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Melatonin improves the efficiency of super-ovulation and timed artificial insemination in sheep

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It has been well proved 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 this study, the effects of melatonin on the number of embryo s harvested from superovulation, and the pregnant rate in recipients after embryo transferred have been investigated. Hu sheep with synchronous estrus treatment were given melatonin subcutaneously injection (0, 5, and 10 mg/ewe, respectively). It was found that t he estrogen level in the group of 5 mg melatonin was significantly higher than that of other two groups a t the 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 another 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 (7.40±1.25/ewe vs. 3.96±0.73/ewe, P<0.05) and the pregnant rate (72.3±4.6% vs.54.7±4.0%, 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 36 hours after CIDR withdrawal 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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Melatonin Improves the Efficiency of Super-ovulation and Timed Artificial Insemination in Sheep

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Abstract: It has been well proved 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 this study, the effects of melatonin on the number of embryos harvested from superovulation, and the pregnant rate in recipients after embryo transferred have been investigated. Hu sheep with synchronous estrus treatment were given melatonin subcutaneously injection (0, 5, and 10 mg/ewe, respectively). It was found that the estrogen level in the group of 5 mg melatonin was significantly higher than that of other two groups at the 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 another 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 (7.40±1.25/ewe vs. 3.96±0.73/ewe, P<0.05) and the pregnant rate (72.3±4.6% vs.54.7±4.0%, 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 36 hours after CIDR withdrawal could promote the number and quality of embryos in the *in vivo* condition and increased the pregnant rate and number of lambs.

67 **Introduction**

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

87 Melatonin also plays an important role in the establishment and maintenance of pregnancy in animals. In
88 mouse, extremely high expression of melatonin receptor 1 (MT1) was observed in granulosa cells after
89 human chorionic gonadotropin (hCG) treatment [12]. At the same time, melatonin synthetic enzyme in
90 cumulus cells was upregulated by hCG injection and high level of melatonin in follicle fluid was detected
91 [13] and this was observed also in porcine follicle fluid [14]. Further study demonstrated that melatonin
92 and its receptor MT1 regulated the downstream signaling pathway of hCG (LH) including the luteinization
93 of granulosa cells in mice [12]. The deletion of MT1 receptor severely impairs the fertility of mice due to
94 reduced oocyte number and quality [15].

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

101 **Materials and methods**

102 **Chemicals**

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

108 **Animals**

109 Female sheep (Hu, Suffolk and Small-tailed Han) from Aoxin Animal Husbandry (Beijing, China) and
110 Zhenxin Farmers's Professional Association Organization (Uygur Autonomous Region, China), at the age
111 of 2-4 years old with normal reproductive cycle, healthy and generally the similar body weight were
112 selected for the experiments. All experimental protocols concerning the handling of animals were
113 performed in accordance with the requirements of the Institutional Animal Care and Use Committee at the
114 Xinjiang Agricultural University(permition number:2017003).

115 **Experiment design**

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

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

132 **Experiment 3**: twenty-eight Suffolk ewes as donors and 44 Small-tailed Han ewes as recipient were
133 selected and divided into two groups randomizedly. The donor ewes were treated to induce superovulation,

134 and 36 hours after CIDR removal LH and melatonin (5 mg) was subcutaneously injected into donors at the
135 same time. Six days after artificial insemination by laparoscopic, morula or blastocyst were surgically
136 harvested from the uterine horn of the donor, and the number of CL and embryos were recorded. The
137 recipient was surgically operated with the laparoscopic surgery to find the uterine horn, finally the embryo
138 was transplanted into the uterine horn from the 5cm junction between the uterus and the fallopian tube by
139 the the transplantation pipette. The examination of pregnancy was performed using B-ultrasound 45 days
140 after embryo transfer.

141 **Semen preparation**

142 In this study, fresh semen was collected from male sheep of black Suffolk using a vaginal prosthesis， and
143 then the semen was diluted and store at 36℃. Fresh semen is injected into the uterine horn when its vitality
144 reaches 0.6 or more by laparoscopic insemination.

145 **Blood sample collection and hormones analyze**

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

149 **Statistical analysis**

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

153 **Results**

154 **Effects of melatonin injection on hormones**

155 Firstly, the effects of melatonin on FSH, LH and E2 were evaluated at 3 different time points (Table 1). At
156 the time of artificial insemination, the melatonin in the serum of ewes in 5 mg group (509.0±67.5pg/ml)
157 was significantly higher than that of the other control group (330.2±38.7pg/ml) ($p<0.05$). But there was no
158 difference in melatonin levels between the three groups at the time of CIDR withdrawal and estrus. The
159 FSH level in the control group was always in a high level, but the increase of FSH from estrus to
160 insemination (0.2 mIU/ml) in the 5 mg group was significantly higher than that in the other two groups
161 (0.03 mIU/ml and 0.09 mIU/ml, $p<0.05$). LH level was increased from CIDR withdrawal to estrus, and
162 gradually decreased after the peak level. LH level in the 5mg group (4.59±0.4 mIU/ml) was significantly
163 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
164 ($p<0.05$). Progesterone concentration in the 10 mg group was the highest, followed by a downward trend,
165 significantly higher than that in the control group at the time of withdrawal CIDR and estrus ($p<0.05$), and
166 significantly higher than 5 mg group at the time of estrus ($p<0.05$). The concentration of progesterone of

167 the 10 mg treated group was highest at the time of insemination, but there was no significant difference
168 between the two groups (p>0.05). There was no difference in estradiol level between the two groups at the
169 time of CIDR withdrawal and estrus. At the time of insemination, 5mg group (19.2±2.7pg/ml) was
170 significantly higher than the other two groups (13.8±1.8 pg/ml, 14.1±2.9 pg/ml, $p < 0.05$).

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

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

179 **Effects of melatonin on embryo production and pregnancy rate in sheep**

180 To know how melatonin affects sheep reproductive activity, the pronuclear embryos and blastocysts of the
181 donors were harvested and then transferred to recipients. It was found that melatonin injection slightly
182 increased the number of CL (8.78±1.78 vs 8.44±1.13) and pronuclear embryos (8.8±1.9/ewe vs
183 8.3±1.0/ewe), (p>0.05) (Table 3). However, the pregnancy rate of embryos and birth rate of lambs in
184 melatonin 5 mg group were significantly higher than that of the control group (43.3±6.1% vs 25.3±4.9%,
185 and 54.0±4.0% vs 27.7±9.0% p<0.05) (Table 4). During the period of estrus, the donor who received
186 melatonin treatment significantly increased the total number of morula embryo/blastocyst at 6 days after
187 insemination (7.4 ± 1.3/ewe, 4.0± 0.7/ewe, p<0.05) (Table 5), and also the pregnancy rate was significantly
188 increased after embryo transfer compared to the control group (72.3±4.6% vs 54.7±4.0%, p<0.05), for
189 the number of lambs the ewes born, there was no difference among two groups (p>0.05) (Table 6).

190

191 **Discussion**

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

199 Ovulation is similar to inflammatory reaction that produces a large amount of ROS and reactive nitrogen
200 (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 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.

Conclusion:

Melatonin at 5 mg subcutaneously injected into the neck during estrus would increase the level of melatonin and estradiol in the blood of ewe, and melatonin promoted embryo production and the pregnancy rate in the awes naturally mated or embryo transfer. Meanwhile, melatonin had beneficial effects on recipients for embryo transfer. Altogether, melatonin could be used to the improve the number of lambs.

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Table 1(on next page)

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

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1 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]

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

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Table 2 (on next page)

Effect of melatonin treatment on pregnancy of ewes

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1 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]

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

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Table 3 (on next page)

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

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1 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]

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

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Table 4(on next page)

Table_4_Effect_of_different_treatments_on_pregnancy_rate_after_pronuclear_embryo_transplantation_in_recipient_sheep_and_lambs_born-

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

Group	Recipient	Embryos transferred	Pregnancy rate	Lambs/ewes
5 mg	45	2.5±0.2 (115/45)	43.3±6.1% (20/45)[a]	24/45 (54.0±4.0%)[a]
Control	58	2.6±0.1 (150/58)	25.3±4.9% (14/58)[b]	15/58 (27.7±9.0%)[b]

3 Note: different letters in the same column indicate significant difference ($p < 0.05$).
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Table 5 (on next page)

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

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1 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]

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

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Table 6 (on next page)

Table_6_Effect_of_different_treatments_on_pregnancy_rate_after_morula-

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

Group	Recipient	Embryos transferred	Pregnancy rate	Lambs/ewes
5 mg	25	1.88±0.13 (47/25) [a]	72.3±4.6% (18/25)[a]	19/18 (105.7±10.0%)[a]
Control	22	1.86±0.15 (41/22) [a]	54.7±4.0%(12/22)[b]	14/12 (116.7±14.4%)[a]

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