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Current topic

Expression of aquaporin water channels in canine fetal adnexa in respect to the regulation of amniotic fluid production and absorption

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ABSTRACT

Amniotic fluid (AF) is created by the flow of fluid from the fetal lung and bladder and reabsorbed in part by fetal swallowing and partly by the transfer across the amnion to the fetal circulation. Placental water flux is an important factor in determining AF volume and fetal hydration. In addition the fetal membranes might be involved in the regulation of fluid composition. To understand the mechanisms responsible for maintaining a correct balance of AF volume we evaluated the expression of aquaporins (AQPs) in canine fetal adnexa. AQPs are a family of integral membrane proteins permitting passive but physiologically rapid transcellular water movement. The presence of AQP1, 3, 5, 8 and −9 was immunohistochemically assessed in canine fetal adnexa, collected in early, middle and late-gestation during ovario-hysterectomies performed with fully informed owners' consent. Changes in AF volume and biochemical composition were also evaluated throughout pregnancy. Our results show distinct aquaporin expression patterns in maternal and extraembryonic tissues in relation to pregnancy period. AQP1 was localized in placental endothelia, allantochorion, amnion, allantois and yolk sac. AQP3 was present in the placental labyrinth, amnion, allantois and yolk sac. AQP8 was especially evident on the epithelia lining the glandular chambers, the amniotic and allantois sacs. AQP9, a channel highly permeable to water and urea, was observed in epithelia of amnion, allantois and yolk sac. In summary, AQP1, 3, 5, 8 and −9 have distinct expression patterns in canine fetal membranes and placenta in relation to pregnancy period, suggesting an involvement in mediating the AF changes during gestation.
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1. Introduction

Aquaporins (AQPs) are a family of small, integral membrane proteins that are able to make the cell membrane 10–100 fold more water permeable compared to membranes lacking such channels. They are organized in the plasma membranes as tetrameric assemblies, each monomer forming a hydrophilic pore in its center as an independent water channel. Water selectivity is a central property of the channel [1,2]. To date up to 14 different types of AQPs have been identified in mammals, a number significantly lower than the 100 related proteins found in plants and bacteria [3]. On the basis of their permeability characteristics, corresponding to the amino acid sequence in their molecules, aquaporin water channels are divided into aquaporins (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8) and aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10). With the exception of AQP0, which doesn't have a definite channel function, aquaporins are only permeated by water. Even if included in this group because of the sequence analysis [4,5], AQP6 is an intracellular channel gated and permeated also by anions [6] and AQP8 might be permeated by water and urea. The aquaglyceroporins are permeable to small uncharged solutes such as lactate, glycerol and urea in addition to water [7].

AQPs regulate water flux dependent upon cellular localization and the prevailing osmotic gradient. Recent studies have demonstrated the presence of AQP1, 3, 8 and −9 in human, sheep and mice fetal membranes [8–13], suggesting that these channels may all contribute to the regulation of gestational water flow.

We have already shown the presence of AQPs in the bitch uterus [14] and verified their importance for water homeostasis in

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this organ. Bitches are monoestrous, typically non-seasonal, polytocous, spontaneous ovulators and have a 65 ± 1 days long pregnancy [15]. Dogs placenta includes a prominent region of exchange that forms a girdle completely surrounding the fetus [16,17]. A second zone consists of a pigmented ring and is made up of small hematomas (marginal hematoma or paraplacenta). A third region is the transparent one on the distal ends of the chorion that has poor vascularity and could be involved in absorption of materials straight from the uterine lumen [16]. The dog placenta is endotheliochorial, characterized as having a complete erosion of the endometrial epithelium and underlying interstitium in such a way to offer a total exposure of maternal capillaries to the chorial epithelial cells. The chorion is part of the fetal contribution to the placenta, which starts its formation with chorionic villi and very soon develops into a system of densely arranged lamellae where fetal and maternal tissues face each other and intimately intermingle [16].

The placenta regulates the exchange between the fetus and dam, involving mechanisms like simple diffusion (gas and water), facilitated diffusion (glucose, amino acids), and active transport (sodium, potassium, calcium pumps), pinocytosis and phagocytosis. Lipids do not cross the placenta, but maternal triglycerides and phospholipids are hydrolyzed and new lipids are synthesized by the placenta for the fetus. Fat soluble vitamins do not cross the placenta easily, while water soluble vitamins pass through the placenta without difficulty [16].

Despite this uniform function dog placental structure varies significantly throughout the gestation. At first, modifications of the endometrial glands and myometrium influence the *conceptus* attachment, thereafter changes occur throughout gestation mainly in the endometrium. Uterine glands provide histiotrophic nutrition for the fetus mostly at mid pregnancy. The placental labyrinth increases its thickness at late pregnancy. The fetal membranes progressively modify also, maintaining the fetus healthy, well fed and hydrated until parturition. The amniotic fluid (AF), essential for healthy fetal movement, growth and development, during embryogenesis increases its volume faster than embryonic size [18]. The amnion increases the number of microvilli as pregnancy progresses, allowing fluid exchange [17]. After 35 days post-breeding, in the dog, increased amniotic fluid volume distends the amniotic sac of each fetus, making them confluent and obscuring their characteristic shape and turgidity [19].

The regulation of water transfer and intramembranous resorption are imperfectly understood. Amniotic fluid is created by the flow of fluid from the fetal lung and bladder and reabsorbed in part by fetal swallowing and in part by the transfer across the amnion to the fetal circulation. Transfer of fluid and solutes from the amniotic cavity to the fetal circulation across the amniotic membranes is supposed to take place by passive diffusion due to an osmotic gradient [18,20].

Furthermore, many solutes diffuse in the opposite direction (from fetus to AF). It is possible that trans-cellular transport mechanisms are involved in the transfer of fluid and solutes from AF into the fetal circulation [20]. This is where aquaporin water channels could play a pivotal role. Alteration of aquaporin expression in fetal membranes and placenta may be important in the pathophysiology of disorders of AF volume associated with significant perinatal morbidity and mortality [12].

In order to understand the mechanisms responsible for maintaining amniotic fluid volume in the dog our aim was to determine the expression and cellular localization of AQP1, 3, 5, 8, 9 in canine placenta and fetal membranes. Furthermore, we sought to establish a correlation between AQP expression and AF volume and composition changes across gestation in the dog, a species with endotheliochorial placenta.

2. Materials & methods

2.1. Tissue collection and processing

The canine placenta and fetal membranes were obtained with the owners' approval during natural delivery, or during ovario-hysterectomies performed at any stage of undesired pregnancies or in case of caesarean sections, at the Reproduction Unit of the Faculty of Veterinary Medicine of Milan (Italy). The specimens were collected from 12 pregnant medium sized bitches. Gestational age estimate was obtained by the day of mating and by ultrasonography measurement of uterine and fetal diameters, according to Yeager et al. [21], England et al. [22] and Kutzler et al. [23]. As such, the specimens were included into three groups, according to the day of gestation (DG): aged up to 35 DG (early gestation: $N = 4$), from 36 to 45 DG (middle gestation: $N = 3$), from 46 DG to delivery (late gestation: $N = 5$).

Immediately after ovario-hysterectomy and AF gathering by needle aspiration (see below), tissue specimens were collected from placenta and from fetal membranes after careful separation into amnion, allantois, allantochorion and yolk sac. Specimens were fixed in formalin 10% for 24 h at 4 °C. After fixation, fragments were dehydrated in a graded series of ethanol and embedded in paraffin. Serial sections were cut at 4 μm thickness, de-waxed, and stained with routine hematoxylin and eosin (H&E) for histological examination.

2.1.1. Immunohistochemistry

Immunohistochemistry was performed using a DakoCytomation EnVision + Dual Link System-HRP (DAB+) kit (code #K4065, DakoCytomation, Denmark), a two-step technique that takes advantage of the superior sensitivity of a horseradish peroxidase-labeled polymer conjugated with secondary antibodies. The labeled polymer does not contain avidin or biotin so that non-specific staining resulting from endogenous avidin–biotin activity in tissues is eliminated.

After de-waxing, formalin-fixed serial sections (4 μm thick) were washed and immersed in a freshly prepared 3% H_2O_2 solution for 15 min to block the endogenous peroxidase activity, followed by incubation for 30 min at room temperature with 2% protease-free bovine serum albumin (BSA) (Sigma–Aldrich) in PBS-Tween to block non-specific antibody binding. Sections were then incubated overnight in a humidity chamber at 4 °C using rabbit polyclonal antibodies against rat AQP1 (Alpha Diagnostic International, San Antonio, TX, USA; cat # AQP11-A), AQP3 (Chemicon International, Inc, Temecula, CA, USA), AQP5 (Alpha Diagnostic International, San Antonio, TX, USA; cat # AQP15-A), AQP8 (developed in the laboratories of Prof. Ali Mobasheri, Nottingham University and Dr. David Marples, Leeds University, in partnership with Sigma-Genosys, Poole, Dorset, UK) and AQP9 (Alpha Diagnostic International, San Antonio, TX, USA; cat # AQP19-A) diluted 1:200 in PBS containing 2% of BSA. Subsequently, the sections were rinsed 3X 5 min in PBS-Tween before incubation with horseradish peroxidase-labeled polymer conjugated to affinity purified goat anti-rabbit and goat anti-mouse immunoglobulins for 30 min at room temperature. The sections were washed 3X 5 min with PBS before applying liquid DAB + Chromogen (DAKO; 3,3′-diaminobenzidine solution) for up to 10 min. Sections were counterstained with Mayers' hematoxylin, dehydrated, and mounted using Eukitt (Bioptica, Milan, Italy).

2.1.2. Staining controls

Sections of mouse kidney, sheep salivary gland and mouse liver, similarly processed as above, were used as positive controls for immunoreactions respectively of AQP1 and AQP3, AQP5, AQP8 and AQP9 antisera [3,5,24]. All of them gave the expected immunoreactivity (Figs. 1G, 2A and 3A, 4E, 5G).

The specificity of the immunostaining was tested by including negative controls, performed by (1) use of non-immune rabbit serum (DakoCytomation; code #X0903) in place of specific antisera; and (2) omission of the primary antibody. All of them resulted in the absence of immunoreactivity (Figs. 1F and 2G).

The evaluation of staining intensities was based on subjective estimates of at least two blinded authors. Slides were observed and photographed under an Olympus BX51 photomicroscope equipped with a digital camera and DP software (Olympus, Tokyo, Japan) for computer-assisted image acquisition and analysis.

2.2. Amniotic fluid biochemical analysis

AF was collected from the amniotic cavity at post-mating day 30, 38, 40, 55, 57, 60 and 62.

After AF collection, a part of the samples (2–3 mL) were centrifuged (177.4 × g, 22 °C, 10 min) and supernatants collected and stored at −70 °C for up to 1 month until determination of urea, electrolytes and total protein content using the Cobas Mira Plus Chemistry Analyzer (Roche Diagnostic, Italy). AF Na^+, K^+ and protein content and osmolality were not evaluated at day 19 as the AF volume was insufficient at early pregnancy.

The other part of the AF samples (1 mL) were not centrifuged and used for measuring osmolality by freezing-point depression (Automatic cryoscopic osmometer Osmomat 030, Gonotec, Berlin, Germany).

All samples were analyzed in one batch.

Data obtained were evaluated with ANOVA using the PROC GLM of SAS package (9.2 version).

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