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ORIGINAL PAPER

Expression and Immunolocalization of Aquaporins in the Buffalo Liver and Adipose Tissue

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Abstract

Increasing incidences of fatty liver in humans and animals worldwide is the leading cause of liver related morbidities. Currently, in the face of the growing global increase in fatty liver, and the necessity to explore new factors significantly affecting it, aquaporins (AQPs) have become the focus of interest for many researchers. AQPs are membrane integral proteins involved in the transport of water, glycerol and other small solutes. These are expressed in all tissues and play multiple roles under normal and pathophysiological conditions. Despite ongoing advancements in understanding the involvement of aquaporins in metabolic processes, there remains a notable lack of knowledge concerning cellular and subcellular localization of the AQPs in bovine tissues and organs. Understanding this could provide a new therapeutic target for metabolic syndromes such as fatty liver disease in bovine. In this study, AQPs in bovine liver, adipose tissue and gall bladder are examined using immunohistochemistry. AQP9 immunoreactivity is predominantly detected at the sinusoidal surfaces of hepatocytes. AQP8 is mostly intracellular and localized to the central vein and sinusoid, whereas AQP7 is found around the portal vein. Notably, AQP3 is observed in the bovine gall bladder and adipose tissue but not in the liver. In adipose tissue, AQP7 is also detected in the cytoplasmic membranes of adipocytes. AQPs in liver and adipose tissue were also studied using the western blotting technique. Higher AQP9 and AQP3 expression is observed in the liver and adipose tissue, respectively, indicating they are the dominant aquaporins in these tissues. This suggests they could be potential therapeutic targets for treating fatty liver disease and other metabolic disorders in bovine.

Keywords Aquaporins · Immunolocalization · Liver · Adipose tissue · Fatty Liver · Bovine

Introduction

Aquaporins play multifaceted roles beyond bile formation, influencing energy balance through hepatic gluconeogenesis and lipid metabolism. Implicated in various clinical disorders including fatty liver disease, diabetes, obesity, cholestasis, hepatic cirrhosis, and hepatocarcinoma, their significance extends across diverse physiological contexts (Rodríguez et al. 2014; Lehmann et al. 2008; Marinelli et al. 2011). Aquaporins are small, integral membrane proteins

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that play a crucial role in facilitating the bidirectional transport of water and various small solutes across the cell membranes. In mammals, 13 AQPs (AQP0-AQP12) have been identified so far, and their presence has been observed in many tissues and organs (Gena et al. 2011). Based on their permeability and phylogenesis, mammalian AQPs are grouped into: 1) orthodox aquaporins, permeable to water (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8); 2) aquaglyceroporins, permeable to water and other small solutes such as glycerol (AQP3. AQP7, AQP9, AQP10); and 3) unorthodox aquaporins AQP11 and AQP12, which are homologous to AQPs due to their distinct evolutionary pathway and their permeability is still unclear (Gomes et al. 2009).

In the human liver, several aquaglyceroporins have been identified, including AQP3, AQP7, AQP9, and AQP10 (Ishibashi et al. 1995, 1998; Catalán et al. 2008; Rodriguez et al. 2011). Among these, AQP9 plays a significant role as the main channel responsible for glycerol uptake by hepatocytes (Lindskog et al. 2016). It is also the most abundantly expressed aquaglyceroporin in the sinusoidal plasma membrane, and its presence is crucial for hepatic glycerol uptake and gluconeogenesis. Particularly during fasting, AQP9 becomes the primary channel for glycerol influx into hepatocytes (Calamita et al. 2012; Rodríguez et al. 2014). Illustrating the significance of AQP9 in glucose and lipid metabolism alongside energy balance, AQP9-deficient mice exhibited reduced liver glycerol permeability, leading to elevated plasma glycerol and triacylglycerol (TAG) levels (Calamita et al. 2012; Rojek et al. 2007). Moreover, obese mouse models, display reduced hepatocyte AQP9 levels, correlating with a marked decrease in liver glycerol permeability (Gena et al. 2013).

In adipose tissue, AQP7 was the first glycerol channel to be identified in humans and rodents (Ishibashi et al. 1998). Subsequently, the presence of other aquaglyceroporins, such as AQP3, AQP9 (Rodriguez et al. 2011), AQP10 (Laforenza et al. 2013), and AQP11 (Madeira et al. 2014), was also discovered. It is worth mentioning that while the AQP10 protein is expressed in humans, it exists as a pseudogene in cattle and mice (Morinaga et al. 2002; Ishibashi et al. 2011; Tanaka et al. 2015). In human adipose tissue, both AQP3 and AQP7 are localized in the membranes of lipid droplets and the plasma membranes of adipocytes, while AQP9 is constitutively expressed in the plasma membranes (Rodríguez et al. 2011). AQP10 has been identified at both the mRNA and protein levels in human subcutaneous adipose tissue and is found in the membranes of lipid droplets and the plasma membranes (Laforenza et al. 2013).

In human, mice, and murine gall bladder, AQP1 and AQP8 are expressed in epithelial cells. AQP1 is present both at the apical and at the basolateral plasma membrane of the epithelial cells (Ambe et al. 2016; Calamita et al. 2005) as well as in the corpus portion in subapical vesicles and plasma membrane (Van Erpecum et al. 2006). AQP8 is present at the plasma membrane and in intracellular vesicles of the gallbladder epithelium of various species (Nielsen et al. 1993; Calamita et al. 2005). In mice, some studies (Van Erpecum et al. 2006; Swartz-Basile et al. 2007; Goldblatt et al. 2002) have found correlations between AQP1 and AQP8 expression levels and the gallbladder's reduced contractility which is one of the reasons for gallstone disease (cholecystolithiasis). However, no such relationship was observed in human gall bladder (Ambe et al. 2016).

The aforementioned discussion highlights the varied distribution and expression of AQPs in the liver, adipose tissue, and gall bladder of humans and mice, highlighting their crucial roles in water and glycerol exchange and their significance in metabolism. Despite advances in bovine metabolism research, there is still limited knowledge about aquaporins (AQPs) in the liver, adipose tissue, and gall bladder of bovine.

Fatty liver is a common metabolic disorder in dairy cattle during early lactation. It occurs when the liver's lipid uptake exceeds its ability to oxidize and secrete them, often following elevated plasma NEFA levels mobilized from adipose tissue (Baba et al. 1995). This condition is linked to declines in health, reproduction, and in severe cases, reduced milk production and feed intake. Targeting aquaporins for preventative treatments of fatty liver could lead to significant savings for dairy farmers by cutting treatment costs and mitigating production losses. Currently, there is a limited literature on the distribution of aquaporins in bovines. Understanding protein functions requires a thorough examination of their localization, which is essential for guiding further research. To date, no comprehensive studies have been conducted to verify the presence and distribution of individual aquaporins in the liver, adipose tissue, and gall bladder of bovines. The results discussed here form a part of a broader investigation exploring whether future scenarios could see the quantification of aquaporin (AQP) expression becoming a modern biomarker for evaluating the metabolic status of animals. By understanding the expression and localization of AQPs could provide insights into the health and metabolic status of animals, paving the way for new therapeutic approaches.

In pursuit of answers to these questions, the current study embarks on research aimed at elucidating the precise localization of AQP3, AQP7, AQP8 and AQP9 in liver, AQP3 and AQP7 in adipose tissue and AQP3, AQP7, AQP8 and AQP9 in gall bladder. To comprehensively evaluate the distribution of AQPs, the current study incorporates morphological analysis and localizes aquaporins in liver, adipose tissue and gall bladder in buffalo using immunohistochemistry.

Material and Methods

Animals

Six healthy Murrah buffaloes were selected for the study following approval from the Institutional Animal Ethics Committee (IAEC) of the National Dairy Research Institute, Karnal (Approval no. 48-IAEC-23-07), and were managed in accordance with the standard conditions of the Livestock Research Center, NDRI, India.

Liver and adipose tissue biopsy

Liver biopsies were performed on heifers by a well-trained veterinarian using 14 G × 6" disposable Clear Needle™ biopsy needle (NewTech Medical devices, New Delhi, India) following standard minor surgical and postsurgical procedures (Singh 2017; Singh et al. 2019).

Tissues for protein isolation were collected in a dissection buffer with a Halt^{TM} protease inhibitor cocktail (Thermo Fisher Scientific, USA) and stored in a deep refrigerator at −80°C until use. The tissue destined for immunohistochemistry was immediately immersed in cold fixative. Blood samples were collected in vacutainers and processed immediately. The buffalo gall bladder tissue was collected from Meem Agro Food Pvt. Ltd., Shamli, U.P, India. Initial processing of the tissues was done at the location of sample collection and was immediately immersed in formalin.

Membrane fractionation and immunoblotting

Liver and adipose tissues were homogenized in ice-cold lysis buffer in a solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, supplemented with 8.5 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) along with Halt^{TM} protease inhibitor cocktail (Thermo Scientific^{TM}; Thermo Fisher Scientific, USA). Following homogenization, the homogenate was centrifuged in an eppendorf centrifuge at 4000 xg for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. The resulting supernatant was then subjected to further centrifugation at 2,00000 xg for 1.5 h to generate a pellet containing membrane fractions enriched for both plasma membranes and intracellular vesicles. Protein concentrations of supernatants were determined by the BCA method. Samples of supernatants containing 50 μg protein were separated by 12% SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membrane using the wet transfer method. After 3 h of blocking with tris buffered saline containing 5% nonfat dry milk and 0.1% Tween 20, the membranes were incubated overnight with Antibodies targeting AQP3 (catalog no. AQP3-301AP), AQP7 (catalog no. AQP7-701AP), and AQP9 (catalog no. AQP9-901AP) (Invitrogen; Thermo Fisher Scientific, USA) at dilution 1:300 and AQP8 (catalog no. AQP8-801AP) (dilution 1:200, Invitrogen; Thermo Fisher Scientific, USA)), and β-actin (dilution 1:1000; Catalog no. MA5-11869, Invitrogen; Thermo Fisher Scientific, USA) diluted in the blocking solution were employed for immunostaining. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Finally, the signals were detected by the DAB Substrate detection kit (Thermo Fisher Scientific, USA). The signal was captured by a UVP BioSpectrum500 imaging system (UVP, CA, USA.)

Immunohistochemistry

The indirect immunoperoxidase method was used to detect AQP in histological sections of liver, adipose tissue, and gall bladderrodriguez 2011 insulin \cite. Sections (4 µm) of formalin fixed, paraffin-embedded sections were dewaxed in xylene, followed by rehydration in decreasing concentrations of ethanol. Subsequently, they were treated with 3% H_2O_2 (Sigma, Aldrich) for 10 min at room temperature to quench endogenous peroxidase activity. Sections were blocked and incubated overnight at 4°C with antibodies to AQP3 (catalog no. AQP3-301AP), AQP7 (catalog no. AQP7-701AP), AQP8 (catalog no. AQP8-801AP), AQP9 (catalog no. AQP9-901AP), (dilution 1:300; Thermo Fisher Scientific, USA) washed in PBS and incubated with secondary antibody (biotinylated anti-rabbit IgG, 45 min at 25°C) followed by HRP-conjugated streptavidin. Negative controls were performed by omitting the primary antibody. The reactions were visualized by incubation with a 3,3-diaminobenzidine chromogen solution (Invitrogen, USA). The sections were then mounted with glass coverslips and visualized with Nikon Eclipse 600 photomicroscope equipped with a Nikon DMX 1200 camera (Nikon Instruments SpA, Calenzano, Italy).

Results

Animal status and morphological studies of liver, adipose tissue and gall bladder

In this study, heifers aged 1-2 years were included. Parameters such as Alanine Transaminase (ALT), Asparate Aminotranferase (AST), Gamman Glutamyl Tansferase (GGT), and other blood parameters for these heifers were experimentally evaluated and were found to be within normal range values in the animals as evidenced from the values listed in Appendix A.

Hematoxylin and eosin (H&E) staining of tissue sections was done which revealed the typical histological appearance

of the liver, adipose tissue, and gall bladder. In the liver, the parenchymal architecture displayed typical features with hepatocyte cords radiating from the centrilobular vein and separated by hepatic sinusoids (Fig. 1A). Adipose tissue sections exhibited large spherical adipocytes, with peripheral cell nuclei and a thin cell membrane enclosing cytoplasmic lipid (Fig. 1B). The gallbladder wall has mucosa composed of columnar epithelium that secretes mucus (Fig. 1C). Overall, the blood profiling and morphological study revealed the healthy status of the animals involved in this study.

Localization of aquaporins

Localization of AQPs in the liver, adipose tissue, and gall bladder were analyzed using respective antibodies. Antibodies targeting AQP3, AQP7, AQP9 (dilution 1:300; USA) and AQP8 (dilution 1:200, Invitrogen; Thermo Fisher Scientific, USA) were employed for immunostaining. Liver tissue exhibited robust immunostaining for AQP9 (Fig. 2C, D), AQP7 (Fig. 2E, F) and AQP8 (Fig. 2G, F) antibodies. AQP9 staining was observed along the sinusoidal surfaces of hepatocyte plates (Fig. 2C, D) whereas AQP7 is intracellular in hepatocytes. The staining intensity corresponding to AQP9 and AQP8 (Fig. 2C, D and G, H, respectively) was highest around the central vein (perivenous zone), while the sinusoids surrounding the portal vein (periportal zone) showed weaker staining (AQP7 in Fig. 2E, F), consistent with previous findings (Carbrey et al. 2003). Staining for AQP8 immunolabeling was predominantly intracellular and localized to the surface of the central vein and sinusoid, indicating likely interaction with the endothelial cells lining these structures. This observation is consistent with findings from studies on other species. Notably, hepatocytes displayed no reactivity to AQP3 antibody (Fig).

The histological distribution of AQP3 and AQP7 in adipose tissue by immunohistochemistry was examined. For AQP3 antibodies staining is observed on the membrane (Fig. 4 A, B), marking its presence in the cytoplasmic membrane of adipocytes. And, as illustrated in Fig. 4 (C, D) the immunostaining of AQP7 was also mainly detected in the cytoplasmic membranes of adipocytes.

Similarly, through the immunohistochemistry technique, AQP3, AQP7, AQP8 and AQP9 were detected in gall bladder (Fig. 4). Specifically, staining corresponding to AQP3 antibody is detected around the hepatic artery (Fig. 4C, D). AQP7 staining was also detected around the hepatic artery (Fig. 4E, F). AQP8 staining is observed in the region of mucosal folds (Fig. 3G, H) and AQP9 staining is observed around the bile duct (Fig. 3I, J) and villi region (Fig. 3K, L).

Immunoblotting and densitometric analysis of aquaporins

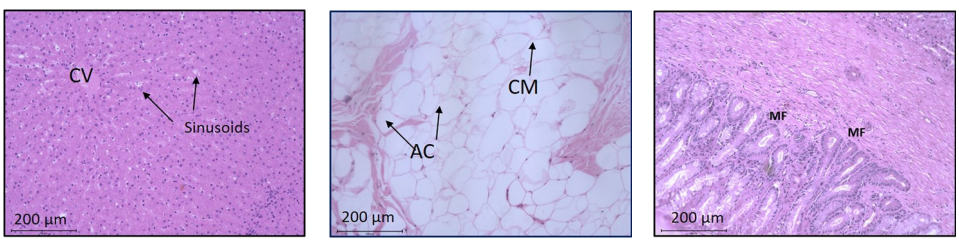
The Immunoblotting technique was further used to validate the expression of the AQP3, AQP7, AQP8, and AQP9 in bovine liver and AQP3 and AQP7 in bovine adipose tissue. Western blot analysis confirmed the presence of AQP7, AQP8, and AQP9 in the bovine liver (Fig. 5). As observed in Fig. 5A, the AQP9 was detected as a 29 kDa band. The AQP7 was detected as a band of 30 kDa in the bovine liver (Fig. 5 A), and the AQP8 was detected as a band of 30 kDa (Fig. 5A). Similarly, in bovine adipose tissue, immunoblotting confirmed the presence of the AQP7 and AQP3. The AQP3 was detected as 32 kDa band and AQP7 was detected as 30 kDa band as shown in Fig. 5B.

Summary of aquaporins' expression

Based on the results presented in the previous sections, the expression of the studied aquaporins AQP3, AQP7, AQP8, AQP9 in liver, adipose tissue and gall bladder are summarized in Table 1.

Discussion

The liver serves as the primary organ responsible for orchestrating the metabolic flexibility of the organism, effectively maintaining the delicate balance between immune function and metabolic homeostasis (Robinson et al. 2016). There exists a substantial interplay between the liver and gall bladder, supported by bile and products of digestion and absorption. Likewise, a connection is observed between the liver and adipose tissue, facilitated by glycerol, notably during both fasting and fed states. Non-alcoholic fatty liver disease



(a) Liver (b) Adipose tissue (c) Gall bladder

Fig. 1 Histological analysis of Liver, subcutaneous adipose tissue, Gall bladder assessed in H&E stained sections. (Abbreviations: CV- Central vein, AC- adipocytes, CM- cytoplasmic membrane, MF- mucosal folds.)

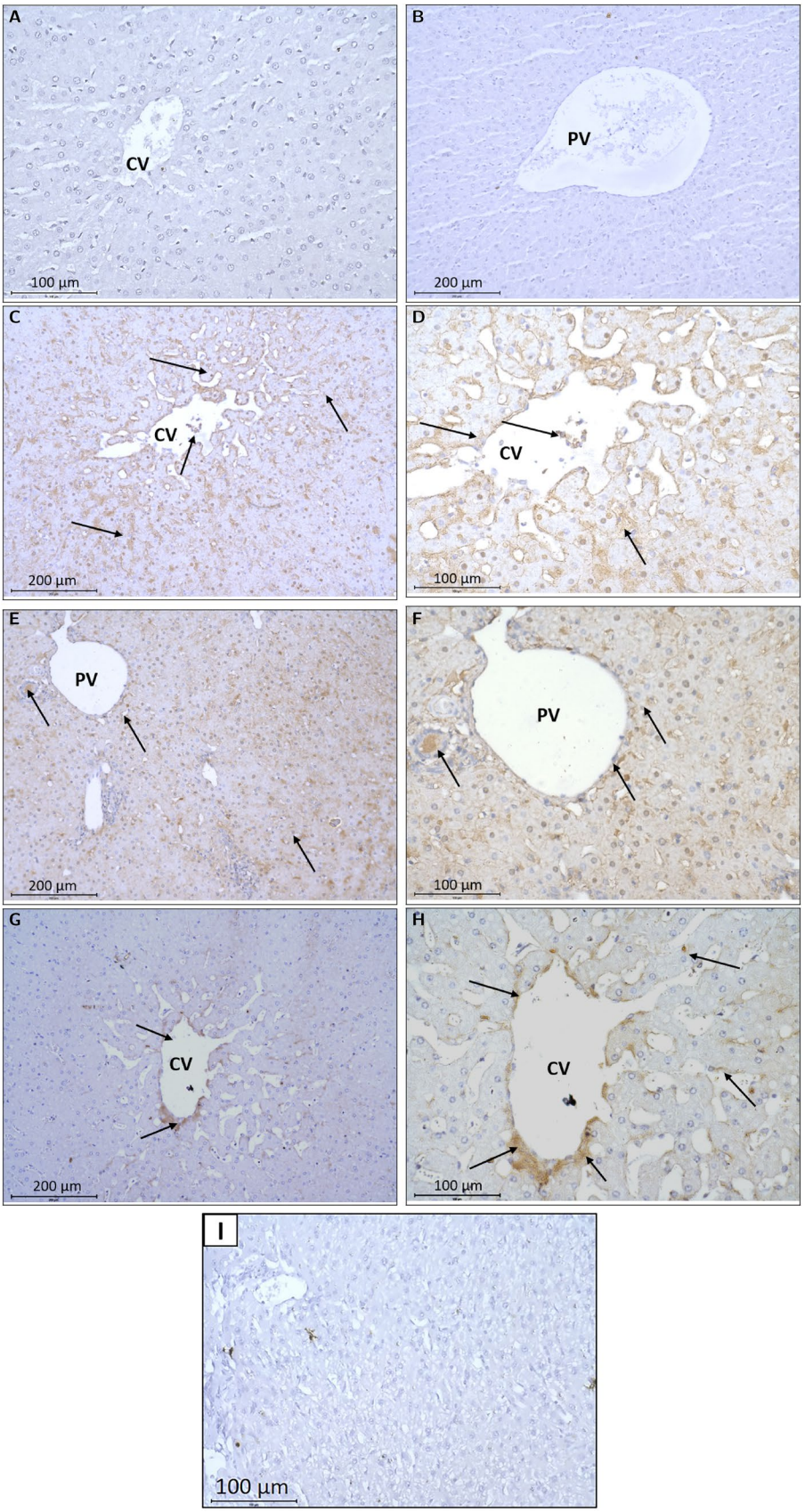


Fig. 2 Immunohistochemical localization of AQPs in liver stained with AQP antibodies. [A] and [B] Negative controls, no staining was observed in experiments omitting the AQP7 and AQP9 primary antibody respectively. [C] and [D] AQP9 staining was observed along the sinusoidal surfaces of hepatocyte plates, magnification 20X, and 40X respectively. [E] and [F] AQP7 antibody at dilution 1:300, staining around the portal vein is visible, magnification 20X and 40X respectively. [G] and [H] AQP8 immunolabeling was predominantly intracellular and localized to the surface of the central vein and sinusoid, magnification 20X and 40X respectively. (Abbreviations: CV- Central vein, PV- Portal vein)

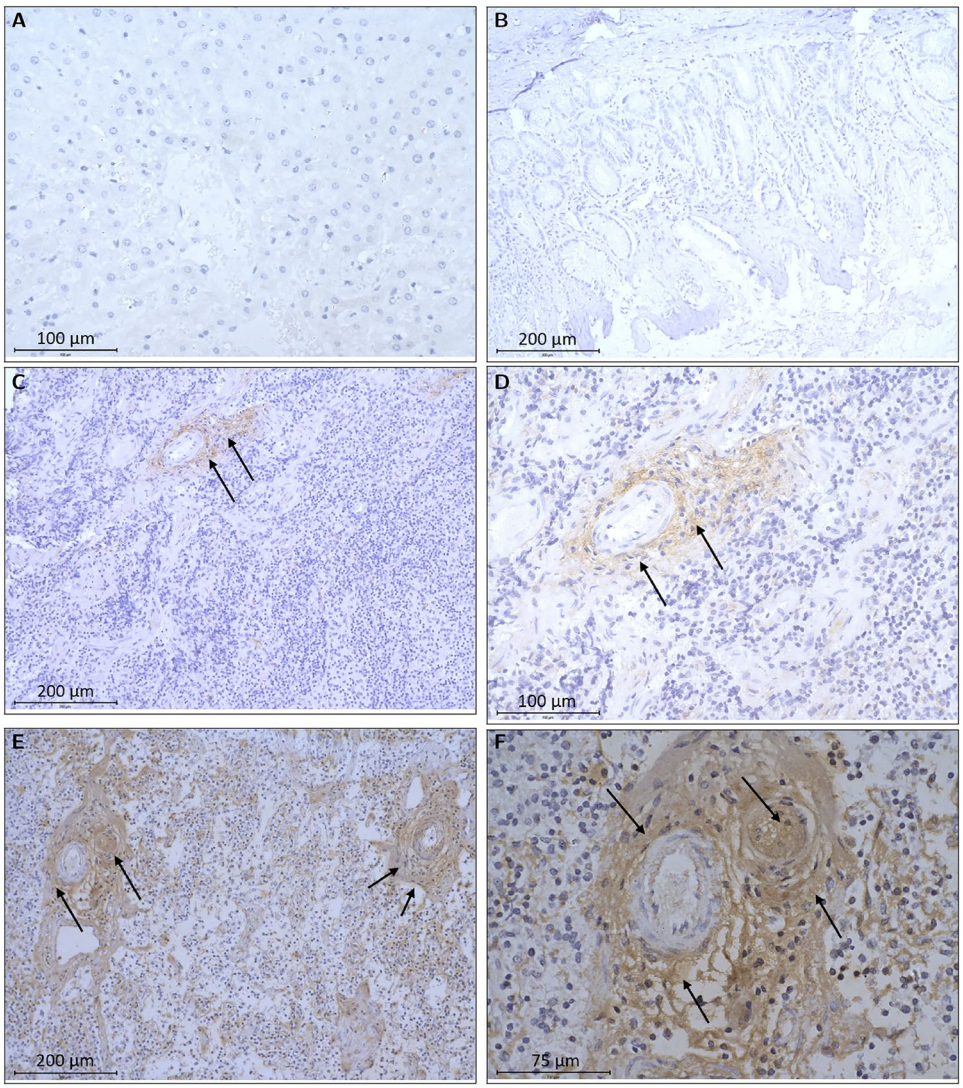


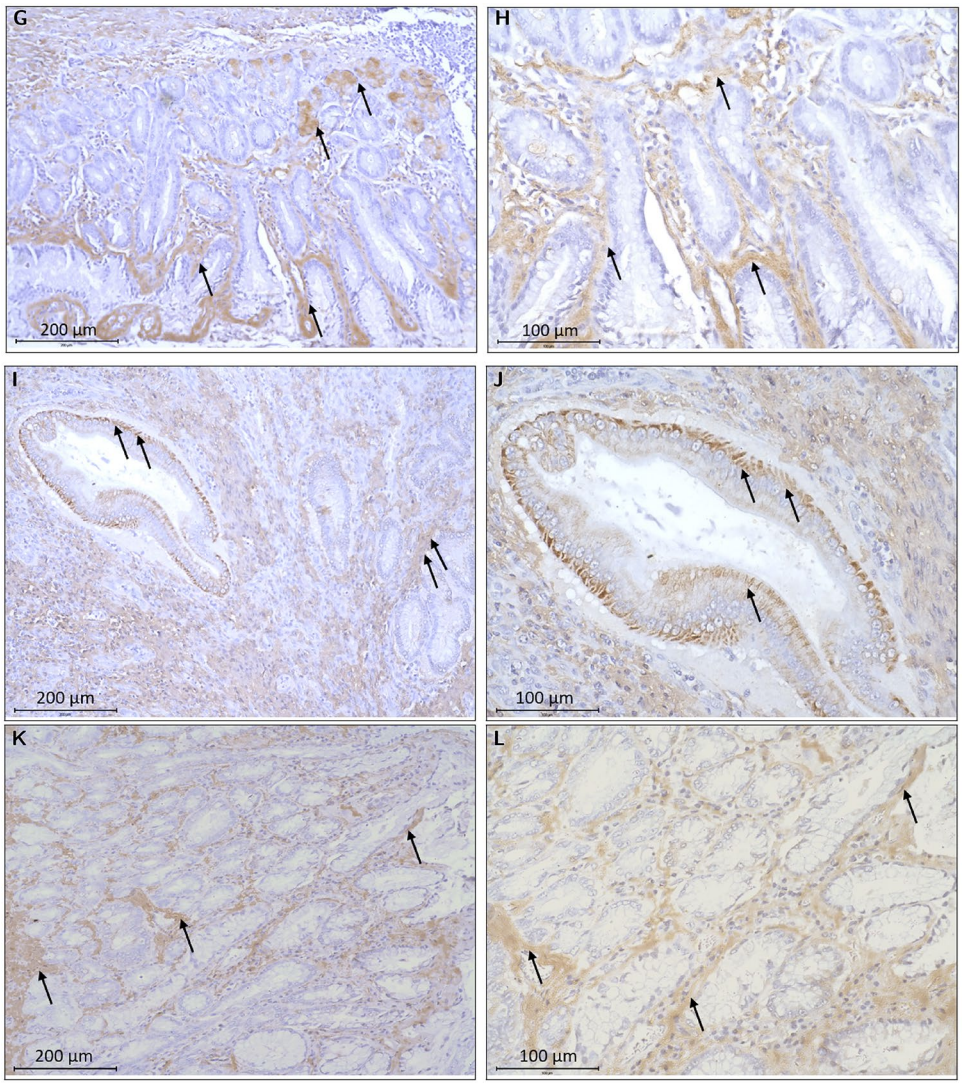
Fig. 3 Immunohistochemical localization of AQPs in Gall Bladder stained with polyclonal antibodies. [A] and [B] Negative controls, No staining was observed in experiments omitting AQP3 and AQP8 primary antibodies, respectively; [C] and [D] AQP3 antibody at dilution 1:300, staining around the hepatic artery is visible. [E] and [F] AQP7 antibody at dilution 1:300, staining around the hepatic artery, Magnification 20X [E] and 60X [F]; [G] and [H] AQP8 antibody at dilution 1:300, staining around mucosal folds is visible, Magnification 20X [G] and 40X [H] ; [I] - [L] AQP9 at dilution 1:300, staining around bile duct and microvilli is visible. Magnification 20X [I], [K] and 40X [J], [L]

(NAFLD) is a chronic liver disorder characterized by liver fat content equal to or exceeding 5% of hepatocytes by histology or intrahepatic triglyceride content equal to or surpassing 5.5% by MRI in non-alcoholic individuals (Chalasani et al. 2012). It is the predominant chronic liver disease, globally spanning a spectrum of histological features from simple steatosis, marked by the accumulation of liver fat, to non-alcoholic steatohepatitis (NASH), characterized by hepatocyte ballooning, inflammation, and/or fibrosis. Over time, this progression can lead to liver cirrhosis and hepatocellular carcinoma (HCC) (Sayiner et al. 2016).

Glycerol is an intermediate metabolite and a connecting link between carbohydrate and fat metabolism and aquaporins are pivotal channels in facilitating the import of glycerol (Baba et al. 1995). To our knowledge, this study represents the first initial characterization of cellular and subcellular localization aquaporins namely AQP3, AQP7, AQP8, and AQP9 in the liver, and AQP3 and AQP7in the adipose tissue of buffalo ovines. In addition to this, the localization of AQP3, AQP7, AQP8, and AQP9 in the gall bladder is also studied and presented in Fig. 6.

In the liver, AQP9 has been suggested to serve as the entry route for plasma glycerol originating from adipose tissue lipolysis, which is a primary substrate for hepatic gluconeogenesis. Kishida et al. (2000); Kuriyama et al. (2002); Carbrey et al. (2003); Rojek et al. (2007). The presence of liver steatosis may impact both the expression of AQP9 in the liver and vice versa (Gena et al. 2013). In rats AQP9 expression is confined to the hepatocyte basolateral membrane, the plasma membrane facing the sinusoids (Nicchia et al. 2001). Also, AQP9 is predominantly expressed in the liver, specifically at the sinusoidal domain of hepatocyte plasma membranes (Elkjær et al. 2000). As discussed earlier in the results section, the liver tissue in bovine exhibited robust immunostaining for AQP9, AQP7 and AQP8 antibodies. Staining for AQP9 was detected on the sinusoidal

Fig. 3 (continued)



surfaces of hepatocyte plates, exhibiting the strongest intensity around the central vein (perivenous zone) (Fig. 2 E, F). This pattern aligns with earlier studies (Carbrey et al. 2003; Elkjær et al. 2000). On the other hand, it is observed that AQP9 null mice exhibit elevated plasma glycerol and TG levels, indicating a decrease in liver glycerol permeability (Rojek et al. 2007; Calamita et al. 2012). In rodents, insulin transcriptionally represses hepatic AQP9 (Kuriyama et al. 2002), while AQP9 levels increase in insulin-resistant states (Carbrey et al. 2003; Rojek et al. 2007).

The subcellular distribution of AQP8 in hepatocytes varies with species. In mouse hepatocytes, different groups reported different AQP8 localization, that is, widespread expression in intracellular membranes including smooth endoplasmic reticulum, subapical vesicles, and mitochondria, in contrast, strong localization on the plasma membrane with weak intracellular localization (Huebert et al. 2002). Rat hepatocytes express three AQPs, namely AQP0, AQP8, and AQP9, in varying quantities (AQP8 > AQP9 > AQP0). AQP0 and AQP8 are predominantly found intracellularly and to a lesser extent on the canalicular plasma membrane (Kuriyama et al. 2002; Ferri et al. 2003; Elkjær et al. 2000; Nicchia et al. 2001; Garcıa et al. 2001; Huebert et al. 2002; Calamita et al. 2001). In the case of bovines, as we observed in Fig. 2G, H, AQP8 is localized to the surface of the central vein and sinusoids. In the liver, AQP8 primarily facilitates water transport and helps in modulating the bile acid formation. The authors in Xiang et al. (2023), found the expression of AQP8 was reduced in the liver specimens of patients with NAFLD, high-fat diet (HFD)-induced mice, and genetically obese db/db mice. The authors also reported two key findings: 1) Knockdown of AQP8 in hepatocytes reduced the intracellular lipid accumulation induced by free fatty acid (FFA) mixtures, and on the other hand 2) hepatic AQP8 overexpression activated farnesoid X receptor (FXR), inhibiting gene expression associated with lipogenesis,

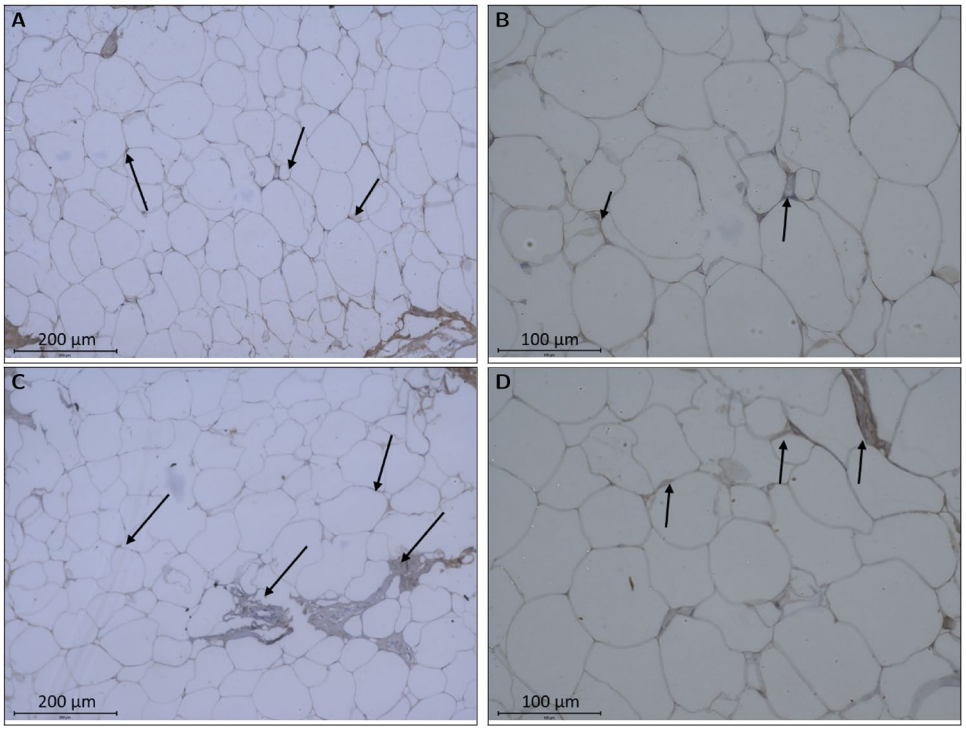


Fig. 4 Representative micrographs of Immuno-localization of AQP3 and AQP7 in Adipose tissue. [A] and [B]: AQP3 antibody at dilution 1:300, Magnification 20X and 40X respectively. [C] and [D]: AQP7 antibody at dilution 1:300, Magnification 20X and 40X respectively

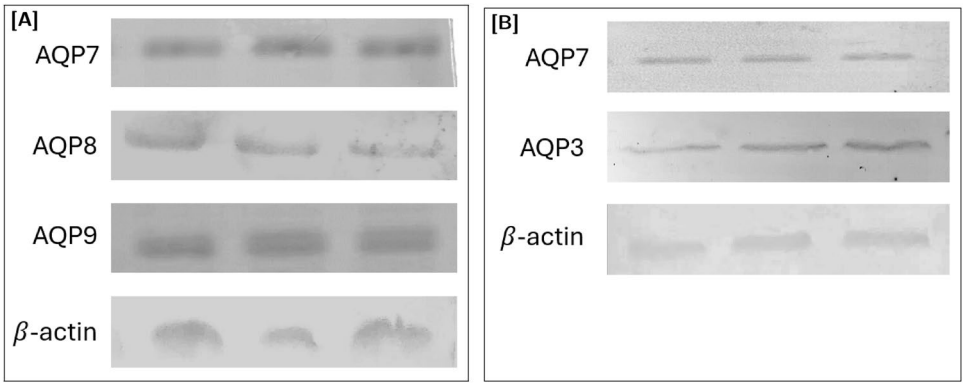


Fig. 5 Representative immunoblotting images showing presence of AQPs: AQP7, AQP8, AQP9 in Liver [A] and AQP7 and AQP 3 in Adipose tissue [B]

Table 1 Expression of Aquaporins

AQP	Liver	Adipose Tissue	Gall Bladder
AQP3	Not detected	Present	Present
AQP7	Present	Present	Present
AQP8	Present	Not determined	Present
AQP9	Present	Not determined	Present

which further improved liver steatosis in obese mice. As AQP8 plays such a crucial role in mice liver steatosis and patients with NAFLD, a similar functional role of AQP8 could likely be observed in the bovine liver. However, a systematic targeted research effort is required to establish such findings in the case of bovines.

As shown earlier in the results section, AQP7 is also expressed in the buffalo liver. Previous studies have also reported the presence of AQP7 in the liver of human (localized in the cytoplasmic domain of hepatocytes (Gregoire et al. 2015; Chen et al. 2016) and the apical plasma membrane domain of cholangiocytes and endothelial cells (Gregoire et al. 2015)) and mice (localized in the cytoplasmic domain of hepatocytes (Gregoire et al. 2015; Fu et al. 2016)). Notably, hepatocytes displayed no reactivity to anti-AQP3 antibodies in the bovine liver.

Various findings in humans have shown the presence of both AQP3 and AQP7 in the membranes of lipid droplets and the plasma membranes of adipocytes (Kishida et al. 2000; Maeda et al. 2004). A similar observation can be made based on Fig. 4, where the immunolabeling of AQP3

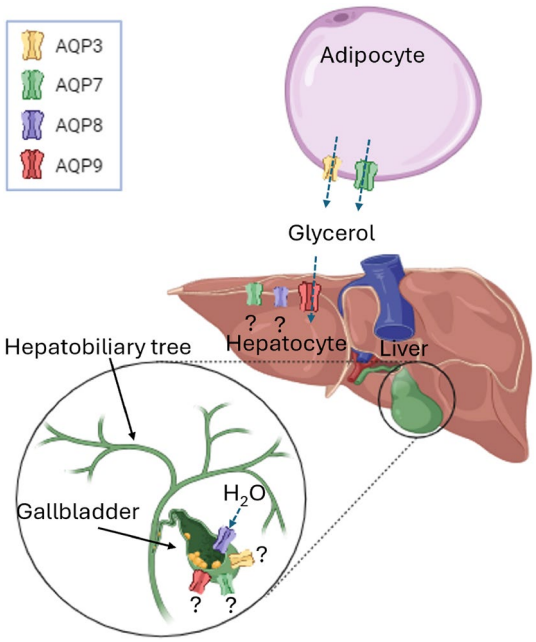


Fig. 6 Distribution of AQPs in the bovine liver, adipose tissue and gall bladder. The figure illustrates the localization of AQP3, AQP7 in adipose tissue AQP7, AQP8, and AQP9 in liver, AQP3, AQP7, AQP8 and AQP9 in gall bladder. The different aquaglyceroporins are identified in different colors

and AQP7 was mainly shown to be detected in cytoplasmic membranes of adipocytes.

Reduced expression of AQP7 in white adipose tissue is associated with excessive lipid accumulation in adipocytes (Hirako et al. 2023). AQP7 is instrumental in regulating the size of adipocytes in the peritoneum during the fasting condition and the expression of AQP7 is observed to have increased in the peritoneal membrane in mice (Costa et al. 2023).

Bile formation begins with hepatocytes and undergoes modifications through secretory and absorptive processes in the epithelial cells that line the lumen of intrahepatic bile ducts, known as cholangiocytes, as well as in the gallbladder. Hepatocytes release osmotically active substances, mainly bile salts and glutathione, into the canaliculus. This creates osmotic gradients that can lead to the passive entry of water through a transcellular pathway, likely mediated by AQPs (Portincasa et al. 2003; Calamita et al. 2005; Masyuk and LaRusso 2020). The AQP8 has been found in the plasma membrane and intracellular vesicles of the gall bladder in previous studies (Nielsen et al. 1993; Calamita et al. 2005). As mentioned in Calamita et al. (2005), AQP8 is likely to be involved in the absorption of water and in the secretion of water into the lumen of mice. However, no conclusion regarding the functional role of AQP8 in the gall bladder is yet reached due to several contradicting results. Further, investigation into the functional role of AQP8 in the gall bladder is needed.

In our work, based on immunohistochemistry results, we have also identified the presence of aquaporins AQP3, AQP7 and AQP9 from the aquaglyceroporins family in the gall bladder of buffalo . However, no such findings have been reported in the literature for any animal. In that sense, the findings in this paper regarding the presence of AQP3, AQP7 and AQP9 in buffalo gall bladder are interesting. These findings open up new directions for research questions pertaining functional role of these aquaporins in gall bladder and their potential role in or impact on gall bladder-related diseases such as gallstone, etc.

Overall, for the studied AQPs namely, AQP3, AQP7, AQP8 and AQP9 some of the expression and localization patterns observed in this work are consistent with the prior results on other animals, while some other patterns are newly observed in the buffalo . The similar-found as well as newly found expression and localization patterns observed in this study serve as a first step toward understanding the possible role of these AQPs in the complex biological processes involving the liver, hepatobiliary system, and adipocytes in bovine. These AQPs thus could be affecting the metabolic status of the bovines. It would be interesting to further investigate these AQPs in the context of bovine liver, adipose tissue and hepatobiliary system in normal as well as diseased animals to compare their specific roles in the metabolism and their impact on the metabolic state of the bovines.

Conclusion

Based on the immunolocalization and immunoblot studies, we found the presence of AQP7, AQP8 and AQP9 in the liver hepatocyte whereas no AQP3 was detected. In adipose tissue, AQP3 and AQP7 were expressed. Based on immunohistochemistry study, AQP3, AQP7, AQP8 and AQP9 were found to be expressed in the gall bladder. The role of these aquaporins in various metabolic diseases associated with liver and hepatobiliary systems is complex and requires further targeted research undertakings. In addition to emerging as a pivotal player in metabolic balance, these aquaporins may present a novel therapeutic target for addressing a prevalent aspect of metabolic syndrome, such as NAFLD. This study sets the premise for future research endeavors targeted at understanding the potential role of aquaporins AQP3, AQP7, AQP8 and AQP9 in the metabolism of the bovines in normal and pathological conditions.

Appendix A Blood parameters of the heifers

The blood parameters of the heifers studied in this work are given in Table 2.

Table 2 Blood parameters and liver function tests of the heifers in the study

Parameter	Mean value	SD	Reference Interval	Unit
ALP	167.22	34.660	80.33 - 364.81	IU/L
SGPT/ALT	24.752	4.610	8.58 - 46.19	IU/L
SGOT/AST	41.264	9.132	22.29 - 68.71	IU/L
BUN	12.4905	1.165	4.74 - 22.89	mMol/dL
Glucose	2.84	0.076	1.24 - 5.41	mMol/L
TC	1.666	0.130	0.69 - 1.97	mMol/dL
WBCs	11.1	2.759	4.0 - 12.0	$10^3/mm^3$
RBCs	6.71	0.714	5.00 - 10.00	$10^6/mm^3$
HGB	10.85	0.964	8.0 - 15.0	g/dL
HCT	30.36	3.517	24.0 - 46.0	%
MCV	45.16	1.572	40 - 60	μm^3
MCH	16.23	0.791	11.0 - 17.0	pg
MCHC	35.9	1.751	30.0 - 36.0	g/dL
PLT	698	166.636	100 - 800	$10^3/mm^3$

Abbreviations in Table 2 are defined as follows. ALP: Alkaline Phosphatase, SGPT/ALT: Serum Glutamic Pyruvic Transaminase/alanine transaminase, SGOT/AST: serum glutamic-oxaloacetic transaminase/aspartate aminotransferase, BUN: blood urea nitrogen, TC: Total cholesterol, WBCs: white blood cells, RBCs: Red blood cells, HGB: Haemoglobin, HCT: hematocrit, MCV: Mean Corpuscular Volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, PLT: platelets, IU/L: International units/ litre.

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Data availability The raw data and python code to post-process the raw numerical data is available on request.

Declarations

Conflict of interest Authors have no conflict of interest.

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