the numbers of apoptotic nuclei and total numbers of nuclei.

Statistical analysis

All percentage data obtained in this study are presented as the means±standard deviation. Moreover, of the results of the H_2DCFDA experiments are presented as the means±standard error of the mean. All experiments were replicated three times,

after which the results were analyzed by one-way analysis of variance followed by Bonferroni's Multiple Comparison Test using *t*-tests. All data analysis was performed using the GraphPad Prism 5.0 software package (San Diego, CA, USA). Differences were considered significant at * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

RESULTS

Effect of *P. amurense* and/or *H. japonicus* extracts on the developmental competence of bovine embryos *in vitro*

Here, we evaluated bovine embryos grown in culture medium supplemented with various concentrations (0.01, 0.05, and 0.1 μg/mL) of *P. amurense* for 6 days. We then confirmed that the developmental competence of bovine embryos increased in response to 0.01 μg/mL *P. aamurense* extract (Table 1). We also used the optimal *H. japonicus* extract concentration as defined in a previous study that effects of *H. japonicus* extract on sperm motility and fertilization status in cattle [20]. Based on this report, we investigated the effects of *P. amurense* and/or *H. japonicus* extracts on bovine embryonic development. As shown in Figure 1, the blastocyst development rates were significantly increased (p<0.05) in embryos from *P. amurense* (28.9%±2.9%) or *H. japonicus* (30.9% ±1.5%) extract treated groups compared with the control (25.4% ±1.6%), respectively. Moreover, blastocysts development was significantly higher (p<0.05) in both the *P. amurense* and *H. japonicus* treated group (34.8%±2.1%) than in the control groups. In addition, this result in both the *P. amurense* and *H. japonicus* treated group appeared similar to that of the melatonin treated group (35.7%±5.1%). These results indicate that *P. amurense* and/or *H. japonicus* extracts enhanced developmental competence of bovine embryos.

Effect of *P. amurense* and/or *H. japonicus* extracts on intracellular ROS levels of bovine blastocysts

Balanced intracellular ROS levels are very important for improving the efficiency of IVP bovine embryos [22]. Therefore, we measured the intracellular ROS levels in blastocysts from the bovine embryos of IVP treated with *P. amurense* and/or *H. japonicus* extract by dichlorodihydrofluorescein diacetate (H_2DCFDA) staining to determine if they reduced the intracellular ROS levels

Table 1. Effect of various concentrations of *Phellodendron amurense* extract on preimplantation development of bovine embryos *in vitro*
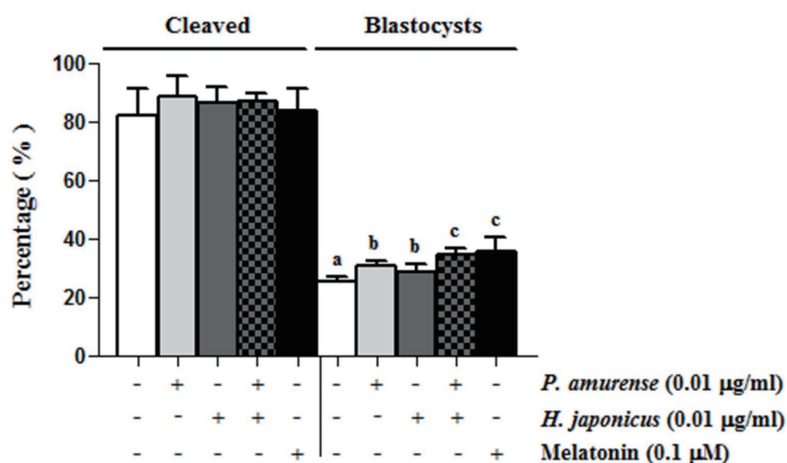
Groups (μg/mL)	No. of embryos examined	% of embryos cleaved (n)	% of blastocysts (n)
0	36	89.4±6.2 (30)	25.0±0.0 (9)[a]
0.01	36	85.6±6.2 (31)	30.6±0.9 (11)[b]
0.05	36	88.8±1.8 (32)	16.3±5.3 (6)[c]
0.1	36	83.8±5.3 (28)	13.8±1.8 (5)[c]

This experiment was replicated three times.
Data are the means ± standard deviation.
Different superscripts denote significant differences compared with other groups (p<0.05).

(A)

(B)

P. amurense (μg/ml)	H. japonicus (μg/ml)	Melatonin (μM)	No. of embryos examined	No. (%) of embryos cleaved	No. (%) of blastocysts produced
0	0	0	194	161 (82.6 ± 9.0)	49 (25.4 ± 1.6)[a]
0.01	0	0	101	90 (88.9 ± 6.9)	31 (30.9 ± 1.5)[b]
0	0.01	0	119	102 (86.6 ± 5.3)	34 (28.9 ± 2.9)[b]
0.01	0.01	0	126	110 (87.4 ± 2.6)	44 (34.8 ± 2.1)[c]
0	0	0.1	114	94 (84.2 ± 7.4)	41 (35.7 ± 5.1)[c]

Figure 1. Effect *Phellodendron amurense* and/or *Humulus japonicus* extracts on preimplantation development of bovine embryos *in vitro*. (A) Diagram of cleavage stage and blastocyst formation pattern after *in vitro* fertilization (IVF). (B) Summary of bovine embryonic development after IVF. Data are the means±standard deviation. [a-c] Values from three times replicates with different superscripts denote a significant difference relative to other groups (p<0.05).

of bovine blastocysts generated by oxidative stress. As shown in Figure 2, the intracellular ROS levels were significantly decreased (p<0.05) in *P. amurense* and *H. japonicus* extract treated groups when compared with the control group. Moreover, the intracellular ROS levels in bovine blastocysts derived from the *P. amurense* and *H. japonicus* extract treated groups were significantly lower (p<0.01) than those of other treatment groups. This result also appeared similar to that of the melatonin treated group. These results suggest that *P. amurense* and/or *H. japonicus* extracts improve developmental competence of bovine embryos through elimination of ROS levels during IVC.

Changes in apoptotic factors in bovine blastocysts after *P. amurense* and/or *H. japonicus* extracts treatment during IVC

Cleaved caspase-3 plays an important role in the apoptotic pathway of animal cells [8]. Therefore, we investigated the expression of cleaved caspase-3 in bovine blastocysts treated with both *P. amurense* and *H. japonicus* extracts. As shown in Figure 3, the expression of cleaved caspase-3 decreased in response to treatment with either *P. amurense* and *H. japonicus* extracts (1.9±1.0) or melatonin (1.8±0.6) relative to the control group (3.1±1.0).

We also confirmed the presence of apoptotic cells in *P. amurense* and *H. japonicus* extract treated blastocysts by a TUNEL assay. As shown in Figure 4, no significant differences were detected in total nuclei among the control (112.5±14.8), *P. amurense* (117.7±14.9) or *H. japonicus* (114.8±11.7) extract treated groups. However, total nuclei increased significantly (p<0.05) in both the *P. amurense* and *H. japonicus* extract treated groups (141.2±15.8). Moreover, apoptotic nuclei significantly decreased (p<0.05) in the *P. amurense* (2.9%±0.7%) and *H. japonicus* (3.1%±1.1%) extract treated groups when compared with the control group (4.4%±1.0%). Notably, the apoptosis rate in both the *P. amurense* and *H. japonicus* extracts treated group (1.4%±0.6%) or the melatonin treated group (1.3%± 0.7%) was significantly lower (p<0.05) than in the other treatment groups. Therefore, simultaneous treatment with two extracts produced results similar to melatonin treated groups with respect to developmental competence, ROS level and apoptotic index in bovine blastocysts of IVP.

Taken together, these results suggest that treatment with *P. amurense* and/or *H. japonicus* extracts improves blastocyst quality by reducing cellular apoptosis.
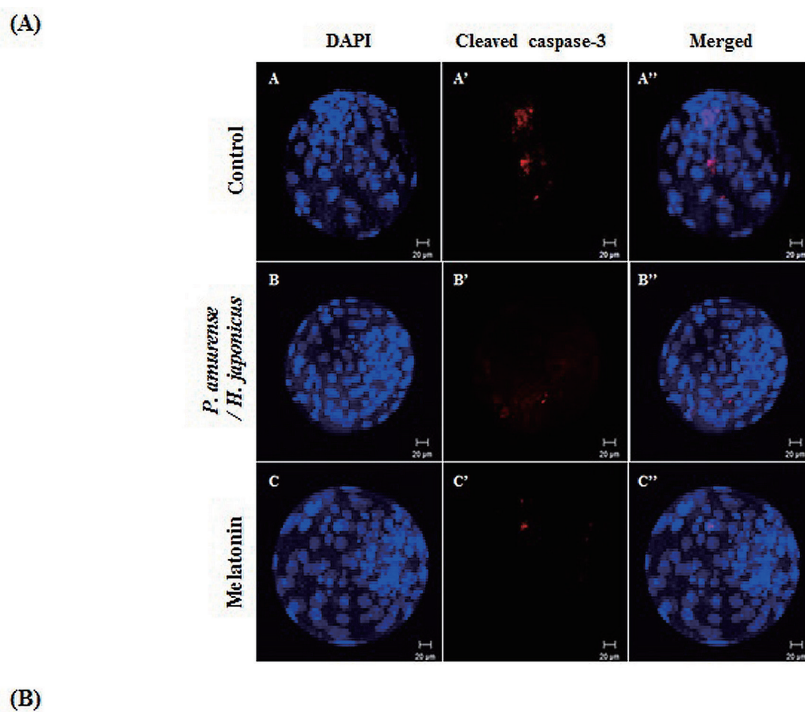
(A)

(B)
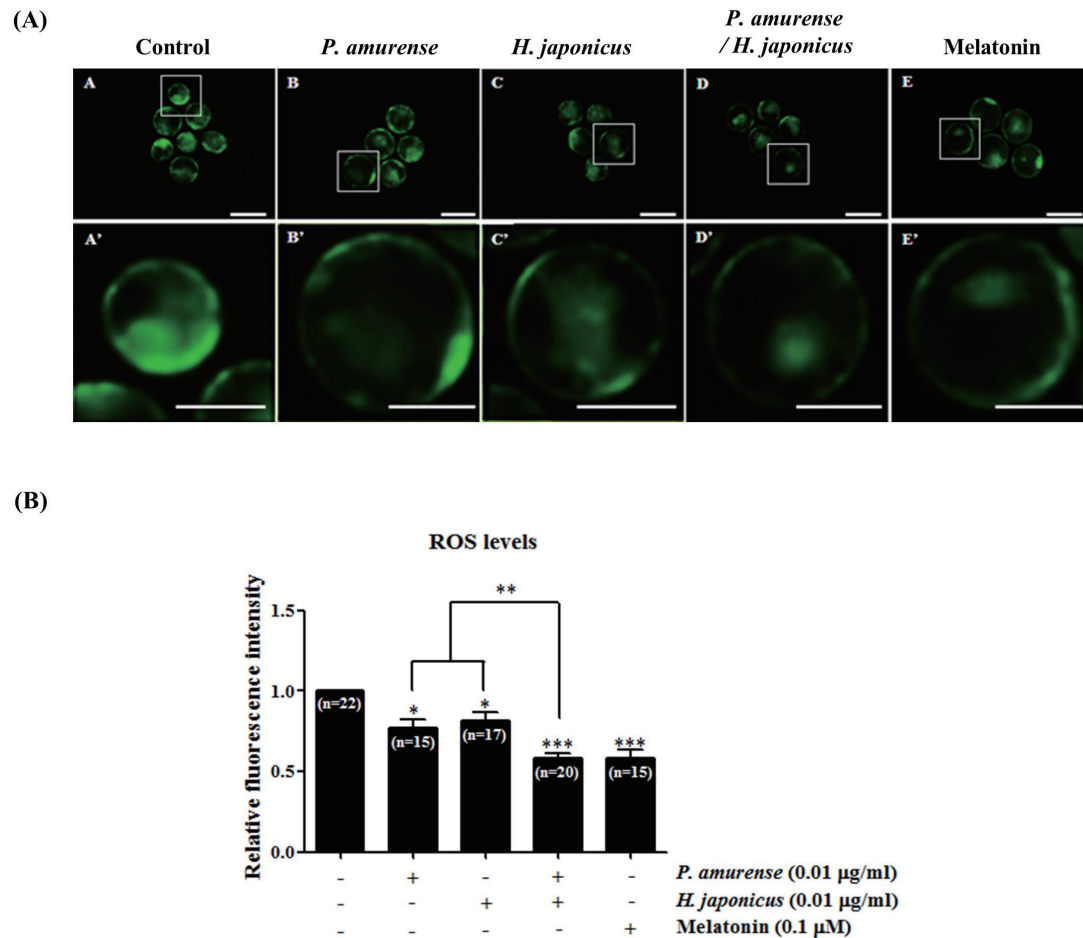
Figure 2. Detection of ROS level in bovine blastocysts derived from *Phellodendron amurense* and/or *Humulus japonicus* extract treated embryos by H_2DCFDA staining. (A) Fluorescence microscopy imaging of ROS production in bovine blastocysts by H_2DCFDA staining. (B) Quantification of ROS levels in bovine blastocysts. Scale bars = 200 μm. Quantification of fluorescence intensity in H_2DCFDA stained bovine blastocysts was obtained using the Image J software. This experiment was replicated at three times. Data in the bar graph represent the means±standard error of the mean of three independent experiments. Statistically significant differences are indicated by asterisks (* p<0.05, ** p<0.01, and *** p<0.001, compared to control group). ROS, reactive oxygen species; H_2DCFDA, dichlorodihydrofluorescein diacetate.

DISCUSSION

This study demonstrated that the *P. amurense* and *H. japonicus* extracts were an effective means to induce developmental competence and blastocyst quality in bovine embryos. We also confirmed that the intracellular ROS levels were reduced by the anti-oxidant effects of *P. amurense* and *H. japonicus* extracts in bovine blastocysts. Moreover, the expression of apoptotic factor (cleaved caspase-3) and apoptotic nuclei decreased in bovine blastocysts derived from *P. amurense* and *H. japonicus* extracts treated embryos *in vitro*. Additionally, we confirmed that these results in both the *P. amurense* and *H. japonicus* extract treated group showed a similar response to the melatonin treated group, a known antioxidant.

Synthetic phenolic antioxidants are widely used as antioxidant additives in various studies because of their effects and cost effectiveness. However, they are known to cause an aversion to composite food and have toxic effects [23]. Therefore, many studies

have investigated antioxidants by using natural products. Natural antioxidants include vitamin C, γ-tocotrienol, flavonoid and phenolic acid. *P. amurense* and *H. japonicus* extracts, which have been used as traditional medicine in Korea and China, include flavonoid and polyphenolic substances [11,12]. According to recent studies, *P. amurense* glycan prevented skin oxidative stress in ultraviolet radiation by reducing lipid peroxidation and increasing antioxidant enzymes activities [13], while *H. japonicus* improved antioxidant enzyme activities in human fibroblast cells [11]. In a previous study, we confirmed that *H. japonicus* extract improves early bovine embryo development [20]. In the present study, we also confirmed that *P. amurense* extract (0.01 μg/mL) enhances preimplantation embryo development in cattle. Therefore, we identified the anti-oxidant effects of treatment with *P. amurense* and *H. japonicus* extracts during IVC of bovine embryos. The rate of blastocyst development was significantly higher in the *P. amurense* or *H. japonicus* extract treatment groups than the control group (Figure 1). Moreover, both the *P. amurense* and

(A)

(B)

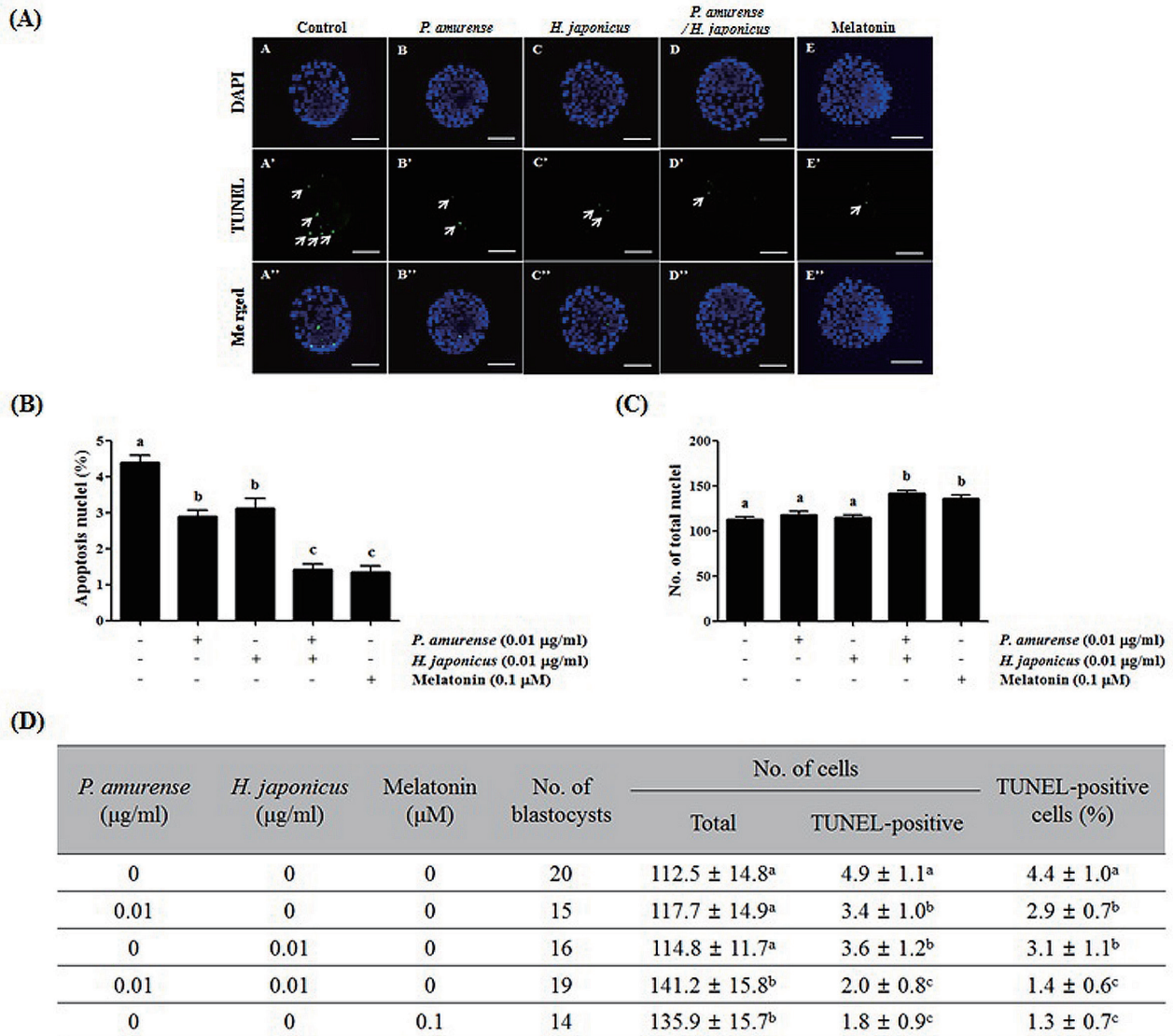| P. amurense (μg/ml)	H. japonicus (μg/ml)	Melatonin (μM)	No. of blastocysts	No. of cells	
				Total	Cleaved caspase-3
0	0	0	13	106.2 ± 7.9^a	3.1 ± 1.0^a
0.01	0.01	0	13	130.9 ± 12.8^b	1.9 ± 1.0^b
0	0	0.1	13	133.0 ± 16.7^b	1.8 ± 0.6^b

Figure 3. Detection of cleaved caspase-3 in bovine blastocysts derived from *Phellodendron amurense* and *Humulus japonicus* extract treated embryos by immunofluorescence staining. (A) A-C: the chromatin content was determined by Hoechst staining (total DNA), A'-C': cleaved caspase-3 (#9664; Cell Signaling) was labeled with Alexa Fluor 555 in fluorescence in bovine blastocysts. Scale bars = 20 μm. (B) Summary of total cells and cleaved caspase-3 cells pattern in bovine blastocysts. Data in the bar graph represent the means± standard deviation of three independent experiments. [a,b] Values from three replicates with different superscripts denote a significant difference relative to other groups (p<0.05).

H. japonicus extract treatment groups were similar to the melatonin treatment group (Figure 1). A previous study reported that melatonin enhanced blastocyst development *in vitro* [21]. Taken together, these results demonstrated the addition of *P. amurense* and *H. japonicus* extracts during IVC enhances the developmental competence of bovine embryos.

Generally, intracellular ROS levels generated in embryos increased during IVC [22]. ROS play important roles as positive or negative factors influencing embryonic development [24]. However, IVP embryo quality is known to be reduced by increasing ROS levels produced by the relatively higher oxygen levels and lower levels of free radical scavengers when compared with *in vivo* conditions [25]. Therefore, we identified the expression of intracellular ROS levels in bovine blastocysts derived from *P. amurense* and/or *H. japonicus* extract treated embryos. In the present study, the intracellular ROS levels decreased significantly in both the *P. amurense* and *H. japonicus* extract treatment groups

relative to the control group (Figure 2). Moreover, the ROS levels in both the *P. amurense* and *H. japonicus* extract treatment groups were similar to those in the melatonin treatment groups (Figure 2). A previous study demonstrated that melatonin was reduced in intracellular ROS levels of blastocysts *in vitro* [26]. These results suggest that treatment with *P. amurense* and *H. japonicus* extract decreases intracellular ROS levels of bovine blastocysts *in vitro*.

Apoptosis is an important indicator used in evaluation of blastocyst quality [8]. In a previous study, we demonstrated that decreasing apoptosis enhanced blastocyst quality, while high apoptosis rate reduced blastocyst quality [27]. Apoptosis, which is known to be one of the mechanisms involved in the mitochondrial pathway, is activated by caspase-8 and -9 via the mitochondrial pathways. Cleaved caspase-3 induced the increasing of cleaved poly (ADP-ribose) polymerase (PARP) involved in DNA repair and induced apoptosis [8]. In the present study, the expression of cleaved caspase-3 was decreased in both the *P. amurense* and

(A)

(B)

P. amurense (0.01 µg/ml)
H. japonicus (0.01 µg/ml)
Melatonin (0.1 µM)

(C)

P. amurense (0.01 µg/ml)
H. japonicus (0.01 µg/ml)
Melatonin (0.1 µM)

(D)

P. amurense (µg/ml)	H. japonicus (µg/ml)	Melatonin (µM)	No. of blastocysts	No. of cells		TUNEL-positive cells (%)
				Total	TUNEL-positive	
0	0	0	20	112.5 ± 14.8^a	4.9 ± 1.1^a	4.4 ± 1.0^a
0.01	0	0	15	117.7 ± 14.9^a	3.4 ± 1.0^b	2.9 ± 0.7^b
0	0.01	0	16	114.8 ± 11.7^a	3.6 ± 1.2^b	3.1 ± 1.1^b
0.01	0.01	0	19	141.2 ± 15.8^b	2.0 ± 0.8^c	1.4 ± 0.6^c
0	0	0.1	14	135.9 ± 15.7^b	1.8 ± 0.9^c	1.3 ± 0.7^c

Figure 4. Evaluation of cellular apoptosis in bovine blastocysts derived from *Phellodendron amurense* and/or *Humulus japonicus* extract treated embryos by TUNEL assay. (A) Detection of apoptosis fluorescent images in bovine blastocysts by TUNEL assay. A–E: the chromatin content was determined by DAPI staining (total DNA); A′–E′: fragmented DNA detected by TUNEL (apoptotic nuclei, white arrows). Scale bars = 100 µm. (B) Percent of TUNEL positive cell per blastocyst and (C) total cell number per blastocyst. (D) Summary of apoptosis pattern in bovine blastocysts. Data in the bar graph represents the means±standard deviation of three independent experiments. [a-c] Values from three replicates with different superscripts denote a significant difference relative to other groups (p<0.05). TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4′,6-diamidino-2-phenylindole.

H. japonicus extracts treatment groups, similar to the melatonin treatment group (Figure 3). The apoptosis rate was significantly decreased in the *P. amurense* or *H. japonicus* extract treatment groups relative to the control group. Moreover, both the *P. amurense* and *H. japonicus* extract treatment groups were similar to the melatonin treatment group (Figure 4). A previous report demonstrated that melatonin reduced apoptosis in IVP blastocysts [28]. These findings suggest that treatment with *P. amurense* and *H. japonicus* extracts reduces apoptotic nuclei of bovine blastocysts through the regulation of caspase-3 expression *in vitro*. In summary, the present study provides the first evidence associating *P. amurense* and *H. japonicus* extracts treatment with the pro-

motion of bovine embryos developmental competence *in vitro*. We also found that the *P. amurense* and *H. japonicus* extracts increased the qualities of bovine blastocysts by reducing intracellular ROS and apoptosis. Moreover, the effects they induced on developmental competence, elimination of ROS, and apoptosis were similar to those induced by melatonin as a known antioxidant. Overall, these results suggest that *P. amurense* and *H. japonicus* extracts improve preimplantation embryos development and quality of bovine blastocysts *in vitro*. Moreover, our results suggest that natural plant extracts may enable improvement of embryo quality for infertile patients.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

ACKNOWLEDGMENTS

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