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Biochemical and expression studies on Aquaporin 9 (AQP9) in wild and AQP9 knockout mice

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ABSTRACT

The objective of this study was to determine the cellular and subcellular localization of AQP9 in different organs of wild-type ($AQP9^{+/+}$) and AQP9 knockout mice ($AQP9^{-/-}$) by immunohistochemistry (IHC), immunofluorescence (IF) and RT-PCR, as well as some biochemical evaluation. Twenty wild and homozygous AQP9 KO-mice (C57 BL/6J) were used. Both $AQP9^{+/+}$ and $AQP9^{-/-}$ were randomly assigned to 4 subgroups based on sex type. IHC analyses revealed AQP9 specific labeling in the hepatocytes, kidney, spleen and epididymis of wild type mice ($AQP9^{+/+}$), but a complete absence of labeling in $AQP9^{-/-}$ mice. In the liver, labeling was strongest at the sinusoidal surface, and there was little intracellular labeling. AQP9 expression was found to be sex-linked. In the liver of females, the expression of AQP9 was mostly confined to perivascular hepatocytes, whereas males showed more homogeneous hepatocyte staining. Confocal immunofluorescence confirmed the localization of AQP9 immunostaining on the basolateral plasma membrane of hepatocytes. Compared with the control mice, serum levels of glycerol and triglycerides were significantly ($P<0.05$) increased, in association with hypoglycemia in the $AQP9^{-/-}$ mice, whereas the total cholesterol, urea, alanine aminotransferase, and alkaline phosphatase were not statistically different.

Key words: aquaporin 9, knockout mice, immunohistochemistry, serum glycerol

Introduction

Aquaporins (AQPs) are members of a large family of integral membrane proteins involved in the rapid movement of water and neutral solutes across cell membranes (CAPERNA et al., 2007; KRANE and GOLDSTEIN, 2007) and are fundamentally important to fluid transport in the bile ducts and ductules of the liver (TALBOT et al., 2003). Various disease states have been associated with alterations in AQP expression and targeting in cells (VAN OS et al., 2000).

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To date, 12 isoforms of AQPs (AQP0-AQP12) have been identified in a wide variety of mammal cells (HUANG et al., 2006; ISHIBASHI et al., 2002; MARINELLI et al., 2004) and have greatly influenced the medical sciences (MAEDA et al., 2008). Among 13 identified mammalian aquaporins, AQP3, AQP7, AQP9, and AQP10 represent the aquaglyceroporin subfamily, on the basis of their amino acid sequences and solute permeabilities (AGRE et al., 2002). In the liver, AQP9 is expressed in hepatocytes within the sinusoidal surfaces of hepatocyte plates, where, during starvation, it is speculated to function in glycerol uptake from the bloodstream for gluconeogenesis (CARBREY et al., 2003; GRADILONE et al., 2005; KURIYAMA et al., 2002). In addition, it has been proposed that AQP9 is involved in urea elimination from hepatocytes (ISHIBASHI et al., 1998). AQP9 expression has been reported in other tissues, including the male reproductive tract, where it localizes to the efferent ductule epithelium, epididymis, and vas deferens of rats (PASTOR-SOLER et al., 2002). It is important to emphasize that, despite an abundance of data from different studies; there are still major discrepancies between the reported expression sites. In speculation, these differences may be due to either heterogeneity of expression in different species, or the existence of different splice variants, or may represent artifacts related to specificity of the anti-AQP9 antibodies used in immunohistochemistry (IHC), immunofluorescence (IF) and immunoblotting methods.

The aims of the present study were: 1) to identify the exact tissue localization of AQP9 in mice, males or females, wild-type and/or knockout- type, 2) to study some biochemical analyses in wild and knockout mice, 3) to study the liver structure of wild and knockout mice by electron microscopy.

Materials and methods

AQP9 knockout mice. The generation of AQP9 knockout (KO) mice was accomplished by completely removal of whole exon5 from AQP9 genomic DNA and substituted for nucleotides sequence encoding a neomycin phosphotransferase expression cassette (Fig. 1). This was done in the Structural Pathology Department, Institute of Nephrology, Niigata University, Japan. For AQP9 genotyping, the mice were tested by PCR using genomic tail DNA and 2 primers set.

Experimental animals. A total of 10 wild type and 10 homozygous AQP9 KO-mice (C57 BL /6J), 2-3 months of age, were used. Both $AQP9^{+/+}$ and $AQP9^{-/-}$ were randomly assigned to 4 subgroups based on sex type. All mice were housed at a controlled temperature, had free access to food and water and were maintained on 12-hourr light-dark cycles. Experiments involving these mice were approved by the Animal Care Committees of Niigata School of Medical and Dental Sciences.

Antibodies. An affinity-purified rabbit polyclonal antibody against an AQP9 C-terminal peptide from rat AQP9 (DAKO, Carpentaria, CA, USA) was used as a primary antibody.

The antibody was diluted 1:100 with sterile phosphate buffer saline (PBS) containing 0.05% sodium azide (NaN_3) before use. A horseradish-peroxidase (HRP) conjugated to goat anti-rabbit immunoglobulins (EnVision, DAKO, Kyoto, Japan) was used as a secondary antibody in dilution 1:5 with sterile PBS containing 0.02% NaN_3 for immunohistochemistry (IHC). For immunofluorescence (IF), fluorescein isothiocyanate (FITC) - labeled goat anti-rabbit polyclonal IgG from IBL (Immuno-Biological Laboratories, Takasaki, Gunma, Japan) in dilution 1:100 with sterile PBS containing 0.05% NaN_3 was used as a secondary antibody.

Primers. The primer sets were obtained from Nihon Gene Res. Lab., Miyagi, Sendai city, Japan.

For AQP9 genotyping, 2 primers were used: 5'-CAAATCCCAGCAACCATCTG-3' and 5'-GTAGCACATGCTTGCAATGC-3'. The expected PCR product for homozygous AQP9 KO- mice is 350 bp, for wild-type mice is 605 bp and both for heterozygous mice ($AQP9^{+/-}$).

To amplify specifically AQP9 transcript, 2 primers were designed from the sequence of full-length cDNA (GenBank accession no. 022026): A sense primer: 5'-CTCATCACGGGAGAAAATGG-3' and an antisense primer: 5'-GCTGGTTCTGCCTTCACTTC-3' (the expected PCR product is 437bp). For a positive control, glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was used as a house-keeping gene. The sense primer was 5'-CAAGATGGTGAAGGTCGGTG-3' and the antisense primer 5'-GAAGATGGTGATGGGTTTCC-3'(PCR product is 239bp).

Serum analysis. Blood was drawn from the supra-orbital venous plexus of $AQP9^{+/+}$ and $AQP9^{-/-}$ mice under light ether anesthesia, left for clotting, and clear sera were separated after centrifugation at 2000 ×g for 20 min at 4 °C for blood chemistry using diagnostic kits (Biomeria /France). Serum glycerol and triglycerides were determined according to the method of HERCULES and SHEEHAN (1978). Serum total cholesterol (RICHMOND, 1973), glucose using glucose oxidase method (TRINDER, 1969) and urea (ORSONNEAU et al., 1992) were estimated. The serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined colorimetrically according to REITMAN and FRANKEL (1957).

Immunohistochemistry (IHC). Immunoperoxidase staining was carried out according to the procedure of KOYAMA et al. (1999). The tissues (liver, kidney, spleen, pancreas, stomach, intestine, heart, lung, skin, brain, testis, epididymis, uterus and ovary) from wild type and $AQP9^{-/-}$ mice were fixed with methyl-Carnoy's fixative (60% methanol, 30% chloroform, 10% acetic acid), embedded in paraffin, and sectioned at 4 μm thickness. Sections were incubated overnight at 4 °C with primary rabbit anti-rat AQP9 antibody (2 μg/mL). After being washed (PBS for 3 × 5 min), slides were incubated for 1 hour at room temperature with secondary antibody, HRP-coupled goat anti-rabbit immunoglobulins (EnVision), and washed again as described above. The peroxidase reaction was developed

with 0.05% 3, 3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with Mayer's hematoxylin for 3 min. The immunostained sections were dehydrated in ethanol series and mounted with mounting medium. Then the immunocytochemical signal was examined with an Eclipse E800 light microscope (Nikon, Niigata, Japan). For the control, sections were incubated without primary antibody and showed no staining.

Immunofluorescence (IF). Liver tissues were removed from mice and frozen at -80 °C in n-hexane for 30 minutes and kept at -80 °C until sectioning. They were embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Tissue-Tek, Miles Inc., Torrance, CA) and mounted on a cutting block. After freezing in a Reichert Frigocut microtome, the tissue was cut at 3-4 μm thickness and sections were picked up on Superfrost Plus microscope slides (Matsunami Glass Ind., LTD., Japan). For immunostaining, sections were incubated overnight at 4 °C in rabbit anti-rat AQP9 primary antibody. Slides were washed twice for 5 minutes in high-salt PBS (2.7% [w/v] NaCl) to reduce nonspecific staining (ELKJAER et al., 2000; MARÍA et al., 2005), and once in normal PBS. They were then incubated for 1 hour at room temperature with secondary antibody, a goat anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC). After being washed (PBS for 3 × 5 min), sections were coverslipped, mounted in Vectashield (Vector Labs, Burlingame, CA) and examined under a Zeiss LSM 510 confocal fluorescence microscope.

RNA isolation. Total cellular RNA was also isolated from the same tissues previously used for IHC by a modified acid guanidinium thiocyanate phenol-chloroform extraction method (Trizol, GIBCO BRL, Life Technologies, Rockville, MD). Total RNA concentration was quantified by spectrophotometry, and its quality (purity) was checked by electrophoresis in agarose gels (CHOMCZYNSKI and SACCHI, 2006).

Reverse Transcription - Polymerase chain Reaction (RT-PCR). For detection of AQP9 mRNA, reverse transcription was performed using total RNA (5 μg); previously treated with DNase I buffer and DNase I amplification grade for 15 minutes at 25 °C; and SuperScript II reverse transcriptase enzymes. Reverse transcription was achieved by heating the reaction mixture for 1 hour at 42 °C and then for 5 minutes at 95 °C, then chilling it on ice for 5 minutes to stop the reaction. The PCR experiment was performed using 1 μL of the cDNA product (template) plus 4 μL of PCR mix containing 10 X PCR buffer (0.5 μL), 2.5 mM dNTPs (0.4 μL), Milli Q. (1 μL), Taq polymerase (0.1 μL), and specific primers (1 pmol/μL), forward (1 μL) and reverse (1 μL). The PCR amplification cycle using a PCR thermal cycler machine (TakaRa Co., Otsu, Shiga, Japan) was 35 PCR cycles, one PCR cycle consisted of initial denaturing at 95 °C - 5min, denaturing at 95 °C - 1 min, annealing at 62 °C - 1 min and extension reaction at 72 °C - 1min. After 35 cycles, final products were extended for 5 min at 72 °C. AQP9 amplified cDNA fragments were resolved in 1.5% agarose gel containing ethidium bromide, and their electrophoretic

migration was compared against a 1 kp plus DNA ladder (Marker) and the products were photographed under ultraviolet illumination.

Electron microscopy (EM). For transmission EM, the livers of wild type and knockout mice were removed and fixed in 2.5% glutaraldehyde at 4 °C overnight. They were then osmicated and embedded in Epon 812. Seventy-nm thick sections were cut on a Reichert-E ultramicrotome and collected on formvar-coated EM grids. The ultrathin sections were stained in 5% uranyl acetate for 3 minutes, then in lead citrate for 1 minute, dried and finally examined using the electron microscope (Philips CM 400).

Statistical analysis. Data reported as the mean ± SE, were computer analyzed using SPSS 8.0 software (Statistical Package for the Social Sciences Inc, Chicago, Illinois, USA). Statistical comparisons between investigated groups were accomplished by unpaired t test (equal variances). $P < 0.05$ was considered statistically significant.

Results

AQP9 knockout mice were generated as shown in Fig. 1. Under physiological conditions, knockout mice have normal embryonic survival, fertility, appearance, behavior, and plasma parameters, except for a significant change in the level of some biochemical parameters.

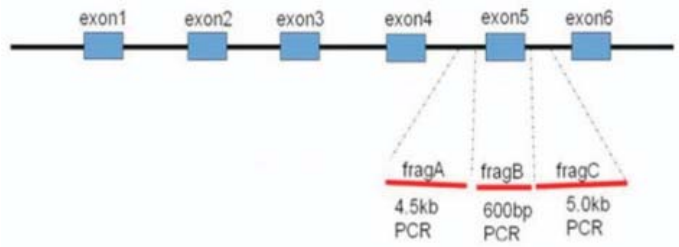

Fig. 1. Genomic structure of mouse AQP9 DNA and knockout construct. The exon 5 was completely removed and substituted by a nucleotides sequence encoding a neomycin phosphotransferase expression cassette.

Expression of AQP9 protein in mouse organs. Immunohistochemistry staining was used to localize AQP9 in different tissues of mice, males, females, wild, and/or KO-type. In wild mice, using rabbit anti-rat AQP9 antibodies, only the liver, epididymis and kidney (not previously identified expression site) showed strong immunostaining, whereas there was a complete absence of staining in $AQP9^{-/-}$ mice. At high concentration, the AQP9 antibody also stained different structures in the spleen and pancreas (not shown).

Immunocytochemical localization of AQP9 in mice liver. Liver AQP9 expression was analyzed to determine cell distribution and membrane localization and whether AQP9

expression/distribution was dependent on sex and the knockout effect. AQP9$^{+/+}$ male mice showed a more homogeneous hepatocyte staining by AQP9 (Fig. 2, A, C), whereas in female livers, the expression of AQP9 was mostly confined to perivascular hepatocytes (Fig. 2, B, D). Staining was restricted to the sinusoidal surfaces of hepatocyte plates of the liver. In some cases, there was little intracellular labeling. In AQP9$^{-/-}$ mice, there was no staining of the liver (Fig. 2, E, F).

Immunofluorescence. Immunofluorescence staining of the liver was used to confirm the subcellular localization and distribution of AQP9 in hepatocytes. Affinity-purified antibodies were used to stain cryostat liver sections from mice (Fig. 3). AQP9 was only found expressed in the hepatocytes of control mice (AQP9$^{+/+}$). In male mice, the expression of AQP9 was found to be more homogeneous and involved all the hepatocytes (Fig. 3, A). However, in female mice, the expression was strongest in the cells closest to the central vein (Fig. 3, B). At higher magnification, the staining was localized in the basolateral membrane of the cells in both male and female mice, whereas the apical bile canalicular membrane was not labeled (Fig. 3, C). The staining was completely absent in the AQP9$^{-/-}$ mouse (Fig. 3, D).

Immunocytochemical localization of AQP9 in mice kidney. Immunoperoxidase labeling of kidney sections showing anti-AQP9 labeling in the distal and collecting tubules of AQP9$^{+/+}$ mice of both sexes (a previously unidentified expression site). In addition, very weak labeling was observed in the proximal tubules, whereas other nephron segments and glomeruli did not exhibit labeling in excess of background. In the distal tubule, the labeling was confined to cytoplasmic domains (in the apical, central, and basal parts of the cells) (Fig. 4, A, B) and the absence of, or very weak, labeling of basolateral plasma membrane domains. Similarly, in collecting duct cells, strong labeling was associated with intracellular structures (arrow in Fig. 4, B, C). The AQP9$^{-/-}$ mice of both sexes showed a complete absence of AQP9 staining in the kidney tissues (Fig. 4, E, F).

Immunohistochemical localization of AQP9 in the spleen. The AQP9$^{+/+}$ mice, males and females showed AQP9 immunolabeling in the cells of white pulp (Fig. 5, A, B), whereas there was no labeling of cells in the red pulp.

Immunohistochemical localization of AQP9 in reproductive organs. The testis, seminiferous epithelium, uterus and ovary of AQP$^{+/+}$ mice showed no AQP9 expression, whereas there was only expression of anti-AQP9 labeling in the epididymis of male mice. In the epididymis, AQP9 was uniformly distributed over the microvilli of the principal cells of all regions, with the most intense reaction being noted in the initial segment and cauda regions. Intense labeling of the apical stereocilia was observed in the principal cells of the initial (Fig. 6, A, B) and distal (Fig. 6, C) segments of the epididymis. This staining was completely absent in AQP9$^{-/-}$ mice (Fig. 6, D).

Electron microscopy. Transmission electron microscopy showed no apparent differences in the structures of the liver in wild-type (Fig. 7, A) and AQP9 null (Fig. 7, B) mice. However, the bile canaliculi in KO-mice were shorter than in the wild-type.

RT-PCR analysis of the expression of AQP9 in mouse organs. For AQP9 genotyping, the mice were tested for the presence of disrupted AQP9 allele by PCR using genomic tail DNA and 2 primers. The 2 primers set produced the correct wild-type 605-bp-PCR product in the $AQP9^{+/+}$, while in the $AQP9^{-/-}$ mice it produced 350-bp-PCR product. Heterogeneous-type mice ($AQP9^{+/-}$) have both PCR product sizes (Fig. 8). We examined the expression of AQP9 in a range of mice organs using mRNA encoding AQP9 of these tissues and AQP9-specific primer set. A house- keeping gene, GAPDH, was used as a positive control (Fig. 9, A, B, C, D). In $AQP9^{+/+}$ mice of both sexes, all organs showed AQP9 expression by RT-PCR, except the pancreas, stomach, intestine and ovary (Fig. 9, E, F). The tissues already reported containing AQP9 in wild-type mice were found as amplified bands at the expected 437-bp position. $AQP9^{-/-}$ mice showed small PCR product size at 220 bp (Fig. 9, G, H).

Biochemical analysis. To examine whether $AQP9^{-/-}$ mice exhibit defects in the metabolism of various solutes, the concentrations of glycerol, triglycerides, total cholesterol, glucose, urea, alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in serum samples from $AQP9^{+/+}$ and $AQP9^{-/-}$ mice were determined (shown in Table 1). $AQP9^{-/-}$ mice exhibited a marked increase in serum glycerol and triglyceride levels with a decrease in serum glucose (only after fasting) compared with $AQP9^{+/+}$ mice. There were no significant differences in the other measured parameters. Moreover, there were no statistical changes between male and female, wild and/or KO-type mice.

Table1. Serum values for some biochemical analyses in $AQP9^{+/+}$ and $AQP9^{-/-}$ mice

Parameter		Wild-type mice ($AQP9^{+/+}$)		KO-type mice ($AQP9^{-/-}$)	
		male (n = 5)	female (n = 5)	male (n = 5)	female (n = 5)
Glycerol (μmol/L)		322.00 ± 18.95	319.55 ± 19.00	479.88* ±22.50	481.00* ± 24.14
Triglycerides (mmol/L)		75.00 ± 4.00	80.55 ± 6.13	98.77* ± 7.40	110.00 * ± 6.95
Total cholesterol (mmol/L)		180 .00 ± 8.10	174.18 ± 9.00	199.00 ± 11.54	187.55 ± 9.75
Glucose (mmol/L)	Random	120 .0 ± 4.88	118.00 ± 5.11	117.55 ± 4.16	118.22 ± 3.75
	9 hs after fasting	115 .00 ± 4.13	113 .00 ± 4.00	80.00* ± 3.50	85.00* ± 4.05
Urea (mmol/L)		76.42 ± 4.19	73.00 ± 4.50	77.00 ± 3.00	75.22 ± 3.55
ALT (U/L)		85.42 ± 5.22	84.00 ± 4.05	87.66 ± 4.14	85.53 ±.17
ALP (U/L)		144.33 ± 8.11	145.00 ± 10.00	142.00 ± 9.34	141.00 ± 8.50

Values are means ± SE; n = number of mice; *P<0.05; $A^{-/-}$QP compared with the control ($AQP^{+/+}$).

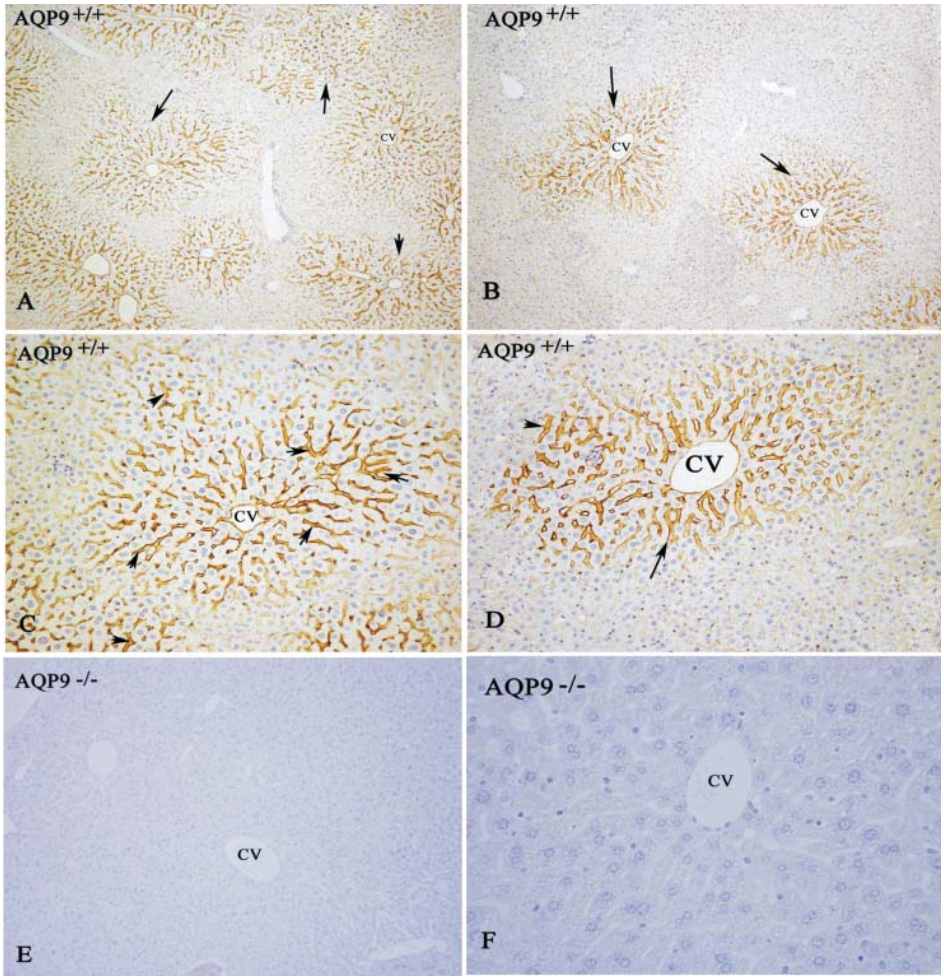

Fig. 2. Immunohistochemistry for AQP9 in liver of AQP9$^{+/+}$ and AQP9$^{-/-}$ mice stained with rabbit polyclonal antibody at dilution 1:100. In male's liver (A), immunoreactivity of AQP9 (brown staining) was more homogenous and widely distributed in hepatocytes than female's liver (B) in which staining was mostly confined to perivascular hepatocytes. Higher magnification showed staining of the sinusoidal plates (arrow heads) surrounding the central vein (CV) in the AQP9$^{+/+}$ male (C) and female mice (D). The label appears to be more uniformly distributed in all the hepatocytes of male mice. However, AQP9$^{-/-}$ mice showed completely absent of AQP9 labeling in the liver (E, F). Original magnifications: A, B, E ×100; C, D, F ×200.

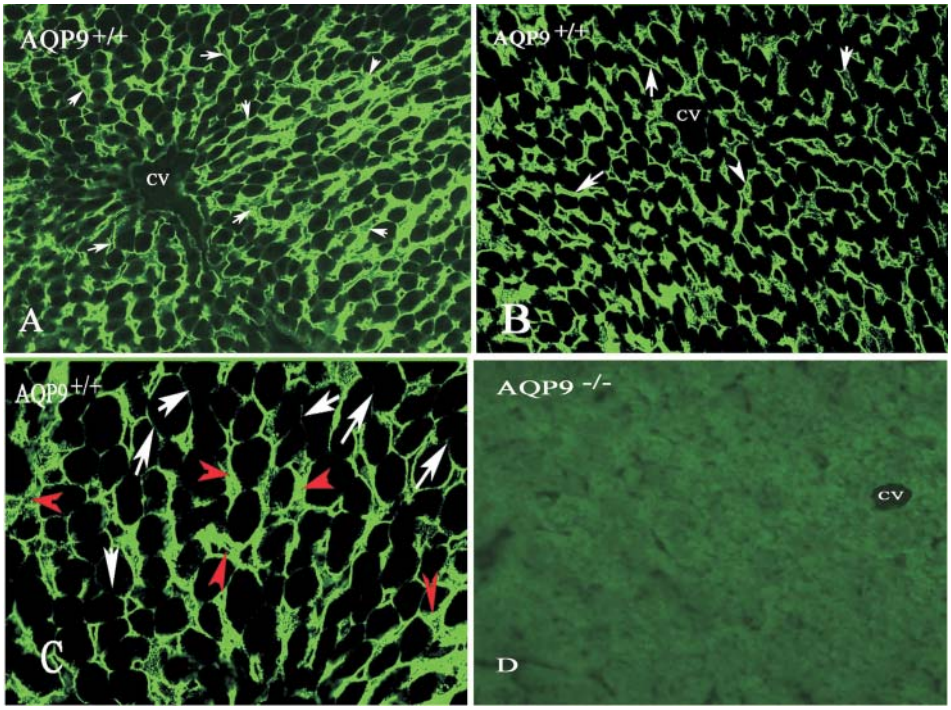

Fig. 3. Immunofluorescence localization of AQP9 in mice liver using anti-AQP9 antibodies. Frozen sections from liver of male (A) and female (B) wild-type mice ($AQP9^{+/+}$) showing hepatocytes close to the central vein (CV) intensely labeled. The label appears to be more uniformly distributed in all the hepatocytes in male mice. (C) High magnification showing the basolateral membrane expression of AQP9 (red arrows). A white arrow indicates the apical membrane of two hepatocytes. (D) $AQP9^{-/-}$ mice showed completely absence of labeling. Original magnifications: A, B, D ×200; C×400.

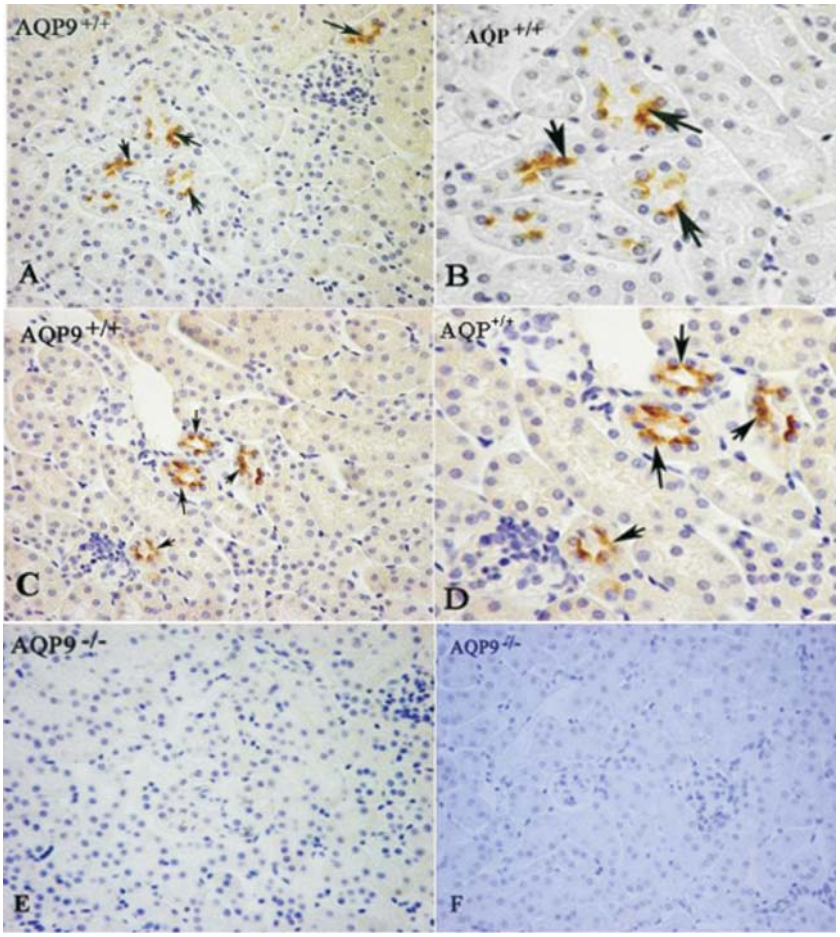

Fig. 4. Immunocytochemistry localization of AQP9 in kidney using anti-AQP9 antibodies. $AQP9^{+/+}$ mice showing anti-AQP9 labeling in the distal (A, B) and collecting (C, D) tubules. The labeling is mainly intracellular (arrows). E and F: immunolabeling $AQP9^{-/-}$ mice showing absence of AQP9 staining in all kidney tubules. Original magnifications: A, C, E ×200; B, D, F ×400.

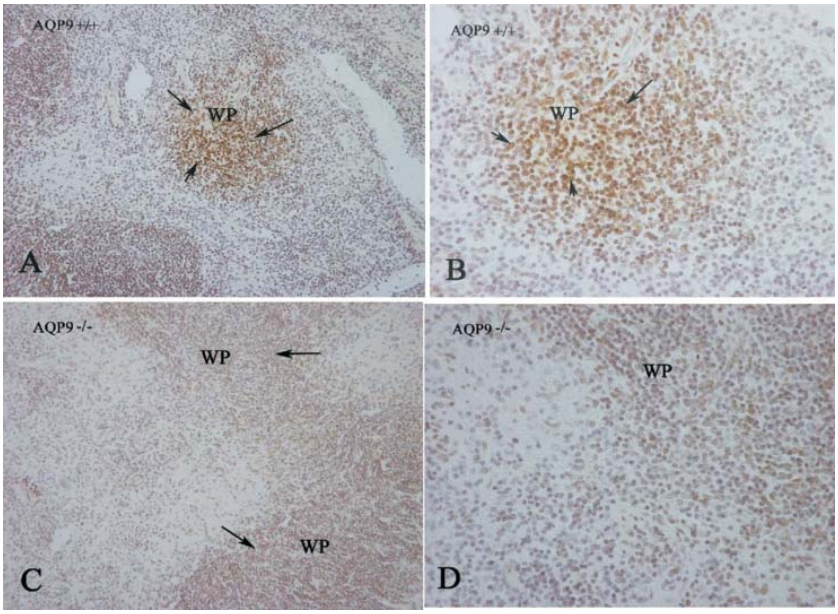

Fig. 5. Immunocytochemistry localization of AQP9 in spleen using anti-AQP9 antibodies. A and B: $AQP9^{+/+}$ mice showing anti-AQP9 labeling in the white pulp (WP). The labeling is mainly in the leukocytes (arrows). C and D: $AQP9^{-/-}$ mice showing complete absence of AQP9 immunoreactivity. Original magnifications: A, C ×200; B, D ×400.

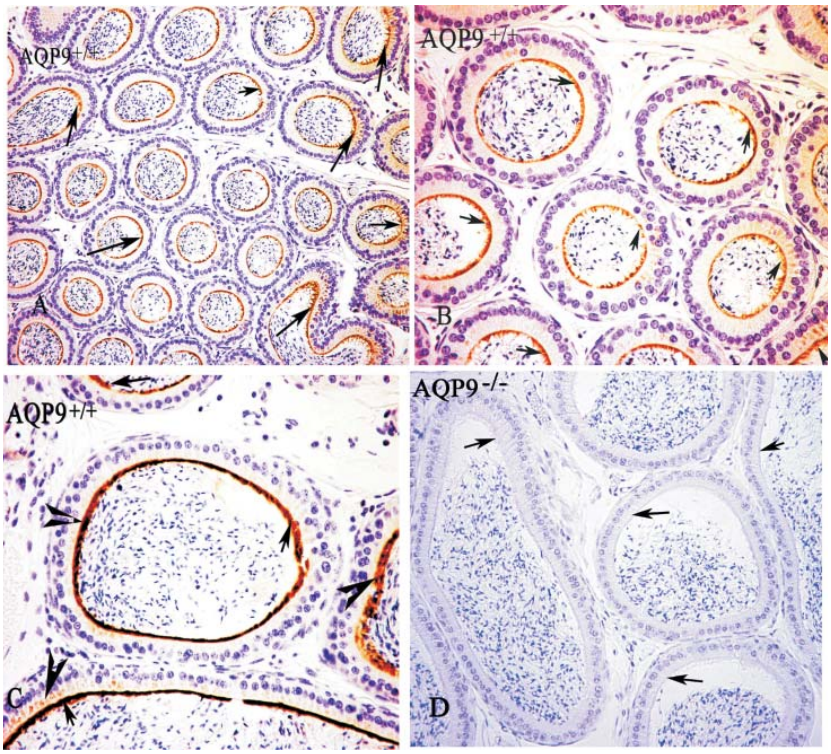

Fig. 6. Immunohistochemistry localization of AQP9 staining in the epididymis. In AQP9$^{+/+}$ mice (AC), AQP9 staining is concentrated at the apical pole (microvilli) of principal cells of initial segment (A, B). In cauda epididymidis (C), intense reaction is seen over the microvilli (thin arrows) of principal cells and cytoplasm of clear cells (thick arrows). Sperms in the lumen are unreactive. The staining is completely absent in the AQP9$^{-/-}$ mouse (D). Original magnifications: A ×200; B, C, D ×400.

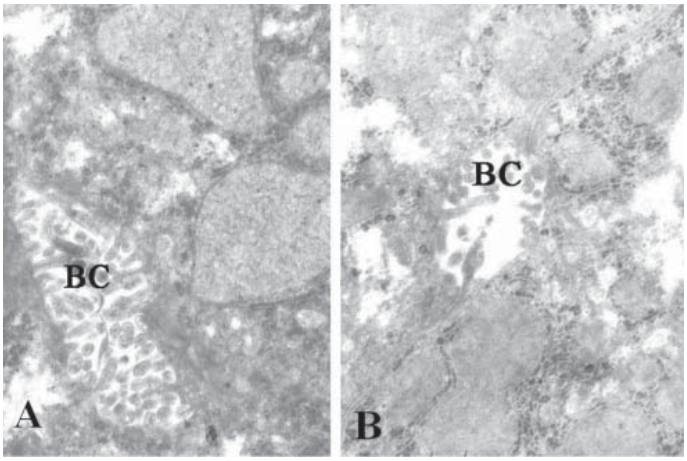

Fig. 7. Electron microscopy examination of liver in $AQP^{+/+}$ and $AQP^{-/-}$ mice. $AQP^{-/-}$ mice (B) showed short and small bile canaliculi (BC) comparatively with the $AQP^{+/+}$ control mice (A).

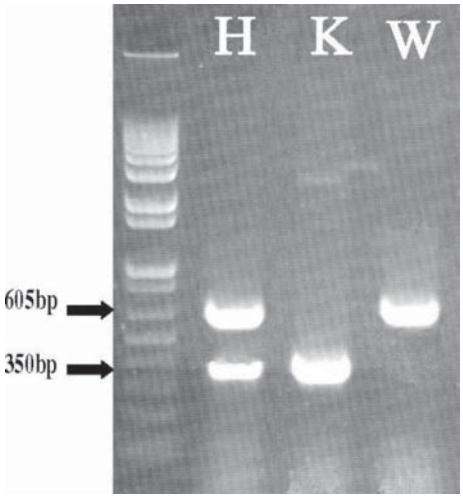

Fig. 8. AQP9 genotyping. A 2Primers set were produced the correct PCR product at 605-bp for the wild-type (W) and at 350-bp for the knockout-type mice (K), while heterozygous-type mice have both PCR product size positions (H).

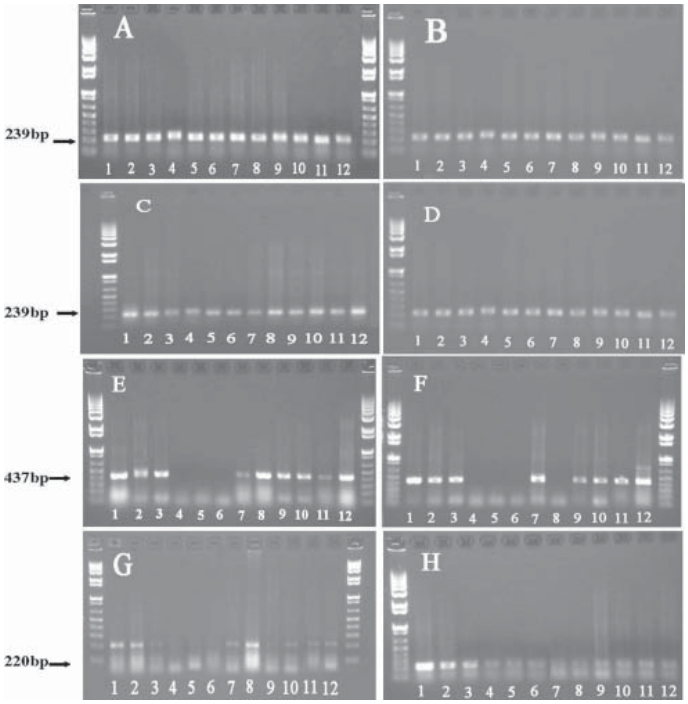

Fig. 9. AQP9 transcript analysis by RT-PCR of RNA isolated from different organs of mice. A house keeping gene, GAPDH, was used as a control positive with PCR product at 239-bp for male (A) and female; (B) wild-type mice and for KO-mice respectively (C&D). Primer pair "sense" and "antisense" produced the correct wild-type 437-bp-long product in the $AQP9^{+/+}$ for male (E) and female (F) mice which is absent in the $AQP9^{-/-}$ mice. However, in $AQP9^{-/-}$ male (G) and female (H) mice, these primers amplified 220-bplong PCR product, because whole exon5 containing the neo expression cassette is lost. Lane1-liver, 2-kidney, 3-spleen, 4-pancreas, 5-stomach, 6-intestine, 7-testis or uterus, 8-epididymis or ovary, 9-heart, 10-lung, 11-skin, 12-brain.

Discussion

The goal of this study was to identify the main expression sites of AQP9 in wild and AQP9 knockout mice of both sexes and to determine whether knockout for AQP9 plays a role or affects the biochemical analyses in mice that thus far have remained undefined.

Our data determined that AQP9 is more highly expressed in the liver, which represents its main expression site, than in the epididymis, kidney and spleen. The major finding reported here relates to the expression and subcellular localization of AQP9. Our results demonstrated that AQP9 expression is confined to the hepatocyte basolateral membrane, the plasma membrane facing the sinusoids. This pattern of staining may be consistent with CAPERNA et al. (2007), CARBREY et al. (2003), ELKJAER et al. (2000), KURIYAMA et al. (2002), MARINELLI et al. (2004), ROJEK et al. (2007). A new and interesting finding in the present study is the different expression observed between male and female $AQP9^{+/+}$ mice. In the livers of female mice, the immunohistochemistry (IHC) signal was progressively more intense in hepatocytes close to the central vein (perivenous hepatocytes), whereas in the livers of male mice, the label appeared to be more uniformly distributed in all the hepatocytes. This indicates that differences in the liver may be gender-dependent (PASTOR-SOLER et al., 2002). Moreover, our immunofluorescence signal confirmed the basolateral localization of AQP9 with IHC results and this is in agreement with HUEBERT et al. (2002). It is well established that many hepatic functions are expressed in a sexually dimorphic fashion (NICCHIA et al., 2001). A number of hepatic enzymes, plasma membrane receptors and transcription factors have been shown to be sexually dimorphic (SIMON et al., 1996). In contrast, $AQP9^{-/-}$ mice did not show any immunostaining. A recently identified member of the AQP family, AQP9, was expressed specifically in the distal and collecting tubules of the kidneys. This indicates a key role of AQP9 function in the kidney as a water channel protein. Moreover, the principal renal cells in some parts of the collecting duct have high levels of both AQP3 and AQP4 on their basolateral plasma membranes (ISHIBASHI et al., 1997). In this case also, one of the expressed channels (AQP4) is conductive only to water, while the others (AQP3, AQP9) are promiscuous water channels with high urea and glycerol permeability. At high concentration, the AQP9 antibody also stained different structures in the spleen and pancreas, but these results may be attributed to non specific binding of the antibody, especially for the pancreas, which showed no band correspondence to a specific AQP9 fragment with RT- PCR. Our results were in coincidence with ELKJAER et al. (2000) and ISHIBASHI et al. (1998).

Concerning IHC for the male reproductive tract, AQP9 is abundantly expressed in different segments of $AQP9^{+/+}$ mice, where it could represent an apical pathway for transepithelial water flow. In the epididymis, AQP9 was localized on the microvilli of

the principal cells of all regions (ELKJAER et al., 2000; HUANG et al., 2006; PASTOR-SOLER et al., 2002; RUZ et al., 2006). However, in the present study, AQP9 expression on the microvilli of the principal cells was noted to be region-specific, with the most intense reaction being noted in the initial segment and cauda regions. Similar results were obtained by BADRAN and HERMO (2002). Therefore, in the proximal portion, AQP9 is the only AQP so far detected on principal cells, whereas in the distal portion it is coexpressed with AQP2 on principal cell apical membranes (PASTOR-SOLER et al., 2001). The testes did not show any AQP9 labeling, although other authors have observed it**.** The difference may be due to the use of single anti-AQP9 antibody in this study and/or probably because its expression level from AQP9 is very low. These results are consistent with localization of AQP9 in the testis by weak intensity than in the epididymis, which revealed strong PCR product band as shown by RT-PCR. Our data now show that AQP9 is an abundant apical membrane protein in mice epididymis and other regions of the reproductive tract; it appears to be a constitutive apical membrane protein that may be responsible for apical membrane water and/or solute permeability of these epithelia**.** In female wild-type mice, AQP9, which had not been identified by IHC analysis, probably because its expression level was low, was found to be present in the uterus as shown by RT-PCR.

Using RT-PCR, we showed that most tissues of wild-type mice expressed AQP9-PCR product at 437bp position. However, a smaller 220-bp product was amplified from cDNA from KO-mice because the whole exon5 containing the neoexpression cassette was lost. In KO-mice, DNA sequencing showed that the smaller product is an alternatively spliced transcript, where exon4 is spliced directly to exon6. Our data were in harmony with CAPERNA et al. (2007).

With regard to the biochemical study, serum levels of glycerol and triglycerides were markedly increased, in association with hypoglycemia in AQP9$^{-/-}$ mice, revealing a role of AQP9 in glycerol metabolism. Aquaporin subtypes 7 and 9 have vital roles in the control of glycerol channels in adipocytes and hepatocytes, and thereby the balance between glycerol release and uptake by adipocytes and the liver, respectively (MAEDA et al., 2008). Glycerol, a product of adipose tissue lipolysis, is an important substrate for hepatic gluconeogenesis. As the substrate for hepatic glucose production, glycerol accounts for 90% in the prolonged fasting state and 50% in the postabsorptive state in rodents (KURIYAMA et al., 2002). Its abundant expression in the liver of normal mice strongly suggests that the increased serum glycerol levels in AQP9$^{-/-}$ mice are caused by an absence of hepatic AQP9 and an impaired uptake of glycerol through the hepatocyte plasma membrane. Although the increased serum glycerol level in AQP9 null mice is likely to be mediated by absence of AQP9, it cannot be excluded that the uptake and release of glycerol by other organs expressing aquaglyceroporins, e.g., kidney cortex (AQP3 and AQP7), fat tissue (AQP7), or intestine (AQP3 and AQP10), may also influence the final plasma glycerol level (ROJEK et al., 2007). Interestingly, according

to the model of metabolic zonation of the liver, gluconeogenesis takes place predominantly in the hepatocytes surrounding the portal vein (periportal) that express little AQP9, whereas relatively less gluconeogenesis takes place in the hepatocytes surrounding the central vein (perivenous), where highest AQP9 levels are detected (JUNGERMANN and KIETZMANN, 1997). Thus, it cannot be excluded that AQP9 may have additional functions in mouse liver other than glycerol transport. The hypoglycemia in our results suggesting that the absence of AQP9 in the hepatocyte membrane reduced the capacity for glycerol entrance for gluconeogenesis, leading to reduced serum glucose levels specially during fasting of AQP-null mice (CARBREY et al., 2003; KURIYAMA et al., 2002; ROJEK et al., 2007).Therefore, our studies of AQP9 protein strongly support the role for AQP9 expression in the liver as a molecular mechanism for maximizing glycerol influx during states requiring increased gluconeogenesis.

Our data concluded that AQP9 is a major water channel protein that is expressed throughout different organs, with high expression in the liver of male mice. In this study the kidney was shown to be a newly identified AQP9 expression site a. Hence, this recent identification of intracellular AQP will open new areas of research into cell biology and expand the scope of AQP. AQP9 is essential for hepatic glycerol metabolism, and an important influence on hepatic glucose production.

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References

AGRE, P., L. S. KING, M. YASUI, W. B. GUGGINO, O. P. OTTERSEN, Y. FUJIYOSHI, A. ENGEL, S. NIELSEN (2002): Aquaporin water channels-from atomic structure to clinical medicine. J. Physiol. 542, 3-16.

AGRE, P., D. KOZONO (2003): Aquaporin water channels: molecular mechanisms for human diseases. FEBS Lett 555, 72-78.

BADRAN, H. H., L. S. HERMO (2002): Expression and regulation of Aquaporins 1, 8, and 9 in the testis, efferent ducts, and epididymis of adult rats and during postnatal development. J. Androl. 23, 358- 373.

CAPERNA, T., A. SHANNON, M. RICHARDS, W. GARRETT, N. TALBOT (2007): Identification and characterization of aquaporin-9 (AQP9) in porcine hepatic tissue and hepatocytes in monolayer culture. Domest. Anim. Endocrinol. 32, 273-286.

CARBREY, J. M., D. A. GORELICK-FELDMAN, D. KOZONO, J. PRAETORIUS, S. NIELSEN, P. AGRE (2003): Aquaglyceroporin AQP9: solute permeation and metabolic control of expression in liver. Proc. Natl. Acad. Sci. USA. 100, 2945-2950.

CHOMCZYNSKI, P., N. SACCHI (2006): The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat. Protoc. 1, 581-585.

ELKJAER, M., Z. VAJDA, L. NEJSUM, T. KWON, U. JENSEN, M. AMIRY-MOGHADDAM, J. FROKIAER, S. NIELSEN (2000): Immunolocalization of AQP9 in liver, epididymis, testis, spleen, and brain. Biochem. Biophys. Res. Commun. 276, 1118-1128.

GRADILONE, S. A., P. S. TIETZ, P. L. SPLINTER, R. A. MARINELLI, N. F. LARUSSO (2005): Expression and subcellular localization of aquaporin water channels in the polarized hepatocyte cell line,WIF-B. BioMed. Central Physiol. 5, 13-17.

HERCULES, D. M., T. L. SHEEHAN (1978): Chemiluminescent determination of serum glycerol and triglycerides. Anal. Chem. 50, 22-25.

HUANG, H., R. HE, C. SUN, Y. ZHANG, Q. MENG, Y. MA (2006): Function of aquaporins in female and male reproductive systems. Human Reproduction Update 12, 785-795.

HUEBERT, R. C., P. L. SPLINTER, F. GARCIA, R. A. MARINELLI, N. F. LARUSSO (2002): Expression and localization of aquaporin water channels in rat hepatocytes. J. Biol. Chem. 277, 22710-22717.

ISHIBASHI, K., M. KUWAHARA, Y. GU, Y. TANAKA, F. MARUMO, S. SASAKI (1998): Cloning and functional expression of a new aquaporin (AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. Biochem. Biophys. Res. Commun. 244, 268-274.

ISHIBASHI, K., T. MORINAGA, M. KUWAHARA, S. SASAKI, M. IMAI (2002): Cloning and identification of a new member of water channel (AQP10) as an aquaglyceroporin. Biochem. Biophys. Acta. 1576, 335-340.

ISHIBASHI, K., S. SASAKI, K. FUSHIMI, T. YAMAMOTO, M. KUWAHARA, F. MARUMO (1997): Immunolocalization and effect of dehydration on AQP3, a basolateral water channel of kidney collecting ducts. Am. J. Physiol. 272, 235-241.

JUNGERMANN, K., T. KIETZMANN (1997): Role of oxygen in the zonation of carbohydrate metabolism and gene expression in liver. Kidney Intern. 51, 402-412.

KOYAMA, Y., T. YAMAMOTO, T. TANIT, K. NIHEI, D. KONDO, H. FUNAKI, E. YAOITA, K. KAWASAKI, N. SATO, K. HATAKEYAMA, I. KIHARA (1999): Expression and localization of aquaporins in rat gastrointestinal tract. Am. J. Physiol. Cell. Physiol. 276, 621-627.

KRANE, C., D. GOLDSTEIN (2007): Comparative functional analysis of aquaporins /glyceroporins in mammals and anurans. Mamm. Genome 18, 452-462.

KURIYAMA, H., I. SHIMOMURA, K. KISHIDA, H. KONDO, N. FURUYAMA, H. NISHIZAWA, N. MAEDA, M. MATSUDA, H. NAGARETANI, S. KIHARA, T. NAKAMURA, Y. TOCHINO, T. FUNAHASHI, Y. MATSUZAWA (2002): Coordinated regulation of fat-specific and liver-specific glycerol channels, aquaporin adipose and aquaporin 9. Diabetes 51, 2915-2921.

MAEDA, N., T. FUNAHASHI, I. SHIMOMURA (2008): Metabolic impact of adipose and hepatic glycerol channels aquaporin 7 and aquaporin 9. Nat. Clin. Pract. Endocrinol. Metab. 4, 627-634.

MARÍA, C., B. MORALES, M. RÍOS, M. VILLALÓN (2005): Regulation of the immunoexpression of AQP9 by ovarian hormones in the rat oviductal epithelium. Am. J. Physiol. Cell. Physiol. 288, 1048-1057.

MARINELLI, R. A., S. A. GRADILONE, F. I. CARRERAS, G. CALAMITA, G. L. LEHMANN (2004): Liver aquaporins: significance in canalicular and ductal bile formation. Ann. Hepatol. 3, 130-136.

NICCHIA, G. P., A. FRIGERI, B. NICO, D. RIBATTI, M. SVELTO (2001): Tissue distribution and membrane localization of Aquaporin-9 water channel: evidence for sex-linked differences in liver. J. Histochem. Cytochem. 49, 1547-1556.

ORSONNEAU, J. L., C. MASSOUBRE, M. CABANES, P. LUSTENBERGER (1992): Simple and sensitive determination of urea in serum and urine. Clin. Chem. 38, 619-623

PASTOR-SOLER, N., C. BAGNIS, I. SABOLIC, R. TYSZKOWSKI, M. MCKEE, A. VAN HOEK, S. BRETON, D. BROWN (2001): Aquaporin 9 expression along the male reproductive tract. Biol. Reprod. 65, 384-393.

PASTOR-SOLER, N., C. ISNARD-BAGNIS, I. HERAK-KRAMBERGER, I. SABOLIC, A.VAN HOEK, D. BROWN, S. BRETON (2002): Expression of Aquaporin 9 in the adult rat epididymal epithelium is modulated by androgens. Biol. Reprod. 66, 1716-1722.

REITMAN, S., S. FRANKEL (1957): A colorimetric method for determination of serum glutamic oxalacetic and pyruvic transaminases. Am. J. Clin. Pathol., 28, 56 -63.

RICHMOND, W. (1973): Enzymatic colorimetric method for determination of cholesterol. Clin. Chem. 20, 470.

ROJEK, A. M., M. T. SKOWRONSKI, E. FÜCHTBAUER, A. C. FÜCHTBAUER, R. A. FENTON, P. AGRE, J. FRØKIÆR, S. NIELSEN (2007): Defective glycerol metabolism in aquaporin 9 (AQP9) knockout mice. Proc. Natl. Acad. Sci. USA. 104, 3609-3614.

RUZ, R., G. MARY, C. SMITH, G. DANIEL, B. DENNIS, A. REX, H. LOUIS (2006): Expression of Aquaporins in the efferent ductules, sperm counts, and sperm motility in estrogen receptor-α deficient mice fed lab chow versus casein. Mol. Reprod. Dev. 73, 226-237.

SIMON, F.R., J. FORTUNE, M. IWAHASHI, P. GUZELIAN, E. SUTHERLAND (1996): Gender differences in alcohol dehydrogenase are regulated by growth hormone secretory patterns. Gastroenterology 110, A1327.

TALBOT, N., W. GARRETT, T. CAPERNA (2003): Analysis of the expression of Aquaporin-1 and Aquaporin-9 in pig liver tissue: comparison with rat liver tissue. Cells Tissues Organs 174, 117-128.

TRINDER, P. (1969): Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann. Clin. Biochem. 6, 24-27.

VAN OS, C. H., E. J. KAMSTEEG, N. MARR, P. M. DEEN (2000): Physiological relevance of aquaporins: luxury or necessity? European J. Physiol. 440, 513-520.

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SAŽETAK

Svrha ovog istraživanja bila je odrediti stanični i supstanični nalaz akvaporina AQP9 u različitim organima miševa divljeg tipa ($AQP9^{+/+}$) i miševa s odstranjenim genom za AQP9 ($AQP9^{-/-}$) imunohistokemijski, imunoflurescencijom i RT-PCR-om kao i biokemijskim pretragama. Upotrijebljeno je dvadeset miševa divljeg tipa i homozigotnih AQP9 „knockout" miševa (C57 BL/6J). I jedna $AQP9^{+/+}$ i druga $AQP9^{-/-}$ skupina miševa bile su nasumce podijeljene u četiri podskupine na osnovi spola. Imunohistokemijske pretrage pokazale su da se specifično obilježen AQP9 nalazi u hepatocitima, bubregu, slezeni i epididimisu miševa divljeg tipa ($AQP9^{+/+}$), a da ga nema u miševa $AQP9^{-/-}$. U jetri je imunohistokemijska reakcija bila najjača u sinusoidnoj površini, a slaba intracelularno. Ustanovljeno je da je ekspresija AQP9 vezana uz spol. U jetri ženki ekspresija AQP9 bila je pretežito ograničena na perivaskularne hepatocite, dok je u mužjaka obojenost hepatocita bila homogena. Konfokalnom imunofluorescencijom potvrđena je lokalizacija AQP9 na bazolateralnoj plazminoj membrani hepatocita. U usporedbi s kontrolnim miševima serumske razine glicerola i triglicerida bile su značajno ($P<0,05$) povišene u pokusnih miševa te povezane s hipoglikemijom u miševa $AQP9^{-/-}$, dok vrijednosti za ukupni kolesterol, ureju, alanin aminotransferazu i alkalnu fosfatazu nisu bile statistički promijenjene.

Ključne riječi: akvaporin 9, miševi s odstranjenim genom, imunohistokemija, serumski glicerol