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Characterization of paneth cells in alpacas (*Vicugna pacos*, Mammalia, Camelidae)

María Vásquez.[a,*], Boris Lira.[a], José Rodríguez[b], Néstor Falcón[c], Jorge Ocampo[d], Fabián Nishida[e], Claudio Barbeito[f], Carolina Zanuzzi[f]

[a] *Animal Physiology Laboratory, Veterinary Medicine School, National University of San Marcos, (UNMSM), San Borja, Lima, Peru*
[b] *Veterinary Institute of Tropical and Highland Research Experimental Station IVITA-Huancayo, El Mantaro, Jauja, Junín, Peru*
[c] *Veterinary Medicine School, Cayetano Heredia University (UPCH), Lima, Peru*
[d] *National Agrarian Health Service (SENASA), La Molina, Lima, Peru*
[e] *Image Analysis Laboratory (LAI), School of Veterinary Sciences, National University of La Plata (UNLP), La Plata, Buenos Aires, Argentina*
[f] *Histology and Embryology Department, Veterinary Medicine School, National University of La Plata (UNLP), La Plata, Buenos Aires, National Scientific and Technical Research Council (CONICET), Argentina*

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ABSTRACT

Paneth cells are secretory epithelial cells of the innate immune system of the intestine of several mammals, including alpacas. Little is known about the latter; thus, in the present study we described the morphology and histochemical characteristics of Paneth cells in healthy fetuses, and young and adult alpacas. For this purpose, samples of duodenum, jejunum and ileum were taken from 6 fetuses at different days of pregnancy (between days 221–330), 66 offsprings (between 0 and 45-days-old) and 5 adult alpacas (>2-years-old). Samples were fixed in 10% buffered formalin and processed for histological and morphometrical analysis using HE and Masson Trichomicís technique. Immunohistochemistry was used to identify Paneth cells using anti-lysozyme antibody. In addition, the lectinhistochemichal binding-pattern of Paneth cellís granules was evaluated. Lyzozyme was immunohistochemically detected in the granules of Paneth cells from day 283 of pregnancy in all the small intestinal sections of the studied fetuses. In newborn alpacas Paneth cells were initially found in the duodenum, but the following days (days 18–21 after birth) they were also found in the ileum. Their size gradually increased after birth, but then no significant differences were found. In adult alpacas the number was lower than offsprings. We suggest that Paneth cells early differentiate in the small intestine of alpacas, and the increase in their number during the first two weeks of life strongly support their possible involvement in the intestinal defensive functions against the enteric diseases that occur during the lactancy stage.

1. Introduction

Paneth cells (PC) are one of the secretory epithelial cell lineages of the intestine in mammals. They contribute to the innate immune system (Clevers and Bevins, 2013; Bevins and Salzman, 2011), and play a vital role in regulating intestinal microbiota (Zhang and Liu, 2016) by secreting a great number of antimicrobial substances, such as lysozyme (Otto, 1973; Deckx et al., 1967), defensins (Poindexter et al., 2009; Wehkamp et al., 2006; Ghosh et al., 2002; Porter et al., 1997; Ouellette et al., 1989), trypsin (Ghosh et al., 2002; Bohe et al., 1984), immunoglobulin A (Tang et al., 2006), angiogenins (Eckmann, 2005) and secretory phospholipases A2 (Keshav, 2006). PC degranulation occurs by exocytosis and it is triggered by the increase in cytosolic calcium (Satoh et al., 1995), by neuronal cholinergic stimuli, products derived from bacteria, such as, lipopolysaccharide, lipid A, muramyl dipeptide and lipoteichoic acid (Ayabe et al., 2002), and hormones, such as gastrin and cholecystokinin (Porter et al., 2002; Satoh et al., 1989). In addition, the secretory response also occurs after exposure to macromolecules and signals produced by other epithelial or connective tissue cells (Porter et al., 2002) and vary along the intestine (Karlsson et al., 2008). The secretion of these substances regulates the endogenous microbiota composition, cell proliferation and differentiation, and it also protects the stem cell niche. PC may also contribute to digestive and detoxification processes (Bevins, 2005, 2004; Ouellette, 2005; Porter et al., 2002).

* Corresponding author at: Facultad de Medicina Veterinaria,Universidad Nacional Mayor de San Marcos, Av. Circunvalación 2800, San Borja, Lima, Perú.
E-mail address: evasquezc@gmail.com (M. Vásquez.).

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Paneth cells were identified in different species, including human being (Ehrmann et al., 1990; Erlandesen et al., 1974), rat (Rodning et al., 1982; Sundstrom and Heleander, 1980; Behnke and Moe, 1964), mouse (Satoh et al., 1992), guinea pig (Vásquez et al., 2014; Satoh et al., 1990), squirrel (Toth, 1980), hamster, monkey (Satoh et al., 1990), rabbit (Zanuzzi et al., 2008; Abdel-Magied and Taha, 1995), horse (Takehana et al., 1998), sheep (Ergün et al., 2003) and camel (Abdel-Magied and Taha, 1995). In most of the species studied, they are located at the bottom of the Lieberkhün glands of the small intestine where they stay for 18–21 days and then they are renewed (Porter et al., 2002). They are easily recognized by their shape and location, and the staining of their granules using different histological techniques (Klaus, 1969a). In general, PC are pyramidal in shape, with an oval or spherical nucleus, basophilic basal cytoplasm and eosinophilic apical granules (Porter et al., 2002; Ouellette et al., 2000).

Regarding PC in alpacas little and controversial data is known. Montalvo (1966) did not identify PC in the intestine of adult alpacas, whereas Lira et al. (2012) found them from birth up to the first three weeks of life. Since the number, size, shape and location of PC may change under pathological conditions (Liu et al., 2014; Elphick and Mahida, 2005; Kelly et al., 2004; Lewin, 1969; Klaus, 1969a, 1969b), in the present study we described for the first time the morphology and histochemical characteristics of PC in healthy fetuses, and young and adult alpacas. These data may be useful to understand their possible changes under several enteric neonatal diseases caused by *Escherichia coli*, *Clostridium perfringes*, *Eimeria*, rotavirus and coronavirus, the main infectious agents that frequently affect alpacas (Rosadio et al., 2012; Rosadio et al., 2010; Bustinza, 2001).

2. Materials and methods

2.1. Animals

In the present study five adult alpacas (>2-years-old), 66 offsprings (4 newborns –NB-, 12 offsprings between 1–7-days-old, 9 between 8–15-days-old, 10 between 16–21-days-old, 9 between 22–27-days-old, 9 between 28–36-days-old and 13 between 37–45-days-old) were used, and from pregnant alpacas –according to Olivera et al. (2003) the average gestation period of alpacas is 345 ± 5 days- six fetuses from gestational days 221, 230, 293, 317, 325 and 330-one for each time point- were used. Newborn alpacas did not feed colostrum. Adults and offsprings were sacrificed at slaughterhouse, and fetuses were obtained after the sacrifice of their mother at slaughterhouse. All the animals studied were clinically healthy and none of them showed intestinal lesions at the necropsy.

All the management and procedures were carried out according to the 'Guide for the Care and Use of Laboratory Animals' (National Academy Press, 1996, Washington, DC, USA).

2.2. Histological and histochemical techniques

Animals were carefully necropsied and 2 cm long-samples were taken from the middle of the duodenum, jejunum and ileum of each animal, rinsed in PBS, fixed in 10% neutral buffered formalin and embedded in paraffin (A.F.I.P., 1995). Then, section of 5 μm thickness were cut and stained with Hematoxylin and Eosin (HE) (Stevens, 1990), Masson's trichrome (Bradbury and Gordon, (1990)), Alcian blue/periodic acid–Schiff (AB-PAS) and Floxine Tartrazine (A.F.I.P., 1995).

2.3. Immunohistochemistry

Five μm sections were mounted on slides coated with poli-L-lysin- (P 8920, Sigma-Aldrich), and passed through a decreasing graded alcohol scale, and incubated with 3% H_2O_2 in methanol for 30 min at room temperature to inhibit endogenous peroxidase activity. Antigen retrieval was done twice for 5 min using 750 W microwave irradiation in citrate buffer (pH 6.0). Nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) for 30 min. For PC quantification slides were incubated overnight with the primary biotinylated rabbit polyclonal anti-human lysozyme antibody (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) diluted at 1:50. Then, they were rinsed in PBS three times, and incubated with the detection system Horseradish Peroxidase streptavidin SA 704 (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min. Lacrymal gland, which contains lysozyme in other species, (Rennie and Parsons, 1981; Pinard et al., 2003) of an adult alpaca was included as a positive control (Fig. 3B). To identify the proliferating cells of the crypt some slides were incubated with the primary antibody anti-PCNA (anti-proliferation cell nuclear antigen, PC10 clon, Santa Cruz Biotechnology, Inc, Ca, USA), diluted at 1:1000, and then they were rinsed in PBS three times, and incubated with Mouse ABC immunostaining detection system (Santa Cruz Biotechnology, Inc, Ca, USA), according to the manufacturerís instructions. Sections were rinsed in PBS, revealed with liquid 3,3-diaminobenzidine tetrahydrochloride as chromogen (Vector Laboratories, Inc., Burlingame, CA, USA) and counterstained with Harris' hematoxylin. The dark, golden brown DAB hydrogen peroxide reaction product showed the positively stained structures. Negative controls included exposure to horseradish-peroxidase and substrate medium without primary antibody.

2.4. Lectinhistochemistry

The initial steps of the technique were similar to those described for immunohistochemistry. After blocking the nonspecific binding sites with bovine serum albumin (BSA, 1% in PBS) the slides were incubated overnight with the following seven biotinylated lectins (Lectin Kit BK 1000, Vector Laboratories, Inc., Burlingame, CA, USA): Con-A (*Concanavalia ensiformis,* specifically binding α-D-Man and α-D-Glc); DBA (*Dolichus biflorus*, with binding specificity to α-D-GalNAc); SBA (*Glycine maximus*, binding specificity to α-D-GalNAc, β-D-galNAc and α and β-Gal); PNA (*Arachis hypogea*, that specifically binds β-D-Gal and (1–3) GalNAc); RCA-1 (*Ricinus communis*-1, binding specificity β-D-Gal and α-D-Gal); UEA-1 (*Ulex europaeus*-1, binding specificity α-L-Fuc) and WGA (*Triticum vulgaris,* binding specificity α-D GlcNAc and NeuNAc) (Yasui et al., 2006; Goldstein and Hayes, 1978). The optimal lectin concentration was 30 μg/ml in PBS for all lectins, except for PNA (10 μg/ml). The horseradish peroxidase streptavidin SA-5704 (Vector Laboratories, Inc., Burlingame, CA, USA), used as a detection system, was incubated for 30 min. Slides were rinsed three times in PBS for five min each, revealed with liquid 3,3-diaminobenzidine tetrahydrochloride as chromogen (Vector Laboratories, Inc., Burlingame, CA, USA) and counterstained with Harris' hematoxylin. The dark, golden brown DAB hydrogen peroxide reaction product showed the positively stained structures and the results were evaluated according to previous studies (Martínez et al., 2009; Zanuzzi, 2010; Zanuzzi et al., 2008). Negative controls for lectin staining included exposure to horseradish-peroxidase and substrate medium without lectin. Lectin controls were performed by the addition of inhibitory sugars at a final concentration of 0.01 M.