Prevention of adhesions in ewes submitted to consecutive surgical embryo collections

Prevenção de aderências em ovelhas submetidas a sucessivas coletas cirúrgicas de embriões

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Summary: The aim of this study was to evaluate the effect of two solutions on the prevention of adhesions formation in ewes submitted to successive surgical embryo collection. Eight cross-bred ewes were superovulated with 250IU of FSH and embryo collection was performed on day 7. In G1 ewes (n=4), immediately before abdominal wall closure, dexamethasone (25 mg/kg) diluted in Ringer with lactate (qs 75 mL) was infused in the abdominal cavity and in G2 (n=4), vitamin E (100 mg/kg) diluted in olive-oil (qs 75 mL). In G1 ewes, the adhesions were visually thinner, with minimal tenacity and vascularization, and in less amount than in G2 animals, with a positive and significant correlation between amount of adhesions and uterine exteriorization degree (r = 0.34). Uterine exteriorization was possible in all animals and evaluations from G1, but impossible in 25% of G2 animals during the second embryo collection and in 75% of animals on third embryo collection and at necropsy. The average rates of embryo recovery were different only in M2 (P <0.04) between G1 and G2. Results suggest that dexamethasone in Ringer with lactate prevents abdominal adhesions formation in ewes submitted to successive surgical embryo collection.

Keywords: adhesions prevention, dexamethasone, ewe, embryo transfer.

Introduction

Since human population continues to increase, a higher production of animal protein is required, especially originating from meat. Thus, sheep husbandry represents a big potential to contribute in the minimization of this new challenge. In this case, the embryo transfer (ET) technology is an excellent tool to maximize the use of ewes with high genetic merit (Freitas e Simplício, 2001).

The surgical technique, instead of transcervical ones, is still the first choice for embryo collections in the ovine species. However, the exteriorization of the reproductive system frequently leads to post-operative adhesions, which make successive embryo recoveries difficult. Thus, the surgical procedure is not routinely repeated more than two or three times (Cordeiro et al., 2003).

Adhesion formation consists of a series of local events at the trauma site, and can be enhanced by hypoxia of the mesothelial cells, reactive oxygen species, mesothelial dessication and mesothelial trauma by manipulation (Binda e Koninckx, 2009). After abdominal surgeries in various animal species, infusion and/or washing solutions are used in the abdominal cavity as a form to prevent these adhesion formations. Some of the solutions employed are 0.9% NaCl, Ringer with lactate (Elkelani et al., 2002), methylene blue.
solution (Silva et al., 2013), heparinized saline solution (Cordeiro et al., 2003), dexamethasone diluted in Ringer with lactate (Pacheco et al., 2003) and vitamin E diluted in olive oil (Corrales et al., 2008). However, in ET programs, some of the aforementioned solutions, despite of have not been that strictly characterized, have been used empirically, leading to inconsistent results, such as the heparinized saline solution.

Acute phase proteins are a class of heterogeneous structural and functional proteins, released into the bloodstream in response to a variety of stressors such as local inflammation or bacterial infection (Ceciliani et al., 2012). They contribute to the host’s defense, and may neutralize inflammatory agents, minimize the extension of tissue damage and debris produced after tissue damage, besides participating in the repair and regeneration of tissues, and restoration of homeostasis (Murata et al., 2004; Kaya et al., 2014). Among them, fibrinogen, mucoprotein and C-reactive protein are noteworthy, all of which have been related to inflammatory and/or infectious processes in ovine species (Vojtcic e Krajcic, 2000; Eckersall et al., 2007; Costa et al., 2010).

Few studies have been done to study and characterize methods for adhesions prevention in small ruminants used in ET programs. Therefore, the objective of this study was to evaluate the effect of dexamethasone diluted in Ringer with lactate, and of vitamin E diluted in olive oil, in the prevention of adhesions formation in ewes submitted to successive surgical embryo recoveries.

**Material and methods**

The experiment was carried out at the Federal University of Espirito Santo, Alegre Campus, Alegre – ES, southeastern region of Brazil, latitude of 20° 45’ 49'', longitude 41° 31’ 59” and approximately 250 m above sea level. The mean annual temperature was 22 °C. All procedures were performed according to the Brazilian College of Animal Experimentation (COBEA). Eight cross-bred ewes, 2-5 y, 30 ± 6.3 Kg of body weight were used as embryo donors. The animals were kept in pasture of Cynodon dactylon, receiving water and mineral salt ad libitum, and supplemented with corn silage and concentrate.

Each ewe was submitted to three surgical embryo collections (M1, M2 and M3), at 60 d intervals, approximately. The ewes were synchronized by the insertion of an intra-vaginal sponge containing 60 mg of medroxyprogesterone (Progespon®, Intervet/Schering-Plough, Brasil) for 14 d. During days 12-15 of the treatment, donor ewes were subjected to superovulation using 250 IU of pFSH (Pluset®, Calier, Spain) administered intramuscularly at 12 h intervals in decreasing doses (50/50, 37.5/37.5, 25/25 and 12.5/12.5 IU), during 4 d. Sponges were removed at the time of the sixth FSH dose, and the animals received 150 µg of D-Cloprostenol (Sincrosin®, Vallée, Brazil) and 400 IU of eCG (Novormon®, Intervet/Schering-Plough, Brasil), intramuscularly. Estrus detection was performed by a teaser, at 12 h intervals, beginning 24 h after the sponge removal, until the female allowed to be mounted. All ewes in estrus were mated three times with the same ram. The embryo collections were performed seven days after the first mating.

Feed and water were withdrawn from the ewes 24 h and 12 h prior to surgery, respectively. Prior to surgery the animals received oxytetracycline (1.0 mg/kg), intramuscularly (Terramicina LA®, Pfizer Saúde Animal, Brasil). The anaesthetic protocol used was constituted of bupivacaine (0.5 mg/kg) (Neocaina®, Cristália, Brasil) and 2% lidocaine (Xylestesin 2%, Cristália, Brasil) (q.s. 7 mL), via epidural, and then 0.05 mg/Kg of 2% xylazine hydrochloride (Rompun®, Bayer, Brasil) associated to 5.0 mg/Kg of ketamine (Cetamin®, Syntec, Brasil), both IV.

Immediately prior to surgery, surgical gloves were put on and washed in 0.9% NaCl solution, so that the glove talcum could be removed (Numanoglu et al., 2007). A retro-umbilical median incision (4.0 cm) was performed on the abdominal wall, cranial to the udder. After the uterus and ovaries were located, the ovaries were exposed in order to determine the number of corpora lutea present in the ovaries. For embryo recovery, a urethral catheter (n=4) was inserted near the ureteric bifurcation and a peripheral venous catheter was inserted near the utero-tubal junction. The utero-tubal junction and the contralateral uterine horn (near the uterine body) were manually occluded in order to prevent loss of the flush solution and embryos. Each uterine horn was flushed with 60 ml of Dulbecco’s phosphate buffer saline (DMPBS flush – Nutricell, Brasil) (through the peripheral venous catheter previously inserted in uterus’ lumen), with the recovery of the flush by the urethral catheter (inserted near the uterine body) directly to Petri dishes. At the end of the flushing, the peripheral venous catheter and the urethral catheter were removed, without any suture being performed in the uterus.

After the end of each embryo recovery, the ewes from G1 (n=4) had their abdominal cavities full-filled with dexamethasone (Dexamethason®, Lab. Teuto, Brasil) (0.25 mg/kg) (Franko et al., 2007) diluted in Ringer with lactate (q.s. 75 mL) (Hidroglou et al., 1990). In G2 ewes, vitamin E (Vitamin E®, Labovet, Brazil) (100 mg/kg) (Toutain et al., 1992) diluted in olive oil (Aceite de Oliva, Carbonell, Spain) (q.s. 75 mL) was infused. Both solutions were prepared immediately before use. In both groups, the solution infused promoted hydrofloblation of the internal organs (Henderson, 1996). Immediately after the end of surgery, the ewes received a single dose of flunixin meglumine (Injectable Flunixin, Chemitec®, Brasil) (1.1 mg/kg/IM), and luteolysis was induced with 150 µg D-Cloprostenol, intramuscularly (Sincrosin®, Vallée, Brasil). Recovered embryos were examined under stereo-microscope (Nikon, Japan), at 40 x magnification (Stringfellow e Seidel, 1999).
Each ewe was submitted to three evaluations for abdominal adhesions scoring, at moments: A1 - second embryo recovery; A2 - third embryo recovery; and A3 – at necropsy. The qualitative and quantitative scoring system assessed amount, extension, tenacity, vascularization and localization (Oliveira et al., 2001), with modifications (Table 1).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Description</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quantity</strong></td>
<td>Adhesions manually separable</td>
<td>1 point</td>
</tr>
<tr>
<td></td>
<td>Adhesions smaller than 1.0 cm</td>
<td>1 point</td>
</tr>
<tr>
<td></td>
<td>Adhesions between 1.0 and 2.0 cm</td>
<td>2 points</td>
</tr>
<tr>
<td></td>
<td>Adhesions bigger than 2.0 cm</td>
<td>3 points</td>
</tr>
<tr>
<td><strong>Extension</strong></td>
<td>Type I – Thin, filmy adhesions</td>
<td>1 point</td>
</tr>
<tr>
<td></td>
<td>Type II - Adhesions separated from tissue without dissection</td>
<td>2 points</td>
</tr>
<tr>
<td></td>
<td>Type III - Adhesions separated from tissue with dissection</td>
<td>3 points</td>
</tr>
<tr>
<td><strong>Tenacity</strong></td>
<td>Absence of blood vessels visible with the naked eye</td>
<td>0 point</td>
</tr>
<tr>
<td></td>
<td>Presence of neoformed vessels</td>
<td>1 point</td>
</tr>
<tr>
<td><strong>Vascularization</strong></td>
<td>Adhesions between the surgical wound and peritoneal fat</td>
<td>1 point</td>
</tr>
<tr>
<td></td>
<td>Adhesions between omentum and surgical wound</td>
<td>2 points</td>
</tr>
<tr>
<td></td>
<td>Adhesions between a visceral segment (except uterus and ovaries) and peritoneum</td>
<td>3 points</td>
</tr>
<tr>
<td><strong>Localization</strong></td>
<td>Adhesions between omentum or peritoneal fat and uterus and/or ovaries</td>
<td>4 points</td>
</tr>
<tr>
<td></td>
<td>Adhesions between uterine horns and ovaries</td>
<td>5 points</td>
</tr>
<tr>
<td><strong>Level of uterine exteriorization</strong></td>
<td>Easy exteriorization</td>
<td>0 point</td>
</tr>
<tr>
<td></td>
<td>Moderate exteriorization</td>
<td>1 point</td>
</tr>
<tr>
<td></td>
<td>Impossible exteriorization</td>
<td>2 points</td>
</tr>
</tbody>
</table>

* Modified from Oliveira et al. (2001).

The levels of uterine exteriorization were classified as: easy exteriorization – 0 point; moderate exteriorization – 1 point; impossible exteriorization – 2 points. The final total score was the sum of the adhesions points and the level of uterine exteriorization.

Plasma and blood serum samples were collected on days -1, 3, 6 and 15 (day 0 = embryo recovery) for mucoprotein (Winzler, 1955), C-reactive protein (Ribeiro, 1997) and fibrinogen assay (Foster et al., 1959).

A descriptive analysis of the macroscopic findings was performed for the variables amount, extension, tenacity, vascularization, localization of the adhesions and level of uterine exteriorization. The Pearson correlation test was performed between the variables quantity, extension and location of the adhesions. The Spearman correlation test was performed for the vascularization, tenacity and level of uterine exteriorization level variables. In order to evaluate the embryo recovery rates, a Chi-square test was performed. The correlation between the uterine exteriorization level and the embryo recovery rate was evaluated through the Pearson test. The acute phase proteins were evaluated through the Student’s t-test and the Tukey test. All statistical analysis were performed using the SAEG® computer software (SAEG 9.1, Viçosa, Brazil). The confidence level of \( P < 0.05 \) was considered significantly different.

**Results and discussion**

In G1 ewes the adhesions were visually thinner, with minimal tenacity and vascularization, and in lower amount (Fig. 1, A-B) when compared to those in G2. This finding was in agreement to the results described by Kucukozkan et al. (2004), with decrease of adhesions after anastomosis of uterine horns in rabbits with the use of solutions with dexamethasone for abdominal infusion. The decrease in adhesions observed in G1 was probably due to the abdominal solution infused, as reported by Elkelani et al. (2002) the Ringer with lactate decreases the adhesiogenic activity, protecting the damaged cells from hypoxia. Besides this, dexamethasone reduces the initial inflammatory response, preventing the fibrin exsudate, as described by Attard e MacLean (2007). Another important factor that may have contributed to the beneficial effect of dexamethasone was the proper dose for the species of 0.25 mg/kg (Numanoglu et al., 2007), since the corticosteroids, when used in proper doses and concentrations, can reduce intraperitoneal adhesions (Kucukozkan et al., 2004).

Conversely, in the G2 animals, there was extensive adhesion formation (Fig. 1, C-D). This result differs from those described by Corrales et al. (2008), who demonstrated that the intraperitoneal use of vitamin E diluted in olive oil was efficient in the adhesion prevention in rats submitted to abdominal surgical procedures. Possibly, the incapacity of this solution in inhibiting the adhesion formation in ewes, unlike what was observed in rats, is due to the fact that the absorption, transport and distribution of vitamin E are different among species. According to Lodge et al. (2004), the action of this vitamin is influenced by the animal’s diet,
biochemistry and genetic factors inherent to the species.

In all animals studied there were adhesions formation only between the omentum and the surgical wound, or between the uterus, ovaries and uterine tubes, differing from Andrioli et al. (1999), in goats. The main factors that must have contributed to the restriction of the adhesions to the reproductive system and abdominal wall was the careful handling of the tissues and the removal of the talcum from the surgical gloves after flushing them with 0.9% NaCl solution prior to the surgeries (Numanoglu et al., 2007), besides the minimal abdominal incision performed for the celiotomy. Also, the infusion of 75 mL of the solutions into the abdominal cavity promoted a hydrofloation effect, and consequently, the physical isolation of the potential adhesiogenic focuses, thus preventing adhesions between the organs (Liakakos et al., 2001).

Positive correlations were observed in G1 and G2 as to the amount and location (r = 0.67 and r = 0.98), location and tenacity (r = 0.83 and r = 0.98), location and vascularization (r = 0.78 and r = 0.88), location and tenacity and vascularization (r = 0.67 and r = 0.71), amount and tenacity (r = 0.62 and r = 0.72), amount and vascularization (r = 0.56 and r = 0.63), extension and tenacity (r = 0.72 and r = 0.70) and between tenacity and vascularization (r = 0.79 and r = 0.65) of the adhesions, respectively. However, the values found in G2, when compared to G1, suggest that the use of solution of vitamin E diluted in olive oil and adhesions formation are highly correlated. The positive correlation of all variables may have occurred due to the fact that the peritoneal adhesions formation occurs mainly due to serosa trauma of the abdominal viscera, which triggers an inflammatory response that leads to the formation of fibrin matrix. It is then replaced by a collagen matrix and elastin (fibrosis), which promotes an increase in tenacity and activates neovascularization in the trauma site. Besides, the local of the adhesions formation is generally related to the place and extension of tissue injury, as the trauma site requires greater vascularization so that the repair of the lesion may occur (Ceciliani et al., 2012).

In G1, there was a positive and significant correlation between the amount of adhesions and the uterine exteriorization level (r = 0.34), reflecting the possibility of uterine exteriorization in all evaluations (A1, A2 and A3). Conversely, in G2 ewes, uterine exteriorization was not possible in 25% of the animals in the second embryo recovery (A1), and in 75% of the animals in the third recovery and at necropsies (A2 and A3, respectively). Uterine exteriorization in all moments in G1 may have resulted from the protective effect of the...
intrabdominal solution infused, which provided a minimal adhesion formation and consequently, an easier uterine exteriorization. A similar result was reported by Cordeiro et al. (2003) after two consecutive embryo surgical recoveries in ewes, washing the genital tract with heparinized saline solution during the trans-operative period.

The highly negative correlation \( r = -0.99 \) between the uterine exteriorization level and the embryo recovery rate in G1, demonstrated that the easy exteriorization of the uterus allows for better handling (Brebion et al., 1992). Also, the use of this solution in ewes participating in ET programs would allow for a minimum of four consecutive embryo surgical recoveries, maximizing the in vivo recovery of embryos and optimizing the use of the donors.

The fibrinogen values (Table 2) did not differ statistically among the groups and the observation days (D-1, D3, D6 and D15). However, 50% of the animals in G1 presented high, but physiological values of fibrinogen for the species (Kramer, 2000) on day 6 of post-operative period, possibly related to the larger incision in the celiotomy, since it was necessary to expand the surgical incision in some animals in order to facilitate the localization of the uterus. However, even with these fibrinogen values, suggesting an inflammatory process, no increase in the adhesion formation in G1 animals was observed.

The values of mucoprotein (Table 2) did not differ statistically among the groups and the observation days, with values below 10 mg/dl, within the range of normality for the species (Eckersall et al., 2007). This finding, especially on day 3 (three days after the surgery), was possibly due to the use of flunixin meglumine, with a half-life of 24 h (Guibault et al., 1987), used in a parenteral manner immediately after surgery, which controlled the inflammatory process, inhibiting the synthesis of this protein, and also the intrabdominal use of dexamethasone, which has a half-life of 36-72 h (Brunton et al., 2006). Thus, we speculate that the parenteral use of flunixin meglumine for 3 consecutive days, instead of a single administration, associated to the use of the abdominal solution used, would be even more effective in controlling the inflammatory process, and may be indicated for ewes in ET programs. Thus, the prevention of adhesion formation must be multifactorial, combining for example, the use of systemic drugs and intraperitoneal mechanical barriers (Kaya et al., 2014).

C-reactive protein concentrations lower than 6 mg/l were detected in all moments evaluated in both groups, and therefore, also within the values of normality for ewes (Kaya et al., 2014). However, as described by Morimatsu et al. (1991), the C-reactive protein may not be an acute phase inflammatory protein in ruminants, a fact that may justify the negative values found for this protein in this study.

Embryo recovery rates of 40.0%, 83.3% and 50.0% were obtained in G1 (M1, M2 and M3, respectively), and of 44.4%, 37.5%, and 0.0% in G2 (M1, M2 and M3, respectively), without a significant variation between moments for each group. However, only in M2 there was a variation between groups \( P < 0.04 \). In the third superovulation treatment, 50% of the G1 ewes did not superovulate (Cordeiro et al., 2003), and therefore a third embryo recovery was not performed in such animals, a fact that was also described by Cognie (1999), characterizing one of the obstacles of the ET technique in sheep. The third embryo recovery (M3) was also not possible in 100% of the G2 animals, due to the impossibility of uterine exteriorization because of the presence of adhesions, similar to what was described by Andrioli et al. (1999) in goats.

The mean embryo recovery rates in G1 and G2 (57.7% and 40.1%, respectively) were lower than those described by Cordeiro et al. (2003) and Simonetta et al. (2008) with the same species. This may have occurred due to the difference between the technique used in this study and the one used by the aforementioned authors\(^{a}\), who performed the uterine flushing from the uterine bifurcation to the utero-tubal junction. Another possibility was the manual occlusion of the utero-tubal junction and the contralateral horn during flushing, performed in this study, which may have allowed for the loose of small quantities of PBS and embryos, and thus negatively influencing recovery rates. Although a minimally invasive surgical technique was used, with minimal abdominal incision and without hysterotomy, Table 2 - Evaluation of fibrinogen (mg/dl) and mucoprotein (mg/dl) between ewes from G1 and G2, at moments D-1, D3, D6 and D15 (D0 = embryo collection).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Group</th>
<th>D-1</th>
<th>D3</th>
<th>D6</th>
<th>D15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>G1</td>
<td>283.3±183.5(^{aA})</td>
<td>333.3±141.4(^{aA})</td>
<td>422.2±299.1(^{bA})</td>
<td>271.4±125.4(^{bA})</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>216.7±132.9(^{bA})</td>
<td>325.0±183.2(^{bA})</td>
<td>337.5±213.4(^{bA})</td>
<td>175.0±116.5(^{bA})</td>
</tr>
<tr>
<td>Mucoprotein</td>
<td>G1</td>
<td>7.1±1.6(^{aA})</td>
<td>8.9±4.6(^{aA})</td>
<td>6.5±2.3(^{aA})</td>
<td>7.3±2.4(^{aA})</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>5.6±2.1(^{bA})</td>
<td>6.1±3.0(^{bA})</td>
<td>6.9±3.1(^{bA})</td>
<td>6.2±2.1(^{bA})</td>
</tr>
</tbody>
</table>

Numbers followed by the same letter (superscript vertically and lowercase horizontally) are not different \( P > 0.05 \).
thus avoiding uterine sutures, whose threads used in the suture might act as a foreign body and consequently become an additional adhesiogenic factor, the low embryo recovery rates reflected a uterine flushing which was not effective.

In conclusion, this study suggests that dexamethasone (0.25 mg/kg) diluted in Ringer with lactate (q.s. 75 mL) prevents adhesions formation in ewes submitted to successive surgical embryo collection.

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Conflict of Interest: The authors declare no conflict of interest.