Amiloride modifies the progression of lithium-induced renal interstitial fibrosis.

Running title: Amiloride and lithium-induced chronic interstitial fibrosis

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ABSTRACT:

Aim: Long-term administration of lithium has been associated with the development of a chronic interstitial fibrosis in addition to nephrogenic diabetes insipidus (NDI). Earlier studies have demonstrated that amiloride, by blocking the epithelial sodium channel ENaC and thus preventing lithium uptake into the principal cells of the collecting ducts, can partially reverse lithium-induced NDI. However, there are no long-term studies examining whether or not amiloride also modifies the progressive chronic interstitial fibrosis and tubular atrophy often evident with long term lithium exposure.

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Methods: Using an established animal model of lithium-induced chronic interstitial fibrosis, rats were treated with amiloride and lithium for 5 months following one month of exposure to lithium alone and compared with control animals and those given only lithium.

Results and Conclusions: In this study, the 5 months of amiloride therapy partially mitigated the lithium-induced NDI and limited the further progression of lithium-induced kidney fibrosis. This improvement was associated with decreased expression of the pro-fibrotic connective tissue growth factor (CTGF), along with reduced myofibroblast infiltration and decreased collagen deposition around the distended cortical collecting ducts. This may, in part, be mediated by modifying lithium-induced alterations in β-catenin activity through its effects on GSK-3β.

KEY WORDS: Lithium, amiloride, chronic interstitial fibrosis.

Lithium is a widely used and effective therapy to treat individuals with mood disorders. However, lithium therapy has well documented side effects on the kidney, most obviously the development of nephrogenic diabetes insipidus (NDI).[1] Additionally, long term medication with lithium has been associated with the development of a chronic focal interstitial fibrosis, tubular atrophy and focal glomerulosclerosis[2] that may progress to end stage kidney disease.[3] A chronic, 6 months, rat model of lithium-induced renal fibrosis, with minimal active inflammation, which mimics the chronic kidney interstitial fibrosis seen in the human kidney, has been previously described.[4] This was characterised by cystic dilatation of the cortical collecting ducts, a progressive interstitial fibrosis, with increasing numbers of myofibroblasts, enhanced transforming growth factor-β1 (TGF-β1) expression, interstitial collagen deposition and a minimal inflammatory cellular response. The earliest changes were evident at 1 month and progressed over the subsequent 5 months. It has been suggested that further mechanistic validation of this model may make lithium a very useful tool for the study of pathogenesis of chronic interstitial fibrosis with minimal impact on renal function.[5]

The main action of lithium appears to be on the principal cells of the collecting duct, which has been well described by numerous authors, and which leads to NDI.[1,6,7] Lithium enters these cells via the epithelial sodium channel ENaC, situated on the apical (luminal) membranes.[8,9] . Within the principal cells, the initial effect of lithium is to inhibit arginine vasopressin stimulated aquaporin-2 (AQP2) translocation to, and its insertion into, the apical cell membrane. Longer term, lithium also down-regulates AQP2 synthesis.[6] The lack of AQP2 creates a barrier to the passive movement of water down its osmotic gradient, leading to the excretion of a large volume of dilute urine, manifest as NDI. Amiloride acts to block lithium uptake through ENaC.[8,9] The relatively short term administration of amiloride has been shown to partially correct the lithium-induced NDI in both animal and human studies.[10,11] However, to date there are no long term studies examining whether or not partial correction of the lithium-induced NDI with amiloride modifies the progressive chronic interstitial fibrosis, tubular atrophy and glomerular sclerosis often evident with long-term lithium exposure. In a clinical setting, amiloride administration is not usually commenced at the time of initiation of lithium therapy, rather only if the lithium-induced NDI becomes a clinical problem. Therefore, using our rat model of chronic lithium exposure,[4] amiloride treatment was commenced after the establishment of lithium-induced NDI, in order to reflect usual clinical practice. In this study, 5 months of lithium therapy is shown to partially correct the lithium-induced NDI and limit the further progression of lithium-induced kidney fibrosis. This improvement was associated with decreased expression of the profibrotic cytokine connective tissue growth factor (CTGF), along with reduced myofibroblast infiltration and decreased collagen deposition around the distended cortical collecting ducts.

METHODS

Animals and protocols

Wistar male rats (200 g) from the Hercus-Taieri Resource Unit, University of Otago were separated into 3 groups, each of 6 rats (i) a control group, (ii) a lithium group, and (iii) an amiloride group. Ethical approval for the protocols described here was given by the University of Otago Animal Ethics Committee (92/08), under New Zealand National Animal Welfare guidelines.

To induce NDI and subsequent chronic interstitial fibrosis, the protocol of Kwon *et al*.[12] was extended.[4] The control group was kept for 6 months on a standard rodent diet (Speciality Foods, Perth, Australia) and given tap water *ad libitum.* Both experimental groups received tap water and the same standard rodent diet with the addition of 40 mmol lithium/kg dry food (as lithium carbonate) for the first 7 days, followed by 60 mmol lithium/kg dry food (lithium diet) for the rest of the experimental period. To enable adequate mixing of the diet with the lithium, all food pellets were pulverised to a fine powder in a blender. There was no difference in weight gain between control rats fed whole or powdered pellets. It has been well established that this protocol results in plasma lithium levels comparable to the therapeutic levels in human plasma (0.8-1.3 mmol/l) and minimises the reduced weight gain that can be caused by lithium. The amiloride group was given the same diet as the rats in the lithium group but after 1 month their drinking water was supplemented with 0.2 mmol/l amiloride.[10] The NDI caused by lithium can lead to dehydration, salt wasting and lithium intoxication. Consequently, all rats administered lithium were given access to a salt block. Body weight was measured weekly and water intake daily.

To verify previously examined physiological and morphological changes after 1 month exposure to lithium,[10] a further 4 rats were given a lithium diet as above. All rats prior to euthanasia were placed singly in metabolic cages and allowed to settle for 24 hours. In the subsequent 24 hours, water uptake (mls) was measured, and urine was collected under mineral oil, the volume measured (mls) and then stored at -20^{o}C for later analysis. Animals were then narcotised with carbon dioxide, killed by decapitation, and the kidneys removed. The kidneys were sectioned longitudinally: one half was placed in 10% neutral buffered formalin for 4 hours, microwaved to 55^{o}C for 5 minutes, then placed in 70% alcohol for immunohistochemistry, and the remaining halves were placed in RNALater® (Ambion, Life Technologies, ThermoFisher Scientific, Waltham, MA, USA). At the time of decapitation, blood was collected from the neck, spun immediately, and the plasma decanted and frozen for later analysis. The control, lithium and amiloride groups were maintained for a further 5 months and then treated similarly.

Plasma and urine analysis

The osmolality of the urine and plasma samples was determined using vapour pressure osmometry (Wescor 5500, Logan, UT, USA). Sodium, potassium and lithium concentrations in the urine and plasma of each rat were determined by flame photometry (Radiometer FLM3, Bronshoj, Denmark). Chloride ions in the plasma were measured electrometrically using a Cotlove chloride titrator (American Instrument Co., Silver Spring, MD, USA). Creatinine concentrations in the plasma were measured spectrophotometrically after appropriate dilution (Randox Laboratory, London, UK), and urea was also measured in the plasma (Pointe Scientific, Canton, MI, USA) to further assess renal function. Urinary protein was determined using bicinchoninic acid (BCA kit, Pierce Chemical Co., ThermoFisher Scientific, Rockford, IL, USA) using bovine serum albumin (BSA) (2mg/ml) as a standard. Amiloride was measured in both urine and plasma by Liquid Chromatography/Mass Spectrometry (LCMS, Agilent 1200 series LC system with an ABI 3200 QTrap LCMSMS (Applied Biosystems Instruments, Rockford, IL, USA) at the Canterbury Health Laboratories (Christchurch, New Zealand).

Histology and immunohistochemistry

Fixed (neutral buffered formalin) kidney tissue was wax embedded and sections cut at 3µm. For histology, sections were stained with Masson's Trichrome or periodic acid-Schiff's (PAS) stain using conventional protocols. Cortical regions of interest were examined on an Olympus Provis AX70 microscope and photographed with a Spot digital camera (Olympus Corp., Tokyo, Japan). For immunohistochemistry, sections were dewaxed and rehydrated. Antigen retrieval was generated by microwaving in 10 mmol/l citrate buffer, pH 6.0 for 10 mins. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate-buffered saline (PBS). After preincubation in 1% BSA (Sigma Aldrich, St Louis, MO, USA) in phosphate-buffered saline (PBS) to block non-specific binding, sections were incubated with the selected antibody. Antibodies used were against: connective tissue growth factor (CTGF), β-catenin, transforming growth factor β1 (TGFβ1) (all Santa Cruz Biotechnology, Dallas, TX, USA), α-smooth muscle actin (αSMA) (Sigma Aldrich, St Louis, MO, USA), collagen 3 (Rocklands Immunochemicals, Limerick, PA, USA) and AQP2 (Chiron Mimetopes, Melbourne, Australia).[13] Labelling of the tissue was visualised using a

horseradish peroxidise-coupled secondary immunoglobulin (IgG-HRP, DAKO, Glostrup, Denmark), followed by incubation with diaminobenzidine (Sigma-Fast tablets, Sigma Aldrich, St Louis, MO, USA) and the tissue was counterstained with Erhlich's haematoxylin. After dehydration and clearing, the sections were mounted in DPX. Negative controls were carried out either by omitting the primary antibodies or by using appropriate blocking peptides, and positive controls used appropriately chosen tissues.

For quantitative determination of collagen 3, immunostained sections from each rat were examined microscopically and a series of at least 25 images was collected into Photoshop®, during an excursion across the section, either through the cortex or outer medulla. Changes in the measurement of changes in the amount of collagen 3 present could be distorted by recording the presence of collagen surrounding blood vessels. Thus, if a blood vessel came into the field of view the field was adjusted to the right or left to remove it and scanning continued. These images were analysed using a mask of 100 microns square, and a subroutine in ImageJ (IHC Profiler), and the area of DAB staining expressed as a percentage of the field. A similar approach was used to quantify myofibroblasts, but in this case the number of α-SMA positive cells was counted, and expressed as the number in each 100 μ square area.

Quantitative RT-PCR

The medulla and cortex were separated, homogenised in Trizol® (Invitrogen, Life Technologies, ThermoFisher Scientific, Waltham, MA, USA) and the RNA extracted by precipitation. The RNA was reverse transcribed using a Supercript III First Strand Synthesis kit (Invitrogen, Life Technologies, ThermoFisher Scientific, Waltham, MA, USA) with random hexamers. The resulting cDNA was reacted with SYBR® Premix Ex Taq™ Rox Plus kit (Takara Bio, Shiga, Japan) in the CFX Connect Real Time PCR system (BioRad, Berkeley, CA, USA). The housekeeping gene β-actin was used. Relative quantification was carried out using ΔC_ts. Primer sequences are given in Table 1.

Statistics

All quantitative results are expressed as mean ± SEM. Differences among the means of multiple parameters were analysed by one or two way analysis of variance (ANOVA) and Student's t-test. After ANOVA, the Bonferroni's *post hoc* test was performed to compare each of the groups to the others (Prism 5 software, San Diego, CA, USA). Values of $P<0.05$ were considered statistically significant.

RESULTS

Physiological parameters

Rats fed lithium had established NDI by the end of 4 weeks of treatment. At this time their urine output was 429 ± 5 µl/min/kg, significantly more ($P<0.001$) than the control group which was 74 ± 6 µl/kg/min (Table 2). Lithium treated rats remained polyuric over the next 5 months. with almost no change in urine production (413 ± 67 µl/min/kg). The animals given amiloride, however, had a greatly reduced urine volume (128 ± 19 µl/min/kg. The changes in urine volume were associated with corresponding changes in urine osmolality (control group, 2007 ± 51 mOsm/kg water; lithium group, 199 ± 14 mOsm/kg water ($P<0.001$); amiloride group 590 ± 80 mOsm kg/water ($P<0.001$). These results are consistent with partial correction of the lithium-induced NDI with amiloride when compared to the control animals (Table 2). Plasma lithium concentrations in both experimental groups were maintained at the high end of the therapeutic dosage recommended for humans. At 6 months, lithium concentration in the lithium group was 1.1 ± 0.1 mmol/l and in the amiloride group 1.2 ± 0.1 mmol/l. Plasma electrolytes, plasma osmotic pressure and renal function were comparable in all 3 groups (Table 2), although all rats given lithium showed a significant increase in sodium output as the rats used the salt blocks provided. Sodium excretion was less in the amiloride group although it remained significantly higher than in the control group (Table 2). There was a small but significant increase in urinary protein in the lithium group.

Consonant with the measured physiological changes in the urinary concentrating ability, the water channel, aquaporin 2 (AQP2) showed related changes. AQP2 was present throughout the renal collecting ducts of the control group (Fig. 1A,E,I), but was sparse in the experimental groups after 1 month of lithium treatment (Fig. 1B,F,J). The lithium group still showed low levels of AQP2 after a further 5 months on lithium (Fig. 1C,G,K). However the partial recovery of the concentrating ability shown by the amiloride group at 6 months was reflected in the increased presence of AQP2 (Fig.1D,H,L), albeit mainly with an intracellular distribution, and more prominently in the renal medulla. It is notable that the cystic dilatations of the collecting ducts, in the outer cortex, remain without immunohistochemically detectable levels of AQP2 in this group.

Effect of amiloride on the morphological changes induced by lithium

Fig. 2 illustrates the morphological changes in the renal cortex resulting from lithium treatment, and the effect of co-administration of amiloride. Compared with the control group (Fig. 2A,B) after 1 month of lithium exposure, morphological changes have become apparent in the renal cortex (Fig. 2C,D). The collecting ducts are dilated and appear to be either cyst-like, with the epithelium stretched thinly over the enlarged lumen, or to be composed of many greatly enlarged cells, many with prominent nuclei, and some cells appearing to be binucleate.

With an additional 5 months of lithium exposure, these changes had progressed further. The cortical cystic dilatations were increased in size, and many were surrounded by interstitial cells (Fig. 2F). There appeared to be an accumulation of extracellular collagen around the tubules, some of which were compressed and appeared to be non-functional (Fig. 2E). Additionally, there were subtle changes in glomerular morphology (Fig. 3A-C). Many glomeruli showed evidence of a focal glomerulosclerosis, of 142 glomeruli examined from three animals, 36 showed evidence of sclerosis, while 12 appeared to have an attachment to the capsule wall. However, in the animals given amiloride and lithium, only 13 out of 105 glomeruli showed evidence of sclerosis (Fig. 3C). There was no visible evidence of glomerular damage in either the control animals or those sacrificed after one month of lithium exposure. The observed changes in the glomeruli were associated with a small

increase in proteinuria (Table 2). In spite of these observed histological changes, there was no deterioration in renal function as measured by plasma creatinine (Table 2).

In marked contrast to the progressive changes shown by the kidneys of the lithium group, kidneys of rats given amiloride (together with lithium) for 5 months of the experimental period, showed little if any difference in morphology from the appearance of those with only 1 month of lithium treatment (Fig. 2E,F). Although the cortical collecting ducts were dilated, there were few associated accumulations of interstitial cells, and no extensive peritubular collagen deposition.

Effect of amiloride on the profibrotic changes induced by lithium

Localisation of αSMA identifies the smooth muscle fibres enveloping blood vessels and also myofibroblasts, which accumulate in regions of cellular injury and are responsible for the deposition of collagen. Fig. 4A showed that apart from its presence in blood vessels, αSMA is absent from the cortex of control rats. At 1 month (Fig. 4B) myofibroblasts (arrows) can be identified in small numbers, as thin structures closely adpressed to basement membranes of distal tubules and collecting ducts. By 6 months of lithium treatment (Fig. 4C) myofibroblasts were present in considerable numbers in the cortex, mainly adjacent to the cystic dilatations of the collecting ducts. In marked contrast, the kidneys of the amiloride group (Fig. 4D). revealed an almost total absence of fibroblasts. Measurement of the number of myofibroblasts present in the renal cortex, calculated as the mean of 100 random samples, found no significant difference between the abundance of myofibroblasts in the control animals (1.2 ± 0.35/100 μ^2), those treated with lithium for one month (2.09 ± 0.17/100 μ^2) and those given lithium plus amiloride for six months (0.78 ± 0.19/100 μ^2). There was however a significant difference in the abundance of myofibroblasts in the renal cortex of animals given lithium alone for six months (5.44 ± 0.53/100 μ^2, $P<0.0001$). The high standard error in this measurement reflects the fact that the distribution of myofibroblasts in this tissue was irregular, clusters being located adjacent to the collecting ducts.

These results were matched by the amount and distribution of collagen 3, which in control rats occured only in the intima of blood vessels (Fig. 5A), and only sparsely in the interstitium after 1 month of lithium treatment (Fig. 5B). After 6 months of lithium exposure there was an extensive network of collagen fibres in the vicinity of the dilated collecting ducts (Fig. 5C). However, in the amiloride group, the abundance and distribution of collagen 3 was similar to that of the animals exposed to lithium for 1 month (Fig. 5D). Measurement of the extent of collagen (measurement as the percentage of the area of the 100 μ^2 sampling mask) showed little difference between the controls (0.93 ± 0.03), the one month lithium group (0.99 ± 0.08) and the group given amiloride (1.07 ± 0.06), while the 6 month lithium group had substantially more collagen in the renal cortex (3.21 ± 0.04, $P<0.001$).

A key element in the progression of fibrosis is widely held to be CTGF, which may be modulated via TGFβ1 or in some cases independently. The effect of lithium on CTGF distribution in the renal cortex is shown in Fig. 6A-D). CTGF is confined to the basolateral membranes of distal tubules and collecting ducts and there appears to be little change in the distribution or abundance in all 4 groups.

On the other hand, the picture of TGFβ localisation, as shown by immunohistochemistry (Fig. 7) presented unexpected data. TGFβ1 appears to be absent in the control renal cortex, and only barely appears after one month of lithium treatment (Fig. 7B), where it was confined to the Bowman's space of a few glomeruli. After six months exposure to lithium, TGFβ1 was distributed throughout the interstitial spaces and in the glomerular capillaries (Fig. 7C). In marked contrast, in those animals given amiloride in addition to lithium, TGFβ1 was weakly distributed intracellularly in the distal tubules, but absent extracellularly (Fig. 7D).

Localisation of β-catenin in the lithium group appears to be increased in the distal tubules and collecting ducts (Fig. 8C). β-catenin expression was also enhanced in the amiloride group (Fig. 8D) when compared to the control rats (Fig. 8A). There was however some suggestion of a lowered intensity of β-catenin expression lining the basolateral membranes of the epithelial cells in the amiloride treated animals (Fig. 8D) compared with the lithium group.

mRNA expression of collagen 3, CTGF and TGFβ1

Collagen 3 mRNA expression showed a 2.80 ± 0.22 fold increase (P<0.001) in the cortex of the kidneys from lithium treated rats compared to controls. Amiloride administration resulted in a down regulation of collagen 3 mRNA (1.6 ± 0.31, P<0.01, cortex) to values similar to that of the control animals (Fig. 9). TGFβ1 mRNA expression demonstrated a 3.4 ± 0.61 fold increase (P<0.01) in the cortex with lithium treatment only whereas co-treatment with amiloride reduced TGFβ1 mRNA to values similar to control values (1.9 ± 0.67, P<0.01 compared with lithium only). A similar pattern was seen with CTGF mRNA expression with a 3.0 ± 0.4 fold increase (P<0.01) in the cortex of lithium treated animals. Amiloride co-treatment resulted in CTGF mRNA values being similar to that seen in the control group (1.8 ± 0.55, NS). AQP2 mRNA in the cortex was clearly down-regulated with lithium therapy (0.1 ± 0.03. P<0.001) and is not significantly modified by amiloride co-administration.

DISCUSSION

This study has demonstrated that administration of amiloride to rats that had already developed NDI, not only partially restored the concentrating ability of the kidney, but also and more importantly, limited the progressive development of cortical fibrosis resulting from the chronic lithium exposure. In an earlier study, rats showed evidence of renal interstitial fibrosis after 4 weeks exposure to lithium, which had become extensive after 6 months of continued lithium.[4] In the present study, attempts to demonstrate quantitatively the development of fibrosis at one month were not successful. This was due to the fact that deposition of collagen was confined to foci, largely around collecting ducts, so that random measurement of its abundance gave data that was little different from the controls, although the difference was clearly obvious on microscopic examination (Fig. 5 A,B), The changes reported earlier were reproduced in this study but a key additional finding reported here was that amiloride, commenced after 4 weeks of lithium treatment, effectively prevented the further progression of cortical fibrosis as well as a sustained partial reversal of the lithium-induced NDI.

Long term exposure to lithium has been associated with the development of a chronic focal interstitial fibrosis, tubular atrophy and focal glomerulosclerosis,[2] that may progress to end stage renal disease.[3] The pattern of histological injury in this model reflects the changes evident in long-term lithium exposure in humans. Markowitz and colleagues documented a chronic tubulointerstitial nephropathy with focal glomerulosclerosis in a series of 24 patients with biopsy-proven lithium toxicity.[3] In the human biopsies there was a chronic interstitial fibrosis predominately involving the distal and collecting tubules. They also demonstrated the presence of a focal segmental glomerulosclerosis in 50% of the biopsies. In the present study, we found a similar pattern of focal glomerulosclerosis, present in approximately one third of glomeruli observed in each of the lithium-fed animals, which was associated with mild proteinuria in these rats. Of considerable interest was the observation that chronic amiloride administration, started 1 month after the establishment of lithium-induced NDI, limited the development of focal glomerulosclerosis, along with an associated reduction in proteinuria. This study was not designed to investigate glomerular changes related to lithium exposure, so further studies are proposed to explore this intriguing observation. The prevention of the progression of lithium-induced fibrosis, by the co-administration of amiloride, was associated with a reduced expression of the pro-fibrotic cytokine CTGF along with decreased myofibroblast activity as measured by αSMA staining and decreased collagen 3 deposition. Amiloride, by reducing the cellular uptake of lithium, is presumably altering the intracellular regulatory pathways associated with the development of fibrosis.

Earlier animal models demonstrated that lithium inhibits intracellular adenylate cyclase activity leading to lower intracellular concentrations of cAMP.[14] Duncan and co-workers[15] reported that cAMP, both *in vitro* and *in vivo*, inhibited TGFβ-mediated collagen synthesis as well as having an impact on the CTGF mediated pathway. Activation of adenylate cyclase also led to the inhibition of TGFβ and CTGF mediated fibrotic pathways.[15] Based on these findings it is postulated that lithium inhibition of intracellular adenylate cyclase leads to the disruption of the downstream signalling pathways, either directly via cAMP, or indirectly by loss of activation of cAMP response element binding protein (CREB) which normally down-regulates CTGF gene expression. Hence lithium can lead to an upregulation of CTGF that

may or may not be associated with alterations in TGFβ1 expression, that is then associated with an increased fibrosis as demonstrated in this study.

Lithium also increases the phosphorylated (inactive) form of GSK3β, and lithium-induced fibrosis may be mediated in part, via inhibition of GSK3β.[8,16] GSK3β modulates the actions of adenylate cyclase so inhibition of GSK3β may lead to a decrease in adenylate cyclase activity contributing to the profibrotic changes evident with lithium therapy. The lithium-induced inhibition of GSK3β may also be important in the upregulation of the profibrotic processes. In addition, GSK3β constitutively phosphorylates β-catenin, an important transcription factor involved in epithelial-mesenchymal transition (EMT), a phenomenon proposed to be crucial in fibrogenesis.[17] β-catenin expression, as a downstream marker of GSK3β inhibition (Fig. 8) was increased with lithium treatment and partially reduced with the co-administration of amiloride. Clearly, these findings will need to be sustained with further experiments.

Chronic lithium therapy not only produces a chronic interstitial fibrosis. There are a number of published studies demonstrating that lithium induces hyperplastic changes in the medullary collecting ducts, along with dilated late distal tubule and connecting tubule segments with characteristic distal tubule dilatation and microcyst function. These changes are evident after only 3-4 weeks exposure to lithium.[4,18-20] The degree of distal tubule dilatation and other changes of interstitial nephropathy tends to progress with duration of lithium exposure.[4,18,19] Of interest in this study was the finding that, although the co-administration of amiloride prevented the progression of the interstitial fibrosis, there was no alteration of the morphological changes induced by lithium. The mechanisms by which lithium induces the cellular changes are not known. In a previous paper[4] Walker *et al.* suggested that the cortical changes induced by lithium exposure might be due to crosstalk resulting from lithium entry into proximal tubular cells resulting in the release of cytokines causing a fibrotic response in the distal tubules. Marouf *et al.* [21] have recently presented evidence that interstitial fibrosis may be driven by Wnt signalling from the proximal tubules. In the lithium-treated rat, lithium concentration in the proximal tubule is at a concentration near 1 mmol/litre. Alsady *et al.*[22], in discussing lithium handling in the kidney, point out

that, as has been known for many years, about 70% of filtered lithium is reabsorbed in the proximal tubule. Much of this reabsorption must be transcellular. The lithium-transporting pathway is not known, but is thought to be the sodium–hydrogen exchanger NHE3, which can be blocked by amiloride at concentrations of >50-100 μmol[23] It is possible therefore that lithium could enter the proximal tubular cells via NHE3 and leave through the basolateral action of NHE1. Although NHE3 has a low affinity for lithium, it may be sufficient to disrupt cellular metabolism and lead to a release of Wnt1 ligands, in turn activating interstitial fibrosis. Clearly further studies are required to investigate how these cellular changes occur and to see if administration of amiloride at the onset of lithium treatment prevents the development of the dilated cystic tubules and cellular abnormalities.

This study has demonstrated that the progressive development of chronic interstitial fibrosis induced by long term lithium exposure is significantly suppressed by the addition of amiloride 4 weeks after the commencement of lithium therapy. This was associated with the downregulation of the key profibrotic cytokine CTGF, which, may in part, be mediated by lithium-induced changes in GSK3β activity and/or adenyl cyclase. This may have significant clinical interest in limiting progression of lithium-induced renal fibrosis.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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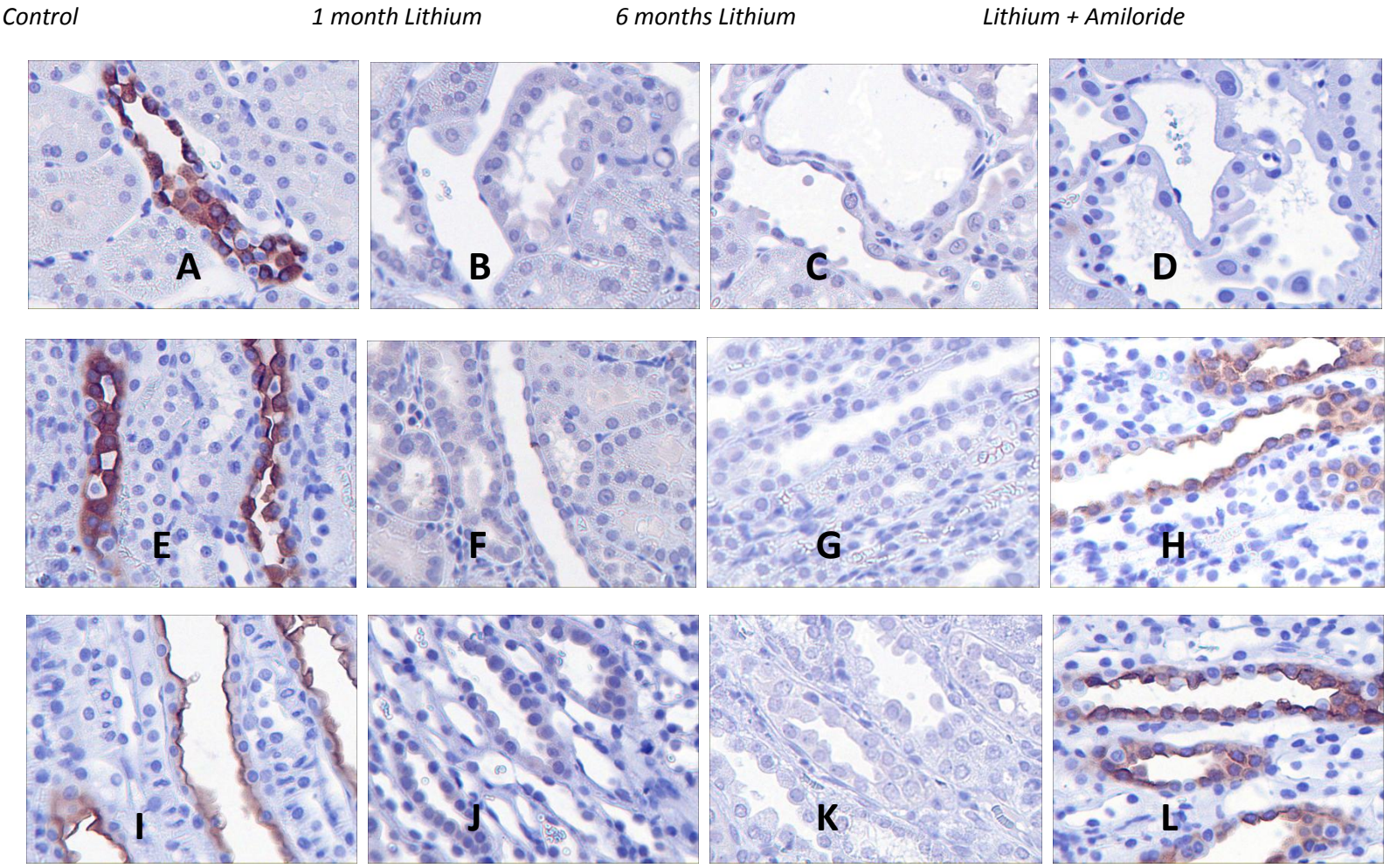

Figure 1. Representative regions of rat kidneys showing the localization of AQP2. Control (A,E,I); after 1 month on a lithium diet (B,F,J); after 6 months on a lithium diet (C,G,K); after 1 month of lithium exposure followed by 5 months on lithium with co-administration of amiloride (D,H,L). Top row, cortex; middle row, outer medulla; bottom row, inner medulla. DAB staining shows localization of AQP2 on the apical membranes of the collecting ducts. Magnification x 400.

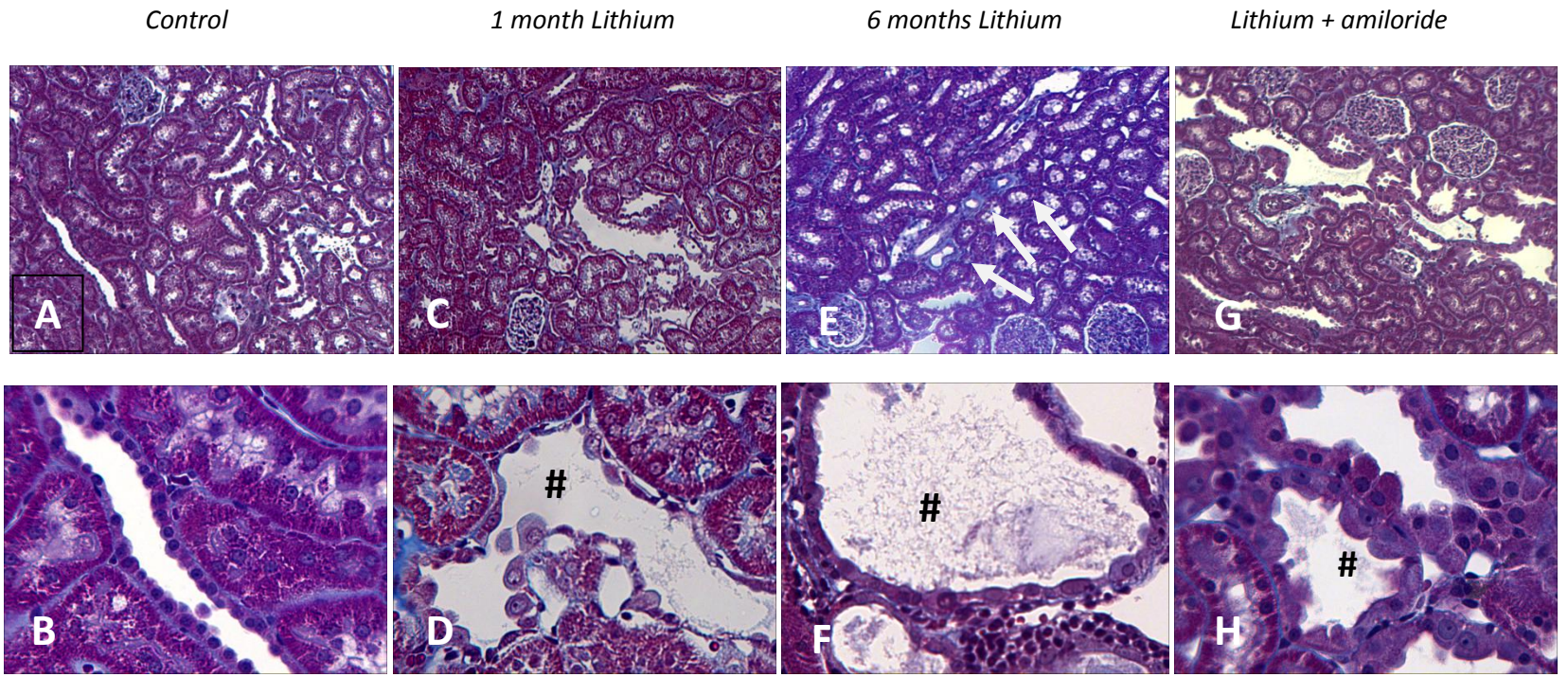

Figure 2. Representative regions of the cortex of rat kidneys stained with Masson's Trichrome. Control (A,B); after 1 month on a lithium diet (C,D); after 6 months on a lithium diet (E,F); after 1 month on a lithium diet followed by 5 months on lithium with co-administration of amiloride (G,H). # indicates dilated collecting ducts; arrows identify compressed (non-functional) tubules. Magnification A,C,E,G x 100; B,D,F,H x 400.

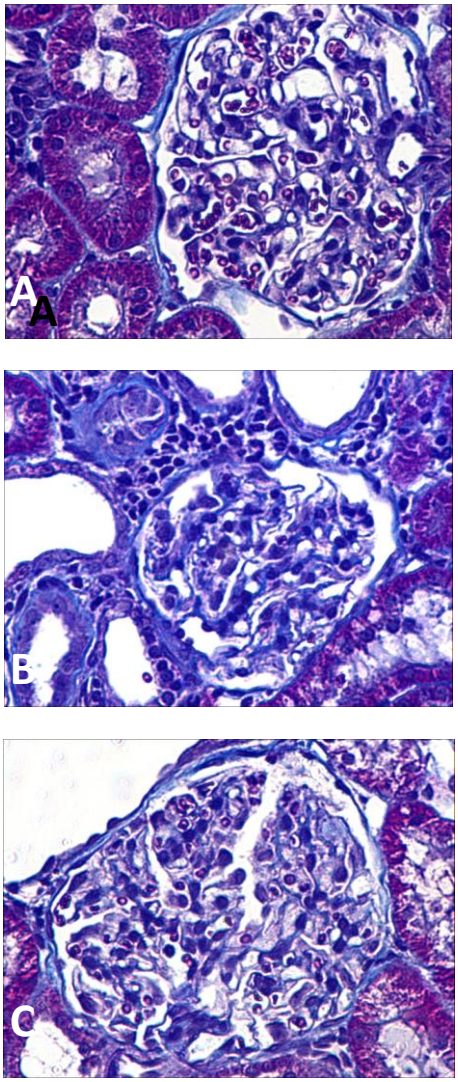

Figure 3. Representative glomeruli from (A) control; (B) after 6 months on a lithium diet - focal glomerulosclerosis ; (C) a glomerulus with adhesions to the capsular wall. Magnification x 400.

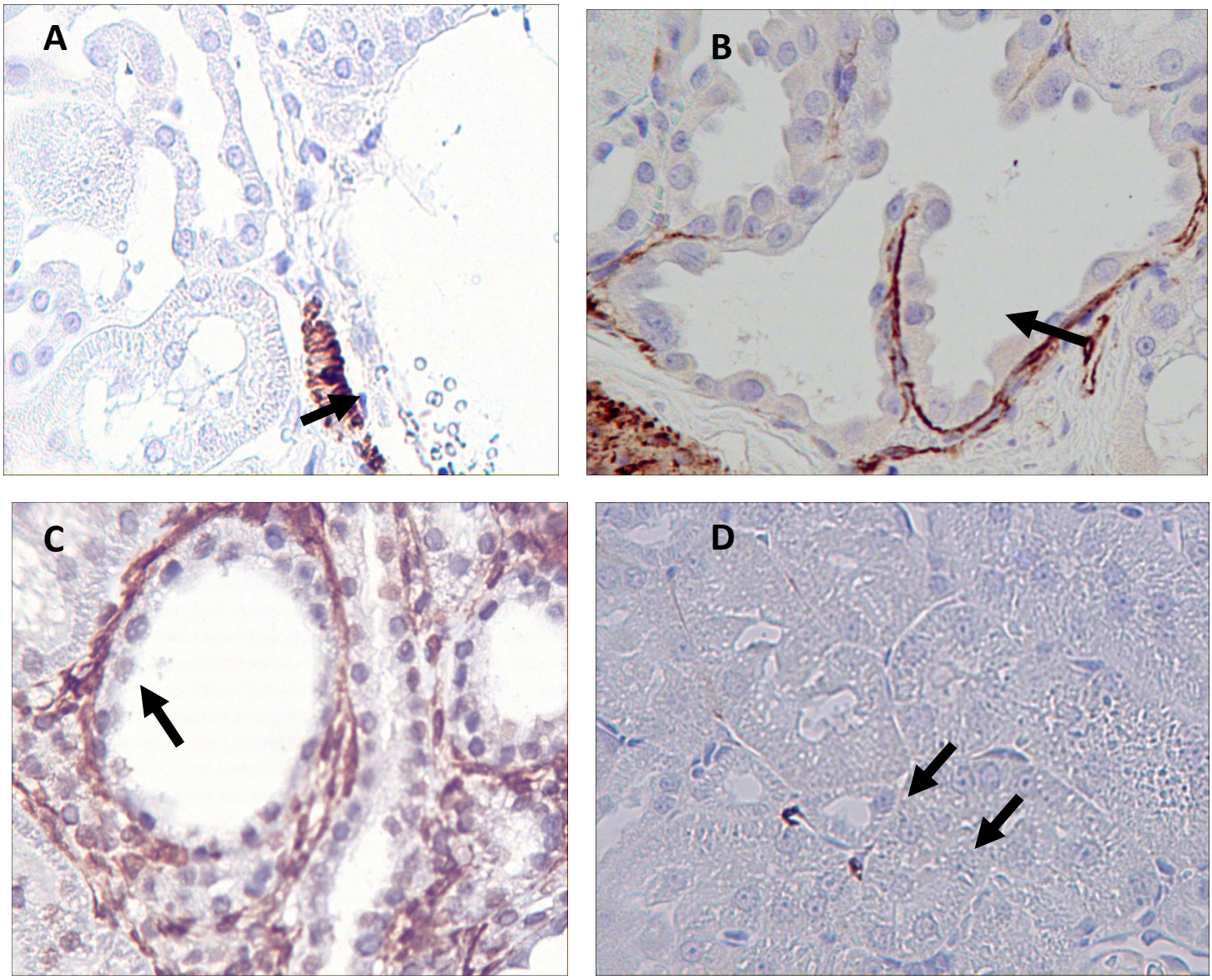

Figure 4. Representative regions of the cortex of rat kidneys showing localization of αSMA. Control (A), after 1 month on a lithium diet (B), after 6 months on a lithium diet (C), 1 month of lithium exposure followed by 5 months on lithium with co-administration of amiloride (D). In A: arrow indicates smooth muscle of arteriole; B,C: arrows point to myofibroblasts beneath the epithelium of the collecting duct; D: only a few myofibroblasts are identifiable. Magnification A-D x 400

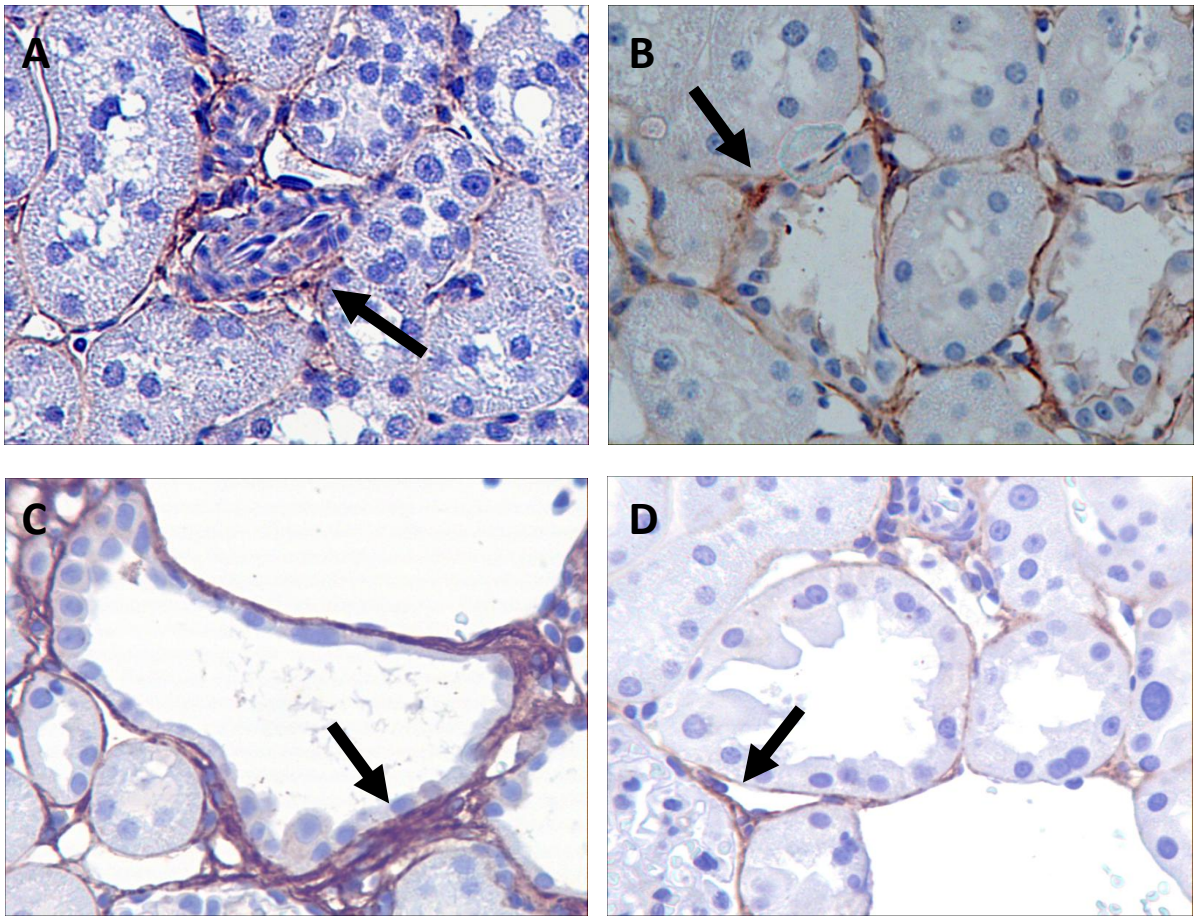

Figure 5. Representative regions of the cortex of rat kidneys showing localization of collagen 3. Control (A) where arrows indicate collagen 3 surrounding a small arteriole; (B) after 1 month on a lithium diet collagen 3 is present around collecing ducts (arrows); (C) after 6 months on a lithium diet collagen 3 deposition is more extensive; (D) after 1 month of lithium exposure followed by 5 months on lithium with co-administration of amiloride shows that collagen 3 is still present around collecting ducts but is comparable in extent to rats exposed to lithium for one month only. Magnification A-D x 400

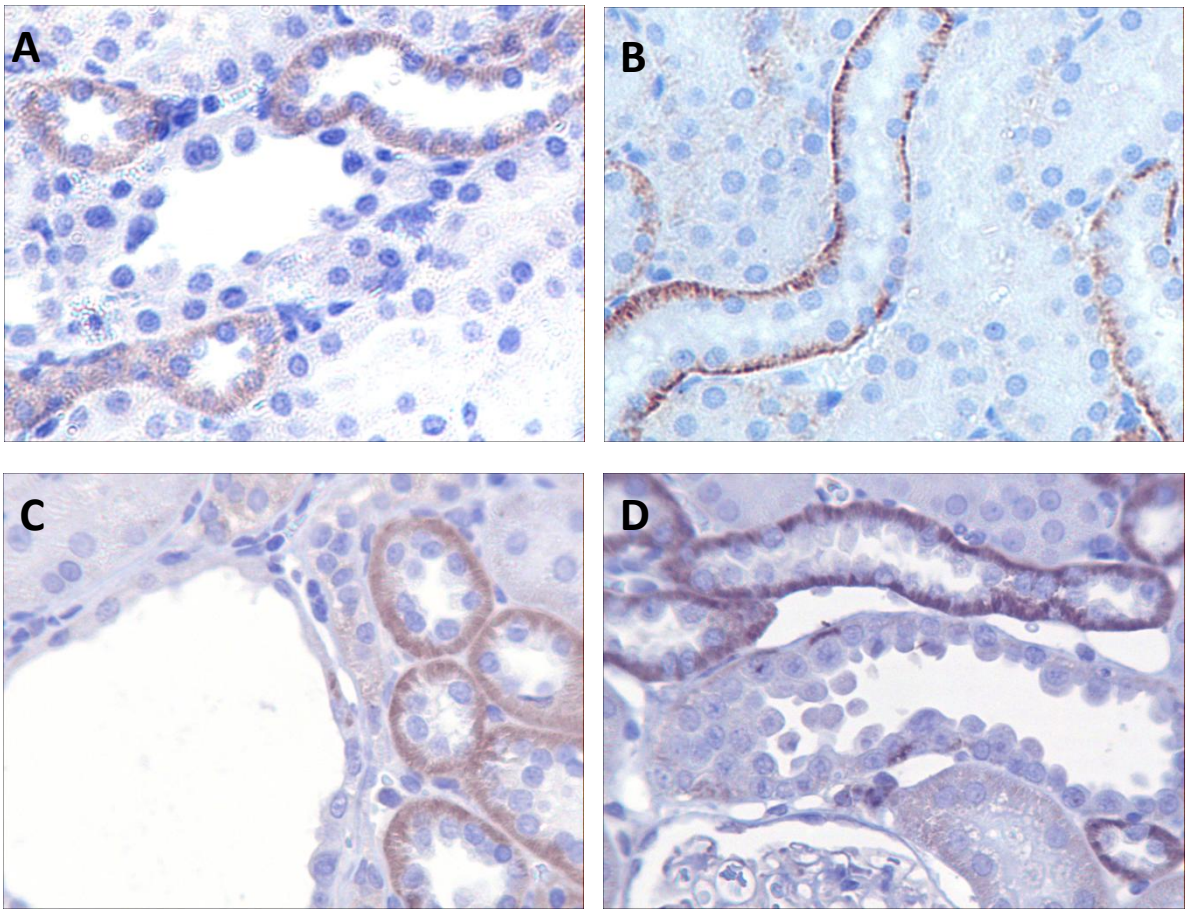

Figure 6. Representative regions of the cortex of rat kidneys showing localization of CTGF. Control (A); after 1 month on a lithium diet (B) after 6 months on a lithium diet (C); after 1 month of lithium exposure followed by 5 months on lithium with co-administration of amiloride (D). CTGF is confined to the basolateral membranes of the thick ascending limb and distal tubules. Magnification A-D x 400

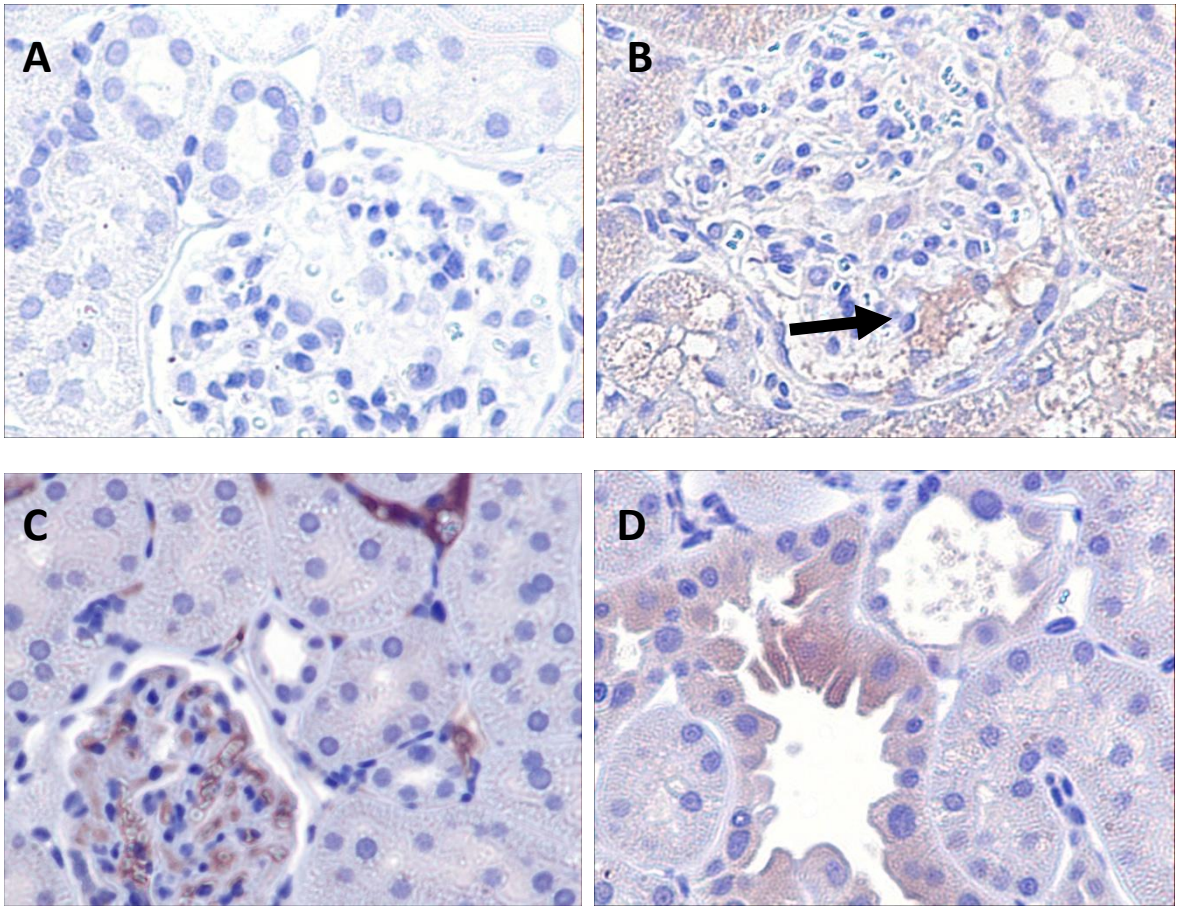

Figure 7. Representative regions of the cortex of rat kidneys showing localization of TGFβ1. Control (A); after 1 month on a lithium diet (B) – arrow points to glomerular distribution of TGFβ1; after 6 months on a lithium diet (C); after 1 month of lithium exposure followed by 5 months on lithium with co-administration of amiloride (D). Magnification A-D x 400

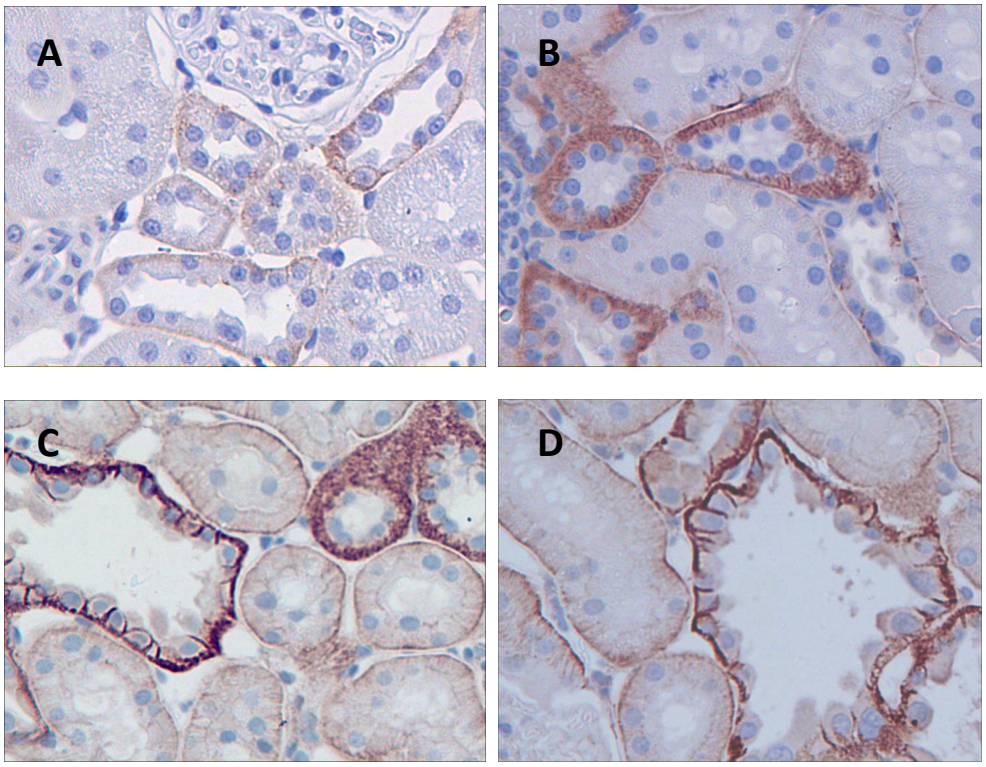

Figure 8. Representative regions of the cortex of rat kidneys showing localization of β-catenin. Control (A); after 1 month on a lithium diet (B); after 6 months on a lithium diet (C); after 1 month of lithium exposure followed by 5 months on lithium with co-administration of amiloride (D). Magnification A-D x 400

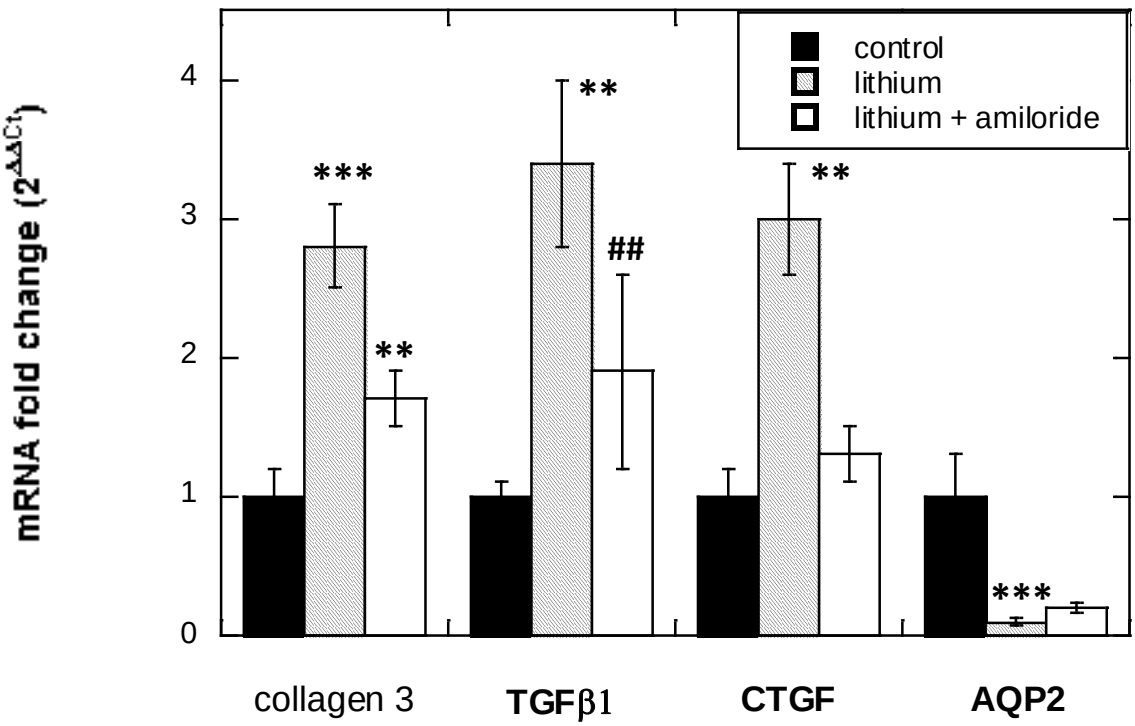

Figure 9. mRNA expression (2ΔΔCT) of collagen 3, TGFβ1, CTGF and AQP2 from extracts from the cortex from the kidneys of rats. Experimental conditions were control, after 6 months on a lithium diet, 1 month of lithium exposure followed by 5 months on lithium with co-administration of amiloride ***P<0.001 and **P<0.01 compared to controls; ##P<0.01 compared to lithium.

Table 1. Forward and reverse primer sequences for TGFβ1, CTGF, collagen 3, AQP2 and β-actin for *Rattus norvegicus*

Gene	Primer
Transforming Growth factor (TGFβ1)	Forward : AAGGCACATCCTTGGACGAAGGTC (24 b) Reverse : TGTGCACAAGCTTCCAGTGGTG (reverse complement: CACCACTGGAAGCTTGTGCACA) (22 b)
Connective Tissue growth Factor (CTGF)	Forward : AGCGCTGACATTCTGATTCCAGTG (24b) Reverse : ACTTGCCACAAGCTGTCCAGTC (reverse complement: GACTGGACAGCTTGTGGCAAGT) (22 b)
Collagen 3	Forward : TGCCTACATGGATCAGGCCAATG (23 b) Reverse : TGCTCCATTCACCAGTGTGTTTAG (reverse complement: CTAAACACACTGGTGAATGGAGCA) (24 b)
Aquaporin 2 AQP2	Forward : CTGCCCTCTCCATTGGTTTCTC (22 b) Reverse : TGGAGCAACCGGTGAAATAGATCC (reverse complement: GGATCTATTTCACCGGTTGCTCCA) (24 b)
B-actin	Forward : ACCCGCGAGTACAACCTTCTTG (22 b) Reverse : TATCGTCATCCATGGCGAACTGG (reverse complement: CCAGTTCGCCATGGATGACGATA) (23 b)

Table 2. Physiological parameters of rats maintained on a lithium diet for 1 and 6 months, and on lithium + amiloride for 6 months.

	control	Lithium 1 month	Lithium 6 months	Lithium + amiloride
Body weight (g)	428 ± 14[A]	304 ± 2***	414 ± 13***	442 ± 10***
Plasma composition				
Na^+ (mmol/l)	134 ± 1	145 ± 5	138 ± 1	138 ± 1
K^+ (mmol/l)	5.6 ± 0.1	5.3 ± 0.1	5.6 ± 0.1	5.5 ± 0.1
Li^+ (mmol/l)	0	1.4 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Cl^- (mmol/l)	97 ± 2	100 ± 3	96 ± 2	98 ± 2
Osmotic pressure (mOsm/kg water)	289 ± 1	295 ± 2	291 ± 1	290 ± 1
Urea (mM/l)	6.9 ± 0.8	6.5 ± 0.8	5.9 ± 0.5	5.8 ± 0.5
Creatinine (µmol/l)	108 ± 17	120 ± 15	155 ± 22	110 ± 20
Amiloride (mmol/l)	ND	ND	ND	0.65 ± 0.07
Urine composition				
Urine output (µl/min/kg)	74 ± 6	429 ± 30***	413 ± 67***	128 ± 19**###
Na^+ (mmol/l)	39 ± 6	18 ± 2**	14 ± 4***	22 ± 4*##
K^+ (mmol/l)	185 ± 18	15 ± 1***	11 ± 3***	30 ± 15
Li^+ (mmol/l)	0	3.0 ± 0.3	2.6 ± 0.8	4.2 ± 0.3
Osmotic pressure (mOsm/kg water)	2007 ± 51	178 ± 10***	199 ± 14***	590 ± 80###
Protein (mg/hr/100g)	0.4 ± 0.2	3.2 ± 0.5***	2.1 ± 0.1***	0.2 ± 0.3###

A = weight at 1 month after commencement of experiment, 6 month weight = 600 ± 19 g
Values are means ± SEM. *** $P < 0.001$/ ** $P < 0.001$ / * $P < 0.05$ lithium treated and amiloride rats compared to control values. $^{###}P < 0.001$/ $^{##}P < 0.001$/ $^{#}P < 0.05$ lithium treated rats compared to the rats administered lithium and amiloride. One-way ANOVA, Bonferroni's post-hoc test was used to compare the groups lithium vs lithium + amiloride and each of these against the controls. n = 7 (lithium), n = 6 (control and lithium + amiloride group). ND = not determined.