Melatonin Improves the Efficiency of Superovulation and Timed Artificial Inseminationin Sheep

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It has been well documented that melatonin participates in the regulation of the seasonal reproduction of ewes. However, the effects of short term treatment of melatonin on ewe's ovulation are still to be clarified. In the current study, the effects of melatonin on the embryo number harvested from superovulation, and the pregnant rate in recipients after embryo transferring have been investigated. Duolang sheep with synchronous estrus treatment were then given melatonin subcutaneously injection (0, 5, and 10 mg/ewe, respectively) and). It was found that the estrogen level in the group of 5mg melatonin was significantly higher than that of other two groups at time of sperm insemination ($p < 0.05$). The pregnant rate and number of lambs in the group of 5 mg melatonin treatment was also significantly higher than that of the rests of the groups ($P < 0.05$). In a separated study, 31 Suffolk ewes as donors and 103 small-tailed han sheep ewes as recipients were used to produce pronuclear embryo and embryo transfer. Melatonin (5 mg) was given to the donors during estrus. The results showed that, the number of pronuclear embryos and the pregnancy rate were also significantly higher in melatonin group than that in the control group. In addition, 28 donors and 44 recipient ewes were used to produce morula/blastocyst and embryo transferring. Melatonin (5 mg) was given during estrus. The total number of embryos harvested (7.4 ±1.3/ewe*vs.*4.0 ±0.7/ewe, $P<0.05$) and the pregnant rate (72.0% *vs.*63.4%, $P<0.05$) and number of lambs were also increased in melatonin group compared to the control group. Collectively, the results have suggested that melatonin treatment at 36 hours after withdrawal CIDR could promote the number and quality of embryos in the *in vivo*condition and increased the pregnant rate and number of lambs.

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Abstract: It has been well documented that melatonin participates in the regulation of the seasonal reproduction of ewes. However, the effects of short term treatment of melatonin on ewe's ovulation are still to be clarified. In the current study, the effects of melatonin on the embryo number harvested from superovulation, and the pregnant rate in recipients after embryo transferring have been investigated. Duolang sheep with synchronous estrus treatment were then given melatonin subcutaneously injection (0, 5, and 10 mg/ewe, respectively) and). It was found that the estrogen level in the group of 5mg melatonin was significantly higher than that of other two groups at time of sperm insemination ($p < 0.05$). The pregnant rate and number of lambs in the group of 5 mg melatonin treatment was also significantly higher than that of the rests of the groups ($P < 0.05$). In a separated study, 31 Suffolk ewes as donors and 103 small-tailed han sheep ewes as recipients were used to produce pronuclear embryo and embryo transfer. Melatonin (5 mg) was given to the donors during estrus. The results showed that, the number of pronuclear embryos and the pregnancy rate were also significantly higher in melatonin group than that in the control group. In addition, 28 donors and 44 recipient ewes were used to produce morula/blastocyst and embryo transferring. Melatonin (5 mg) was given during estrus. The total number of embryos harvested (7.4 ±1.3/ewe *vs.* 4.0 ± 0.7/ewe, P<0.05) and the pregnant rate (72.0% *vs.*63.4%, P<0.05) and number of lambs were also increased in melatonin group compared to the control group. Collectively, the results have suggested that melatonin treatment at 36 hours after withdrawal CIDR could promote the number and quality of embryos in the *in vivo* condition and increased the pregnant rate and number of lambs.

Key words: Melatonin, Sheep, Superovulation, Embryo transfer, Pregnancy

Introduction

The estrous cycles of most sheep are regulated by the switch of seasons. With the reduced photoperiod and increased melatonin level, ewes adjust GnRH level and estrus cycles [1]. Thus, melatonin as a photoperiodic signal molecule to regulates the reproductive activity of ewes. Melatonin is also a potent antioxidant and free radical scavenger [2]. As this consideration, melatonin can also protect the reproductive tissues and organs. A various of ROS (reactive oxygen species such as $OH_{\cdot}^{-}$ and $O_2^{\cdot -}$) will damage the DNA and the lipid of cell membrane, accelerate apoptosis in reproductive system [3]. For example, oxidative stress induces two cell developmental block, apoptosis and infertility [4-5]. The reason is that oxidative stress reduces the quality of oocytes which is an important factor for sheep fertility. Melatonin scavenges OH^-, H_2O_2 and other reactive oxygen species, therefore, it contributes to effectively reduce oxidative DNA damage and cell apoptosis during ovulation [6]. Melatonin also reduces oxidative damage to mitochondrial DNA [7]. It was reported that, melatonin preserved the normal distribution of mitochondria, mitochondrial DNA copy number membrane potential (MMP), and ATP level [8]. Melatonin improves the quality of bovine oocyte, oocyte maturation, efficiency of *in vitro* fertilization and embryo development [9]. Melatonin administration improved the conception rates in mice and cows [10]. Melatonin added during culture of mouse prokaryotic embryos significantly increased blastocyst rate, pregnancy rate after transplantation, average number of offspring and survival rate of offspring [11]. For deer, melatonin subcutaneous implantation also improved the quantity and quality of super ovulatory oocytes [12]. Therefore, melatonin is probably the key factor to improve fecundity by improving the quality of oocytes in mammals.

Melatonin also plays an important role in the establishment and maintenance of pregnancy in animals. In mouse, extremely high expression of melatonin receptor 1 (MT1) was observed in granulosa cells after human chorionic gonadotropin (hCG) treatment [12]. At the same time, melatonin synthetic enzyme in cumulus cells was upregulated by hCG injection and high level of melatonin in follicle fluid was detected [13] and this was observed also in porcine follicle fluid [14]. Further study demonstrated that melatonin and its receptor MT1 regulated the downstream signaling pathway of hCG (LH) including the luteinization of granulosa cells in mice [12]. The deletion of MT1 receptor severely impairs the fertility of mice due to reduced oocyte number and quality [15].

In addition, melatonin subcutaneous implantation in sheep increase the number of corpus luteum and pregnancy rate [16]. However, there is no report regarding the effect of melatonin short term administration during estrus on the reproductive efficiency and embryo production in animals, particularly in ewes. Therefore, this study was conducted to test whether melatonin short term administration can improve the synchronized estrus pregnant rate and the production of both pronuclear and morula/blastocyst embryos in ewes.

Materials and methods

Chemicals

CIDRs (Controlled Internal Drug Release) which contains 300 mg progesterone were purchased from Pfizer Animal Health (New Zealand). Follicle stimulating hormone (FSH), luteinizing hormone (LH) and pregnant mare serum gonadotropin (PMSG) were from Ningbo Sansheng Pharmaceutical Industry Co., Ltd.(China). Melatonin and all other reagents, unless specified, were purchased from Sigma-Aldrich Co. (USA).

Animals

Female sheep (Duolang, Suffolk and Small-tailed Han) from Aoxin Animal Husbandry (Beijing, China) and Zhenxin Farmers's Professional Association Organization (Uygur Autonomous Region, China), at the age of 2-4 years old with normal reproductive cycle, healthy and generally the similar body weight were selected for the experiments. 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 Xinjiang Agricultural University (permission number:2017003).

Experiment design

Experiment 1: fifty-seven ewes were randomly divided into three groups and then treated with CIDRs to induce synchronized estrus. All the ewes assess freely to the same feed and drink water. The CIDRs were removed 13 days later, blood samples were collected and the ewes were injected with PMSG. The second blood collection was conducted 36 hours after the CIDR removal and melatonin (0, 5, 10mg,) was subcutaneously injected at the same time, respectively. Artificial insemination was conducted 48 hours after the removal of the CIDR, and the third blood collection was conducted. B-ultrasound examination was conducted 45 days after artificial insemination and ewes with pregnancy were recoded.

Experiment 2: thirty-one Suffolk ewes as donors and 103 Small-tailed Han ewes as recipients were selected, and the donors were divided into two groups and underwent the stimulated ovulation procedure. Among the donors, 13 ewes were subcutaneously injected with 5mg melatonin 36 hours after the CIDR was removed and 18 ewes served as control group, and LH was injected to all the donors at the same time. Then the donors were inseminated by laparoscope, and 10 hours later pronuclear embryos were surgically collected, and the pronuclear embryos were observed by stereomicroscope and selected depends on the morphology. The number of corpora luteal (CL) and embryos harvested were recorded. The embryos with normal morphology were transferred into the oviduct of recipients. The examination of pregnancy was performed using B-ultrasound 45 days after embryo transfer.

Experiment 3: twenty-eight Suffolk ewes as donors and 44 Small-tailed Han ewes as recipient were selected and divided into two groups randomizedly. The donor ewes were treated to induce superovulation, and 36 hours after CIDR removal LH and melatonin (5 mg) was subcutaneously injected into donors at the same time. Six days after artificial insemination by laparoscopic, morula or blastocyst were surgically harvested from the uterine horn of the donor, and the number of CL and embryos were recorded. Embryos were transferred into the uterine horn of the recipient. The examination of pregnancy was performed using B-ultrasound 45 days after embryo transfer.

Semen preparation

In this study, the method of peritoneal endoscopy was used to inseminate uterine horn. Semen of male sheep of black Suffolk was collected, and it was used for artificial insemination when its vitality reaches 0.6 or more by microscopic examination.

Blood sample collection and hormones analyze

For **Experiment 1,** 5ml blood samples was collected and stored in -80℃ freezer for measurement. Hormones level were determined was conducted by Beijing North Biotechnology Research Institute by radioimmunoassay.

Statistical analysis

All data were presented as means ± SEM. The data were analyzed using ANOVA and followed by LSD and Duncan tests for the differences between treatments (SPSS software), $P < 0.05$ was used as the criterion for the significance of the difference.

Results

Effects of melatonin injection on hormones

Firstly, the effects of melatonin on FSH, LH and E2 were evaluated at 3 different time points (Table 1). At the time of artificial insemination, the melatonin in the serum of ewes in 5 mg group (509.0±67.5pg/ml) was significantly higher than that of the other control group (330.2±38.7pg/ml) ($p<0.05$). But there was no difference in melatonin levels between the three groups at the time of withdrawal CIDR and estrus. The FSH level in the control group was always in a high level, but the increase of FSH from estrus to insemination (0.2 mIU/ml) in the 5 mg group was significantly higher than that in the other two groups (0.03 mIU/ml and 0.09 mIU/ml, $p<0.05$). LH level was increased from withdrawal CIDR to estrus, and gradually decreased after the peak level. LH level in the 5mg group (4.59±0.4 mIU/ml) was significantly higher than that in the other two groups (4.02±0.3 mIU/ml, 4.27±0.3 mIU/ml) at the time of insemination ($p<0.05$). Progesterone concentration in the 10 mg group was the highest, followed by a downward trend, but significantly higher than that in the control group at the time of withdrawal CIDR and estrus ($p<0.05$), and significantly higher than 5 mg group at the time of estrus ($p<0.05$). The concentration of progesterone of the 10 mg treated group was highest at the time of insemination, but there was no significant difference between the two groups ($p>0.05$). There was no difference in estradiol level between the two groups at the time of withdrawal CIDR and estrus. At the time of insemination, 5mg group (19.2±2.7pg/ml) was significantly higher than the other two groups (13.8±1.8 pg/ml, 14.1±2.9 pg/ml, $p < 0.05$).

Effects of melatonin on the pregnancy and number of lambs born in sheep

Melatonin were subcutaneously injected to the ewes 36 hours after the withdrawal of CIDRs, at the dosage of 5 mg (21 ewes), 10 mg (20 ewes), and control group (18 ewes). Then the ewes received artificial insemination and the pregnancy was examined 45 days later. As shown in Table 2, the pregnancy rate of ewes with melatonin 5 mg injection (66.67 ± 4.76%) was significantly higher than that of the other two groups, respectively (40.48 ± 6.30%, 37.62 ± 5.78%, $p<0.05$), and there was no significant difference between the 10 mg group and the control group. As for the number of lambs the ewes born, there was no difference among all three groups ($p>0.05$).

Effects of melatonin on embryo production and pregnancy rate in sheep

To know how melatonin affects sheep reproductive activity, the pronuclear embryos and blastocysts of the donors were harvested and then transferred to recipients. It was found that melatonin injection slightly increased the number of CL (8.78±1.78 vs 8.44±1.13) and pronuclear embryos (8.8±1.9/ewe vs 8.3±1.0/ewe), ($p>0.05$) (Table 3). However, the pregnancy rate of embryos and birth rate of lambs in melatonin 5 mg group was significantly higher than that of the control group (44.4% vs 24.1%, and 53.3% vs 25.7% $p<0.05$) (Table 4). During the period of estrus, the donor who received melatonin treatment significantly increased the total number of morula embryo/blastocyst at 6 days after insemination (7.4 ± 1.3/ewe, 4.0± 0.7/ewe, $p<0.05$) (Table 5), and also the pregnancy rate was significantly increased after embryo transfer compared to the control group (72.0% vs 63.4%, $p<0.05$), but for the number of lambs the ewes born, there was no difference among two groups ($p>0.05$) (Table 6).

Discussion

Current study has demonstrated that short term melatonin treatment during estrus elevates estradiol and LH levels, improves oocyte quality and leads to the increase of the pregnant rate in ewes. These observations are consistent with previous studies which also indicated that melatonin treatment increased s serum LH [17] and progesterone levels in sheep [18]. Wang *et al* [19] found that the melatonin level decreased after the removal of CIDR in deer and this is similar to our observation in the current study. We also observed that melatonin administration significantly increased serum melatonin level, in turn, it enhanced the LH and progesterone levels, subsequently led to increase in the numbers of embryos and better pregnant rate.

Ovulation is similar to inflammatory reaction that produces a large amount of ROS and reactive nitrogen (RNS) [20]. In the early pregnancy, ROS inhibits progesterone production from corpus luteal cells causes luteal CL regression [21], and also induces ovarian cell apoptosis [22]. As a potent antioxidant, melatonin detoxifies ROS including OH^-, O^{2-} and H_2O_2 and reduces the oxidative damage of ovarian cells [23] and thus, improves the fertility and fecundity of sheep by improving the survival rate of corpus luteum and embryos [24]. Numerous studies have proved that melatonin promotes the development of oocytes and embryos in sheep [25], pigs [26], cattle [27], mice [28] and humans [29] in the *in vitro* environment by scavenging ROS or.

Luridiana *et al* reported that the melatonin implantation in ewe at the age of 5-6 with 3.5-4.0 body condition score (BSC) in spring improved fertility of ewes [30]. In our study, the pregnant rate of ewes received single melatonin injection was significantly increased as well as both pronuclear embryos and blastocysts compared with the control group. Yang *et al* [31] observed that the melatonin injection before mating improved the pregnancy rate of Holstein cows preluded with elevated serum melatonin and progesterone levels. In our experiment, the progesterone level in serum did not change after melatonin treatment, but the estradiol was increased. Melatonin may benefit follicular development, and then increases estradiol synthesis to promote ovulation in female sheep.

In this study, we observed the effects of melatonin to synchronize estrous in ewe, especially in the donor ewes to benefit the embryo transplantation. Short term of melatonin treatment during estrus might improve the uterine environment of ewe and significantly increased the pregnant rate. In addition, melatonin also improves the quality and quantity of embryos and this may also contribute to the increased pregnant rate. These data provide strong support for the application of melatonin in sheep to improve the reproductive outcome industrially.

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Table 1 (on next page)

Effects of melatonin injection on FSH, LH, P_4 and E_2.

Table 1: Effects of melatonin injection on FSH, LH, P_4 and E_2.

Time	Group	MT (pg/ml)	FSH (mIU/ml)	LH (mIU/ml)	P_4 (mIU/ml)	E_2 (mIU/ml)
Withdrew CIDR	5 mg	363.59 ± 63.87^{a}	2.04 ± 0.08^{a}	4.93 ± 0.25^{a}	0.26 ± 0.04^{ab}	14.61 ± 2.56^{a}
	10 mg	390.69 ± 64.22^{a}	2.05 ± 0.07^{a}	4.93 ± 0.34^{a}	0.30 ± 0.05^{a}	15.06 ± 1.30^{a}
	Control	350.95 ± 63.16^{a}	2.16 ± 0.14^{a}	4.64 ± 0.29^{a}	0.21 ± 0.02^{b}	15.04 ± 1.39^{a}
Estrus	5 mg	458.69 ± 48.40^{a}	1.86 ± 0.09^{b}	5.39 ± 0.52^{a}	0.17 ± 0.02^{b}	23.11 ± 3.62^{a}
	10 mg	458.09 ± 60.60^{a}	1.79 ± 0.13^{b}	5.41 ± 0.49^{a}	0.22 ± 0.02^{a}	24.49 ± 2.50^{a}
	Control	393.37 ± 51.53^{a}	2.05 ± 0.15^{a}	5.26 ± 0.48^{a}	0.16 ± 0.02^{b}	22.96 ± 2.01^{a}
Insemination	5 mg	509.00 ± 67.52^{a}	2.06 ± 0.13^{a}	4.59 ± 0.38^{a}	0.14 ± 0.02^{a}	19.23 ± 2.66^{a}
	10 mg	457.24 ± 40.65^{ab}	1.76 ± 0.14^{b}	4.02 ± 0.27^{b}	0.18 ± 0.03^{a}	13.78 ± 1.77^{b}
	Control	330.23 ± 38.72^{b}	2.14 ± 0.26^{a}	4.27 ± 0.31^{b}	0.14 ± 0.02^{a}	14.07 ± 2.86^{b}

Note: different letters in the same column at same time point indicate significant difference ($p < 0.05$).

Table 2(on next page)

Effect of melatonin treatment on pregnancy of ewes

Table 2: Effect of melatonin treatment on pregnancy of ewes

Group	Ewes	Pregnant Ewes	Pregnancy rate	Lambs/ewes (%)
5 mg	21	14	66.67±4.76 [a]	40/14 (285.7%) [a]
10 mg	20	8	40.48±6.30 [b]	22/8 (275.0%) [a]
Control	18	7	37.62±5.78 [b]	22/7 (314.3%) [a]

Note: different letters in the same column indicate significant difference ($p < 0.05$).

Table 3(on next page)

Effect of different treatments on pronuclear embryo production after superovulation of donor ewes

Table 3 Effect of different treatments on pronuclear embryo production after superovulation of donor ewes

Group	Donors	Corpus Luteum	Embryos	Normal Embryos
5 mg	13	8.78±1.78[a]	8.81±1.86[a]	8.81±1.86[a]
Control	18	8.44±1.13[a]	8.31±1.00[a]	8.31±1.00[a]

Note: different letters in the same column indicate significant difference ($p<0.05$).

Table 4(on next page)

Effect of different treatments on pregnancy rate after pronuclear embryo transplantation in recipient sheep and lambs born

Table 4 Effect of different treatments on pregnancy rate after pronuclear embryo transplantation in recipient sheep and lambs born

Group	Recipient	Embryos transferred	Pregnancy rate	Lambs/ewes (%)
5 mg	45	2.4（108/45）	44.4% (20/45)[a]	24/45 (53.3%)[a]
Control	58	2.9（168/58）	24.1% (14/58)[b]	15/58 (25.7%)[b]

Note: different letters in the same column indicate significant difference ($p < 0.05$).

Table 5
(on next page)

Effect of different treatments on morula/blastocyst production after superovulation of donor

Table 5 Effect of different treatments on morula/blastocyst production after superovulation of donor

	Donors	Average Luteum	Average Embryos	Normal embryos
5 mg	15	10.33±1.37[a]	7.80±1.25[a]	7.40±1.25[a]
Control	13	8.08±1.13[a]	4.08±0.70[b]	3.96±0.73[b]

Note: different letters in the same column indicate significant difference ($p < 0.05$).

Table 6 (on next page)

(on next page)

Effect of different treatments on pregnancy rate after morula/blastocyst transplantation in recipient sheep

Table 6 Effect of different treatments on pregnancy rate after morula/blastocyst transplantation in recipient sheep

Group	Recipient	Embryos transferred	Pregnancy rate	Lambs/ewes (%)
5 mg	25	1.9（47/25）	72.0% (18/25)[a]	19/18 (105.6%)[a]
Control	22	1.9（41/22）	63.4%b(12/22)[b]	14/12 (116.7%)[a]

Note: different letters in the same column indicate significant difference ($p < 0.05$).