Eurasian Journal
of Veterinary Sciences

www.eurasianjvetsci.org

RESEARCH ARTICLE

Expression profile of cannabinoid receptor 2 in ovine ileum during prenatal development

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Received:22.06.2019, Accepted 26.09.2019
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Prenatal gelişim boyunca koyun ileumunda kannabinoid reseptör 2'nin ekspresyon profili

Eurasian J Vet Sci, 2019, 35, 4, 217-223
DOI: 10.15312/EurasianJVetSci.2019.246

Öz

Amaç: Bu çalışmada prenatal gelişim boyunca koyun ileumundaki kannabinoid reseptör 2 (CB2)'nin ekspresyonunu araştırmayı amaçladık.

Gereç ve Yöntem: Çalışmamızda fötal yaşı 63 ile 147 gün arasında değişen 20 adet koyun fötüsünün ileum örnekleri kullanıldı. CB2 ekspresyonunu göstermek için Streptavidin-Biotin peroksidaz immunohistokimya yöntemini kullandık.

Bulgular: Bağırsak epitelinin çoğunluğu CB2 için zayıf bir pozitif reaksiyon gösterdi. İlginç bir şekilde, hem villus hem de kriptlerin bazı epitel hücrelerinde yoğun intrasitoplazmik boyanma gözlemledik. Ayrıca, submukozal ve miyenterik pleksustaki ganglion hücrelerinde CB2 immun reaktivitesi gözlendi. Üstelik, lamina ve tunica muskularisteki düz kas hücrelerinde de pozitif boyanma tespit ettik. Prenatal dönemin sonuna doğru, histolojik olarak olgun Peyer plaklarına rastladık. Follikül ve dom bölgesini oluşturan immune sistem hücrelerinin çoğunda CB2 boyanması tespit edildi. Ayrıca, folliküle ilişkili epitelde CB2 ile pozitif boyandı.

Öneri: Bu çalışma fötal koyun ileumda CB2 ekspresyonunu tanımlayan ilk çalışmadır. Fizyolojik koşullar altında, CB2 ekspresyonu fetal koyun ileumdaki epitel hücrelerinde, düz kaslarda, enterik nöronlarda ve Peyer plaklarında belirlendi. CB2 kuvvetli boyanan epitel hücrelerinin morfolojisi ve önceki araştırmacıların bulguları göz önüne alındığında, CB2 prenatal dönemde enteroendokrin hücreler için biyolojik bir belirteç olabilir.

Anahtar kelimeler: Koyun, CB2, ileum, prenatal

Abstract

Aim: Here, we aimed to explore cannabinoid receptor 2 (CB2) expression in ovine ileum during prenatal development using immunohistochemistry.

Materials and Methods: We used 20 ileal samples from fetal sheep whose gestational ages range from 63 to 147 days. To visualize CB2 expression in ileum, we applied labeled Streptavidin Biotin method to paraffin sections.

Results: The majority of the intestinal epithelium showed a weak positive reaction for CB2. Interestingly, we observed intense intracytoplasmic staining in some epithelial cells of both intestinal villi and crypts. Moreover, CB2 immunoreaction was observed in ganglion cells in submucosal and myenteric plexus. We also determined positive staining in smooth muscle cells of lamina and tunica muscularis. Towards the end of the prenatal period, we found histologically mature Peyer's patches. CB2 staining was detected in the majority of immune cells forming the follicle and dome region. Follicle associated epithelium also showed CB2 immunoreaction.

Conclusion: The present study is the first to describe CB2 expression in fetal sheep ileum. CB2 expression was determined in epithelial cells, smooth muscles, enteric neurons and Peyer's patches in ileum fetal sheep under physiological conditions. Considering the morphology of epithelial cells with intense CB2 staining and previous findings, CB2 may be a possible marker for enteroendocrine cells during the prenatal period.

Keywords: Sheep, CB2, ileum, prenatal

Introduction

For centuries, plant and herbal-based remedies has been used to treat gastrointestinal (GI) tract disorders (Di Carlo and Izzo 2003, Comar and Kirby 2005). Preparations obtained from the marijuana plant *Cannabis sp*. are salient amongst them (Di Carlo and Izzo 2003). The *Cannabis sativa* has more than 60 aromatic hydrocarbon compounds called cannabinoids, of which delta-9-tetrahydrocannabinol (Δ9-THC) is the most plentiful and is the major psychotropic component (Gaoni and Mechoulam 1964). Cannabis has been used in the treatment of enteric infections, motility disorders, abdominal pain, emesis and inflammatory conditions such as inflammatory bowel disease (Izzo and Sharkey 2010). The understanding of the mechanism by which Cannabis exerts its pharmacological effects has seen remarkable progress following the discovery of specific membrane, G-protein-coupled receptors for Δ9-THC, namely cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) in the early 1990s (De Petrocellis et al. 2004). The identification of CB1 and CB2 has led to demonstration of endogenous cannabinoid ligands, anandamide and 2-arachidonylglycerol. The endocannabinoids, their degradative and biosynthetic enzymes and cannabinoid receptors constitute endocannabinoid system (ECS, Devane et al. 1992, Di Marzo and Fontana 1995, Sugiura et al. 1995).

In addition to the brain, the GI tract is one of the organs that play an important role in ECS. (Hasenoehrl et al. 2016). While CB1 expression is intensely found in the central nervous system, it is also expressed in immune, respiratory, cardiovascular, reproductive, gastrointestinal, integumentary, muscular and skeletal tissues (Matias and Di Marzo 2007, Maccarrone et al. 2015). However, CB2 is primarily seen in myeloid cells and lymphoid tissues but is also found at low levels in non-neuronal and neuronal (for instance, activated microglia) brain cells (Navarro et al. 2016). In GI tract, CB2 is found on enteric neurons and epithelial cells under physiological conditions (Duncan et al. 2008, Wright et al. 2008). Increasing evidence has indicated that the levels of cannabinoid receptors and/or endocannabinoids are changed in the biopsies of patients with intestinal diseases, such as celiac disease, diverticulitis, inflammatory bowel disease, irritable bowel syndrome, and colon cancer, suggesting important function of the ECS in gut pathophysiology (Alhouayek and Muccioli 2012, Izzo and Camilleri 2009, Izzo and Sharkey 2010).

Significant differences between laboratory animals (for example, mouse) and man, including physical size, limit these animals to use as a model of human disease (Bruce et al. 2016). Therefore, the focus is increasingly on larger animals, with pigs and dogs seeing greatest use in addition to primates (Casal and Haskins 2006, Camus et al. 2015). We thought it would be more appropriate to choose sheep as an experimental animal for present study. Cannabinoids perform significant physiological and pathophysiological effects in the GI tract including emesis, appetite regulation, intestinal ion transport, intestinal motility, protection of the gastric mucosa and gastric emptying (Izzo and Sharkey 2010). However, the ontogeny of CB2 has not been characterized in sheep intestine. Considering that CB2 is primarily expressed in the immune system cells, we reason that ileum containing large lymphoid aggregates such as Peyer's patches would be more appropriate for this study. We suggest that detection of CB2 expression in fetal sheep intestine would contribute substantially to the field of gastrointestinal physiology. Therefore, we sought to investigate the CB2 expression in ovine ileum during prenatal development.

Material and Methods

Animal samples and tissue processing

We use 20 fetal sheep ileum at varying gestational ages range from 63 to 147 days (Figure 1). All of the specimens were obtained from Akkaraman breed in slaughterhouses. We applied Richardson's x=2.1(17+y) formula to estimate age of the fetuses (Richardson et al., 1976). According to this formula, where x indicates the age of the fetus in days, and y is the space between the anus and forehead of the fetus in cm. The procedure involving all the animal experiments was conducted according to the guidelines of Ankara University Animal Care and Use Committee.

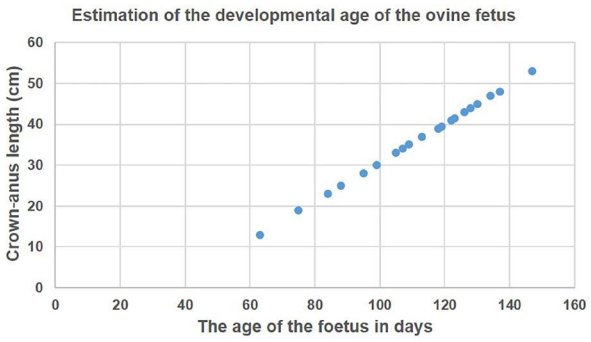


Figure 1. Estimation of the developmental age of the ovine fetus according to Richardson's x=2.1(17+y) formula (Richardson et al. 1976).

As a result of dissection of abdominal organs, we found the cecum. Tissues from the terminal ileum attached to the cecum were removed and fixed in Bouin's solution. After fixation, ileal samples were kept in 70% alcohol at overnight and then passed in grade alcohols (80%, 90% and 100%). After dehydration, ileal tissues were passed through the methyl benzoate and benzol series for clearing and embedded in the paraplast.

Immunohistochemistry

To visualize CB2 expression in ileum, we applied labeled Streptavidin Biotin method to paraffin sections as previously described (Öztop et al. 2019). Briefly, the slides were deparaffinized and rehydrated using routine histological methods. The samples then were transferred into phosphate buffer saline (PBS). For antigen unmasking/epitope recovery, the slides were immersed in 0.1 M, pH 6.0 citrate buffer in a microwave oven (#AP-9003-500, Thermo Fisher Scientific) for 20 min followed by washing with PBS. Then, the sections were incubated with 0.2% Triton X-100 in PBS for 15 min. To quench endogen preoxidase, the slides were immersed in 3% hydrogen peroxide for 20 min. Later, the sections were transferred into PBS for 15 minutes and encircled with a hydrophobic PAP pen. For blocking nonspecific antigenic reactions, the samples were treated with Ultra V Block solution (#TP-125-HL, Thermo Fisher Scientific) for 10 min. After this step, the slides were incubated for 16 h at 4 °C with 1:100 diluted anti-CB2 primary antibodies (#ab45942, Abcam). This step was followed by washing in PBS for 15 min. The sections were incubated with biotinylated secondary antibody (#TP-125-HL, Thermo Fisher Scientific) for 30 min at RT followed by washing in PBS for 15 min. To visualize the resulting signal, we applied AEC (#TA-125-HA, Thermo Fisher Scientific) as a chromogen. We used Gill's II haematoxylin for nuclear staining and the sections then were coverslipped with a hydrophilic mounting medium.

We examined the prenatal period in three different stages (prenatal 60-100 days, prenatal 101-125 days, prenatal 126-150 days) considering the data on development of Peyer's patches as previously reported in our study (Özbek and Bayraktaroğlu, 2019). To validate immunohistochemical staining, the slides were incubated with PBS instead of primary antibodies. Furthermore, slides were treated with nonimmune rabbit IgG (sc-2027, Santa Cruz Bio Inc.) or goat IgG (sc-2028, Santa Cruz Bio Inc.) for isotype control. For the positive control, we used rat ileum section (Figure 2). All procedure involving negative control was carried out in the same way. Immunohistochemical staining was repeated at least 3 times for each sample. Immunohistochemical staining was evaluated under a light microscope (BX51, Olympus, Japan) with digital camera (DP74, Olympus, Japan) and photographed with the aid of olympus cellsens software.

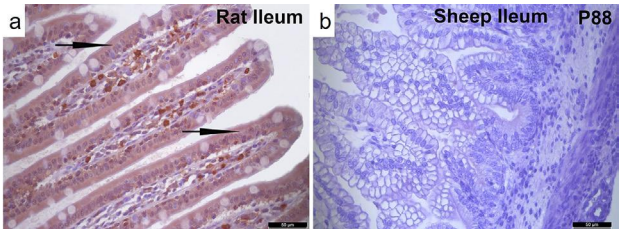


Figure 2. Representative figures showing rat ileum sections as positive control and fetal sheep ileum sections as negative control for immunostaining. Strept-ABC, AEC, Paraffin. CB2 staining were detected in intestinal epithelium of rat ileum (arrows). No immunoreaction was detected in fetal sheep ileum sections that were incubated without the primary antibody. P88; day of 88 prenatal. Bars: 50 µm (a, b).

Results

The rat ileum were used as a positive control in order to detect the specificity of the antibodies. We observed CB2 staining in intestinal epithelial cells in rat ileum (Figure 2a). Fetal sheep ileum was applied for negative control. We did not observe any nonspecific immunoreaction in the negative control sections (Figure 2b). Immunohistochemical results were evaluated and summarized in Table 1.

Prenatal 60-100 days

In the early stages of the fetal period, we observed weak staining in the majority of the intestinal epithelium. These CB2 positive cell are mostly present in basal region of intestinal villi. Interestingly, we determined that some epithelial cells showed intense intracytoplasmic immunoreaction (Figure 3a, b, and c). We also observed positive staining in smooth muscle cells of the tunica muscularis and vessels wall. Ganglion cells of submucosal and myenteric plexus also showed positive immune reaction for CB2. We also observed a positive reaction in some cells in the submucosa (Figure 3d and 4a).

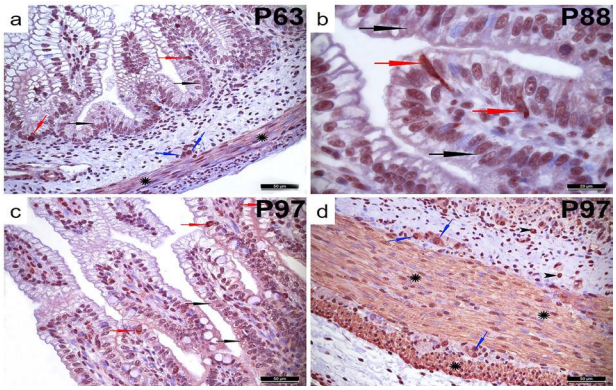


Figure 3. Representative figures showing CB2 in ileum of day 63 prenatal (a; P63), day 88 prenatal (b; P88) and day 97 prenatal (c, d; P97). AEC, Strept-ABC, Paraffin. A weak immunoreaction was observed in the majority of the intestinal epithelial cells (black arrows). Some epithelial cells showed intense intracytoplasmic immunoreaction (red arrows). Positive staining in smooth muscle cells of the tunica muscularis (asterisk) and some cells (black arrow heads) in the submucosa. Ganglion cells (blue arrows) in submucosal and myenteric plexus showed positive immune reaction for CB2. Bars: 50 µm (a, c, d), 20 µm (b).

Prenatal 100-125 days

In this period, intestinal epithelial cells showed a weak reaction for CB2. As in the previous period, we observed intense intracytoplasmic staining in some epithelial cells of intestinal

Table 1. A semiquantitative summary of the results obtained from present study)

		Prenatal 60-100 days n: 6	Prenatal 100-125 days n: 8	Prenatal 125-150 days n:6
Intestinal epithelium	Villus intestinalis	+	+	+
	Crypts	+	+	+
Smooth muscles	Lamina muscularis	+++	+++	+++
	Tunica muscularis	+++	+++	+++
	Vessel walls	+++	+++	+++
Enteric ganglions	Plexus Submucosus	++	++	+
	Plexus myentericus	++	++	+
Peyer's Patches	FAE	+	+	+
	Dome region	++	++	++
	Interfollicular Region	-	++	++
	Follicle	+	++	++

Proportion of CB2 staining cells was scored on a scale of - to +++ (- = no cells with staining; + = weak; ++ = medium; +++= intense). The number of staining cells was evaluated subjectively.

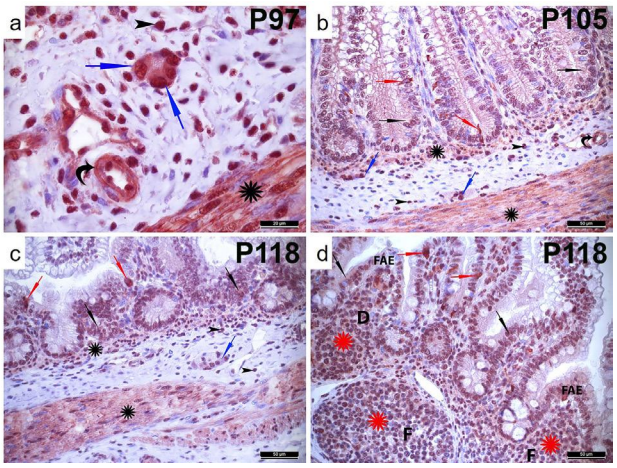


Figure 4. Representative figures showing CB2 in ileum of day 97 prenatal (a; P97), day 105 prenatal (b; P105) and day 118 prenatal (c, d; P118). AEC, Strept-ABC, Paraffin. A weak immunoreaction in the majority of the intestinal epithelial cells (black arrows) and some cells (black arrow heads) in the submucosa. Some epithelial cells showed intense intracytoplasmic immunoreaction (red arrows). Positive staining in smooth muscle cells of the tunica muscularis (asterisk), lamina muscularis (asterisk) and vessel walls (black curved arrows). CB2 expression in ganglion cells (blue arrows) of submucosal and myenteric plexus. CB2 staining in immune cells of primordial Peyer's patches (red asterisk). F: follicle, FAE: follicle associated epithelium, D: dome region. Bars: 20 µm (a), 50 µm (b, c, d).

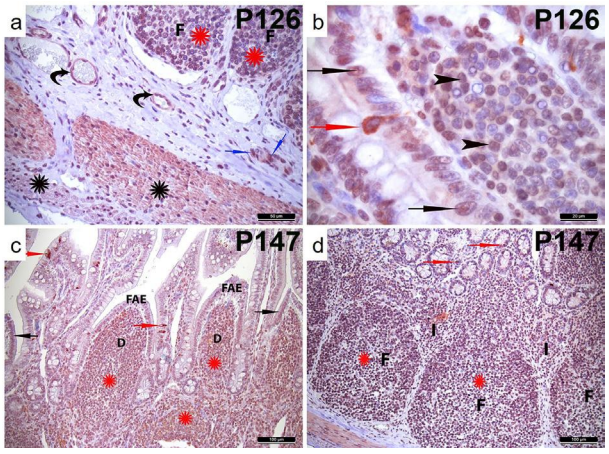


Figure 5. Representative figures showing CB2 in ileum of day 126 prenatal (a, b; P126), day 147 prenatal (c, d; P147). AEC, Strept-ABC, Paraffin. Positive immunostaining in smooth muscle cells of the tunica muscularis (asterisk) and vessel walls (black curved arrows). A weak CB2 staining in the majority of the intestinal epithelial cells (black arrows). Some epithelial cells showed intense intracytoplasmic immunoreaction (red arrows). CB2 staining in ganglion cells (blue arrows) of submucosal and myenteric plexus. CB2 immunoreaction in immune cells of Peyer's patches (red asterisk). F: follicle, FAE: follicle associated epithelium, D: dome region. I: interfollicular area. Bars: 20 µm (a), 50 µm (b), 100 µm (c, d).

villi and crypts (Figure 4b). Ganglion cells in submucosal and myenteric plexus also showed positive immune reaction for CB2 (Figure 4c). During this period, we observed primordial Peyer's patches consisting of primordial follicles and domes. The FAE, which overlies the dome region, also showed positive staining. In addition, we observed an immunostaining in the majority of immune cells in the follicle and dome region (Figure 4d).

Prenatal 125-150 days

Towards the end of the prenatal period, we found histologically mature Peyer's patches. CB2 staining was detected in the majority of cells forming the follicle and dome region. FAE is also positive for CB2 (Figure 5). As in the previous periods, we generally observed a weak immune reaction in most of the intestinal epithelium but an intense immune reaction in some epithelial cells. The intensively stained cells were found in intestinal villi and crypts, but we did not detect these cells in FAE. Moreover, we observed differences in the morphology of these intense stained cells in intestinal villi and crypts. As in the previous periods, we also detected a positive staining in smooth muscle cells of the tunica muscularis and ganglion cells of submucosal and myenteric plexus (Figure 6).

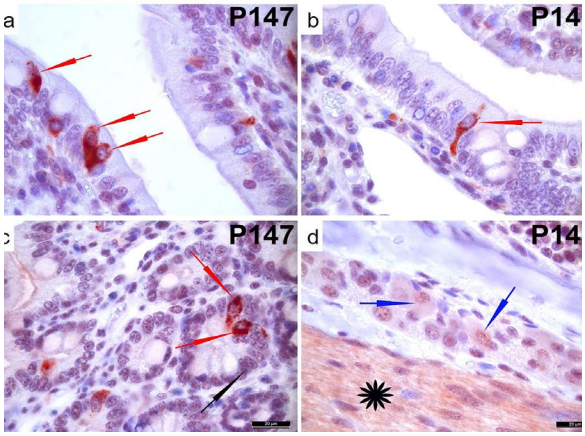


Figure 6. Representative figures showing CB2 in ileum of day 147 prenatal (a, b, c, d; P147). AEC, Strept-ABC, Paraffin. Positive immunostaining in smooth muscle cells of the tunica muscularis (asterisk). Some epithelial cells in intestinal villi (a, b) and cyrpts (c) showed intense immunoreaction (red arrows). CB2 staining in ganglioncells(bluearrows)ofsubmucosalplexus.Bars:20µm(a,b,c,d).

Discussion

Here, we demonstrated CB2 expression in the ileum of ovine fetuses between days 60 and 150 of gestation by using immunohistochemistry. During the fetal period, we observed CB2 expression in intestinal epithelium, ganglion cells, smooth muscle cells, and Peyer's patches.

Cannabinoids have been seen to perform pharmacological effect on epithelial cells. These cells play an important role in host defense against microorganisms in the gastrointestinal lumen, and they are also involved in inflammatory responses. Although epithelial cells prevent absorption of potentially detrimental luminal material as barriers, they also express a variety of pro-inflammatory cytokines (Sturm and Dignass 2008). In patients with inflammatory diseases, including celiac disease, Crohn's disease, ulcerative colitis and diverticulitis, increased cannabinoid receptor expression and/or enhanced endocannabinoid levels have been mostly determined in gastrointestinal specimens (Izzo and Camilleri 2009). Wright et al. (2005) reported that CB2 are either absent or weakly expressed in human intestinal epithelial cells under physiological conditions. However, CB2 expression was upregulated in intestinal bowel disease and was expressed in the apical membranes at ulcerative margins. Moreover, the same researchers observed CB2 expression in the immune system cells such as plasma cell and macrophage in lamina propria (Wright et al. 2005). CB2 expression was also found epithelial cell lines derived from human colorectal tumours, including DLD-1, Caco2 and HT29 (Ihenetu et al. 2003, Ligresti et al. 2003). While CB2 mRNA expression did not found in mucosal samples of rat ileum (Storr et al. 2002), Grill et al. (2019) determined only little CB2 gene expression in colon epithelium. Approximately 1% of the entire intestinal epithelium consists of enteroendocrine cells. Enteroendocrine cells constitute the biggest endocrine organ due to secrete upwards of 20 different types of hormones (Sternini et al. 2008, Gribble and Reimann 2019).These hormones act in concert to regulate multiple important functions including secretion, gastrointestinal motility, appetite, and glucose homeostasis. Enteroendocrine cells also have specific receptors that detect intestinal specimens, and in response, secrete bioactive peptides serving endocrine, paracrine and neural functions (Moran-Ramos et al. 2012). Moss et al. (2012) reported that murine K cell expressed high level mRNA of CB1. Expression of CB2 has, to our knowledge, not previously been reported in ovine gastrointestinal tract in both prenatal and postnatal periods. For the first time, our research showed that weak CB2 expression was observed in health ovine intestinal epithelium during prenatal periods. However, some of the intestinal epithelial cells showed intense CB2 expression. These intensely stained cells were located in both intestinal villi and crypts. We also found that these cells are morphologically heterogeneous. Considering the stained cell morphology and previous findings, we reason that some of these cells are enteroendocrine cells. However, further studies are needed to prove that these cells are enteroendocrine cells.
In both human and animal GI tract, the endocannabinods exert remarkable antipropulsive effects (Pesce et al. 2018). The agonists of cannabinoid receptors influence motility of the isolated intestinal segments in a way that is similar to the neuromodulatory response to prejunctional m-opioid receptor or a2-adrenoceptor activation of cholinergic, postganglionic parasympathetic neurones. Therefore, many cannabinoid receptor agonists (via CB1 activation) have been

demonstrated to block or diminish excitatory transmission, neural acetylcholine release and peristalsis efficiency in isolated intestinal segments (Di Carlo and Izzo 2003). Cannabinoids – via CB1 activation have been seen to decrease electrically-induced contractions in the human colon (Hinds et al., 2006), rat (Storr et al., 2002) or mouse stomach (Mulè et al. 2007), human (Manara et al. 2002) and guinea pig ileum (Abalo et al. 2005). In addition to CB1, CB2 was also involved in the regulation of abnormal intestinal motility. (Wright et al. 2008, Izzo 2007). In intestinal hypermotility induced via lipopolysaccharide (LPS) in rats, the intestinal motility is controlled almost totally through CB2 signalling pathway; hypermotility is normalized via a CB2, but not by a CB1 agonist (Mathison et al. 2004). In vitro, JWH133 that is CB2 agonist did not influence the electrically evoked twitch response of the ileum under basal conditions. However, in the LPS-induced tissues, JWH133 was able to diminish the exaggerated contractile response in a concentration-dependent manner (Duncan et al. 2008). Similarly, JHW015 which is CB2 agonist decreased intestinal motility in the inflamed intestine, but not in healthy mice (Capasso et al. 2008). While cannabinoid receptors have a wide distribution in the GI tract, their expression are mostly seen in the enteric nervous system (Duncan et al. 2008). The expression of both CB1 and CB2 are determined on nerve fibres, nerve terminals and enteric neurons in the enteric nervous system by using immunohistochemistry. CB1 is expressed on nerve fibres through the gut wall, but with the highest intensity in the the submucosal and myenteric plexus (Duncan et al. 2008, Wright et al. 2008). Moreover, CB2 mRNA expression was found in longitudinal muscle with the adherent myenteric plexus in rat ileum (Storr et al. 2002). Consistent with previous findings, we observed CB2 expression in ganglion cells of submucosal and myenteric plexus. Moreover, we determined CB2 immunoreactivity in smooth muscles in the lamina and tunica muscularis. The expression of CB2 in both enteric neurons and smooth muscles suggests that it is involved in GI motility. However, further experimental studies need to investigate the effect of CB2 agonists on GI tract motility in sheep under physiological and pathological conditions.

Essentially, CB2 are expressed predominantly on immune cells such as neutrophils, macrophages, and T and B lymphocytes (Galiègue et al. 1995). As is known, Peyer's patches are lymphoid aggregates in ileum and jejunum. The mature Peyer's patches consists of corona, dome region, germinal centre and interfollicular area. The luminal side of the Peyer's patches are covered with specialized epithelium namely the follicle-associated epithelium (FAE) (Yasuda et al. 2006). The development of Peyer's patches in sheep occurs in prenatal period (Özbek and Bayraktaroğlu 2019). Wright et al. (2005) reported that lymphoid follicles were negative for CB2 in human colon, while we observed CB2 immunostaining in almost all cells in the Peyer's patches. This seems to be due to the species diversity or prenatal period.

Conclusion

In conclusion, the present study is the first to describe CB2 expression in fetal sheep ileum. CB2 expression was determined in epithelial cells, smooth muscles and enteric neurons, Peyer's patches in ileum fetal sheep under physiological conditions. Some epithelial cells showed intense CB2 expression and the morphology of these cells was heterogeneous. Considering the morphology of epithelial cells with intense CB2 staining and previous findings, CB2 may be a possible marker for enteroendocrine cell during the prenatal period.

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