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Research Article

Investigation of Aquaporin Molecules in the Placentas of Pregnant Women with Premature Rupture of Membranes

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Abstract

Aim: This study aimed to investigate the immunohistochemical expression of Aquaporin 3 (AQP3) in placentas of pregnant women with premature rupture of membrane (PROM) and to explore AQP3-related interactors and signaling pathways using in silico approaches.

Material and Method: Placental samples from 21 healthy (control) pregnant women and 21 pregnant women diagnosed with PROM were processed for routine histological tissue preparation. Sections were immunostained with AQP3 and analyzed under light microscope via ImageJ software. Protein-protein interaction (PPI) network of AQP3 was constructed with Cytoscape (version 3.10.2). Nodal centrality indexes (degree, closeness and betweenness) were computed through CentiScaPe plugin. The Enrichr tool was utilized to perform pathway enrichment analysis for 15 central genes.

Results: AQP3 immune activity was significantly decreased in the plasma membrane of the trophoblastic cell layer of the PROM group compared to control group. According to network centrality analysis, AQP subfamily proteins predominantly play central roles in the AQP3 network; Major Intrinsic Protein of Lens Fiber (MIP), Glycerol-3-Phosphate Dehydrogenase 2 (GPD2), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Glycerol Kinase 2 (GK2), GK, and Actin Beta (ACTB) with additional central interactors including proteins. Peroxisome proliferator-activated receptors (PPAR) signaling pathway was obtained as the most significantly enriched pathway.

Conclusion: Alterations in AQP3 expression level in the PROM group compared with the control group may contribute to disturbances in water transport and cellular homeostasis in placental tissues and in silico potential interaction between AQP3 expression and PPAR signaling suggest the role of AQP3 in cell metabolism in PROM.

Keywords: Premature rupture of membranes, aquaporin 3, placenta, immunohistochemistry

INTRODUCTION

Premature rupture of membranes (PROM) a common obstetric complication causing perinatal mortality and morbidity, affecting 5-10 % of all pregnancies (1,2). PROM can significantly have adverse impacts on the health of neonates, comprising main reasons for neonatal survival and stay in the intensive care unit (3). Maternal, neonatal and environmental factors are etiologically risk factors. Many predisposing factors such as preeclampsia, cervical effacement, congenital malformations, autoimmunity, previous high-risk pregnancies, exposure to heavy metal poisoning, use of assisted reproductive techniques, and stress are strongly associated with PROM (4,5).

Water transport in the cell membrane structure is facilitated by integral membrane proteins called aquaporins (AQPs) (6). AQPs are channel proteins that use osmotic charge to help water transport (7). So far, 13 aquaporins have been discovered. Aquaporin 3 (AQP3) is one of the well-investigated of AQPs and it is mainly found in epithelial layer of the skin. Ear, eye, intestine and human placenta also have AQP3 expression. Although, AQPs has been known to take role in transport of water and various substances,

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recently, the role of AQPs has been a subject of curiosity in placentas. Damiano et al showed that AQP3 was located to on the apical surface of syncytiotrophoblasts since these cells played an important role in transport of metabolites, ions and water from mother to fetus (8). The authors speculated that AQP3 may be an important regulator of these changes. Another study also revealed that AQP3 level was decreased in placentas of preeclamptic patients. The authors claimed that the reduction in AQP3 level was an adaptive response to trophoblastic apoptosis (8,9).

There are many studies on AQP3 and placentas, yet none include association between AQP3 and placentas of patients with PROM. In this study, we examined the level of aquaporin 3 and investigated whether it is a possible regulator in the molecular mechanism of PROM.

MATERIAL AND METHOD

Study Design

Ethical approval was taken from Non-interventional Clinical Research Ethical Committee of Faculty of Medicine, Dicle University (approval no: 188). The placentas of 21 healthy (control) pregnant women and 21 pregnant women who diagnosed with PROM were collected. Small samples were dissected and objected to routine histological tissue preparation.

Tissue Preparation

The placenta tissues were placed in 10% neutral formalin solution for fixation. The tissues were then placed under running tap water for 12 hours and dehydrated by passing through increasing alcohol series (50%, 70%, 80%, 90%, 96% and absolute ethyl alcohol). Samples were kept in xylene for 3 x 15 minutes and placed in paraffin incubation. 5 µm thick sections were cut from paraffin blocks using a microtome (catalog number: LeicaRM2265, Wetzlar, Germany).

Immunohistochemical Staining

Placental sections were dewaxed, ran through a decreasing series of alcohol series, and then cleaned with distilled water. The endogenous peroxidase activity was inhibited by using hydrogen peroxide (H2O2). After being cleansed in PBS, the sections were treated with blocking solution. The sections were incubated with the primary antibody Aquaporin 3 (catalogue no: Santa Cruz 518001 Biotechnology Inc. CA, USA) for an overnight period at +4°C. The sections were incubated with a secondary antibody the next morning, and then were treated with streptavidin peroxidase reaction for 15 minutes. Diaminobenzidine (DAB) chromogen was applied following PBS washing. Sections were counter-stained with Hematoxylin. All sections were imaged by Zeiss Imager A2 light microscope.

ImageJ Analysis

ImageJ software was used to process and quantify each image. The AQP3 staining intensity was determined using the Image J software (version 1.53, http://imagej.nih.gov/ij). The measurement was computed using Crowe et al.'s approach (10). In order to record the quantification, ten fields from each specimen in each group were examined (11,12). In specimens, the color blue denotes a negative expression of the target antibody, whereas the color brown indicates a positive expression of the antibody. The calculation of signal intensity (expression) from a field involved dividing the intensity of the target antibody by the total area of the sample. For every sample from 10 fields, a staining area/whole area value was computed. Measured for each group, an average value was examined for semi-quantitative immunohistochemical scoring. Zeiss Imager A2 was used to image the slides.

Network Topology Analysis

Using Cytoscape (http://www.cytoscape.org/, version 3.10.2), we constructed the protein-protein interaction (PPI) network of AQP3 by querying the Search Tool for the Retrieval of Interacting Genes (STRING) database with a reliability threshold set to less than 0.4, and subsequently selecting 50 additional interactors. The network topology was analysed by calculating nodal centralities using the CentiScaPe 2.2 plugin. Degree centrality was computed to identify central regulator interactors, closeness centrality was calculated to assess functional relevance probability of interactors, and betweenness centrality was computed to evaluate organizational regulator characteristics of interactors (13). The nodes were colored based on closeness values, while node sizes were set according to degree values.

Pathway Enrichment Analysis

The proteins within the network were initially ranked according to their degree values. To include additional interactors beyond different AQPs, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted using Enrichr for the top 15 proteins (14). It was conducted to observe which signaling pathways could be affected by the expression difference obtained in healthy and PROM pregnant women for AQP3. KEGG (https://www.kegg.jp), an extensively utilized repository, contains extensive information on human cell signaling and metabolic pathways, genomes, diseases, and pharmaceuticals. A significance threshold of $p<0.05$ was applied for statistical significance. Significant terms were ranked based on their p values, and a bar graph depicting the top ten enriched terms was generated.

Statistical Analysis

IBM SPSS 25.0 (IBM, Armonk, New York, US) was used for statistical analysis. The median (Quartiles 1–3) was used to record the data. Using the Shapiro-Wilk test, statistical distribution was assessed. The Mann-Whitney U test was used for binary group comparisons. $p<0.05$ were regarded as significant. Using G Power analysis (version 3.1), the number of patients for each group was determined. Alviggi et al.'s work served as the basis for the definition of Cohen's criterion (15).

RESULTS

Demographical Features

Maternal and fetal parameters belonging to control and PROM groups were listed in Table 1. Compared to control group, gestational age at delivery week was earlier and cesarean ration was higher in PROM group.

Table 1. Demographical properties of patients by groups

	Control (n=21)	PROM (n=21)
Maternal age, year	27.35±2.59	31.28±5.57
Gravida, n	1.36±0.85	2.93±1.58
Parity, n	1.13±0.92	1.73±1.84
Gestational age at delivery (week)	38.76±1.56	32.43±2.05
Birth week,	39.33±1.25	38.22 ±2.50
Birth weight, g	3462.29±287.28	3043.30±329.83
BMI, kg/m²	27.48±3.28	28.35±4.20
Cesarian birth, %	42	61

PROM: premature rupture of membranes, BMI: body mass index

Immunoexpression of AQP3

AQP3 immune staining of placental tissue sections is shown in Figure 1. In the images of the control group, AQP3 expression was abundant in plasma membrane of cytotrophoblast and syncytiotrophoblast and plasma membrane of cells of connective tissue in placental villi. (Figure 1a-c). When compared to control group, PROM group showed abundant negative AQP3 immune activity in plasma membrane of trophoblastic cell layer and in mesoderm of placental villi (Figure 1d-f). This alteration in AQP3 expression may have implications for water transport or homeostasis within these cells and tissues between groups, suggesting deterioration of cellular homeostasis in PROM group.

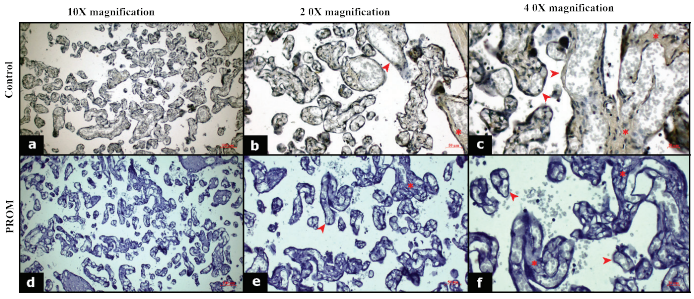

Figure 1. Placental sections of control group (a-c); positive expression of AQP3 in plasma membrane of cyto- and syncytiotrophoblast cells (arrowhead) and in plasma membrane of connective tissue cells (asterisk). Placental sections of PROM group (d-f); negative expression of AQP3 in plasma membrane of cyto- and syncytiotrophoblast cells (arrowhead) and in plasma membrane of connective tissue cells (asterisk); aquaproin3 immune staining, Scale Bar: 100 µm (10X), 50 µm (20X), and 50 µm (40X)

Table 2 displayed the semiquantitative measurement of AQP3 immune staining.

Table 2. AQP3 signal level in groups

	Control	PROM	p value
	Median (Q1-Q3)		
AQP3 signal	27 (25-31)	5.8 (5.3-6.7)	<0.0001*

* Mann Whitney U test, PROM: premature rupture of membrane

Figure 2 displayed a graphic representation of signal intensity and its importance.

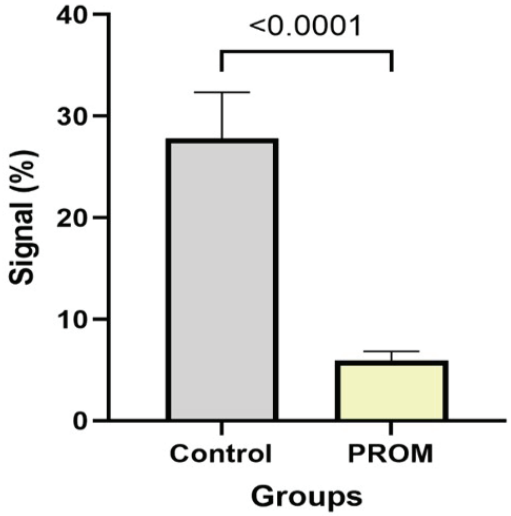

Figure 2. Graphical illustration of aqp3 signal intensity and significance between groups

In-silico findings

The AQP3 protein-protein interaction (PPI) network consists of a total of 51 nodes and 361 edges, as illustrated in Figure 3A. Using CentiScaPe, degree, closeness, and betweenness centrality values were calculated for the AQP3 PPI network, and the top 15 interactor proteins based on degree values were listed in Table 3. Closeness centrality values exhibited a similar pattern to degree values, decreasing from high to low for top central interactors. Additionally, betweenness centrality values, with some exceptions, revealed the same trend as degree and closeness centrality values. Centrality analysis revealed that members of the AQP subfamily (AQP9, AQP2, AQP1, AQP7, AQP10, AQP5, AQP8, AQP10, AQP6) were significant remarkable central genes within the AQP3 network. Additionally, proteins including Major Intrinsic Protein of Lens Fiber (MIP), Glycerol-3-Phosphate Dehydrogenase 2 (GPD2), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Glycerol Kinase 2 (GK2), GK, and Actin Beta (ACTB) were identified as central interactors. Furthermore, KEGG pathway analysis indicated that the top 15 central genes are enriched in the peroxisome proliferator-activated receptors (PPAR) signaling pathway ($p<0.05$) (Figure 3B).

Table 3 degree, closeness, and betweenness values for the top 15 central interactors in the AQP3 PPI network.

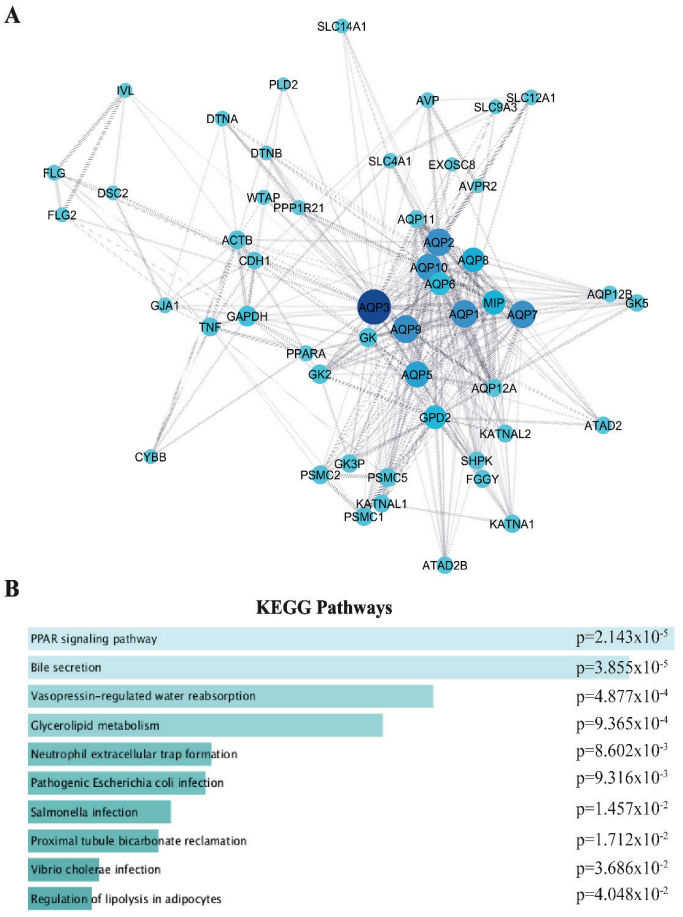

Figure 3. AQP3 PPI network and pathway enrichment; **A.** network centralities analysis was performed by computing degree, closeness and betweenness through CentiScape; the nodes were colored from light blue to dark blue, representing decreasing to increasing closeness values, respectively. Node sizes represent degree values, with larger nodes indicating higher degree values; **B.** significantly enriched KEGG pathway terms for the central 15 proteins of AQP3 PPI network (p<0.05)

Table 3. Degree, closeness, and betweenness values for the top 15 central interactors in the AQP3 PPI network

Gene symbol	Degree	Closeness	Betweenness
AQP3	50.0	0.02	866.97
AQP9	34.0	0.0151	128.84
AQP2	33.0	0.0149	141.32
AQP1	33.0	0.0149	130.65
AQP7	33.0	0.0149	100.39
AQP10	32.0	0.0147	88.42
AQP5	30.0	0.0143	71.93
AQP8	27.0	0.0137	25.72
MIP	26.0	0.0135	25.36
GPD2	25.0	0.0133	21.50
AQP6	23.0	0.0130	24.86
GAPDH	18.0	0.0122	62.29
GK2	15.0	0.0117	6.66
GK	15.0	0.0117	6.66
ACTB	14.0	0.0116	27.71

DISCUSSION

In this study, we reported AQP3 expression in placenta with PROM. The pathophysiology of PROM is multifactorial. The most important cause of term PROM is physiological changes occurring in the membranes (16). Membrane weakening is caused by an increase in local cytokines, collagenase and protease activity, an imbalance between matrix metalloproteinases and tissue inhibitors, and other factors that may lead to increased intrauterine pressure (17).

AQPs are small membrane proteins (30 kDa) found in different organs and responsible for permeability of water. Maternal-fetal fluid balance has critical importance during pregnancy. Therefore, many molecules, including AQPs, have been shown to play important roles in maternal-fetal fluid balance. When the homeostasis of maternal-fetal fluid exchange is disrupted, abortion, premature birth, abnormality in amniotic fluid volume, malformation and fetal growth restrictions may occur (18). Therefore, AQPs may involve in many cellular processes throughout placentation.

Studies demonstrated that AQP3 is present in the placenta and fetal membranes not only during normal pregnancy but also in pathological conditions such as polyhydramnios and oligohydramnios (19). In an animal model study of intrauterine growth restriction, Seo et al. (20) showed that AQP3 deficiency in placental componenets was associate with impared placental function, reduced amniotic fluid volume and fetal growth. Zhu et al. (7) examined the normal amniotic fluid and oligohydramnios patients to investigate role of AQP3.The authors found that APQ3 expression was localized in amnion, chorion and placental structures such as trophoblast cells. Moreover, AQP3 expression was significantly increased in oligohydramnios placentas compared to normal amniotic fluid volume patients.The authors suggest that alterations in AQP3 expression may be important in the pathophysiology of oligohydramnios. Another study indicated that AQP3 expression was significantly reduced in placentas of patients with preeclampsia compared to placentas from healthy pregnant women (21). Similarly, reduced AQP3 was found in gestational diabetes mellitus patient, causing increased risk for pregnancy anomalies such as cesarean section, macrosomia, fetal distress and neonatal asphyxia (22).

In our study, we analyzed AQP3 expression in the placentas of pregnant women with PROM comparing to placentas from healthy pregnant women. Consistent with previous studies, we detected AQP3 expression in syncytiotrophoblasts. Compared with the control group, we showed that AQP3 expression was decreased in the plasma membrane of the trophoblastic cell layer and mesoderm of the placental villi in the PROM group. This change in AQP3 expression may have effects on water transport or homeostasis in these cells and tissues between the groups, suggesting that cellular homeostasis was impaired in the PROM group.

In network topology analysis, it was observed that proteins belonging to the AQP subfamily predominantly play regulatory roles as central interactors associated with AQP3. Additionally, AQP3 was shown to be related to MIP, GPD2, GAPDH, GK, GK2, and ACTB proteins. The PPAR signaling pathway emerged as the most highly annotated pathway in the analysis of potential pathways affected by the expression difference observed for AQP3 in healthy and PROM pregnant women. PPARs play significant roles in regulating lipid and glucose metabolism, inflammation, and angiogenesis, facilitating the mother's adaptation to the nutritional and perfusion needs of the fetus. The regulation of the PPAR pathway has been shown to be involved in trophoblast invasion and placental development, and it is crucial in the risk of pregnancy-related conditions such as gestational diabetes mellitus, intrauterine growth restriction, and preeclampsia (23,24). Additionally, it influences apoptotic mechanisms implicated in fetal membrane rupture and may contribute to preterm delivery (25). One of the studies was demonstrated that mono-2-ethylhexyl phthalate reduces PPARγ activity and its anti-inflammatory properties. As a result, it has been emphasized that amniotic PPARγ disruptors could potentially cause premature rupture of fetal membranes (26). Based on the results of in silico analysis, we suggest that the potential regulation of PPAR, which is crucial in various aspects of maternal-fetal health, may be influenced by the effects of AQP3. This highlights the importance of further investigation of the interplay between AQP3 expression and PPAR signaling in the context of pregnancy-related conditions and pregnant women with PROM, as well.

CONCLUSION

The results strongly suggest that the changes in AQP3 expression in the PROM group compared to the control group may contribute to disruptions in water transport and cellular homeostasis within the placental tissues. This alteration in AQP3 expression could be a relevant factor in understanding the mechanisms underlying the pathophysiology of PROM, highlighting the importance of aquaporin-mediated water transport in maintaining placental health and function. Further research and exploration of the specific implications of AQP3 dysregulation in PROM may provide valuable insights into the development and progression of this condition.

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Ethical approval: *Ethics Committee of Dicle University Faculty of Medicine, Non-Interventional Clinical Researches with decision no. 188 on 2023/06.*

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