ORIGINAL ARTICLE

Effects of melatonin on superovulation and transgenic embryo transplantation in small-tailed han sheep (*Ovis aries*)

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Abstract

OBJECTIVE: In this study, the effects of melatonin on superovulation and the transplantation of transgenic embryos were investigated in Small-Tailed Han sheep.
DESIGN: Different doses of melatonin (0, 40 or 80 mg/animal) were subcutaneously implanted into both multiparous (4–5 years old) donors and recipients before superovulation and estrus synchronization. The one-year-old young ewes without melatonin treatment served to evaluate the reproductive efficiency of the adult multiparous ewes. Ewes with superovulation were used as embryo donors. The estrus were induced in embryo recipients after embryo transplantation.
RESULTS: The results showed that the number of corpora lutea of the ewes received subcutaneous 40 or 80 mg melatonin implant (13.4±1.05/ewe, 15.1±1.62/ewe) were significantly higher than that of in control group (8.8±0.37/ewe) ($p<0.05$). Similarily the number of recovered embryos from the ewes received subcutaneous 40 or 80 mg melatonin implant (10.3±0.84/ewe, 10.9±1.21/ewe) was significantly higher than the control group (6.2±0.60/ewe) ($p<0.05$). The transfered embryos from 40 or 80 mg melatonin treated donors dramatically improved the pregnancy and birth rates compared to control ewes. In addition, both 40 mg and 80 mg melatonin implatation lead to more lambs born per embryo.
CONCLUSIONS: These observations provide valuable information for the application of melatonin in increasing superovulation and transgenic embryo transplantation efficiency in sheep.

INTRODUCTION

Since 1985, the first transgenic livestock has been produced by microinjecting foreign DNA into zygotic pronuclei (Hammer *et al.* 1985), thereafter, numerous animals with important husbandry-related transgenic traits have been generated. Transgenic technology has been used to improve carcass composition, lactational performance, and wool production, as well as to enhance disease resistance (Wheeler 2007). Pronuclear embryo microinjection is one of the most frequently utilized methods for generating transgenic sheep. In this method, donor sheep are subjected to superovulation, mating or artificial insemination and oviduct flushing to harvest zygotes, which are transplanted to synchronizationally induced estrous recipients for lambing (Menchaca *et al.* 2009; Gonz *et al.* 2004). The reproductive efficiency of high-quality pronuclear-stage embryos and the uterine environment of the recipient are important for transgenic efficiency in sheep.

The reproductive activity of sheep is governed by seasonal variations, where photoperiodic information is conveyed to the reproductive neuroendocrine system by the circadian secretion of melatonin from the pineal gland (Bittman *et al.* 1983). Melatonin is released at night and acts in the mediobasal hypothalamus to modulate the pulsatile secretion of GnRH (Vázquez *et al.* 2009). A large amount of scientific evidence supports a local role for melatonin in the reproductive process. Melatonin also acts directly on the ovary (Nakamura *et al.* 2003; Tamura *et al.* 2007), thereby affecting ovarian function (Tamura *et al.* 2009). The quality of oocyte remains a profound problem for the multiple ovulation and embryo transplantation (MOET) technique. ROS are produced within the follicle, especially during the ovulatory process (Agarwal *et al.* 2005). Thus, oxidative stress may result in poor oocyte quality, as ROS such as $^{\bullet}OH$, $O^{\bullet-}$ and H_2O_2 are detrimental to the oocyte (Zuelke *et al.* 1997). ROS deteriorate the cell membrane lipids, damade DNA, and accelerate apoptosis (Kowaltowski & Vercesi 1999). Moreover, these induce a two-cell block, promote apoptosis, and inhibit fertilization (Noda *et al.* 2005). Melatonin and its metabolites are considered direct free radical scavengers as well as indirect antioxidants that up-regulate the gene expression of several antioxidant enzymes such as glutathione peroxidase (GSH) and superoxide dismutase (SOD) (Antolín *et al.* 1996; Mayo *et al.* 2002; Reiter *et al.* 2009; Galano *et al.* 2011; Galano *et al.* 2013). In particular, melatonin has been demonstrated to preserve optimal mitochondrial function and homeostasis by reducing, arresting and preventing mitochondrial oxidative stress, thereby curtailing subsequent apoptotic events and cell death (Kang *et al.* 2008; Papis *et al.* 2007; Ishizuka *et al.* 2002; Tian *et al.* 2010; Paradies *et al.* 2010). Melatonin may become the molecule of choice for improving oocyte quality in sheep superovulation.

Currently, the *in vivo* mechanisms by which melatonin acts to affect oocyte and pronuclear embryo quality, transgenic embryo development and the MOET recipient remain elusive. Therefore, this study was undertaken to examine the effects of melatonin on superovulation and transgenic embryo implantation competence. The effects of exogenous melatonin on the recipient were also investigated.

Tab. 1. Experimental design.

Exp.	Donors	Recipients
1	Young ewes **(Y)**	Adult multiparous ewes
	Adult multiparous ewes **(C)**	
	Adult multiparous ewes received a subcutaneous 40 mg melatonin implant **(M40)**	
	Adult multiparous ewes received a subcutaneous 80 mg melatonin implant **(M80)**	
2	Young ewes **(Y)**	Young ewes **(Y)**
		Adult multiparous ewes **(C)**
		Adult multiparous ewes received a subcutaneous 40 mg melatonin implant **(M40^{R})**
		Adult multiparous ewes received a subcutaneous 80 mg melatonin implant **(M80^{R})**

MATERIALS AND METHODS

Animals and experimental design

The experiment was conducted using adult Small-Tailed Han sheep (*Ovis aries*) fed with a live weight maintenance ration at the experimental facilities of the Institute of Animal Husbandry and Veterinary, Academy of Agricultural Sciences of Tianjin, China (latitude 39°13' N, longitude 117° 20'). All experimental protocols concerning the handling of animals were performed in accordance with the requirements of the Institutional Animal Care and Use Committee at the China Agricultural University.

In August 20, 2011, a total of 21 adult multiparous ewes (4–5 years old) received a subcutaneous 40 mg melatonin implant (Melatonin powders purchased from Sigma, Chemical Co., St. Louis, MO), while another 20 adult multiparous ewes (4–5 years old) received a subcutaneous 80 mg melatonin implant. Twenty six adult multiparous ewes without treatment were used as control group. A total of 27 one-year-old young ewes without treatment were used as donors and recipients to evaluate the reproductive efficiency of the adult multiparous ewes. The 1-year-old young ewes were superovulated or induced estrus individually in January, 2012.

The first expeiment was designed to determine the effects of melatonin supplementation on oocytes and embryo quality in donor. The embryos were collected from the superovulated multiparous ewes who received a subcutaneous 40 (**M40**) or 80 mg (**M80**) melatonin implantation , while the young ewes (**Y**) and adult multiparous ewes (**C**) without melatonin treatment were used as control. After pronuclear microinjection, these embryos were tansplanted into adult multiparous ewes to assess their viability *in vivo*.

The second expeiment were designed to study the effects of melatonin supplementation on the recipients. The embryos harvested from youth ewes group donors were transferred into young ewes (**Y**) and multiparous ewes without melatonin treatment (**C**), multiparous ewes received subcutaneous a 40 (**M40^{R}**)or 80 mg (**M80^{R}**) melatonin implant (Table 1).

Superovulation and endoscopic-assisted insemination

The weight of the ewes at the beginning of the study was 60±4.3 kg, and the animals were primed for 12 d with progesterone by using a controlled internal drug release (CIDR) device which contains 300 mg progesterone (EAZI-BREED®CIDR® Sheep and Goats Device Pfizer Animal Health, New Zealand). The superovulation of donor ewes and the corresponding recipients were given consecutive injections of 45, 45, 40, 35, 35, 30 and 30 IU of follicle stimulating hormone (FSH; Ning-bo Hormone Products CO., LTD, China) every 12 h, commencing 12 h before the CIDR was removed. A single intramasclar injection of 350 IU pregnant mare serum gonadotropin (PMSG) (Ning-bo Hormone Products CO., LTD, China) at the time of CIDR removal was performed to induce ovulation in the donors. CIDRs in recipients were removed 10 hrs in advance of donor's CIDR withdrawal and then recipients were injected with a single dose of 280 IU PMSG. At the time of 12 h after CIDR withdrawal, all ewes were exposed at 6-h intervals to a vasectomized ram to detect estrous onset.

Each ewe was restrained on an operation cradle in dorsal recumbency with the head down at an angle of 45°. An endoscope (30 forward oblique, Karl Storz Endoskope GmbH, Tuttlingen, Germany) was introduced into the abdominal cavity at the ventral midline approximately 5–10 cm cranial to the mammary gland as previous described (Kuehholzer *et al.* 2007). The endoscope intrauterine horn method was used to inseminate donor ewes with 0.2 mL of fresh semen from 4 different rams (motility >0.6, diluted 2-fold with saline) at 55 h after CIDR removal.

Ovarian response and embryo collection

All females were deprived of food for 12 h before embryo recovery to facilitate surgery and reduce post-operative intestinal adhesions. A medial ventral incision was made to expose the reproductive tract. Before embryo collction, an ovulatory response was verified by laparoscopy. The females that displayed corpora lutea (CL) were classified as responsive to superovulation treatment and were chosen for embryo collection, which occurred 72 h after CIDR removal. The embryos were collected by oviduct flushing with a cannula that attached to a syringe and inserted into the lumen near the uterotubal junction. The uterine horn was flushed with 20 mL of a warm solution (PBS+0.3% BSA) (Fraktion, 735078; Roche Diagnostics, GmbH, Mannheim, Germany).

The flush was observed with a stereomicroscope (SZ61; Olympus, Kawasaki, Japan) to search for embryos at magnifications of 10–40. Next, the ova were transfer into holding medium (Immuno-Chemical Products Ltd., Auckland, New Zealand) and qualitatively evaluated with an inverted microscope (LX71; Olympus, Kawasaki, Japan). The evaluation criteria were based on previous studies with goat embryos (Ishwar & Memon 1996; Baril & Vallet 1990). The number of corpora lutea (CL) and the total number of recovered embryos (RE) were recorded for each ewe.

Embryo microinjection

The collected embryos were immediately microinjected or they were cultured. *In vitro* culture was performed at 38.5 °C in a humidified atmosphere in a chamber with 5% CO_2 within a period of 30 min. Microinjection was performed with an inverted microscope equipped with DIC and a pair of micro manipulators (Narishige, Tokyo, Japan). Fertilized eggs were collected and microinjected with a linearized pTLR4-3S vector, which was constructed to overexpress the TLR4 gene Toll-like receptors 4TLR4, which recognizes LPS lipopolysaccharide and initiates a series of intracellular responses, invokes a vigorous cytokine response among immune cells against Gram-negative bacteria (Deng *et al.* 2012). Well-fertilized embryos were transplanted into the recipient oviducts within 1 hour, and each recipient was transimplanted with 3–5 embryos.

Embryo transfer

The surgically removed embryos were transplanted into recipients whose CIDRs were removed 84 hours ago. The reproductive tract of the recipient was exteriorized with minimal manipulation to assess the number and quality of the corpora lutea (CL). The uterine wall was punctured with a 17-ga trocar l cm cranial to the uterotubal junction. A transfer catheter (Agtech, Inc., Manhattan, KS, USA) attached to a 1-mL syringe was advanced approximately 2 cm into the uterine lumen. Then, one or two embryos in 0.3 to 0.5 mL of holding medium were deposited into the uterine horn without insufflating air. The transfer catheter was then released. Three to five embryos were transferred into the uterine horn ipsilateral to the ovary with a good CL.

Pregnancy diagnosis

The recipients' pregnancy status was assessed by ultrasonography 60 days after ET. A real-time B-mode ultrasonic machine (Vetko Plus, Noveko, Quebec, Canada)

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with a 3.5-MHz mechanical sector-scan probe was used to conduct the diagnosis. Pregnancy was diagnosed by the identification of at least one fetal image.

Lambing

At 146 d of pregnancy, ewes were placed indoors (2 ewes/pen) to monitor the onset of lambing. Newborn lambs were weighed within 6 h after birth.

Blood sampling and progesterone assays

To evaluate serum melatonin concentrations, blood samples were collected from all ewes just before melatonin was implanted (T1) and at the time of CIDR was inserted (T2) and removed (T3). Serum melatonin concentrations were measured using a commercially available kit (Melatonin ELISA; IBL International GMBH, Hamburg, Germany) and performed following the instructions provided by the company.

Statistical analysis

The data were expressed as means ± SEM. The data were analyzed with a univariate analysis of variance (ANOVA) followed by Duncan's test using SPSS 18.0 statistical software. The significant difference between treatments was set at $p<0.05$.

RESULTS

Serum melatonin concentrations

In all groups, before melatonin was implanted (**T1**), the baseline serum melatonin concentrations were similar. Five months after melatonin implantation, the concentration of serum melaton in the group **M40** (27.3±4.81 pg/ml) was higher than that in group **C** (23.9±3.08 pg/ml) ($p<0.05$). However, after the sponges were removed (**T3**) these differences were no longer significant (Table 2).

Effect of melatonin on the number of sheep corpora lutea and embryos collected

Exogenous melatonin administration increased the number of corpora lutea and recovered embryos. The number of corpora lutea per ewe in the M80 group (15.1±1.62) was higher than that in the group C (8.8±0.37) ($p<0.05$), and the difference between the group Y (18.8± 2.49) and group C was also significant ($p<0.01$); however, the difference between group Y and groups M40 (13.4±1.05) was not significant. The numbers of recovered embryos per ewe in groups M40 (10.3±0.84) and M80 (10.9±0.84) were illustrated In Figure 1A which were higher than that in the group C (6.2±0.60) ($p<0.05$). The difference between group Y (14.4±7.93) and group C was highly significant ($p<0.01$). The recovery rates (the number of recovered embryos/corpus luteum) in group **Y (**76.6±2.05%**)** and im group **M40 (**76.6±1.56%**)** were higher than that in the group **C** (69.88±1.86%**)** ($p<0.05$). All of the recovered embryos were fertilized.

Tab. 2. Sheep serum melatonin concentrations.

	T1 (pg/ml)	T2 (pg/ml)	T3 (pg/ml)
Y	23.4±1.45[a]	24.4±3.49[ab]	23.4±2.97[a]
C	22.9±4.33[a]	23.9±3.08[a]	22.9±3.57[a]
M40	23.1±2.41[a]	27.3±4.81[b]	24.3±3.62[a]
M80	23.4±2.72[a]	25.6±6.40[ab]	24.9±4.31[a]

The mean (±SEM) (n=9) melatonin concentrations (pg/ml) of Small-Tailed Han sheep serum. Values with different letters in the same row (a, b) are significantly different; $p<0.05$.

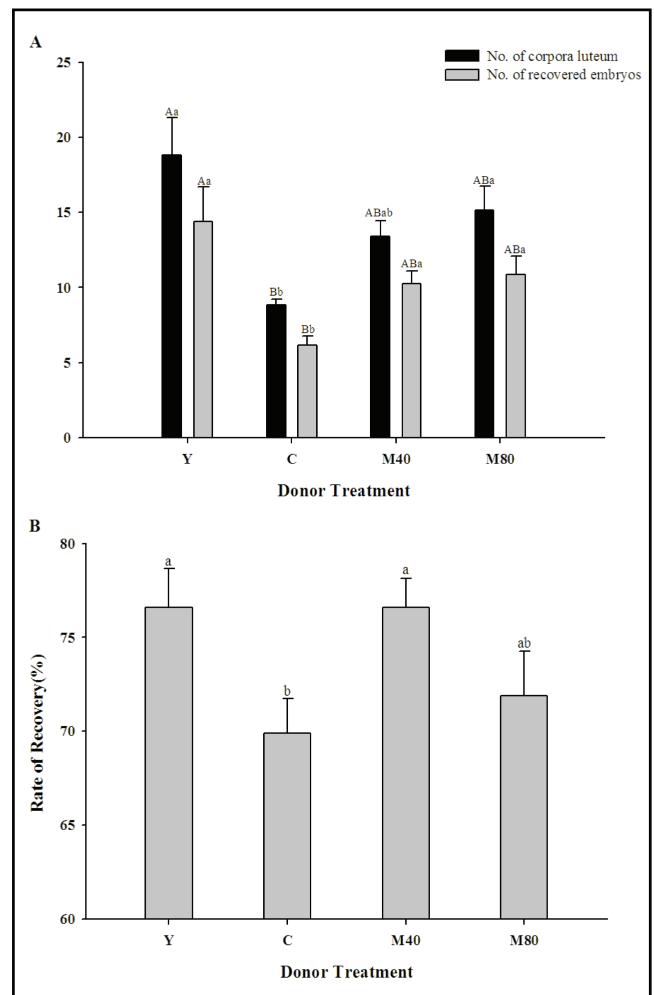

Fig. 1. Effect of melatonin treatment on the number of sheep corpora lutea and recovered embryos. Panel A: Number of the corpora lutea and the recovered embryos 72 h after CIDR removal. **Y**: one year old ewe; **C**: adult multiparous ewes; **M40:** adult multiparous ewes implanted 40 mg melatonin; and **M80:** adult multiparous ewes implanted 80 mg melatonin (n=17, 13, 8 and 8 donor ewes, respectively). Panel B: Embroy recovery rate (the number of recovered embryos/corpus luteum). Data were expressed as mean ± SEM. Different letters indicate differences within groups where a and b represent $p<0.05$, and A and B represent $p<0.01$.

Effect of melatonin administration on in vivo embryo development after pronuclear microinjection

To evaluate the melatonin-implantation competence of the embryos produced in the four groups, the embryos were transplanted into recipient ewes after pronuclear microinjection. The embryos recovered from the groups **Y**, **C**, **M40** and **M80** (donors) were transplanted into multiparous adults. The pregnancy rate in the group **M40** (31.25±2.08%) was significantly higher than those in groups **C** (14.3±0.67%) and **Y** (18.3±0.96%, 22.5±3.70%) (p<0.01). The pregnancy rate in group **M80** (24.0±1.04%) was also higher than that in group **C** (p<0.01). In addition to the pregnancy rate, the number of lambs born per embryo was also investigated. The data was listed in Table 3. The number of lambs born per embryo from the total embryos in groups **M40** (5.9±0.32%) and **M80** (6.77±0.17%) were higher than that born per embryo from group **C** (3.8±0.21%) (p<0.05). There were no significant difference in the birth weight of lambs among all groups.

Effect of melatonin administration on in vivo transgenic embryonic development in recipient ewes

To assess the recipient's ability to support embryonic development *in vivo*, embryos collected from group **Y** were transimplanted into the 4 different group recipients, $\mathbf{Y^R}$, $\mathbf{C^R}$, $\mathbf{M40^R}$ and $\mathbf{M80^R}$, respectively. As demonstrated in Table 4, the pregnancy rates for groups $\mathbf{Y^R}$ (41.7±4.81%), $\mathbf{M40^R}$ (35.4±5.24%) and $\mathbf{M80^R}$ (20.6±2.42%) were higher than that in group $\mathbf{C^R}$ (18.3±0.96%) (p<0.01). The lambing rate in groups $\mathbf{Y^R}$ (41.7±4.81%), $\mathbf{M40^R}$ (35.42±5.24%) and $\mathbf{M80^R}$ (31.67±5.28%) was higher than that in group $\mathbf{C^R}$ (22.50±3.70%) (p<0.05). The number of lambs born per pregnant ewe did not significantly differ among the groups; however, the number of lambs born per embryo from the groups $\mathbf{Y^R}$ (10.4±1.20%) and $\mathbf{M80^R}$ (10.9±1.95%) was higher than that in group $\mathbf{C^R}$ (5.3±0.66%) (p<0.05). There were no differences regarding the birth weight of lambs among the groups. The data were listed in Table 4.

DISCUSSION

This study demonstrates that exogenous melatonin application exerts highly beneficial effects on corpus luteal function, quantity and viability of embryo after microinjection. In addition, melatonin improves the ability of recipients to support transgenic embryonic development in the Small-Tailed Han sheep.

The assessment of superovulation showed that melatonin implantation in sheep increased the number of corpora lutea and recovered embryos. Numerous previous studies have documented that melatonin admin-

Tab. 3. The viability of embyos recovered from youth ewes (Y), adult multiparous sheep (C), and adult multiparous ewes received a subcutaneous 40 mg or 80 mg melatonin implant (M40 or M80) after pronuclear microinjectio

Recipient Groups	Y	C	M40	M80
No. of Recipient	22	21	13	21
Pregnant Ewes	4 (18.3±0.96) ^{ABa}	3 (14.3±0.67) ^{Ab}	4 (31.3±2.08) ^{Bc}	5 (24.0±1.04) ^{Bac}
Lambs Born (% of Pregnant Ewes)	5	3	4	5
Embryos per Pregnant Ewes (Lambs % of Embryos)	4.0 (30.8±3.63)^{a}	4.2 (21.7±1.67) ^{b}	3.5 (29.2±2.41) ^{ab}	3.8 (27.3±2.61) ^{ab}
Total Embryos Transplanted (Lambs % of Total Embryos)	40 (5.3±0.66)^{Aa}	95 (3.8±0.21) ^{Aa}	63 (5.9±0.32) ^{ABb}	45 (6.8±0.17) ^{Bb}
Birth Weight (Kg)	4.1±0.22 ^{a}	4.1±0.32 ^{a}	4.1±0.30 ^{a}	3.9±0.30 ^{a}

NOTE: Values with different letters in the same row (a, b, c) are significantly different; p<0.05. Values with different letters in the same row (A, B) are high significantly different; p<0.01.

Tab. 4. The ability of youth ewes (Y^R), adult multiparous sheep (C^R), and adult multiparous ewes received a subcutaneous 40 mg or 80 mg melatonin implant ($M40^R$ or $M80^R$) as recipient to support transgenetic embryos development *in vivo*.

Recipient Groups	Y^R	C^R	$M40^R$	$M80^R$
No. of Recipient	10	22	12	11
Pregnant Ewes	4 (41.7±4.81) ^{Bb}	4 (18.3±0.96) ^{Aa}	4 (35.4±5.24) ^{Bb}	3 (20.6±2.42) ^{ABa}
ambs Born	4 (41.7±4.81) ^{b}	5 (22.5±3.70) ^{a}	4 (35.4±5.24) ^{ab}	5 (31.7±5.28) ^{ab}
Embryos per Pregnant Ewes (Lambs % of Embryos)	4 (25.0±1.00) ^{a}	4 (30.8±3.63) ^{ab}	3.8 (27.1±2.08) ^{ab}	4 (39.4±1.06) ^{b}
Total Embryos Transplanted (Lambs % of Total Embryos)	40 (10.4±1.20) ^{b}	95 (5.3±0.66) ^{a}	63 (6.4±0.19) ^{ab}	45 (10.9±1.95) ^{b}
Birth Weight (Kg)	4.1±0.22 ^{a}	3.9±0.12 ^{a}	4.2±0.13 ^{a}	4.3±0.15 ^{a}

NOTE: Values with different letters in the same row (a, b, c) are significantly different; p<0.05. Values with different letters in the same row (A, B) are high significantly different; p<0.01.

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istration to sheep and goats remarkably increased the serum levels of luteinizing hormone (LH) and progesterone (Zarazaga *et al.* 2009; Abecia *et al.* 2002). Melatonin acts on the ovary to modify its function, especially luteal. High melatonin levels in the luteal phase compared to the follicular phase during the menstrual cycle were documented (Wetterberg *et al.* 1976; Brun *et al.* 1987). In addition to melatonin's stimulation on progesterone production by GCs-luteal cells (Woo *et al.* 2001; Webley & Leidenberger 1986; Brzezinski *et al.* 1992; Yie *et al.* 1995), melatonin also remarkably increases the mRNA expression of the LH (but not FSH) receptor in human GCs/luteal cells while inhibiting the gene expression of GnRH and GnRH receptor (Woo *et al.* 2001). Melatonin might rescue granule cells (GCs) from free radical cytotoxicity in long-term cultured cells by its direct and indirect antioxidant abilities. Reactive oxygen species (ROS) suppress progesterone secretion and induce CL regression in the corpora lutea during the menstrual cycle and in early pregnancy in women (Sugino *et al.* 2000). Melatonin is likely to protect CL from ROS and has important roles in maintaining CL function. The high number of corpora lutea obtained in this study indicates a strong positive response to the superovulation in melatonin treated ewes and result in high number of recovered embtyos. Melatonin treatment prior to oocyte collection hastened the dominant follicle growth phase, thus increasing ovarian follicle turnover (Berlinguer *et al.* 2009). Moreover, melatonin significantly reduced the number and rate of non-viable (degenerate and retarded) embryos (Forcada *et al.* 2006).

The quality of the pronuclear embryo is essential for its implantation after microinjection. Microinjection is a crude process for the embryo and may induce embryonic damage that may result in a developmental deficiency. In our experiment, the rate of lambs born per pregnancy and lambs born per embryo in melatonin implanted group were higher than that of embryos recovered from donors without melatonin implant. Melatonin treatment may increase both the fertility and the fecundity of the sheep through an improvement of luteal and embryonic survival (Abecia *et al.* 2002). Ovulation is similar to a local inflammatory response (Espey 1994), where reactive nitrogen species (RNS) and ROS are generated in this process. These species not only function as regulators for ovulation but also induce apoptosis of ovarian cells (Gupta *et al.* 2006; KORZEKWA *et al.* 2006). Oxidative stress causes poor oocyte quality. That ROS such as $^{\bullet}OH$, $O_2^{\bullet-}$ and H_2O_2 are detrimental to the oocyte has been reported (Zuelke *et al.* 1997). Free radicals deteriorate the cell membrane lipids, destroy DNA, and accelerate apoptosis (Kowaltowski & Vercesi 1999). Melatonin and its metabolites are broad-spectrum antioxidants and free radical scavengers (Reiter *et al.* 2005; Tan *et al.* 2002), and melatonin and its derivatives quench ROS as well as RNS (Kilic 2004; Tan *et al.* 2006). Elevated melatonin in preovulatory follicles is likely to protect GCs and the oocyte from free radicals that are generated during ovulation. Several studies have demonstrated that melatonin promotes the development of oocytes and embryos from sheep, pig, cattle, buffalo, mice, and humans in an *in vitro* setting via its potential antioxidation and antiapoptosis properties (Swarnakar *et al.* 2011; Tajes Orduña *et al.* 2009; Yamamoto & Mohanan 2002; Milczarek *et al.* 2010; Petrosillo *et al.* 2006; Jou *et al.* 2007; Shi *et al.* 2009). We have previously observed melatonin's beneficial effects on the development of fresh and vitrified 2-cell mouse embryos *in vitro*, including the enhancement of the blastocyst formation rate, total blastocyst cell numbers, and the rate of hatched blastocysts (Gao *et al.* 2012). These benificial effects of melatonin on embryo may improve transfer efficency , but to clarify the mechnism of this phenomenon, further study is also needed.

Melatonin implant may improve the uterine environment of the recipient ewe for embryo implantation. In our study, melatonin treated recipients, compared with the control group, the doses of 40 mg melatonin resulted in enhanced pregnancy rates, while both 40 mg and 80 mg melatonin lead to more lambs born per embryo. Elevated melatonin in the luteal phase and during early pregnancy may induce progesterone secretion in luteal cells, which is necessary for a successful pregnancy. Following embryo transfer, pregnant recipient ewes with melatonin administration exhibited consistently higher mean weekly progesterone concentrations than that of controls (McEvoy *et al.* 1998). In the pinealectomized rats, the implantation rates and serum progesterone levels were decreased. This indicates that melatonin may effect on endometrial morphology and embryonic implantation via preserving serum progesterone to normal level (Dair *et al.* 2008). In the current study, we observed the beneficial effects of melatonin implantation in recipient ewes on the survival of transplantation embryo, especially on the embryos with microinjection.

In conclusion, melatonin implantation improves the donor's response to superovulation, which provides more high quality embryos for microinjection. Inaddition, melatonin application also enhances the ability of the recipients to support transgenic embryonic development. As aresult, the pregnancy rates and lambs born per embryo significantly improved with melatonin treatment compared to the control ewes. These data strongly support melatonin application in terms to improve the efficiency of embryo production for microinjection and embryo transplantation in sheep.

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