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In order to study the effect of estradiol-17ß (E2) on estrogen (E) and progesterone (P) receptors expression in oviduct and cervix of lambs, their respective transcripts (ERa mRNA and PR mRNA) were determined by solution hybridization and the receptor proteins (ER and PR) by binding assays after E2 treatments. Lambs (n=4 in each group) were not treated or treated with one, two or three im injections of E2 (1 µg/kg) at 24 h of interval. Tissues were obtained 12 h or 24 h after the last E2 injection. Estradiol treatments increased ERa mRNA and PR mRNA concentrations in an organ-dependent manner: transitory in the oviduct while maintained in the cervix. The E2 effect on the oviductal and cervical ER and PR concentrations were biphasic, with an initial reduction of receptors content that was followed by restoration. The ER restoration in oviduct was earlier than in the cervix. In summary, this study shows that E2 treatments may exert an inductive effect in ERa mRNA and PR mRNA levels and a biphasic effect in ER and PR concentrations in oviduct and cervix of immature ewe. These E2 effects varied in timing and strength depending on the organ of the reproductive tract.

Differential estradiol effects on estrogen and progesterone receptors expression in the oviduct and cervix of immature ewes

Running title: oviductal and cervical steroid receptors of lamb ewes.

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Abstract

In order to study the effect of estradiol-17ß (E2) on estrogen (E) and progesterone (P) receptors expression in oviduct and cervix of lambs, their respective transcripts (ERα mRNA and PR mRNA) were determined by solution hybridization and the receptor proteins (ER and PR) by binding assays after E2

treatments. Lambs (n=4 in each group) were not treated or treated with one, two or three im injections of E2 (1 μg/kg) at 24 h of interval. Tissues were obtained 12 h or 24 h after the last E2 injection. Estradiol treatments increased ERα mRNA and PR mRNA concentrations in an organ-dependent manner: transitory in the oviduct while maintained in the cervix. The E2 effect on the oviductal and cervical ER and PR concentrations were biphasic, with an initial reduction of receptors content that was followed by restoration. The ER restoration in oviduct was earlier than in the cervix. In summary, this study shows that E2 treatments may exert an inductive effect in ERα mRNA and PR mRNA levels and a biphasic effect in ER and PR concentrations in oviduct and cervix of immature ewe. These E2 effects varied in timing and strength depending on the organ of the reproductive tract.

Keywords: cervix; oviduct; lamb; steroid; receptor

1. Introduction

Estrogens (E) and progesterone (P) have deep effects on the growth and the differentiation of the reproductive tract in mammals. The ovarian steroids actions are mainly dependent on the circulating hormone level and on the specific receptor concentration in the target tissues (1). The estrogen and progesterone receptors (ER and PR respectively) are nuclear proteins that act as ligand-dependent transcription factors regulating gene expression (2).

The presence of ER and PR during early development in oviduct, uterus and cervix has been mainly described in rodents (3-6). Some controversy exists regarding the ontogeny and biological activity of the steroid receptors in the reproductive tract. During mouse development, epithelial ER became detectable in the oviduct and cervix at an earlier or at the same age than in the uterus (3,5). It has been suggested that the two types of ER (ERα and ERβ), play an important role in the female reproductive tract development and differentiation (6). However, uterine ERα expression was detected during fetal and postnatal ewe development (7), while the detection of the uterine ERβ expression differ between studies (8, 9). In ewe lambs, both ER and PR transcripts (ERα mRNA and PR mRNA, respectively) and receptors protein contents were higher in the uterus than in the oviduct and cervix (10). These differences in steroid receptors expression along the reproductive tract may determine a differential sensitivity and responsiveness to steroid hormones at an specific stage of development.

Indeed, administration of estradiol-17ß (E2) to prepubertal ewes decreased ER and PR concentrations in uterus, but had no effect in oviduct and cervix (10). On the other hand, the E2 treatment affected only ERα and PR mRNA levels in the cervix (10). A later study performed in uterus revealed that the E2-dependent ER and PR down-regulation was early and transitory, and was followed by synthesis of new receptors (11).

Since E2 regulation of sex steroid receptors expression seems to vary along the

reproductive tract, and E2 effects in oviduct and cervix were confusing, the aim of this work was to study the temporary effects of the estradiol-17ß on ERα and PR transcript and binding proteins levels expression in the oviduct and cervix of prepubertal ewes.

2. Materials and methods

2.1. Animals and treatments

Three-month-old female Corriedale lambs (n=20) (body weight, mean ± SEM: 17.4 ± 0.6 kg) born during the non-breeding season were used. Animal experimentation was performed in compliance with regulations set by the Veterinary Faculty, University of Uruguay. During the experiment, lambs were stayed under natural environmental conditions and were suckling freely. Animals were randomly assigned to five groups, (n=4 in each group). Lambs were not treated (Group 0 h, control) or treated with one (Groups 12 h and 24 h), two (Group 48 h), or three (Group 72 h) im injections of E2 (1 µg/kg, Sigma, St. Louis, MO, USA) in a corn oil vehicle at intervals of 24 h. Animals were painlessly killed by exsanguination 0 h (Group 0 h), 12 h (Group 12 h), 24 h (Group 24 h), 48 h (Group 48 h) or 72 h (Group 72 h) after the first injection. At slaughter, the reproductive tract was dissected at 4 °C and weighed. Both right and left oviduct and the half cranial portion of the cervix were selected for receptor and mRNA determinations. The tissues were frozen in liquid nitrogen and stored

at -80 °C until assayed.

2.2. Hybridization analysis of mRNA

The method used has essentially been described before (11). The hybridization probes used for ERα mRNA and PR mRNA determinations were derived from plasmids containing 360 and 314 bp cDNAs from the ovine ERα and PR, respectively, generously supplied by Dr. N. Ing, Texas A & M University, TX, USA (12). Total nucleic acids (TNA) were obtained by digesting homogenized tissues (200-250 mg) with proteinase K in a SDS-containing buffer, followed by subsequent extraction with phenol-chloroform. The concentration of DNA in the TNA samples was measured fluorometrically at the wavelength 458 nm with Hoechst Dye 33258 (11). The ^{35}S-UTP-labeled cRNA was hybridized overnight at 70^{o}C to TNA samples. The hybridizations were performed at two different concentrations in 40 µl of hybridization formamide-buffer, and samples were then treated with RNase to digest unhybridized RNA for 45 min at 37 ^{o}C. Labeled hybrids protected from RNase digestion were precipitated with trichloroacetic acid and collected on filters, radioactivity was determined in a liquid scintillation counter. The receptor mRNA concentration is expressed as atomol in relation to DNA content (amol/µg DNA).

2.3. Steroid receptors by binding assay

Ligand-binding assays for ER and PR determinations were performed in soluble fractions of oviduct and cervix as described previously (13,14). Steroid receptors are situated predominantly in the nucleus of intact cells and their presence in the soluble fractions is an artifact after cellular disruption during homogenization of the tissues (15,16).

Briefly, the frozen tissues (300 to 500 mg) were sliced and homogenized in TRIS buffer and the soluble fractions were separated by a first centrifugation at 1,000 x g for 15 min and a second at 40,000 x g for 90 min. All these and subsequent procedures were carried out at 0 to 4°C. The soluble fraction was incubated with 5 to 6 increasing concentrations of (2,4,6,7-^{3}H)-estradiol-17ß 86 Ci/mmol (0.3-15 nM), or ^{3}H-ORG-2058, (16α-ethyl-21-hydroxy-19-nor(6,7-^{3}H)pregn-4en-3,20-dione 40 Ci/mmol (0.5-30 nM) (Amersham International, Buckhinghamshire, England) for 18 h with or without 200-fold molar excess of unlabeled diethylstilbestrol or unlabeled ORG-2058, respectively. The separation of free hormone was by dextran-coated charcoal and radioactivity was measured by liquid scintillation counting. The linear regression test of inverse Scatchard model analysis was used to calculate the dissociation constant (Kd, nM) and the concentration of receptor sites, expressed in fmol/mg of protein (13). Protein concentration in the soluble fractions were determined by the method of Lowry (17), using BSA as the standard. Both ER subtypes recently described (ERα and ERβ) exhibit similar ligand binding properties for estradiol, thus in this assay, it was not possible to differentiate the type of ER measured (18,19).

139

140 *2.4. Statistical analysis*

141

142 Data of ER, PR and mRNAs concentrations were analyzed using the General
143 Linear Model procedure for analysis of variance (Statistical Analysis Systems
144 Institute Inc., 1994) according to a model including treatments (Groups I, II, III, IV
145 and V), organ of the reproductive tract (cervix and oviduct) and the interaction
146 between them. The ER, PR and their respective mRNAs concentrations in the
147 control group were defined as 100% and the other groups were compared to the
148 control group in order to express the effects of the E2 treatments. Data are
149 presented as least-square mean ± standard error of the mean (SEM) for each
150 treatment group.

151

152 **3. Results**

153

154 *3.1. Oviductal and cervical weights*

155

156 Estradiol treatments did not modify the lamb body weight (Table 1), but increased
157 approximately 2-fold the oviductal and cervical weights in relation to the body
158 weight, 12 h after the first E2 injection ($P<0.02$) (Table 1). The relative weight
159 increments were maintained during all the treatments.

160

161 *3.2. Oviductal and cervical content of ERα mRNA, PR mRNA, ER and PR in*

untreated prepubertal lambs.

In the non treated lambs (Group 0 h), there were no differences on ERα mRNA, PR mRNA, ER and PR concentrations between oviduct and cervix. The ERα mRNA concentration were 48.0±4.2 and 32.7±7.9 amol/μg DNA and the PR mRNA concentration were 3.16±0.27 and 1.51±0.37 amol/μg DNA, for oviduct and cervix respectively. The ER concentration in oviduct and cervix were 334±44 and 569±155 fmol/mg protein and the PR concentration were 436±4 and 524±97 fmol/mg protein, respectively.

3.3 ERα mRNA and PR mRNA levels

The concentrations of ERα mRNA and PR mRNA in both oviduct and cervix were affected by E2 treatments ($P<0.001$) and by the interaction between treatment and organ ($P<0.05$). In addition, the ERα mRNA concentration was affected by organ ($P<0.02$). Estradiol treatments increased ERα mRNA and PR mRNA concentrations in both oviduct and cervix (Figure A and C). The maximum increase in ERα mRNA levels were detected earlier in the oviduct (12 h after the first E2 injection, Figure A) than in the cervix (24 h after the first E2 injection, Figure C) and were 2 and 4-fold over controls, respectively. The ERα mRNA increase in the oviduct was transient and returned to the control levels 48 h after the first E2 injection (Figure A), while in the cervix the increase was

maintained until the end of the experiment (Figure C). Maximum PR mRNA concentration in the oviduct (3.5-fold increase) was detected 12 h after the first injection (Figure A), while more than 4-fold increase was seen in the cervix (Figure C). The increase in PR mRNA concentration was transient in the oviduct, returning to control levels 72 h after the first E2 injection (Figure A), but was still maintained in the cervix until 72 h (Figure C).

3.4. ER and PR binding protein

A single, saturable and high affinity-binding site for each hormone was found in all cervical and oviductal samples. The dissociation constants (Kd, nM) for ER and PR were: 0.44±0.23 and 0.82±0.24 for cervix, and 0.46±0.12 and 0.56±0.19 for oviduct, n=20, respectively. The ER and PR Kds values were in the range reported for oviduct, uterus and cervix of the ewe (14,20).

The ER and PR concentrations were affected by E2 treatments in both oviduct and cervix ($P<0.001$). A similar biphasic pattern of ER concentration after E2 treatments was observed in both tissues, characterized by an initial 70 % reduction on ER content 12 h after the first E2 injection (Figure B and D) followed by an increase at 24 and 48 h after the first E2 injection, in oviduct and cervix respectively (Figure B and D). In the same way, the PR concentration pattern after E2 treatment was biphasic in oviduct and cervix. The decrease observed 12 h after the first E2 injection was significant in the cervix while in oviduct a tendency was

found (P=0.09) (Figure B and D). The recovery phase of the PR levels was more evident than in ER levels. At 48 and 72 h after the first E2 injection, a significant 2-fold increase over controls in PR concentration could be seen in both oviduct and cervix.

4. Discussion

In the present work, it was demonstrated that E2 treatment of prepubertal ewe affects ERα and PR transcription in oviduct and cervix in an organ-dependent manner. In addition, a transient E2 down-regulation on the oviductal and cervical ER and PR binding proteins is described.

In the non treated 3 month-old lambs, the oviductal and cervical ER and PR concentrations were 50% lower compared to the uterus of the same animals (11) and a similar finding was reported in 2 month-old lambs (10). In the adult ewe, the information is contradictory. During the ovine postpartum, the ER and PR concentrations in cervix were similar or higher than in uterus (20), but in ovariectomized ewes, ER levels were lower in cervix than in uterus (21).

The E2 administered to prepubertal lambs stimulated the steroid receptors transcription in oviduct and cervix 12 h after the first E2 injection. An interesting finding was the earlier and transitory increments of both ERα mRNA and PR mRNA after the E2 treatments in the oviduct, while in the cervix the increments

were higher and maintained over time. This maintained transcription of both steroid receptors in the cervix after the E2 treatments could explain the deep pathological changes of this organ after the acute or chronic exposure to environmental estrogens-like action compounds (xenoestrogens and phytoestrogens) (9,22).

Similar organ-dependent responses to E2 treatments were described for others E regulated mRNAs (IGF-I and thioredoxin) in oviduct, uterus and cervix of the prepubertal lambs (23). Overall, results suggest that E2 is capable to induce the transcription of steroid receptors and growth factors in an organ-dependent manner along the ovine reproductive tract before ovarian cyclicity begins. This tissue specific response may be due to the existence of a variety cell types in each tissue with different receptors concentration, different stages of reproductive organ development and/or cell specific distribution of different ER subtypes. In the cervix-vagina of adults rats (24), in human cervix (25) and bovine oviduct (26), both ER subtypes (ERα and ERβ) have been described, of which ERα is the predominant. In the ovine, the ERβ existence is not clear. The ERβ was reported in uterus of adult (27) and immature ewes (9), but expression of the ERβ gene could not be detected in the neonatal uterus (8), where the ERα was detected at all the ovine developmental stages examined (7).

Conversely of what was found for the transcripts, E2 treatment produced a biphasic effect in oviductal and cervical ER and PR binding proteins

concentration. These data do not agree with what is reported in the adult ewe: E increases the transcript and the binding protein levels of both receptors, (1,28-30). The initial inhibitory phase could be due to a decrease of the synthesis, an increase of the degradation or an inactivation of the ER and PR binding proteins. Since the ER and PR Kd values did not show significant variations after the E2 treatments, the receptors inactivation by loss of their receptor affinities do not seem to be the mechanism that explains the initial decrease phase of the E2 action (31). The fact that the oviductal and cervical ER and PR concentrations were minimal (12 h after the first E2 injection) when their respective ERα and PR mRNAs were high, suggests that the loss of binding proteins was due to an inhibitory posttranscriptional mechanism decreasing receptor synthesis and/or an E-depending mechanism of receptor degradation (32,33). However, it can be also due to an earlier decrease (before 12 h after the first E2 injection) in the transcription rate, as was reported for uterine ERα mRNA in the rat, 6 h (34) or 8 h (32) after E2 treatments. This downregulation might represent a feedback mechanism to limit the duration of the hormone action on the cell.

The oviductal and cervical induction of ERα and PR transcription suggests that the replenishment phase of both receptor proteins is due to the synthesis of new receptors rather than the recycling of inactivated receptors. The results seem to be contradictory with a previous report in 2 month-old lambs using the same E2 treatments, where it was described an increment in the ERα mRNA and PR mRNA levels in cervix but any effect in oviduct, neither in the cervical and

oviductal ER and PR concentrations (10). However, in that study the samples were taken after three E2 injections and thus, it was not possible to detect earlier modifications of steroid receptors expression.

In conclusion, this study demonstrates that E2 treatment exerts an inductive effect on ERα and PR transcription and a biphasic effect on the ER and PR binding proteins concentrations in oviduct and cervix of immature ewe. The effects of the E2 treatments were different in timing and strength depending on the organ of the reproductive tract.

Acknowledgments

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406 TABLE 1

407

408 Table 1

409 Oviductal and cervical weights in relation to the body weight (mean±SEM) of
410 lambs treated with estradiol-17β (E2) at 24 h of interval for three days (n=4 in
411 each group).

412

Group (h after the first E2 injection, number of injections)	Body weight (bw) (kg)	Oviduct (mg/kg bw)	Cervix (mg/kg bw)
0 h, control	17.9±2.0 a	18.0±2.7 a	76.4±17.4 a
12 h, one injection	16.9±1.4 a	28.3±3.4 b	173.7±24.0 b
24 h, one injection	17.0±1.0 a	26.5±2.4 b	162.2±24.4 b
48 h, two injections	18.1±1.3 a	30.2±0.8 b	151.2±19.2 b
72 h, three injections	17.2±0.9 a	32.5±3.0 b	170.7±23.7 b

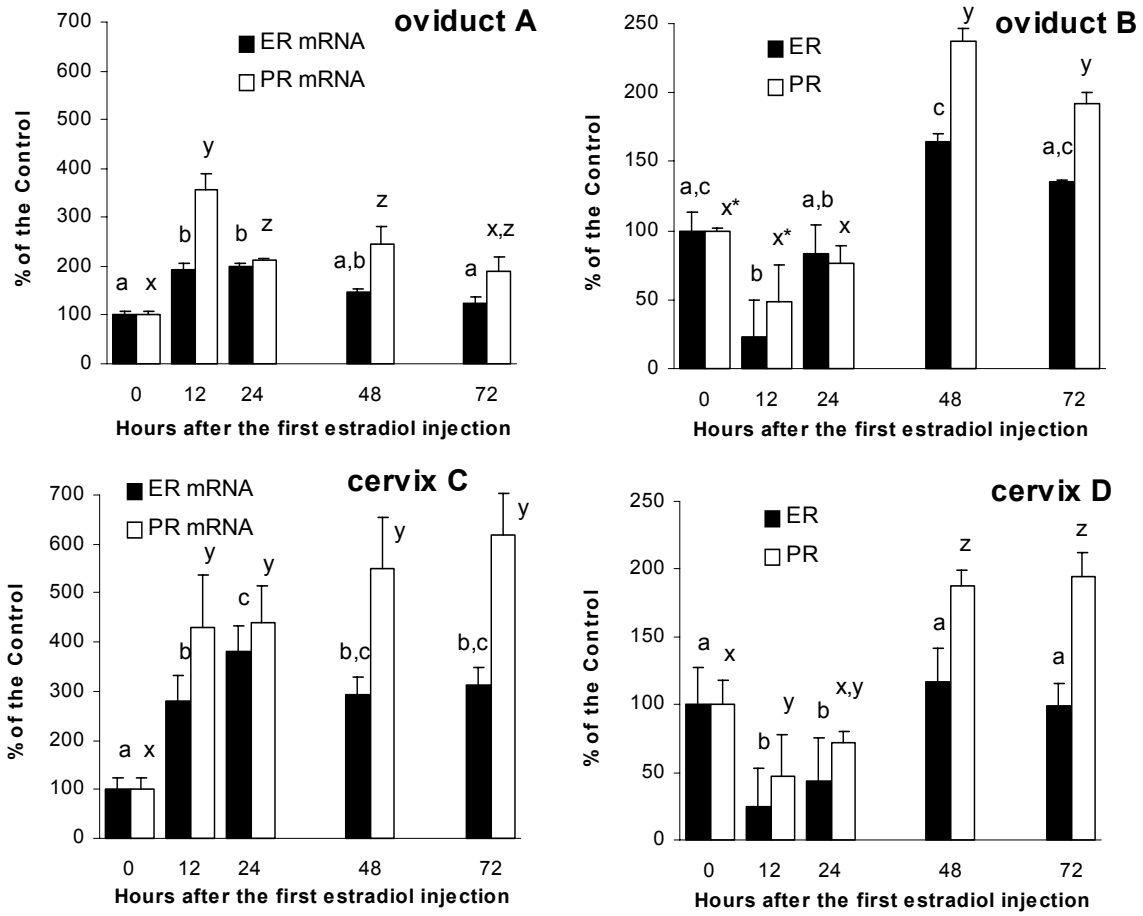
413 Different letters in the same column are significantly different ($P<0.02$).

414

FIGURE CAPTION

Estrogen and progesterone receptors transcript concentrations (ERα mRNA and PR mRNA, left panels) and their respective binding protein levels (ER and PR, right panels) expressed in percentage of the control values in the oviductal (A and B) and cervical (C and D) tissues of control lambs (0 h), and lambs treated with one (12 h, 24 h), two (48 h), or three (72 h) estradiol injections (n=4 in each group). Values (mean±SEM) marked with different letters are significantly different ($P<0.05$) within the same series. Values marked with an asterisk tended to be different ($P=0.09$)

426 FIGURE 1



427

428

Dear Tim D. Braden,

I send you the corrected version of the manuscript entitled "Differential estradiol effects on estrogen and progesterone receptors expression in the oviduct and cervix of immature ewes" (DAE-04-193). The accepted changes and explanations are in blue.

Sincerely,

Marcelo Rodríguez-Piñón
Veterinary Faculty
Montevideo – Uruguay

Reviewer #1:

Introduction

In agreement with the suggestion that the statement "ERβ expression in lamb uterine tissues is undetectable seems to be very risky", we have introduced the expression "differ between studies" with the respective references.

Regarding the article of Morrison et al. (Morrison et al., Dom Anim Endocrinol, 2003: 25, 329-343), although the ERβ was detected by immunohistochemistry in lamb uterus, in the cervix does not determine it, although the title suggest it.

Specific comments:

Page 2, line 25; page 4, line 85: I think that it would be better to write <mu>g/kg instead of 1<mu>g.kg-1 , similarly to mg/kg, Ci/mmol etc. used in the MS. ACCEPTED AND CHANGED

Page 8, line 167: value 436±4 - are you sure that SEM is OK? NOT CHANGED. The SEM is OK, the data of this group were: 436, 425, 446 and 436)

Page 10, line 220: "were similar than in uterus (19)" - I suppose, based on the cited article (19), that the proper version is: were similar or higher than in uterus. By the way, title of the cited article ("19" - p. 16, l. 349-351) is slightly different as compared to the title in proper issue of Theriogenology ("Sex steroid receptors..." but not "Estrogen and progesterone receptors..."). ACCEPTED AND CHANGED

Page 11, line 233, 250, page 12, line 260: I think that instead of E it should be E2. NOT CHANGED. The E symbol was used by Estrogens and the E2 by estradiol-17ß.

Page 12, line 270-275: what is the cause of different results shown in the MS and the cited article (9) written by the same group of authors - an experimental model was very similar but results concerning PR expression in group 72h (the same in both studies) were different. We suppose that the reviewer refers to the E2 effect on the RP binding protein 72

h after the first E2 injection, in both oviduct and cervix tissues. In the present work, the PR binding protein was up-regulate, but in the previous work this effect was not statistically evident, although the RP concentrations were higher than in the control groups. This apparently disagreement could be due to the high coefficients of variation in the previous determinations.

Page 13, line 277: exerts instead of exert. ACCEPTED AND CHANGED

Page 13, line 277-281: In my opinion this summary is too vague. NOT CHANGED.

Fig 1B: Letter "c" in description of bars concerning ER groups 48 and 72h is not necessary (I guess that statistically significant differences exist only between 48 and 72h groups vs. 12h group; if differences concern also 0 and 24h groups - letter "a" is not necessary). ACCEPTED AND CHANGED, differences exist between 0, 48 and 72 vs. 12 h groups; and 24 vs. 48 h groups.

Reviewer #2: Reviewer II

Specific Comments

P 1 - L 4: Is the "running title" necessary? NOT CHANGED, because the DAE guide for authors request it.

P 1 - L 10: Remove the space before "Department of Woman." ACCEPTED AND CHANGED

Abstract

P 1 - L 20: ". on estrogen (E) and progesterone (P)." ACCEPTED AND CHANGED

P 1 - L 23: ". (n=4 in each group)." ACCEPTED AND CHANGED

P 2 - L 36: "Keywords" instead of "Author keywords" ACCEPTED AND CHANGED

Introduction

P 3 - L 55: I suggest that this sentence be changed to ".could not be detected during these periods (or during the same periods)." ACCEPTED AND CHANGED

Materials and Methods

P 4 - L 78: I prefer "Three-month-old female Corriedale lambs (n=20) (body weight.)" ACCEPTED AND CHANGED

P 4 - L 81: "During the experiment, lambs." ACCEPTED AND CHANGED

P 4 - L 83: I prefer ".(n=4 in each group)." ACCEPTED AND CHANGED

P 4 - L 83: ". (Group 0 h, control)." ACCEPTED AND CHANGED

P 4 - L 86: You should say how you killed these animals. ACCEPTED AND CHANGED

P 6 - L 116: "predominantly" instead of "predominately" ACCEPTED AND CHANGED

P 6 - L 117: ". soluble fractions is an artifact." ACCEPTED AND CHANGED

P 6 - L 135: ".thus in this assay, it was not." ACCEPTED AND CHANGED

Results

P 8 - L 174: I prefer "In addition, the ERa mRNA concentration." ACCEPTED AND CHANGED

P 9 - L 185: I suggest that this sentence be changed to ".the increase in PR mRNA concentration was transient in the oviduct, returning to control levels 72 h after the first E2 injection (Figure A), but was still maintained in the cervix until 72 h (Figure C). ACCEPTED AND CHANGED

P 9 - L 201: ". In the same way, the PR concentration." ACCEPTED AND CHANGED

P 9 - L 203: I suggest that you remove ". while in oviduct a tendency was found (P=0.09)". This tendency is not significant, then it is not necessary. NOT CHANGED, because we are sure that this fact have a biological meaning, just as we defended in the discussion.

P 19 - L 405: Table 1: I suggest that you use "E2" instead of "estradiol-17b" ACCEPTED AND CHANGED

P 20 - L 418: Figure Caption: You should remove the sentence: "Values marked with.(P=0.09) from the figure caption and remove the asterisks from figure B, because it is not statistically significant. NOT CHANGED, see P 9 - L 203 suggestion.

Discussion

P 10 - L 211: "In the present work, it was." ACCEPTED AND CHANGED

P 10 - L 213: "In addition" instead of "Also" ACCEPTED AND CHANGED

P 10 - L 216: I suggest that you change "3 mo-old lambs" for "3 month-old lams". This terminology is used many times throughout the manuscript (P 10 - L 218 and P 12 - L 270). ACCEPTED AND CHANGED

P 10 - L 218: "In the adult ewe, the information is contradictory. During the ovine postpartum, the ER." ACCEPTED AND CHANGED

P 11 - L 238: ".to the existence of a variety." ACCEPTED AND CHANGED

P 11 - L 252: ". could be due to a decrease of the." ACCEPTED AND CHANGED

Conclusion

P 13 - L 277: I prefer: "In conclusion, this study." ACCEPTED AND CHANGED

References

P 17 - L 378: "Progesterone" instead of "pogesterone" ACCEPTED AND CHANGED