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ORIGINAL ARTICLE

Expression and Localization of Aquaporin 4 and Aquaporin 5 along the Large Intestine of Colostrum-Suckling Buffalo Calves

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Summary

Aquaporins (AQPs) are membrane channel proteins that play a role in regulating water permeability in many tissues. To date, seven isoforms of AQPs have been reported in the gastrointestinal tract in different mammalian species. In contrast, both tissue distribution and expression of AQPs are unknown in the buffalo. The purpose of this study was to investigate the expression of both AQP4 and AQP5 mRNAs and their relative proteins in the large intestinal tracts of buffalo calves after colostrum suckling using reverse transcriptase polymerase chain reaction (RT-PCR), Western blotting and immunohistochemistry. Our results revealed a diversified tissue AQP4 and AQP5 immunolocalization accompanied by their highest expression in the tissues of colostrum-suckling buffalo calves confirmed by Western blotting. In particular, AQP4 was distributed along the endothelium and enterocytes while AQP5 in the endocrine cells. These findings provide direct evidence for AQP4 and AQP5 expression in the large intestine, suggesting that different AQPs collaborate functionally and distinctively in water handling during intestinal development, especially during the first period after delivery.

Introduction

The intestine in newborn mammals is a tract with considerable ability to adapt to extrauterine life. The transfer of molecules and both the absorption and secretion of fluids across the intestinal wall represent important processes related to intestinal physiology. Therefore, the activity of the intestine and its maturation assume the role of a complex system of integrated regulatory mechanisms, fundamental for the adaptive life strategies of the newborn concerning diet (Henning et al., 1994).

Although the development of intestinal transport functions has been extensively studied, less attention has been paid to the regulatory mechanisms of adaptation and maturation of intestinal transport functions during weaning. It is well known, however, that such processes can be modulated through the complex interaction of many factors (nutrients, receptor activities and other luminal constituents) and that the intestine is part of a complex network comprising the central nervous system and the intestinal microbiota, playing a central role in homoeostatic control of the whole body.

In this respect, in recent decades, the discovery of pore channel systems called aquaporins (AQPs) widely distributed along different tissues has yielded insights into such transport mechanisms as well as homoeostasis control. Such proteins are well known for their particular ability to facilitate and regulate passive exchange either to water alone or to water and other small solutes across membranes. Aquaporins belong to a highly conserved family of major intrinsic membrane proteins (MIPs) with molecular masses between 26 and 30 kDa (Zardoya and Villalba, 2001). At present, 13 members of AQP subtypes have been cloned from mammals (Verkman and Mitra, 2000; Matsuzaki et al., 2004; Itoh et al., 2005; Morishita et al., 2005). Functionally, these proteins have been

classified into two subfamilies: aquaporins and aquaglyceroporins. Aquaporins are specialized in the transport of water and include AQPs 0, 1, 2, 4, 5, 6 and 10. Differently, aquaglyceroporins transport both water and small molecules and include AQPs 3, 7, 8 and 9 (Verkman and Mitra, 2000). At least seven AQP subtypes (AQP1, 3, 4, 7, 8 and 9) have been reported to be widely expressed in the gastrointestinal tract (GIT) and play an important role in a number of physiological and pathological processes (Ma and Verkman, 1999; Matsuzaki et al., 2004). However, the specific function of these proteins during developing processes, in particular along the GIT, is still poorly understood.

This applies especially to ruminant species where the intestine performs several activities due to the secretions of substances and/or absorption processes. A paper (De Luca et al., 2014) from our research team confirmed the presence of aquaporin 1 along the intestine of buffalo calves, suggesting an involvement of this protein in osmoregulation in gastrointestinal physiology, especially during the first week after birth in relation to specific maturation of intestinal tissues and colostrum ingestion. Other proteins besides AQP1 could be expressed in the large intestine of buffalo calves as the presence in young animals of various factors able to stimulate tissue differentiation, hence absorptive capacity, has been reported elsewhere (Jacobi and Odle, 2012).

The present study explores the expression and localization of both AQP4 and AQP5 along the large intestine of neonatal buffalo calves after colostrum suckling. This animal species was chosen as it is now widely bred in Italy. Buffalo farms play a key role in the regional economy in some areas of southern Italy for meat, milk and fresh mozzarella cheese production. The main factor that has contributed to the development of buffalo farming in the past decades has been the increase in mozzarella consumption both in Italy and overseas (Borghese, 2013). The expression of AQP4 and AQP5 mRNA and relative proteins were studied by RT (reverse transcriptase) PCR and immunoblotting, and cellular localization was performed by immunohistochemistry.

AQP4 and AQP5 were chosen for their relative distribution along different regions of the GIT. In particular, mammalian AQP4 is expressed in a number of tissues but is not as ubiquitous as AQP1 (Ishibashi et al., 2009). In the digestive tract, AQP4 is localized in the stomach and intestine. Studies on AQP4-knockout mice have demonstrated that AQP4 is involved in fluid absorption mainly in the proximal colon (Wang et al., 2000). In contrast, AQP5 is expressed particularly in the salivary glands (Scocco et al., 2011) as well as the apical membrane of the pyloric gland secretory cells and in duodenal glands in humans and in rats, suggesting its possible involvement in the secretion and exocytosis of granules (Matsuzaki et al., 2003; Collaco et al., 2013).

Materials and Methods

Animals and tissue sample collection

Fifteen healthy neonatal buffalo male calves from a single dairy farm in southern Italy were used in the study. Five calves were sacrificed at birth. The remaining ten animals were fed for 1 day with 3 l of mother colostrum using a needling bottle according to the farm's standard practice.

On the second day, the animals were divided into two groups (five subjects each): the animals in first group continued to be fed with mother colostrum for 1 week; those in the second group were fed with buffalo milk in the same way. An additional group of five adult male buffaloes (15 months old) was used to evaluate potential age-dependent changes. The animals were killed in a public slaughterhouse. All procedures were conducted in accordance with EU Directive 2010/63 on the protection of animals used for scientific purposes.

The abdominal cavity was opened, and the gastrointestinal tracts (GIT) were removed. Tissue samples of the caecum and colon were immediately harvested and processed for fixing (see Immunohistochemistry) or snap frozen in liquid nitrogen (LN) and subsequently stored at −80°C until use for total RNA isolation and protein extracts. All procedures complied with the Italian legislation regarding the use of animals in research.

Immunohistochemistry

Fresh segments of intestinal tissues were fixed by immersion in Bouin's fixative (6–24 h), processed for paraffin embedding in a vacuum and cut at a thickness of 5–7 μm. The avidin–biotin–peroxidase complex (ABC) method was performed using the Vectastain ABC kit (PK-4000 – Vector Laboratories, Burlingame, CA, USA) as described more fully elsewhere (Liguori et al., 2012).

Primary sera were polyclonal goat anti-AQP4 (sc-9988, diluted 1:200, Santa Cruz Biotechnology, CA, USA) and anti-AQP5 (sc-9891, diluted 1:200, Santa Cruz Biotechnology). The secondary antibodies were biotinylated anti-goat IgG (diluted 1:200; Vector Laboratories). The specificity of the immunoreactions was tested by replacing the primary antibody with buffer. No immunoreaction was detected in control tests. Five slides (one slide selected every ten according sequential thickness) for each intestinal tract (caecum and colon) from each animal were independently evaluated by two observers using a Leica DMRA2 microscope (Leica Microsystems, Wetzlar, Germany).

Western immunoblot analysis

Tissue samples were homogenized in 1X RIPA lysis buffer (0.1 mM PBS, 1% Nodinet P-40, 0.1% sodium dodecyl sulphate 0.05% (SDS) deoxycholate, 1 μg/ml leupeptin and 1 μg/ml phenylmethylsulphonyl fluoride (PMSF) using an Ultra-Turrax homogenizer and centrifuged at 16 000 ***g*** for 20 min at 4°C. Aliquots of the supernatant were subjected to SDS/polyacrylamide gel electrophoresis (SDS/PAGE) (12.5% polyacrylamide) under reducing conditions. Proteins were transferred (2 h at RT, 200 V) onto immunoblot nitrocellulose transfer membrane (PROTRAN, BioScience, Dassel, Germany) using a mini-transblot apparatus (Bio-Rad, Hercules, CA, USA). After 1 h blocking with blocking buffer (5% BSA and 0.3% Tween-20 in PBS), the membrane was incubated for 1 h at room temperature (RT), respectively, with goat anti-AQP4 (diluted 1:1000) (Santa Cruz Biotechnology) or goat anti-AQP5 (diluted 1:1000) (Santa Cruz Biotechnology), and then washed three times and incubated for 1 h at RT with peroxidase-conjugated donkey anti-goat IgG (sc-2020 Santa Cruz Biotechnology, diluted 1:2000 in blocking solution). Marker proteins (coloured protein molecular weight markers; Prosieve, Lonza, Rockland, ME, USA) were used to estimate the molecular weight of each band. To monitor loading of gel lanes, the blots were stripped and re-probed using an anti beta-actin monoclonal antibody (JLA20 CP01; Calbiochem, San Diego, CA, USA). Specific bands were visualized by chemiluminescence (Super Signal West Pico Chemiluminescent Substrate; Thermo Scientific Pierce, Rockford, IL, USA), and the image was acquired with the Kodak Gel Logic 1500 imaging system (Celbio, Milan, Italy).

RNA extraction, cDNA synthesis, RT-PCR and sequencing

Samples of intestinal tracts were taken and immediately frozen by immersion in LN (−173°C) for 10 s, and individually homogenized in ice-cold TRI-Reagent (Sigma, St. Louis, MO, USA) using an Ultra-Turrax homogenizer. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in RNAase-free DEPC water. Total RNA was measured with an Eppendorf Biophotometer (Eppendorf AG, Basel, Switzerland). For cDNA synthesis, 1 μg of total RNA was retrotranscribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) and random hexamers as primers. For PCRs, specific primers for buffalo AQP4 and AQP5 were designed from the published gene sequences (*Bos Taurus* aquaporin 4 Genbank accession number NM_001191160) using the Primer Express™ software (PE Applied Biosystems) as described elsewhere (Squillacioti et al., 2012). The sense and anti-sense AQP4 primers used were 5′-GGAGGATTAGCATCGCCAAGT-3′ and 5′-AAAGCTATGGAACCGGTGACA-3; the sense and anti-sense AQP5 primers used were 5′-ATTGGCCTGTCC GTCACACT-3′ and 5′-CCTCGTCCGGCTCATACGT-3 which amplify a 250-bp and 260-bp fragment, respectively.

The PCR cycle conditions were as follows: 94°C (30 s), 60°C (30 s), 72°C (1 min) for 35 cycles; 72°C (5 min). The PCR products of buffalo AQP4 and AQP5 were purified using GFX PCR DNA and Gel Purification Kit (code 28-9034-70; GE Healthcare, Little Chalfont, Buckinghamshire, UK) and sequenced. In order to verify the efficiency of the reverse transcription (RT) and to exclude genomic DNA contamination, a fragment of β-actin cDNA (Genbank accession number NC_007326) was amplified and sequenced with primers designed to span an intron β-actin for 5′-CAG CTC CTC CCT GGA GAA GA-3′. PCR products were electrophoresed on a 1.5% agarose gel and visualized under UV light. A sample without cDNA template was used to verify that the master mix was free of contaminants.

Results

Aquaporin-4 immunohistochemistry

The results of immunohistochemistry of both aquaporins are summarized according to Table 1. At birth, no AQP4-immunoreactivity (IR) was found in the caecum and colon (Fig. 1a,b). After 1 week of colostrum suckling, AQP4-IR was distributed in the endothelium of the vessels, in enterocytes and in enteric neurons of caecum and lymphoid tissue of large intestine (Fig. 1c–e). After 1 week of milk suckling, AQP4-IR was found only in the lymphoid tissue of the caecum and colon (Fig. 1f,g). The density of AQP4-IR was greater in the colon and caecum of animals suckling colostrum than animals suckling milk. In the adults, AQP4-IR was found in the endothelium, enterocytes, enteric neurons, lymphoid tissue and rarely in the endocrine cells (Fig. 1h,i). The density of AQP4-IR in the endothelium and enterocytes was greater in adults than at other ages.

Aquaporin-5 immunohistochemistry

At birth, AQP5-IR was found in the endocrine cells of all tracts of the large intestine (Fig. 2a,b). After 1 week of colostrum suckling, AQP5-IR was distributed in the endothelium, endocrine cells and in lymphoid cells of the caecum and colon (Fig. 2c,d). After 1 week of milk suckling, AQP5-IR was localized in the endothelium,

Table 1. Distribution of AQP4 and AQP5 immunoreactivity in the buffalo large intestine. – undetectable; –/+ rare; + low density; ++ medium density; +++ high density

	Caecum				Colon			
	Birth	colostrum	milk	adult	Birth	colostrum	milk	adult
(a) AQP4-IR								
Endothelium	–	+	–	++	–	+	–	++
Enterocytes	–	+	–	++	–	+	–	++
Enteric neurons	–	–/+	–	+	–	++	–	+
Endocrine cells	–	–/+	–	–/+	–	–/+	–	–/+
Lymphoid tissue	–	+	+	+	–	+	+	+
(b) AQP5-IR								
Endothelium	–	++	+	++	–	++	+	++
Enterocytes	–	–/+	–	+	–	–/+	–	+
Enteric neurons	–	–	–	–	–	–	–	–
Endocrine cells	+	++	+	++	+	++	+	++
Lymphoid tissue	–	+	+	+	–	+	+	+

endocrine cells and in the lymphoid tissue of all the examined intestinal tracts (Fig. 2e,f). The density of AQP5-IR in the endocrine cells was greater in the colostrum-suckling calves than in both milk suckling and at birth. In the adults, AQP5-IR was distributed in the endothelium, endocrine cells, in some enterocytes and in lymphoid tissue (Fig. 2g,h).

Expression of AQP4 using Western blot analysis

Results of Western blot of AQP4 protein samples prepared from large intestine tissues (colon and caecum) of buffalo calves in different conditions (birth, colostrum and milk) and in adult buffaloes probed with a polyclonal AQP4 antibody raised against a peptide mapping at the C-terminus of AQP4 of human origin are summarized in Fig. 3(a). The immunoblot of AQP4 showed a band at 28 kDa and several bands 45–60 kDa probably corresponding to glycosylated forms of AQP4. Results of densitometric analysis of AQP4 expression considering only the unglycosylated form (28 kDa) are represented in Fig. 3(b).

At birth, AQP4 expression was very weak in both examined intestinal tissues. In particular, analysis revealed only faint bands both at 28 kDa and at approximately 45–60 kDa. By contrast, in colostrum-suckling buffalo calves, AQP4 was expressed in the caecum and colon with intense bands both at 28 kDa and other bands at 45–60 kDa, respectively (Fig. 3a, lane 2). This result was confirmed by densitometric analysis that showed a more intense AQP4 expression in the caecum than in the colon.

A similar profile was observed for the examined tissues in animals which had received milk and in adult buffaloes with a different gradual intensity for each band (Fig. 3a, lanes 3 and 4) from milk suckling to adult subjects, also confirmed by densitometric analysis (Fig. 3b).

Expression of AQP5 using Western blot analysis

Results of AQP5 expression are summarised in Fig. 4(a). As in the case of AQP4, AQP5 antibody recognized in the caecum and colon not only a band at approximately 28 kDa but also other bands approximately at 45–60 kDa.

At birth, in both the colon and caecum, only a faint band at 28 kDa and at approximately 60 kDa, respectively, was observed (Fig. 4a, lane 1). By contrast, a well-defined profile, respectively, in the caecum and colon with intense bands both at 28 and 45–60 kDa, with a more intense band for the latter (Fig. 4a, lane 2), was observed in calves which had suckled colostrum. This result was corroborated by densitometric analysis (Fig. 4b). A similar AQP5 expression band profile was observed for the examined tissues in animals which received milk and in adult buffaloes, albeit with a lower degree of intensity growing from first (milk) to the second condition (adult). This profile is well shown by densitometry (Fig. 4b).

Expression of AQP4 and AQP5 mRNA by using RT-PCR

Reverse transcriptase polymerase chain reaction analysis using AQP4 and AQP5 primer revealed the expression of AQP4 (Fig. 5a) and AQP5 (Fig. 5b) mRNA in the large intestine of buffalo calves, according to different animal conditions (birth, colostrum, milk), and of adult buffaloes. The reaction revealed a single band of the expected size (250-bp AQP4; 260-bp AQP5) in all the intestinal tracts examined. In order to verify the efficiency of the reverse transcription (RT) and to exclude genomic DNA contamination, a fragment of β-actin cDNA (GenBank accession no. NC_007326) was amplified and sequenced with primers designed to span an intron β-actin for 5′-CAG CTC CTC CCT GGA GAA GA-3′. β-actin rev 5′-CTG CTT GCT GAT CCA CAT CTG-3′.

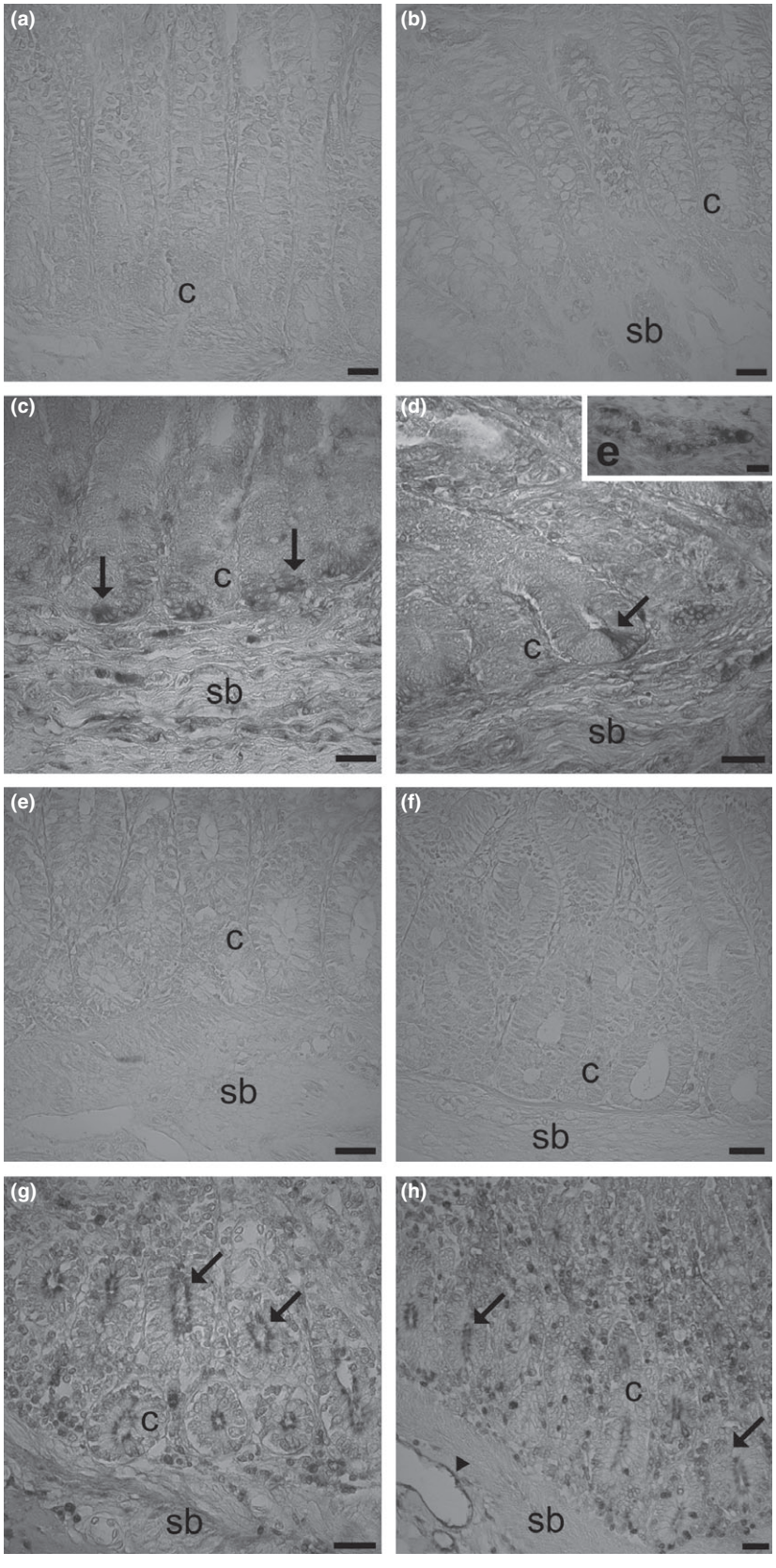

Fig. 1. Distribution of AQP4-immunoreactivity in the large intestine of the buffaloes at different stages: birth (a, b), after 1 week of colostrum (c–e) and milk (f, g) suckling and adult (h, i). Caecum (a, c, f, h) and colon (b, d, e, g, i). c crypts; sb tela submucosa; arrows positive enterocytes; arrow head positive endothelium; scale bar 25 μm.

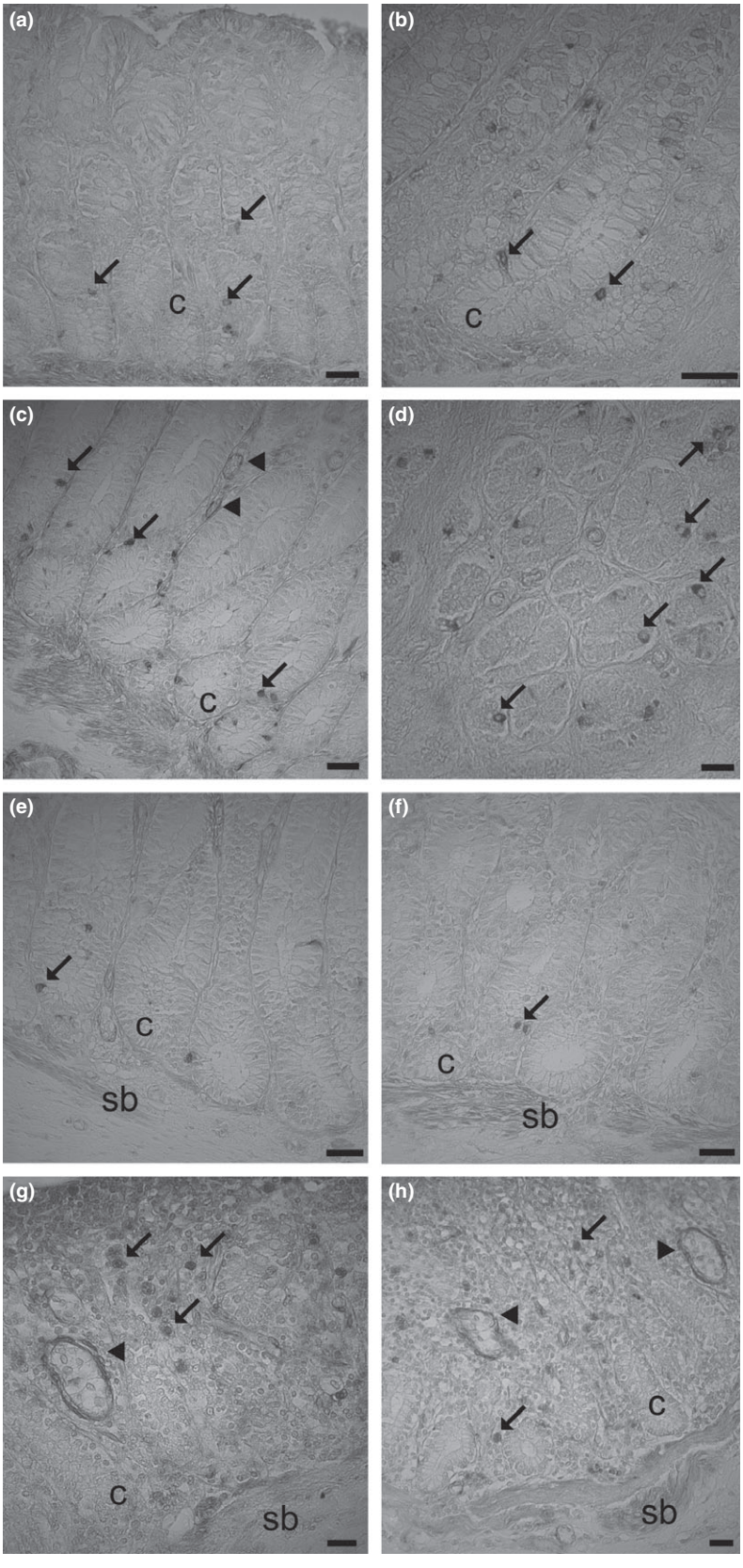

Fig. 2. Distribution of AQP5-immunoreactivity in the large intestine of the buffaloes at birth (a, b), after 1 week of colostrum (c, d) and milk (e, f) suckling and adult (g, h). Caecum (a, c, e, g) and colon (b, d, f, h). c crypts; sb tela submucosa; arrows positive endocrine cells; arrow head positive endothelium; scale bar 25 μm.

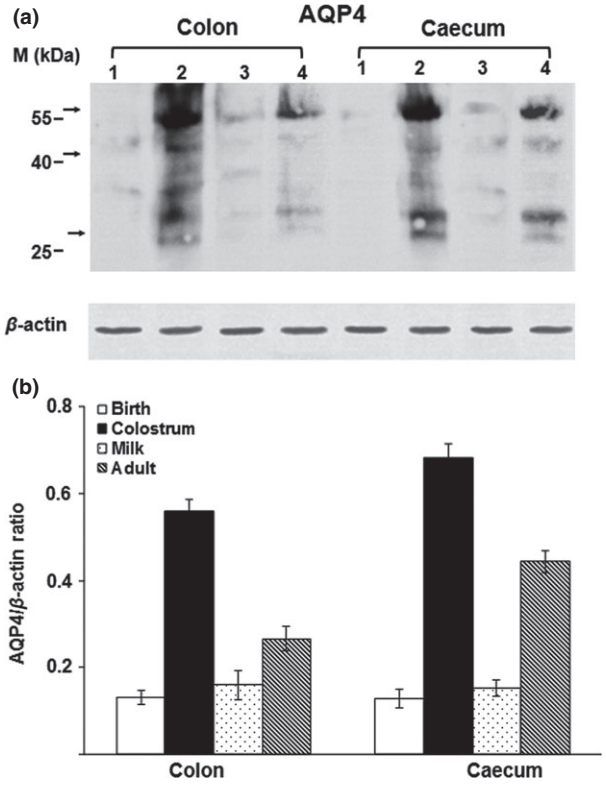

Fig. 3. (a) Western blot analysis of AQP4 expression in the large intestine (caecum and colon) in buffalo calves in different conditions (birth, colostrum, milk) and in adult buffaloes. lane 1. birth; lane 2. colostrum; lane 3. milk; lane 4. adult. Beta-actin was used as a loaded control. (b) Semi-quantitative analysis of AQP4 expression. Averaged data from five animals per group on three different blots for each intestine tract are expressed as ratios with the corresponding value for b-actin. Data are presented as means ± standard error (SE).

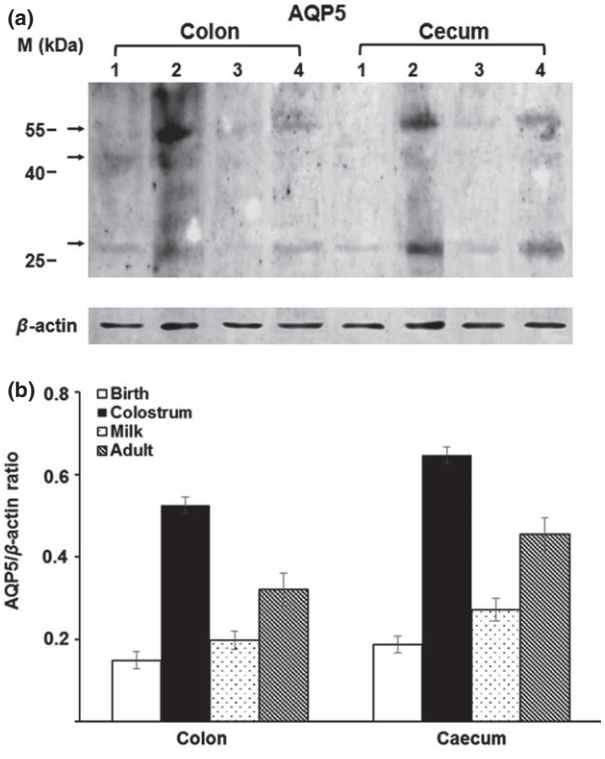

Fig. 4. (a) Western blot analysis of AQP5 expression in the large intestine (caecum and colon) in buffalo calves in different conditions (birth, colostrum, milk) and in adult buffaloes. lane 1. birth; lane 2. colostrum; lane 3. milk; lane 4. adult. Beta-actin was used as a loaded control. (b) Semi-quantitative analysis of AQP5 expression. Averaged data from five animals from each group on three different blots for each intestine tract are expressed as ratios with the corresponding value for b-actin. Data are presented as means ± standard error (SE).

Discussion

To investigate whether colostrum assumption by buffalo calves is associated with altered expression of aquaporins along the large intestine, we examined the changes in the expression of AQP4 and AQP5 mRNAs and relative proteins and their distribution in the caecum and colon of buffalo calves after colostrum suckling. In our previous paper (De Luca et al., 2014), we confirmed the presence of Aquaporin 1 along the gastrointestinal tract in buffalo calves during early post-natal development, suggesting a specific role for this pore channel protein and its probable interaction with others simultaneously present in such tissues.

Results of this immunohistochemical study on AQP4 and AQP5 showed differences in their localization in the large intestine. The antibody against AQP4 immunolocalized in the capillary of the endothelial cells as well as in the enterocytes and in lymphoid tissue, whereas AQP5 was localized particularly in the endocrine cells. Moreover, the intensity of distribution for both AQP4 and AQP5, observed by Western blot analysis, underwent diversification in the examined intestinal tracts of animals according to the different animal conditions, with a maximal expression in colostrum-suckling buffalo calves. In addition, the simultaneous presence of different bands (45–60 kDa) of both AQP4 and AQP5 suggests the expression of possible glycosylated forms as confirmed elsewhere (Cutler et al., 2012; Tiwari et al., 2014).

Our data relative to AQP4 immunoreactivity in the enterocytes are in agreement with the previous report of Wang et al. (2000) in mice which confirms the distribution of this channel protein at the basolateral membrane of surface colonocytes. This particular distribution of AQP4 in caecum and colon enterocytes of colostrum buffalo calves and of adult buffaloes suggests a particular involvement of these cells. These epithelia have two pathways for water transport: the paracellular and transcellular routes. The former occurs through the spaces between cell

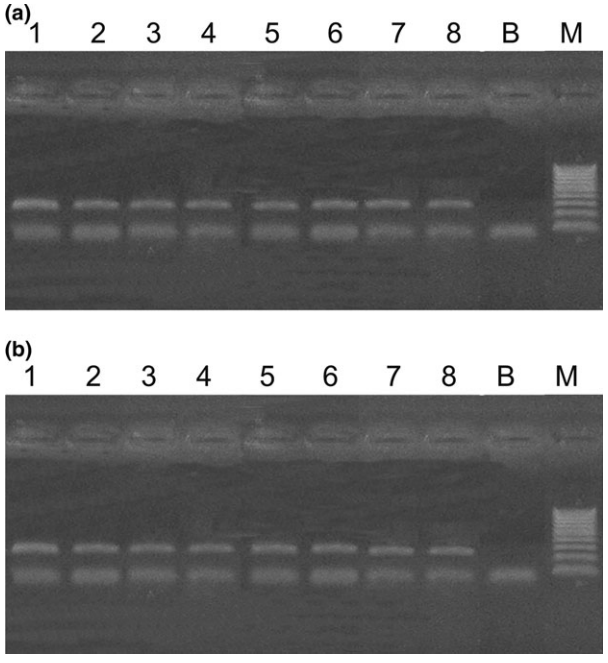

Fig. 5. (a) AQP4 and AQP5 expression by RT-PCR. AQP4 (250-bp fragment) was expressed in the caecum (lanes 1–4) and colon (lanes 5–8) of animals at birth (lanes 1, 5), animals after 1 week of colostrum suckling (lanes 2, 6), animals after 1 week of milk suckling (lanes 3, 7) and adults (lanes 4, 8). (b) AQP5 (260-bp fragment) was expressed in the caecum (lanes 1–4) and colon (lanes 5–8) of animals at birth (lanes 1, 5), animals after 1 week of colostrum suckling (lanes 2, 6), animals after 1 week of milk suckling (lanes 3, 7) and adults (lanes 4, 8). B negative control; M molecular markers of 100-bp ladder.

junctions and the latter through apical and basolateral cell membranes (Field, 2003).

The question of colon permeability in adult animals must take due account of the comments of Naftalin and colleagues in many scientific papers (McKie et al., 1991; Pedley and Naftalin, 1993) who show evident differences between bovine and ovine colon epithelia activities. In particular, bovine colon crypts present a low capacity to absorb fluid, thus indicating the incapacity of the bovine colon to generate hard faeces.

Interestingly, AQP5 has never been found before in the endocrine cells of the large intestine of buffaloes, although already detected in the rat (Matsuzaki et al., 2003). This immunoreactivity could be made to resemble that of protein AQP10 as observed in the intestine by Li et al. (2005). The morphological features, distribution and localization of the immunostained cells were comparable with the GEP (gastroenteropancreatic) endocrine cells or amine precursor uptake decarboxylase cells present in the intestinal glands. From the positivity of AQP5 in the intestinal endocrine cells especially in colostrum-suckling buffalo calves, we may hypothesize an influence on these cells by several agents (trophic factors and bioactive substances) present in colostrum that affect intestinal adaptation by modification induced to different degrees.

Another interesting result of our study, albeit not one of the aims of the present work, was the presence of AQP4 in the enteric neurons, as found for protein AQP1 (De Luca et al., 2014). To our knowledge, this is the first record of AQP4 in a large animal species. Previous studies on AQP4 immunoreactivity in the digestive tract refer only to mice (Thi et al., 2008) and guinea pigs (Jiang et al., 2014). The particularly intense AQP4 positivity in the enteric neurons of colostrum-suckling buffalo calves, shown by immunohistochemistry, thus suggests their possible involvement in regulating water transport functions with regard to food intake. Different reports by the same group of researchers (Oste et al., 2005; van Haver et al., 2008) strengthen this hypothesis by evidencing a modification in density and number of enteric neurons in preterm pigs in response to enteral food (colostral or formulae diets). In particular, their results confirm a rapid increase of enteric neurons and glial cells in the immediate post-natal period, sensitive to diet-dependent and/or developmental factors.

In the present study, the effects of feeding colostrum on intestinal function were indicated by both the enhanced AQP4 and AQP5 protein expression compared with birth condition and both normal milk feeding and adult subjects. Such results indicate a direct involvement of these pore molecules in facilitating absorption of molecules. The same results could be related to a different clinical situation (diarrhoea), a condition in which an observed reduced AQP4 expression in the colon is associated with changes in colonic water transport (Sakai et al., 2014), which leads to a shift in fluid flux from an absorptive to a secretory state. Thus, in our situation (AQP4 increase), an increase in absorption is conceivable, an important physiological function during colostrum assumption. This consideration is reinforced by the fact that colostrum intake influences the development, absorption and maturation of the gastrointestinal tract in the first days after birth (Bessi et al., 2002; Moretti et al., 2010; Machado-Neto et al., 2011; Nordi et al., 2013).

Thus, such changes during development are reflected in aquaporin tissue distribution in a similar manner to that observed for AQP5 during development of the mouse sublingual salivary gland and that suggested by Li et al. (2005) regarding aquaporins in salivary gland development.

In conclusion, the weight of evidence strongly suggests that AQP4 and AQP5 play important roles in water transport in the gastrointestinal tract. Co-expression of several AQP family members in the large intestine makes

it difficult to clarify the specific function of each AQP. Further studies are undoubtedly needed to ascertain the role of each aquaporin during tissue development in order to clarify their possible modulation in relation to feeding and the effect of single factors.

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Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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