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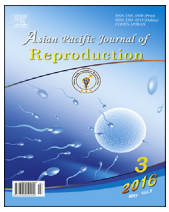

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Superovulatory response and embryonic progressive in Iranian Qezel ewes treated with two different concentrations of bovine somatotropin

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ABSTRACT

Objective: This study was conducted in order to the administration of 50 and 100 mg of bovine somatotropin (bST) at the beginning of the estrus synchronization and natural mating of the sheep to evaluate the improvement of the ovulation rate, embryonic development and pregnancy rate of the transferred embryos.

Methods: Forty eight donors were treated with three different types of treatment; Group A: treated with bST-100 (n = 15), received 100 mg of bST at the beginning of the synchronization and natural mating, Group B: treated with 50 mg of bST (n = 15) same as the previous group and control (n = 18) did not receive any type of bST. Each recipient received two embryos, (n = 108): 30 recipients received the embryos from bST-100s, 45 recipients received the embryos from bST-50 and 33 recipients received embryos from the control group. Using SAS related GENMOD method, rate of superovulatory, recovered structure percentage, cleavage rate, transferable embryo percentage, quality of embryos, rates of pregnancy and embryonic development were analyzed. Using GLM procedure, numbers of corpus luteum and blastocyst cells were analyzed.

Results: The bST administration had no significant effect on rate of superovulatory, number of CL and recovered structures ($P \geq 0.05$). Number of transferable embryos and embryos that had access to the blastocyst in bST-50 ($P \leq 0.01$) was more than bST-100 and control group.

Conclusions: The treatment 50 mg bovine somatotropins enhance the ratio and growth of the transferable embryos. Embryos of bST-50 treatment indicated an improved embryonic development but bST did not affect the pregnancy rates of transferred embryos.

1. Introduction

Multiple ovulation and embryo transfer (MOET) is an implement to maximize the sheep population of fittest race. However, high diversity in superovulatory rate affects the efficiency of high quality embryo production [1]. External administration of bovine somatotropin (bST) increases the circulating concentrations of insulin and insulin-like growth factor 1 (IGF-1) in sheep [2,3]. Studies indicate that insulin and IGF-1 are in charge of bST effects on reproduction [4–6]. These effects include an increase in the recruited follicles [2,7] and development of produced embryos and increase their number [3,8].

Insulin and IGF-1 receptors in follicles of sheep have been identified [9]. In follicular cells, insulin increases the glucose and amino acid metabolism, stimulates cell growth and proliferation, inhibits follicular steroid secretion [10] and regulates the activity of gonadotropin receptors [11,12]. In granulosa cells, IGF-1 co-operates (synergistic) with FSH [13] to improve hormonal activities such as secretion of follistatin, an activator and inhibitor, proliferation and differentiation of granulosa cells, estradiol production and regulation of aromatase activity [14]. Generally, IGF-1 protects the oocyte and improves its maturity [15]. Researches about bST in ruminates indicates that this hormone improves the embryonic development, as a result, increases the reproduction efficiency [16]. bST and obtained concentrations of IGF-1, increase nuclear maturation rate and pyruvate metabolism and have anti-apoptosis effect on *in-vitro*

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bovine embryos [17] and increase the number of blastocyst [18,19]. Moreover, these hormones regulate the PGF2α synthesis [20]. In studies that are related to sheep, commercially available amount of bovine hormone is 500 mg that would be divided to four 125 mg parts. This amount was also used in several other studies before the mating or during the mating. The results of these studies indicate improvement in the embryo size [12] and embryonic development in sheep [3,8]. Also, another study by using the same amount in goats that were at anestrus cycle, showed an increase in pregnancy rate [21].

However, several researches show contradictory effects about superovulatory responses and pregnancy rate after the administration of bST [22–25]. Other scientists suggested that this diversity could be due to several factors such as bST levels, physical condition and serum concentrations of IGF-1 [16,26,27]. For example, treating with bST helps to improve the pregnancy rate in dairy cows [28] decreases the pregnancy rate in non-lactating cows [28]. Detrimental effect of bST on pregnancy rate of non-lactating cows could be related to over-stimulation of blood insulin and IGF-1 secretion [28]. In sheep, various responses may be attributed to the amount of bST. The purpose of this study was to assess the administration of 50 and 100 mg of bovine somatotropin in ewes with multiply ovulation at the beginning of synchronization and during the natural mating to see that if it improves the ovulation rate, embryonic development and pregnancy rate of transferred embryos or not.

2. Materials and methods

2.1. Animals and treatments

This study was conducted in Kashan during the breeding season (autumn) with an average elevation of 982 m and the average annual temperature of 35 °C (The latitude of the location of the experiment was: 32.637 524 532°38′15.09″N), Qezel Ewes Research Center. All experimental procedures were approved by the Kansas State University. Donors 48 cyclic, adult Qezel ewes (3 years old) and recipients include 108 cyclic, adult Qezel ewes (3 years old). Donors and recipients were kept at the same place and they were fed hay, barley, maize, soybean meals and minerals. All of the sheep had the delivery experience (at least once) and their physical condition score was between 3 and 4 (in scale of 5) [29]. Ewes were synchronized using intra-vaginal CIDR (DEC manufacturing, Hamilton, New Zealand), the tool remained inside the vagina for 12 d. Intramuscular injection of selenium (0.05 mg/kg of body weight) and vitamin E (0.05 mg/kg of body weight) was conducted for all of the sheep, simultaneous by application of CIDR (day zero) (Mu-Se, MSD Animal Health, Mexico). After removing the intra-vaginal CIDR, intramuscular injection of 250 IU equine chorionic gonadotropins conducted for all of the sheep (Pregnecol, Bioniche Life Sciences Inc., Australia). At the day 0 (beginning day), donors were treated by three types of different treatments, randomly: (A) treatment with bST (n = 15), received 100 mg of bST as subcutaneous injection at the beginning of synchronization and the second injection was carried out at the time of mating (Boostin-S, MSD Animal Health, Mexico), (B) treatment with bST-50 (n = 15), received a subcutaneous injection of 50 mg of bST with the same program as previous group (Boostin-S, MSD Animal Health, Mexico) and the control group (n = 18) received saline instead of bST. Ten days after placing the CIDR, superovulatory started. Superovulatory was stimulated using 164 mg of pFSH (Folltropin-V, Bioniche, Ontario, Canada) that was conducted as eight reductive doses (1 for every 12 h). Two days after removing the CIDRs, ewes were mated with male sheep that were approved for fertilizing ability, starting at 8 am, each ewe was mated 5 times (with 1 h intervals).

2.2. Embryo transfer

Actually, for each sheep three CLs or more is considered as a response to superovulatory [30]. Embryos were collected using semi-laparoscopic method [31]. Embryos were classified according to the growth level and morphology (Quality grade 1: excellent or good, Quality grade 2: fair, Quality grade 3: weak, Quality grade 4: dead or corrupt) [32].

Collecting and transferring the embryos was conducted 6 d after mating. Embryos were transferred using semi-laparoscopic method [31]. This method introduces two fresh embryos in the presence of CL to the ovary, inside the uterus. Only embryos of the first and second grades of quality were used. Embryos were transferred to 108 recipients: 30 recipients received the embryos from bST-100 treated, 45 recipients received the embryos from bST-50 treated and 33 recipients received the embryos from the control group. At the 40th day after embryo transfer, pregnancies were identified using a 5.0 MHz real-time ultrasound device (Logic 100 PRO VET, GE Medical, Bangalore, India).

2.3. Blood sampling and measurement of hormones

From each group, five female sheep were selected randomly and blood samples were collected inside the clot-activator associated tubes (BD Vacutainer, Becton, DikinSon, Copany, Franklin Lakes, NJ, US). These samples were collected each 48 h after the placement of CIDR (day 0) until the embryo collecting day (day 20), from the jugular vein. All of the samples were collected at 8 am, before feeding the sheep. Blood samples were centrifuged at 1 500 ×g for 10 min in order to denotative the serum, then separated blood serum were stored at −20 °C for further laboratory analysis. Concentrations of blood insulin were measured using commercial radioimmunoassay (RIA) kits (Insulin-CT, Cis-Bio International, Gif sur Yvette, France) with the coefficient of variation of internal testing of 3.5%. Serum IGF-1 concentration was measured for up and down control groups using double antibody radioimmunoassay with the coefficient of variation of internal testing of 0.65% and 14.8%, respectively (IGF-1-RIACT, Cis-Bio international, Gif sur Yvette, France).

2.4. Cell count

Totally, 102 embryos were counted (34 embryos in each group). These cells were counted by making expanded blastocysts permeable and marking them with 0.2% of Triton® X-100 (Cat. No. 93443; Sigma–Aldrich) solvent in preservative solution containing 30 μg/mL propidium iodide in 20 s (EmCare, ICPBio Limited, Auckland, New Zealand). Immediately after the marking, embryos were washed in preservative solution and placed in methanol ice containing 10 μg/mL bisBenzimide H33258 (Cat. No. B1155; Sigma–Aldrich) for 10 min. These embryos were transferred to 50:50 methanol-glycerol solution and

they were mounted on small drops of this solution [33]. Mounted embryos were compacted slowly using a shrouding cover so that they would become spread in order to be counted (number of cells). Cells were counted using a Leica epifluorescent microscope (DMIL Leica Microsystems, Wetzelar, Germany).

2.5. Statistical analysis

The superovulatory rate, recovered structures percentage (oocytes or embryos), cleavage rate, transferrable embryos percentage, classified embryos percentage and their quality, number of embryos in each growth stage and pregnancy rate in transferred embryos were analyzed using GENOD method (SAS/STAT version 9.3, SAS Institute Inc., Cary, NC). Number of CLs and number of blastocyst cells were analyzed using SAS related GLM procedure. Insulin and IGF-1 concentrations among groups were analyzed using ANOVA in order to measure the repeats. The area under the curve (AUC) of insulin and IGF-1 was calculated using trapezoidal rule. AUC data for both hormones was analyzed using ANOVA. The differences between the groups were evaluated using *t*-test.

3. Results

Superovulatory rate (P = 0.14), number of CLs (P = 0.13) and recovered structures percentage (P = 0.07) were not affected by any type of treatments (50 and 100 mg of bST). However, cleavage division rate in bST-100 group (P = 0.000 1) was lower than bST-50 and control group. Transferable embryos percentage in each donor (P = 0.01) and the percentage of embryos reached the blastocyst stage (expanded and hatched) (P < 0.001) in bST-50 group was more than bST-100s and control group. The number of blastocyst cells (P = 0.15) and pregnancy rate of donors were not affected by any type of bST treatment (P = 0.21) (Table 1).

Table 1
Superovulatory rate, embryo development and pregnancy rate of embryos obtained from superovulated ewes treated of 0, 50 and 100 mg of bST at the beginning of estrous synchronization and mating.

Variable	bST Dose		
	0 mg	50 mg	100 mg
Superovulatory rate (%)	77.77[a]	93.34[a]	730.33[a]
Corpora lutea (*n*)	10.78 ± 1.32[a]	15.12 ± 1.39[a]	11.97 ± 1.44[a]
Recovered structures[c] (%)	89.93 ± 4.2[a]	85.1 ± 1.1[a]	86.4 ± 4.3[a]
Cleavage rate (%)	94.5 ± 2.5[a]	97.3 ± 5.1[a]	81.3 ± 8.9[b]
Transferable embryos[d] (%)	86.9 ± 5.1[b]	95.8 ± 3.6[a]	92.6 ± 4.1[b]
Morula (%)	53.98 (61/113)[b]	28.65 (47/164)[a]	59.57 (56/94)[b]
Expanded blastocysts (%)	46.01 (52/113)[b]	59.75 (98/164)[a]	35.01 (33/94)[b]
Hatched blastocysts (%)	3.53 (4/113)[b]	13.41 (22/164)[a]	1.06 (1/94)[b]
Cells per blastocyst (*n*)	109.68 ± 3.66[a]	114.31 ± 4.12[a]	100.12 ± 4.03[a]
Pregnancy rate (%)	55.55 (20/36)[a]	72.00 (36/50)[a]	60.00 (21/35)[a]

Different letters (a, b) within columns indicate significant contrasts (P < 0.05).
[c] Total oocytes or embryos recovered. [d] Quality 1 and 2 embryos.

After 48 h of the first hormone injection, insulin concentration in bST treated sheep increased. Treatment with bST-100 created the highest concentration of insulin (P = 0.02) after treatment with bST-50 (P = 0.02). After 6 d from the first injection in both groups, insulin concentration returned to the baseline. Similarly, the concentration returned to the baseline level 96 h after the second injection (Figure 1). However, after the second injection both groups experienced an increase of IGF-1 concentration, but this increase in comparison to the first injection, was less obvious (Figure 2).

AUC amounts for insulin (P = 0.02) and IGF-1 at the first injection was more than the second injection for both groups. AUC amounts for insulin in bST-50 treated was (121.5 ± 64.6) μUI/(d·mL) and (24.9 ± 25.8) μUI/(d·mL) for the first and second injection, respectively. Similarly, AUC amounts for IGF-1 for the first and second injections were (4.159 0 ± 0.529 0) mg/(d·mL) and (2.529 7 ± 0.815 4) mg/(d·mL), respectively. In bST-100 treated group, AUC amounts for insulin in the first and second injection was (203.5 ± 115.9) μUI/(d·mL) and (46.8 ± 35.9) μUI/(d·mL), respectively. Similarly, AUC amounts for IGF-1 in the first and second injection was

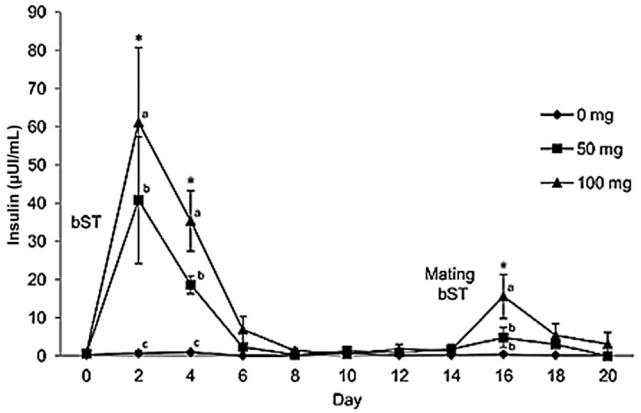

Figure 1. Insulin concentration (mean ± standard error) in ewes of control group, 50 or 100 mg of bST treated at the beginning of estrus synchronization and at the time of mating.
Insulin concentration differ between treatments (literals[a,b,c]) on days marked with *(P = 0.02). The day 0 is related to the beginning of estrus synchronization.

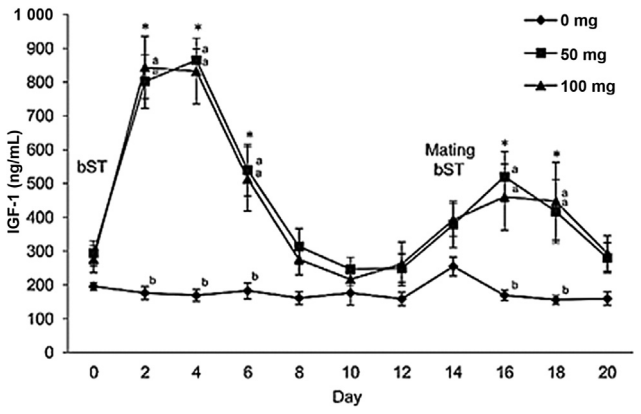

Figure 2. IGF-1 concentration (mean ± standard error) in sheep of control group, 50 or 100 mg of bST treated at the beginning of estrus synchronization and at the time of mating.
Insulin concentration differ between treatments (literals[a,b,c]) on days marked with *(P = 0.01). The day 0 is related to the beginning of estrus synchronization.

(4.1290 ± 0.1051) and (2.4790 ± 0.1021) mg/(d·mL), respectively.

4. Discussion

Administration of bST increased IGF-1 and external insulin concentrations in accordance with the results of other studies [2–6]. Interestingly, in this study serum IGF-1 concentrations for both values (50 and 100 mg) were the same. On the other hand, serum insulin concentration in bST-100s was higher. Generally, insulin and IGF-1 concentration after the first injection was higher than that after the second injection. However, the reason of higher concentrations of insulin and IGF-1 as a response to the first bST injection was not discovered. Some of the researchers have discovered the same pattern for insulin concentration in non-lactating cows [22]. Spencer *et al.* discovered the same pattern for IGF-1 in lambs however the bST hormone had been used daily [34].

These findings indicate that the first injection of bST can stimulate the production of antibodies against this hormone therefore these antibodies can easily surmount the bST of the second injection, easily. Statistical analysis of the recent study didn't show any improvement of superovulatory rate in both amounts of bST (50 and 100 mg) treatment. In contrast, Navarrete-Sierra *et al.* reported an improvement as a response to the administration of 125 mg of bST at the end of the treatment [35].

However, the difference between the groups was not statistically significant, but numerical differences indicated that superovulatory rate in bST-50 group was higher than bST-100 and control group (21% and 13% more). Similarly, number of CLs of donors or recovered structures (oocytes or embryos) had no difference among the groups. In this regard, previous studies suggested that bST has a beneficial effect on number of follicles or CLs [2,8,30,35]. However, other studies indicate that administration of bST does not increase the number of CLs [6,24,36] or recovered structures of ewes [8].

In this study, administration of 100 mg of bST in donors led to a declined percentage of cleavage of embryos, that is an adverse effect and this could be related to the increase in insulin concentration after the administration of 100 mg bST. Reports from the laboratory studies indicate that adding 5 μg/mL of insulin to the follicles medium, decreases the divided embryos (cleavage) percentage [37]. Administration of 50 mg of bST increased the transferable embryos percentage, considerably. This increase was related to the observed percentages of bST-100 and control group. Navarrete-Sierra *et al.* reported an increase of transferable embryos as a response to the administration of 100 mg of bST [35]. However, Montero-Pardo *et al.* [3] and Mejia *et al.* [8] didn't observe this increase of transferable embryos as a response to the administration of 125 mg of bST. Similarly, embryos of bST-50 treatment indicated an improved embryonic development, because in comparison to other groups, most of the embryos were more advanced in term of embryonic stage (developed or hatched blastocyst). This difference, considering the fact that most advanced stage of growth, *i.e.*, hatched blastocyst (hatched or expanded) was evaluated, makes it even more prominent, because the number of hatched blastocysts was approximately 5 times more than bST-50 treated and control group and approximately 11 times more than bST-100 treated group. Mejia *et al.* discovered that administration of 125 mg of bST to each donor at the mating time, increases the number of more advanced embryos in terms of advanced stages of development [8]. This case was similar to the results that Montero-Pardo *et al.* [3] reported. They used the same amount (125 mg) 5 d before the application of progestin.

Anyway, in this study, the number of cells in each blastocyst in both treated groups and the control group was the same. Montero-Pardo *et al.* reported that the administration of 125 mg of bST at 5 d before removing the sponge in sheep with multiplied ovulation increased the number of cells of embryo [3], although these writers reported fewer cells than the number of cells that was observed in the present study. On the contrary, Block *et al.* discovered that adding 100 ng/mL of IGF-1 to the medium had no effect on the total number of cells in bovine embryo [38], which would suggest that IGF-1 effects on *in-vivo* embryo survival, likely is the result of differences in the gene expression, instead of being a result of changing the number of cells. Reports from several studies on the cows [39,40] indicate that administration of bST on the donors increases the pregnancy of the obtained embryos. However, administration of bST did not affect the pregnancy rate. These results are consistent with the reported results of Folch *et al.* [30] about sheep and Neves *et al.* [41] about cattle.

Variability among the experiments could be due to the bST applied amounts and the resultant of IGF-1, because IGF-1 concentration should be kept in a specific physiological range (approximately 200 ng/mL) [27,28]. Threshold concentration of IGF-1 can increase the fertility and pregnancy rates [28] but exceeding from this threshold concentration of IGF-1 can induce negative effects [22]. Recently, Ribeiro *et al.* reported that one time treatment with low amounts of bST (325 mg) at artificial insemination, was not enough to change the embryonic development and pregnancy [16]. However, two sequential therapies of 325 mg of bST at AI and 14 d later increased the pregnancy of the dairy cows and reduced the fertility decline that notes the importance of GH and IGF-1 during the primary growth of the embryo.

In the present study, production of transferable embryos and embryonic development of 50 mg of bST treated cows had better responses in comparison to the 100 mg of bST treated sheep. Embryos that were exposed to high concentrations of insulin and IGF-1, undergo apoptosis. As a result, apoptosis affects the embryo implantation and therefore, embryo would be reabsorbed [42,43]. Chi *et al.* showed that adding high concentrations of insulin to the mouse blastocysts medium, increases the apoptosis by DNA division [43].

Apoptosis is "dose-dependent", because average amount (35 nmol/L) and large amount (700 nmol/L) cause 50% and 70% apoptosis, respectively. Similarly, Mihalik *et al.* reported that adding insulin to the bovine embryo medium has no effect on embryonic development [44]. In a study on cows with normal physical condition (3.4 on the scale of 6), Adamiak *et al.* reported that high concentrations of insulin, produces fewer follicles and blastocysts after the *in-vitro* fertilization [45]. Fouladi-Nashta and Campbell [37] showed that adding 5 μg/mL of insulin to the bovine antral medium, decreases the divided embryo ratio that were grown to transform into blastocyst and quality of embryos has no difference among the groups (evaluated by total cell number). These writers suggested that decrease in the division rate is related to the primary cytoplasmic changes, that indicates oocyte were exposed to over-maturation or they have grown too old and this decreased the fertility rate. However, serum IGF-1

concentration in 50 and 100 mg bST treated groups was similar, while serum insulin concentration among the 100 mg treated group was higher, because insulin and IGF-1 can have cross-reactions with related receptors [46]. These results can show opposite effects, due to over-stimulation by 100 mg of bST that can increase the IGF-1R expression [47] and glucose uptake (insulin-dependent) by embryos [48] to impact reversely on them.

In *in-vitro* systems, consumption and decomposition of IGF-1 takes place without peptide renewal. On the other hand, in settings with high concentrations of IGF-1 (as an example in administration of bST) embryos are exposed to abnormal high concentrations of IGF-1 which may exacerbate apoptosis and hypertrophic ICM [47].

Conflict of interest statement

The authors declare that they have no conflict of interest.

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