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Research article

AQP1 expression in the proximal tubule of diabetic rat kidney

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ABSTRACT

Polyuria is a hallmark symptom and the first clinical manifestation of diabetes mellitus (DM). The glucose that remains in renal tubules was proposed to produce an osmotic effect resulting in polyuria. Although water is reabsorbed in proximal tubules through an aquaporin-1 (AQP1) dependent mechanism, AQP1 role in the genesis of polyuria is unknown. AQP1 expression was studied in a rat model of Type-1 DM at 15-days and 5-months of evolution. A different AQP1 expression pattern was found in both experimental groups, with no changes in AQP1 localization, suggesting that changes in AQP1 may be involved in the development of polyuria.

1. Introduction

One of the main functions of proximal tubule is reabsorption. It reabsorbs 2/3 of all the filtered Na^+, K^+, Cl^- and virtually all the filtered glucose and aminoacids. It also reabsorbs 2/3 of the filtered water [1, 2].

Polyuria is a hallmark symptom and the first clinical manifestation of diabetes mellitus (DM). Polyuria has been explained as a result of the osmotic effect exerted by the glucose that remains in renal tubules. Glucose filters freely through the glomerular barrier and in physiological conditions, is completely reabsorbed in the proximal tubules. Glucose reabsorption is mediated by sodium-dependent glucose cotransporters (SGLTs) [3]. When glucose levels exceed the reabsorption capacity of SGLTs, they become saturated and glucose remains in the tubular lumen. Thus, this glucose cannot be reabsorbed in latter portions of the nephrons and is eliminated, producing an osmotic retention of water in the urine. Accumulated data show that persistent high tubular glucose may result in a primary defect in collecting ducts rendering them impermeable to water [4]. Although roughly 65% of filtered fluid is reabsorbed in the proximal tubule by a mechanism mediated by aquaporin-1 (AQP1), the role of this protein in the development of polyuria is not fully understood.

AQP1 is a water-channel protein, constitutively and abundantly expressed on both the apical and basolateral domains of proximal tubular cells and the descending limb of Henle's loop cells [5]. Increasing evidence suggests that the expression of AQP1 is associated to hypertonicity [6, 7]. However, up to now, the consequences of hyperglycemia in proximal tubule water transport was unexplored.

To the best of our knowledge, there are few previous studies addressing AQP1 changes in the kidney during DM, but their results are contradictory [8, 9].

Here, we analyzed the expression and localization of AQP1 in the kidney of diabetic rats at 15-days and 5-months of evolution.

2. Methods

2.1. Animal model

Adult male Sprague-Dawley rats weighing 150–250 g, were obtained from the animal facility at the Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Argentina.

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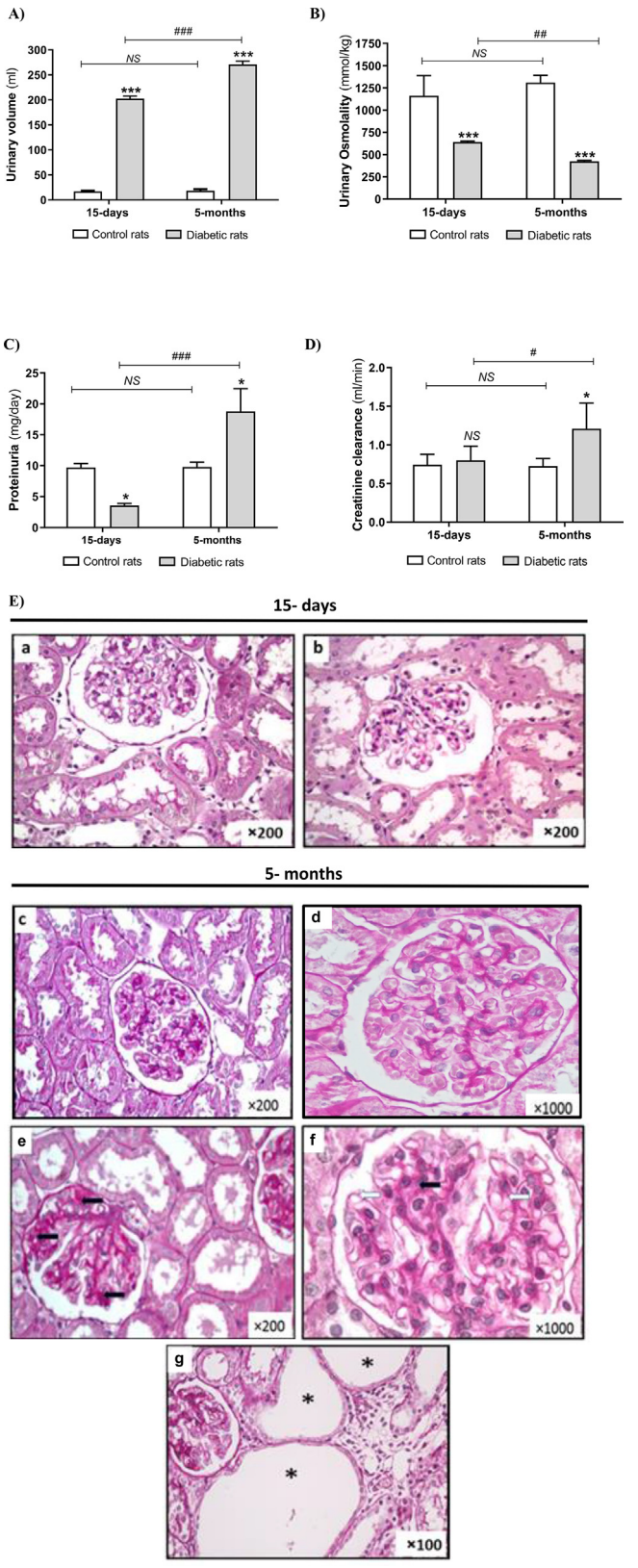

Figure 1. Functional and histological studies in rats after 15 days and 5 months of diabetes induction. As expected, the increase in the urinary volume (A) in both diabetic groups resulted in a significant decreased in urinary osmolarity (B), being lower in animals after 5 months post STZ injection than in those after 15 days post SZT injection. In addition, rats after 15 days of diabetic induction showed no significant alterations in creatinine clearance (D), but proteinuria was surprisingly decreased possible due to a compensatory mechanism of protein reabsorption (C). On the other hand, after 5 months of diabetic induction, both proteinuria (C) and creatinine clearance (D) were significantly increased. Results are expressed as means ± SEM. (n = 6 per group; ***P < 0.001; ##P < 0.01; ###P < 0.001; NS: non-significant). E) Histological studies in kidney of rats. No alterations were observed in rat kidneys after 15 days of diabetes induction (b) compared to control ones (a). However, after 5 months of diabetes induction, mesangial expansion (black arrows) and thickening of the capillary basal membrane (white arrow) were observed (e & f) compared to control ones (c) and (d). Tubular dilatation was also found (asterisks) (g).

(caption on next column)

The experimental protocols and euthanasia procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Facultad de Medicina, Universidad de Buenos Aires, Argentina (CICUAL; CD Resolution No. 2356/11). All the procedures were performed in accordance with the EEC guidelines for care and use of experimental animals (EEC Council 86/609).

Rats were randomly divided into four groups (n = 6 per group). Groups 1 and 2 (experimental groups) were injected intraperitoneally with Streptozotocin (STZ) (Sigma-Aldrich Corp., San Luis, MO, USA) to induce diabetes. Rats were fasted overnight and injected with 65 mg/kg of STZ diluted in freshly prepared citrate buffer (pH 4.5). Induction of diabetes was confirmed 72 h post STZ injection by measuring glycemia in tail vein (Freestyle optium™, Abbot, Oxon, UK). Animals presenting glycemia >350 mg/dL were included in the study. Groups 3 and 4 (control groups) were injected with PBS by the same route. Groups 1 and 3 were studied at 15 days and Groups 2 and 4 at 5 months. The rats were individually housed under controlled conditions of illumination, humidity, and temperature, with food and water being available *ad libitum*. 24 h prior to sacrifice, to study the renal function animals were placed on metabolic cages for urine collection and blood samples were obtained by cardiac punction. Serum and urinary creatinine levels and urinary protein concentration were determined using a Cobas C311 Autoanalyzer (Roche Diagnostics, Basel, Switzerland). Creatinine clearance was also calculated by the following formula:

urinary flow (ml/min) × urinary creatinine (mg/dl) / plasma creatinine (mg/dl)

Urinary osmolality was also measured using a pressure vapor osmometer VAPRO™ (Wescor Inc., Logan, UT, USA).

Finally, rats were anesthetized (100 μg ketamine and 10 μg diazepam per g of body weight, intraperitoneally). Left kidney was removed and conserved at -20 °C to molecular studies and then the animals were perfused with 4% paraformaldehyde. Right kidney was removed, and the tissues were fixed in 10% neutral formalin.

Histological PAS sections of each groups of rat kidneys were also analyzed.

2.2. Real time RT-PCR (qRT-PCR)

Real-time PCR was performed using an iQ™ SYBR® Green Supermix master mix (BioRad, California, USA) on an Applied Biosystems 7500 real-time PCR system (ThermoFisher Scientific, Massachusetts, USA). Samples were run in triplicate to ensure the reproducibility of the results. The thermal cycling program consisted of 95 °C for 3 min for polymerase activation, and then 40 cycles of denaturation (95 °C for 15 s) and annealing and extension (58 °C for 1 min). Reactions were quantified by selecting the amplification cycle when the PCR product of interest was first detected [threshold cycle (Ct)]. Data were analyzed by the comparative Ct method. The primer sequences used in this study were as follows: AQP1, forward 5′- ACCTGCTGGCCATTGACTAC -3′ and reverse

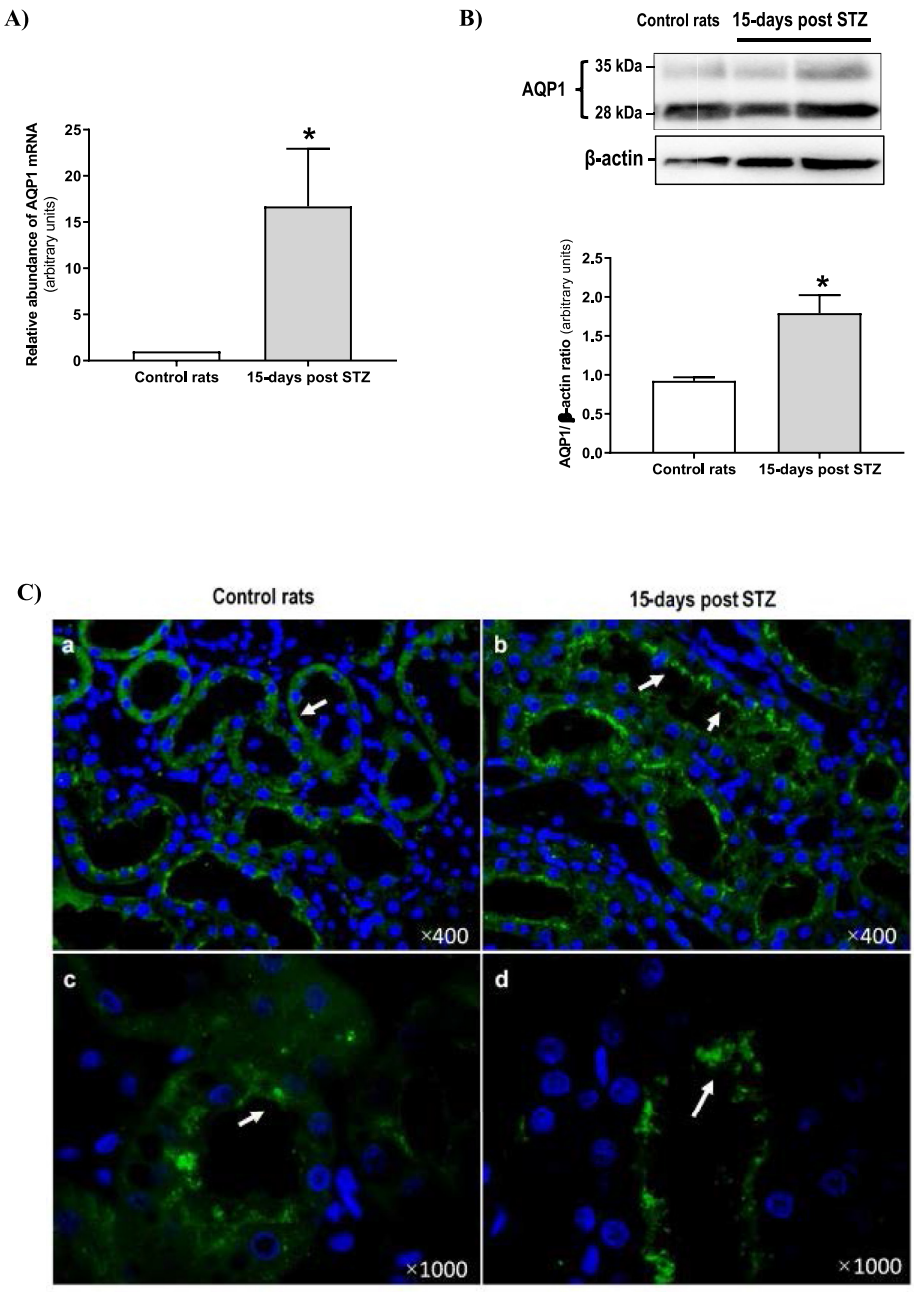

Figure 2. Expression of AQP1 after 15-days of diabetic induction. AQP1 mRNA (A) and protein (B) significantly increased. Results are expressed as means ± SEM. (n = 3 per group; *P < 0.05). The full images of the blots are provided in supplementary material. In agreement immunofluorescence studies (C) showed an increase in AQP1 signal mainly localized on the apical membranes. (a) and (c) Controls; (b) and d) diabetic group. Positive AQP1 is stained in green. Nuclei are stained in blue with DAPI.

5′- CCAGGGCACTCCCAATGAAT-3′; β-actin, forward 5′- CTGTGT GGATTGGTGGCTCT -3′ and reverse 5′- CAGCTCAGTAACAGTCCGCC-3′.

2.3. Western blot

Kidney cortex lysates were obtained in RIPA buffer (50 mM Tris-HCl pH 7,4, 150 mM NaCl, 1% Triton x-100, 1 mM EDTA, 0.1% SDS, 1% sodium deoxycholate) containing 0.2 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich Corp., San Luis, MO, USA) and 0.01x Protease Inhibitor Cocktail Set III (Calbiochem®, EMD Millipore Corporation, Darmstadt, Germany). The total protein concentration was measured using a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific Inc. Waltham, MA, USA). Total protein lysates (~50 μg) were used for immunoblotting studies. After blocking, membranes were incubated overnight with the primary antibody anti-AQP1 (Alpha Diagnostic International Inc., San Antonio, TX, USA; 1:1000) and then with a goat anti-rabbit immunoglobulin G ([IgG] Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; 1:10,000) conjugated to peroxidase. Immunoreactivity was detected using the Enhanced Chemiluminescence (ECL) Western Blotting Analysis System (ECL plus, Amersham Pharmacia Biotech Ltd, Pittsburgh, PA USA) according to the manufacturer's instructions. To confirm equal loading, each membrane was also analyzed for β-actin protein expression. Densitometry was performed and the values were plotted as AQP1/β-actin.

2.4. Immunofluorescent microscopy

Paraffin sections were incubated overnight with the primary antibody (Alpha Diagnostic International Inc., San Antonio, TX, USA; 1:100). Later, the samples were incubated with a secondary antibody conjugated to Alexa Fluor 488 (Abcam, Cambridge, MA, USA). Tissues were mounted with Fluoroshield mounting medium with DAPI (Abcam, Cambridge,

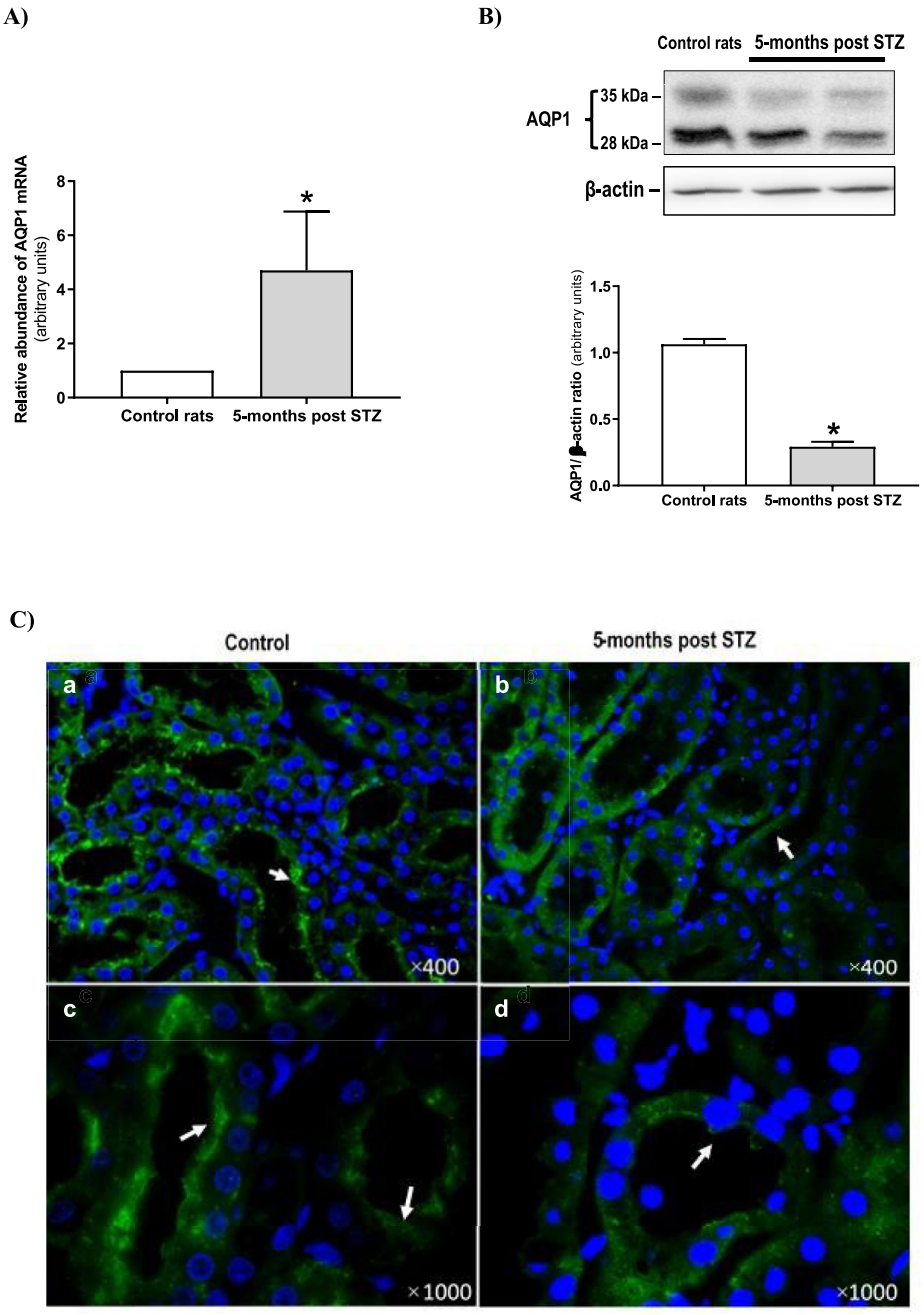

Figure 3. Expression of AQP1 after 5-months of diabetic induction. AQP1 mRNA (A) was increased while the protein levels (B) were significantly decreased. Results are expressed as means ± SEM. (n = 3 per group; *P < 0.05). The full images of the blots are provided in supplementary material. Immunofluorescence experiments (C) showed that AQP1 cellular localization was conserved. (a) and (c) Controls; (b) and d) diabetic group. Positive AQP1 is stained in green. Nuclei are stained in blue with DAPI.

MA, USA) to label nuclei and analyzed with an epifluorescent microscope (Nikon, Eclipse E:200).

2.5. Statistical analysis

Statistical analysis of data was performed by GraphPad Prism v5 software (GraphPad Software, Inc. La Jolla, CA, USA). Results represent mean ± SEM. Comparisons were performed using two-way analysis of variance (ANOVA) followed by Tukey's post-hoc tests where appropriate. Student's t-test was used when the values of two groups were analyzed. Values were considered significantly different when $p < 0.05$.

3. Results

First, we analyzed the presence of polyuria and the urine osmolality in control and diabetic rats. Both groups (15 days and 5 months after diabetes induction) presented polyuria compared to each control group. However, urine volumes were significantly higher in diabetic rats after 5 months of diabetes induction than in those after 15 days of diabetes induction (Figure 1A). Urine osmolalities were also significantly decreased in both groups compared to control but were lower in rats after 5 months of STZ injection (Figure 1B).

After 15 days of STZ treatment, no difference was observed in the creatinine clearance between control and diabetic rats, suggesting that renal function was not altered, while proteinuria was surprisingly decreased in the diabetic group (Figure 1C & D). On the contrary, 5 months after the induction of diabetes, we observed the development of proteinuria (Figure 1C) and the increase of the creatinine clearance in the diabetic rats (Figure 1D). In addition, we also found that after 5 months of diabetes induction rat kidneys showed glomerular alterations typically associated with diabetes such as mesangial expansion, thickening of the capillary basal membrane, and tubular dilatation. No

alterations were observed in rat kidneys after 15 days of diabetes induction (Figure 1E).

Then, we studied AQP1 expression. We found that 15 days after the induction of diabetes, when renal function is still normal, a significant increase of AQP1 mRNA and protein expression was observed (Figure 2A & B). In agreement with these results, immunofluorescence confirmed an increase in the AQP1 signal and showed that it was localized mainly on the apical membranes of renal tubules (Figure 2C).

On the other hand, 5 months after diabetes development when renal function was altered, the expression of AQP1 mRNA was increased while the protein levels were significantly decreased (Figure 3A & B). Immunofluorescence experiments showed that AQP1 cellular localization was unchanged (Figure 3C).

4. Discussion

In this work, our results show that 15 days after diabetes induction, rats presented normal renal function. In these rats, AQP1 mRNA and protein levels were significantly increased. In agreement with previous reports, we propose that the excess of glucose in the proximal tubule could activate an hypertonicity-responsive elements on AQP1 gene which results in the protein increase [6, 7]. This finding could be considered as an adaptive mechanism similar to that previously observed in SGLT2 expression [3, 10].

On the other hand, 5 months after the induction of diabetes, our results confirmed that renal function was impaired by the increase in both proteinuria and creatinine clearance. The increase in the creatinine clearance indicates the development of hyperfiltration, a characteristic feature of the incipient diabetic nephropathy. In this group, we also observed that the transcriptional level of AQP1 was increased while AQP1 protein expression was significantly decreased without changes in its cellular localization. This discrepancy between mRNA and protein levels is not clear yet but it is possible that the decrease of AQP1 expression may be induced by an increased degradation of the protein. In addition, Hassouneh and coworkers recently reported in a mice model that AQP1 protein expression decreased 12 weeks after STZ injection as consequence of kidney damage [8]. Consistent with this work, we also found histological changes in the kidney at 5-month post STZ injection. Taking into consideration these findings, we propose that even though the osmotic effect of glucose induces the increase of AQP1 mRNA, the damage in the kidney may mediate an inadequate cell response and abrogate AQP1 protein expression.

Regarding urine output, both groups (15 days post injection and 5 months post injection) presented polyuria. However, urine production was significantly increased at 5 months post diabetes induction. Accordingly, urine osmolality was also significantly lower in rats at 5 months post diabetes induction compared to rats at 15 days post diabetes induction. Therefore, the increased expression of AQP1 protein observed 15 days post STZ inoculation may correlate with the decrease in urine output and consequently, with the increased urine osmolality. So, we suggest that the altered AQP1 expression could be an adaptive mechanism involved in water management during the increase of osmolality. On the contrary, the decreased AQP1 protein expression may reduce the water reabsorption in the proximal tubule resulting in an increased urine volume and a reduction in the osmolality. This analysis is consistent with previous reports which showed that mice lacking AQP1 are unable to concentrate urine, even after water deprivation [11].

Along with this idea, our results suggest that polyuria may not be only attributed to the osmotic effect of glucose, the classical mechanism, but it also may be associated to a decreased expression of AQP1 protein in the latter period.

Declarations

Author contribution statement

E. Seyahian: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

L. Cacciagiu: Performed the experiments.

A. Damiano: Conceived and designed the experiments; Analyzed and interpreted the data.

E. Zotta: Conceived and designed the experiments; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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