Integrated Optical Density Analysis of the Immunohistochemical Expression of the Progesterone Receptor in the Uterine Endometrium of Prepubertal Araucana Sheep

Análisis por Densidad Óptica Integrada de la Expresión Inmunohistoquímica del Receptor de Progesterona en el Endometrio Uterino de Ovejas Prepúberes de Raza Araucana

Ester Loncoñanco[1]; Felipe Navarrete[2]; Nicolás Cuevas[2]; Adriana Vasconcellos[1] & Marco Paredes[2]

SUMMARY: Progesterone receptors are expressed in the reproductive organs of adult sheep, where they regulate morphofunctional and reproductive development. However, various studies have shown the presence of these receptors in the uterus of prepubertal females. It is not clear what role these receptors have at this level of development in uterine tissue. Therefore, it is relevant as a first step in the investigation, to determine the expression and histological distribution of the progesterone receptor in prepubertal sheep in order to determine possible functions at this level of reproductive development. Immunohistochemical analysis allows visualizing the specific presence of a protein in the cellular and histological context, however, the results displayed through digital images are qualitative data and subject to the observer's criteria. In this work, a quantitative analysis method of immunohistochemical expression of the progesterone receptor in ovine endometrium is presented, using digital analysis of images, by means of integrated optical density of digital photographs of histological sections processed with immunohistochemical methods. The results show the possibility of quantitatively evaluating the expression of progesterone receptors in the endometrial stroma and prepubertal endometrial glands by applying the integrated optical density analysis of digital images.

KEY WORDS: Sheep reproductive development; Araucana sheep; Integrated optical density.

INTRODUCTION

Sheep are characterized by their docility, herd instinct and great ability to graze on natural pastures of varying quality. Reproductive management of sheep is essential to obtain different sub-products such as meat, wool and milk. Sheep have the advantage of adapting to varied geographical conditions while maintaining their productive abilities. They are a seasonally polyestrous species favoring short days, in other words the oestrus cycle only occurs at certain times of year, and they only come into heat at those times (Meikle *et al.*, 2004; Decourt & Beltramo, 2018).

Criolla Araucana is a Chilean breed of sheep which is farmed in southern Chile. The morphostructural and productive features of it have recently been characterized (Bravo & Sepúlveda, 2010). It is used as a dual-purpose animal (meat and wool), suited to local climatic conditions and raised mainly by small-scale farmers in the region.

These animals present a shorter anoestrus in their reproductive cycle than other breeds farmed in the Araucanía Region, which is a feature highly valued by farmers (Sepúlveda *et al.*, 2001).

Reproduction is a complex physical phenomenon, in which each animal species and its various breeds present different characteristics (Vasconcellos & Paredes, 2012). The Criolla Araucana breed presents standard prolificity and is highly adapted to the environment. In southern Chile, it presents 9.6-20 % twin births, reproduction ratio of 109-120 % (Sepúlveda *et al.*, 2007).

The oestrus cycle of sheep varies with the breed but in general terms it lasts between 16 and 17 days; heat may last 30 to 40 hours depending on the age, breed and presence of males. The ewes reach puberty at 5 to 10 months.

[1] Animal Biotechnology Research Laboratory. Basic Sciences Department, Universidad de La Frontera, Temuco, Chile.
[2] Biotechnology Center for Reproduction. Universidad de La Frontera, Temuco, Chile.

Oestrus hormones play a fundamental role in development, sexual differentiation, growth, metabolism and reproduction. The notable differences between the sexes are mainly the result of the effects of testosterone, oestrogen and progesterone (Meikle *et al.*). The importance of hormones in the ewes' reproductive apparatus has been discussed in numerous works. Their action on uterine development and the histomorphological changes which they induce during the foetal and neonatal periods, as well as during reproductive phases, have all been described. Striking results have been achieved in some studies, concluding that no significant histological differences are observed in prepubertal ewes, the appearance of their endometrium being similar to that of adult ewes (Vasconcellos *et al.*, 2008).

Wiley *et al.* (1987), in their works on the morphogenesis and cyto differentiation of ewes' uteri, indicate that endocrine factors act during the perinatal and neonatal periods to stimulate glandular development and proliferation. Theglands absent from the foetal uterus begin to form in the superficial region, with a rather tortuous tubular aspect which is accentuated with the passage of time. Cyclical changes, mainly triggered by oestrogens and progesterone, occur in the endometrium during oestrus (Vasconcellos *et al.*, 2008).

The hormones act by locking into their specific receptors, triggering gene expression as well as metabolic and proliferative cell changes (Meikle *et al.*). Thus, the effect of these hormones is related to the expression and distribution of their receptors in the white tissues (Vasconcellos *et al.*, 2009, 2011). Unlike other mammals, sheep's endometria contain physiologically active oestrogen and progesterone receptors from the prepubertal stage (Meikle *et al.*), although the physiological significance of this early expression of receptors in young ewes is not yet known.

Usually, the analysis of the protein immuno-histochemical expression is of qualitative character, which generates errors in the interpretation of the data. In this sense, the evaluation of the immunohistochemical expression using densitometric techniques allows quantitative evaluation of the protein expression by parameters such as transmittance, optical density or fluorescence intensity. The analysis of the average gray value of each object present in digital image, allows obtaining a quantifiable parameter called optical density (OD) and its correlation with a certain area of an image is known as Integrated Optical Density (IOD) (Jafari *et al.*, 2017). It is measured in pixels per area and allows the estimation of the relative amount of the object of interest relative to the background of the image. In terms of immunohistochemical labelling, the use of IOD, allows the evaluation of color differences in one image, pixel per pixel and convert it into numerical values to obtain a quantitative parameter.

In this way, the objective of this work was to evaluate the expression of the progesterone receptor in uterine endometrium of prepubertal sheep by IOD.

MATERIAL AND METHOD

Animals and preparation of biological samples: Six prepubertal Criolla Araucana ewes were kept at the Maquehue Experimental Farm of Universidad de La Frontera (Araucanía Region, Chile) until slaughter with the agreement of the university's Ethics Committee. Samples of uterine horn (endometrium) were used in the study. They were fixed in formaldehyde 4 % buffered at pH 7.4 (maximum 24-48 hours), then stored in paraffin at ambient temperature.

Immunohistochemical analysis: The samples were prepared in slices 5 µm thick, which were mounted on pre-gelatinized slides. To eliminate the paraffin from the slices, they were submerged in xylol and then dehydrated by incubation in a battery of ethanol at decreasing concentrations, and then washed with saline phosphate (1x PBS). The endogenous peroxidase was deactivated by adding H_2O_2 to the surface and incubating in a humidified chamber for 20 minutes at 37 °C. Next the slices were washed twice with PBS for 5 minutes each time and incubated in blocking solution (normal horse serum) at 37 °C for 2 minutes, then washed in PBS three times for 1 minute. They were incubated overnight in progesterone receptor monoclonal antibody (Alpha PR6)Catalog # MA1-411 (Thermo Fisher Scientific, USA), at dilution 4:1000 at 37 °C. After incubation the samples were washed with 1x PBS buffer twice for 5 minutes and VECTASTAIN Elite ABC Kit, Vector®, was applied, incubating the samples, at 37 °C for 30 minutes in the humidified chamber, then washed in 1x PBS buffer twice for 5 minutes. To determine the presence of PR, the samples were placed in development solution (0.01mg DAB+ 100µL DMSO + 900µL of 1x PBS buffer) for 5 minutes. They were washed 1x PBS for 1 minute and stained for nuclear contrast with haematoxylin and finally washed in running water for 2 minutes.

The digital images were taken with an Olympus CX31 microscope connected to a Canon PC1089 digital camera using Mshot microscope digital software (Micro-shot Technology Limited). The photographs were subjected to qualitative and quantitative analysis.

Integrated optical densitometry analysis: Integrated optical densitometry (IOD), analysis was carried out using the Image-Pro Plus 6.0® image processing program (Media Cybernetics, Inc, USA), using IOD measurement profile installed in the program. A total of 3 sectors per digital image were examined in the preparations from the 5 samples of uterine horn for each animal studied. The result presented is the mean optical density of all the measurements.

Statistical analysis. The statistical significance of the differences in the integrated optical density (IOD) values was evaluated with Student's t-test, with a significance level of 95 % (P<0.05), using the GraphPad Prism 5.0 for Windows program (GraphPad Software Inc., USA).

RESULTS AND DISCUSSION

The Figure 1 shows the immunoreactivity in different areas of the endometrium of prepubertal Criolla Araucana ewes. Immunohistochemical PR expression can be clearly observed in nuclei of stromal zones (Figs. 1a,b) and in nuclei of the basal glandular epithelium. No immune reactivity is observed in the superficial epithelium, or in the superficial glandular epithelium (Figs.1c,d). Figure 2 corroborates the information from the IOD analysis, which shows a statistical difference for the zones studied. An intense immunoreaction can be clearly seen in nuclei of basal glandular cells with high values compared to the stroma and the superficial glands where the immunoreaction is less intense.

The genital tract is very sensitive to the action of the sex hormones, which respond through their specific receptors, modifying its structural characteristics (Spencer & Bazer, 1995).
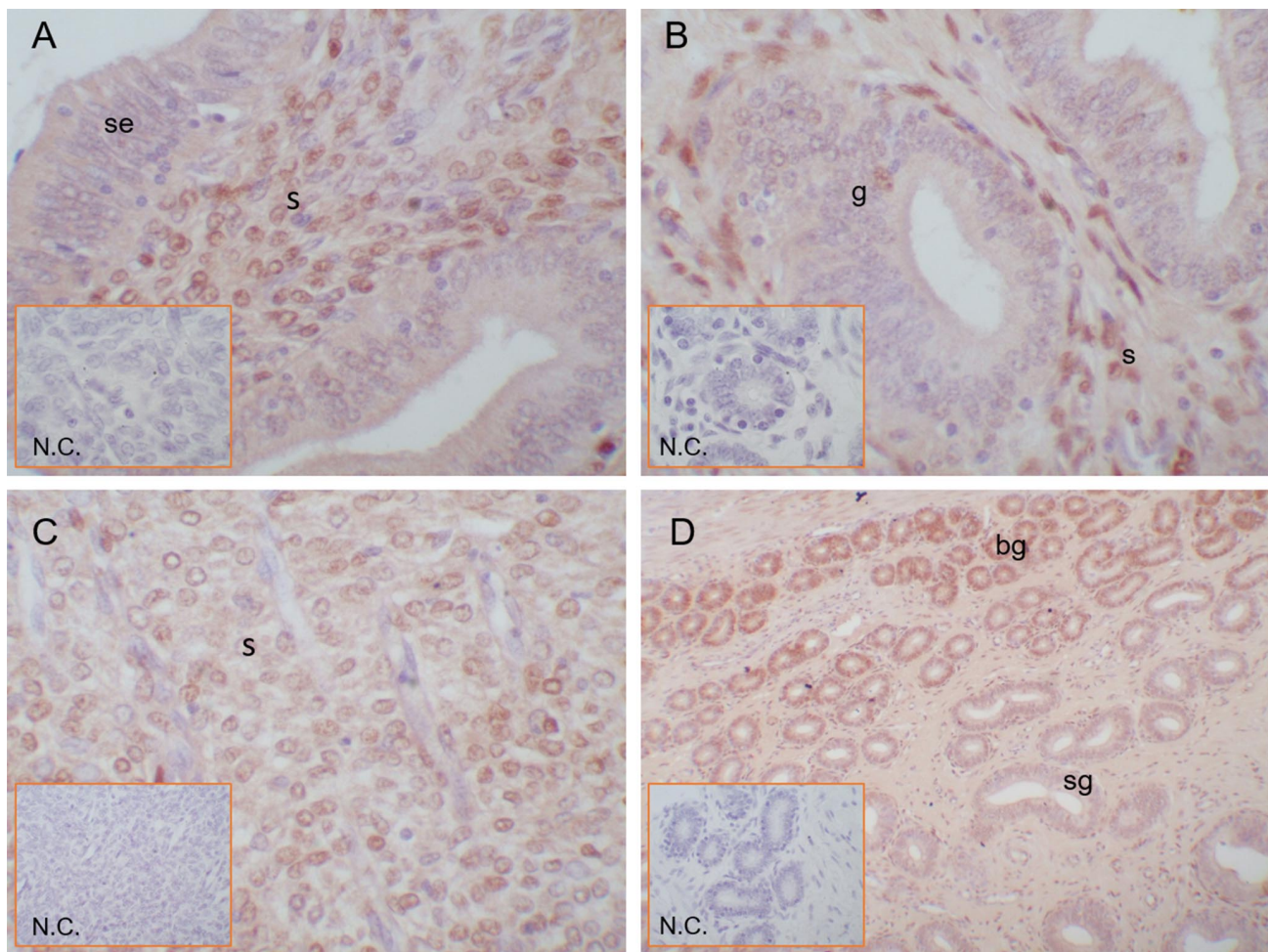

Fig. 1. Immunohistochemistry expression for the progesterone receptor in the uterine endometrium of the Araucana prepubertal sheep. A) Superficial epithelium (se) and stroma (s). 400X. B. Endometrial gland (g) and stroma (e). 400X. C) Endometrial stroma (s) 400x. D) Basal (bg) and superficial glands (sg). 100X. N.C. Negative control.
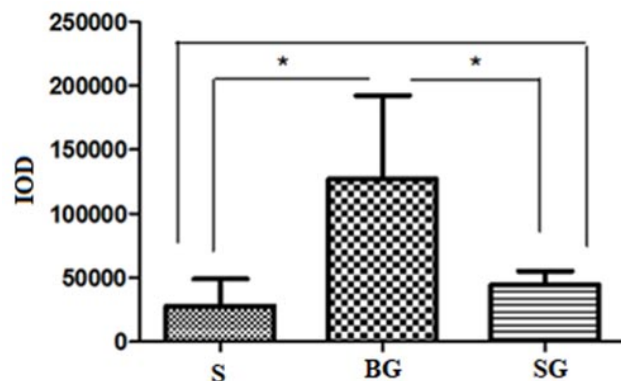
Fig. 2. Quantitative expression level of the progesterone receptor in the uterine endometrium of the Araucana prepubertal sheep. Determined by Integrated Optical Density Analysis (IOD). Stroma (S), basal glands (BG), superficial glands (SG).(*), P value < 0,05.

Integrated optical density provides a quantitative measure of the intensity of expression, since it adds pixels to the area marked in the image, which is more precise and exact than qualitative analysis (Rimm *et al*., 2006). IOD analysis of expression requires one tenth of the time normally used for qualitative analysis; it is also automated, implying less fatigue and fewer errors.

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Various types of analysis, showed that progesterone receptor was present in sheep uterus in oestrus, mainly in the uterine luminal epithelium, stroma, gland and myometrium during the reproductive cycle of adult sheep, however in prepubertal animals it is not clear (Douan *et al*., 2019). Unlike other mammals, such as the dog in which oestrogen receptors and progesterone receptors cannot be shown in the prepubertal stage (Vasconcellos *et al*., 2006), in sheep evidence for the expression of these physiologically active receptors can be shown. This expression in prepubertal and adult animals has been detected in sheep of the Corriedale (Garófalo & Tasende, 1996) and Romey Marsh breeds (Vasconcellos *et al*., 2005). It is known that reproductive response at cell level depends on many factors, including the number and affinity of hormone receptors in the tissues of the reproductive system, the reproductive state of the female and environmental factors (Meikle *et al*.). Vasconcellos *et al*. (2005) propose that the presence of physiologically active receptors in the endometrium of prepubertal females, before they start their ovarian cycles, may be induced and regulated by exogenous hormones in order to increase their efficiency and prevent reproductive dysfunctions. It is also known that oestrogen receptors are present in the reproductive tract of prepubertal ewes. It may therefore be suggested that the presence of receptors in the ovaries of prepubertal ewes may favor a modulated morphofunctional development response by the reproductive system to environmental variations in the photoperiod and temperature which induce the secretion of sex hormones and the start of reproductive activity in these animals. In this way the prepubertal ewe is able to respond immediately and effectively to an increase in oestrogens induced by favorable environmental factors. This may also be associated with the function of the progesterone receptor in the prepubertal stage, since both hormones are directly related with and contribute to the ewe's reproductive characteristics.

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RESUMEN: Los receptores de progesterona se expresan en los órganos reproductores de ovejas adultas, donde regulan el desarrollo morfofuncional y reproductivo. Sin embargo, diversos estudios han demostrado la presencia de estos receptores en útero de hembras prepúberes. No está claro, el papel que estos receptores tienen en este nivel de desarrollo en tejido uterino. Por lo que, es relevante como primer paso en la investigación, determinar la expresión y distribución histológica del receptor de progesterona en ovejas prepúberes con el fin determinar posibles funciones en este nivel de desarrollo reproductivo. El análisis inmunohistoquímico permite visualizar la presencia específica de una proteína en el contexto celular e histológico, sin embargo, los resultados visualizados a través de imágenes digitales, son datos cualitativos y sujeto al criterio del observador. En este trabajo se presenta un método de análisis cuantitativo de expresión inmunohistoquímica del receptor de progesterona en endometrio ovino, utilizando análisis digital de imágenes, mediante densidad óptica integrada de fotografías digitales de cortes histológicos procesados con métodos inmunohistoquímicos. Los resultados muestran la posibilidad de evaluar cuantitativamente la expresión de los receptores de progesterona en el estroma endometrial y las glándulas endometriales prepúberes aplicando el análisis de densidad óptica integrado de imágenes digitales.

PALABRAS CLAVE: Desarrollo reproductivo ovino; Oveja Araucana; Densidad óptica integrada.

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Corresponding autor:
Dr. Marco Paredes H.
Biotechnology Center for Reproduction
Universidad de La Frontera
Temuco
CHILE

E-mail: marco.paredes@ufrontera.cl