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Endogenous interleukin-6 enhances the renal injury, dysfunction and inflammation caused by ischemia/reperfusion.

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RUNNING TITLE: Role of IL-6 in renal I/R injury

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ABSTRACT

Here we investigate the effects of renal ischemia/reperfusion (I/R) on the degree of renal injury, dysfunction and inflammation in (i) interleukin (IL)-6 knock-out (IL-$6^{-/-}$) mice and (ii) mice administered a monoclonal antibody against IL-6. IL-$6^{-/-}$ mice were subjected to bilateral renal artery occlusion (30 min) and reperfusion (24 h). At the end of experiments, indicators and markers of renal dysfunction, injury and inflammation were measured. Kidneys were used for histological evaluation of renal injury. Renal expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and P-selectin, as well as nitration of proteins in the kidney were determined using immunohistochemistry. In addition, wild-type mice were pretreated (24 h and 1 h before ischemia) with an IL-6 antibody to mimic the effects that would be seen in IL-$6^{-/-}$ mice. IL-$6^{-/-}$ mice, and wild-type mice administered the IL-6 antibody, demonstrated significantly reduced plasma urea and creatinine levels indicating reduction of renal dysfunction caused by I/R. Neutrophil infiltration was also significantly reduced in IL-$6^{-/-}$ mice and wild-type mice administered the IL-6 antibody subjected to renal I/R. Pro-inflammatory cytokines (TNF-α and IL-1β) in renal tissues were significantly attenuated in IL-$6^{-/-}$ mice to levels seen in wild-type mice. IL-$6^{-/-}$ mice demonstrated reduced histological evidence of tubular injury and markedly reduced immunohistochemical evidence of ICAM-1, P-selectin, and nitrotyrosine when subjected to renal I/R. We propose that endogenous IL-6 enhances the degree of renal injury, dysfunction and inflammation caused by I/R of the kidney by promoting the expression of adhesion molecules and subsequent oxidative and nitrosative stress.

Interleukins mediate intercellular communication that triggers the immunologic responses of alloantigen recognition and defence, or tolerance. Although certain cytokines display stimulatory effects *in vitro,* others appear to attenuate T-cell responsiveness. The impact of these complementary and apparently competitive effects *in vivo* is still incompletely understood.

Interleukin (IL)-6 is a pleiotropic cytokine, primarily involved in the regulation of immune and inflammatory responses. IL-6 is not only generated by T- and B-lymphocytes, monocytes/macrophages, fibroblasts, vascular smooth muscle cells and endothelial cells, but also by mesangial and tubular epithelial cells (Fukatsu *et al.*, 1993). IL-6 has many biological properties, for instance, it activates signal transducer and activator of transcription (STAT)3 transcription factor, stimulates the expression of tissue factor, monocyte chemotactic protein-1, matrix degrading enzymes, and low-density lipoprotein receptors in macrophages (Hamanaka *et al*., 1992;Biswas *et al*., 1998), causes platelet aggregation, proliferation of vascular smooth muscle cells (Ikeda *et al.*, 1993;Ikeda *et al*., 1991), and formation of C-reactive protein and fibrinogen by hepatocytes. It also regulates the expression of adhesion molecules and other cytokines in endothelial cells including IL-1β and tumor necrosis factor (TNF)-α, which in turn potently enhance the inflammatory response (Mantovani, 1997), and also forms part of a positive feedback cycle in which TNF-α stimulates IL-6 production. In contrast, IL-6 also possess anti-inflammatory properties (Kox *et al.*, 2000), exerting antiapoptotic function as demonstrated in a model of liver transplantation (Sun *et al.*, 2003) . Among the T-cell activators and proliferators, which includes IL-6, are also TNF-α, IL-1 and IL-18. Recently, IL-18 was shown to act in synergy with IL-12 to promote the development of Th1 responses (Blankenberg *et al.*, 2002). IL-8 which can also be stimulated by IL-1β and TNF-α is restricted to the chemotactic and degranulation response of neutrophils (Gerritsma *et al.*, 1996).

However, the role of endogenous IL-6 in the tissue injury and inflammation associated with ischemia/reperfusion (I/R) is controversial. Cerebral I/R leads to the rapid expression of IL-

6 while IL-6 knock-out (IL-$6^{-/-}$) mice subjected to cerebral I/R exhibit reduced survival suggesting that endogenous IL-6 protects the brain against I/R-injury (Herrmann *et al.*, 2003). In contrast, the inflammatory response caused by I/R of the intestine is significantly reduced in IL-$6^{-/-}$ mice, suggesting that an enhanced expression of IL-6 contributes to gut I/R injury (Cuzzocrea *et al.*, 1999). Recent reports now suggest that an enhanced formation of endogenous IL-6 mediates the protective effects of HMG-CoA reductase inhibitors in experimental renal I/R injury, suggesting that IL-6 protects the kidney against I/R injury (Yokota *et al.*, 2003). In contrast to this, suppression of the I/R-induced expression of IL-6 may mediate the renoprotective effects caused by pre-treatment of rats with bacterial lipopolysaccharide (Heemann *et al.*, 2000). Most notably, a recent report has indicated that urinary levels of IL-6 in patients undergoing renal transplantation are predictive for the development of sustained, acute renal failure (Yokota *et al.*, 2003). Experimental renal I/R also results in a significant and sustained increase in the expression of the IL-6 gene (Takada *et al*., 1997), and in cold ischemia changes in IL-6 mRNA expression are limited to the tubules, and do not occur in the glomerulus (Kaminska *et al*., 2003). Thus, the role of endogenous IL-6 in the renal injury, dysfunction and inflammation associated with renal ischemia and reperfusion is unclear.

The expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and selectins, such as P-selectin and E-selectin, (Rabb *et al*., 1997;Chamoun *et al*., 2000), followed by the adhesion, activation, and transmigration of polymorphonuclear leukocytes (PMN) into renal tissues and subsequent production of reactive oxygen species (ROS) and nitric oxide (NO) (Rabb *et al*., 1997), contribute significantly to the development of renal I/R injury and associated ischemic acute renal failure. Furthermore, NO reacts with superoxide anion to from peroxynitrite, which causes injury via direct oxidant injury and protein tyrosine nitration.

This study was designed to investigate the role of endogenous IL-6 in the tissue injury, dysfunction and inflammation caused by renal I/R. Specifically we have investigated the effects

of bilateral renal ischemia (30 min) and reperfusion (24 h) on renal injury and dysfunction. To gain a better insight into the mechanism(s) of action of the beneficial effects following the removal of endogenous IL-6 in mice, we have investigated the expression of adhesion molecules (ICAM-1 and P-selectin), activation of neutrophils, levels of lipid peroxidation and cytokines in IL-$6^{-/-}$ mice and their wild-type littermates. Following this we then proceeded to investigate the role of IL-6 in renal I/R using an antibody directed against IL-6. This study provides evidence that endogenous IL-6 contributes to renal I/R injury and inflammation.

MATERIALS & METHODS

Animals

Forty-two male C57BL/6J x 129/SV/EV and 14 male IL-6$^{-/-}$ mice of a mixed genetic background (20 – 30 g, kindly supplied by Prof. G. Ciliberto, IRBM, Pomezia, Italy) were used to assess the role of IL-6 in the pathogenesis of renal I/R in the mouse. IL-6$^{-/-}$ mice were generated by gene targeting as previously described (Poli *et al.*, 1994). Briefly, embryonic stem cell clones carrying the IL-6 mutation were injected into blastocytes of C57BL/6J and planted into the uteri of C57BL/6J foster mothers. Male chimeras were mated to females and female offspring heterozygous for the mutation were bred once with mice of the 129/SV/EV strain, the strain from which the embryonic stem cells were derived. The resulting heterozygous offspring were bred together to generate mice homozygous for the IL-6 mutation. IL-6$^{-/-}$ mice have previously been shown to develop a normal glomerular architecture and in particular a normal mesangium (Eitner et al., 1997)**.** Mice were allowed access to food and water *ad libitum* and were cared for in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with the European Economic Community regulations (O.J. of E.C. L358/1 12/18/1986).

Renal ischemia/reperfusion

Mice were anesthetized using chloral hydrate (125 mg/kg, i.p.) and core body temperature maintained at 37^{o}C using a homoeothermic blanket. For the first study mice were divided into the following four groups for experiments involving IL-6$^{-/-}$ mice: (i) **I/R wild-type Group**; wild-type mice, which underwent renal ischemia for 30 min followed by reperfusion for 24 h (n = 6), (ii) **I/R IL-6$^{-/-}$ Group**; knock-out mice, which underwent renal ischemia for 30 min followed by reperfusion for 24 h (n = 7), (iii) **Sham wild-type Group** 'sham-operated'; wild-type mice, which were subjected to the surgical procedures described below, but were not subjected to renal I/R (n = 4), (iv) **Sham IL-6$^{-/-}$ Group**; knock-out mice, which were subjected to the surgical

procedures described below, but were not subjected to renal I/R (*n* = 4). No evidence of IL-6 has previously been detected in the plasma of this strain of IL-$6^{-/-}$ mice subsequent to I/R (Cuzzocrea *et al.*, 1999).

In a separate study, another set of C57BL/6J mice were divided into four groups for experiments involving the use of a neutralizing rat (monoclonal) anti-mouse IL-6 antibody (IL-6 MAb, 10 μg/day i.p.; Biosource International Inc., Camarillo, USA): (i) **I/R control Group**; wild-type mice that were administered the isotype control (IgG1, 10 μg/day i.p.; Biosource International Inc.) 24 h and 1 h prior to ischemia, and underwent renal ischemia for 30 min followed by reperfusion for 24 h (*n* = 8), (ii) **I/R IL-6 MAb Group**; wild-type mice that were administered the IL-6 MAb (10 μg/day i.p.) 24 h and 1 h prior to ischemia, and underwent renal ischemia for 30 min followed by reperfusion for 24 h (*n* = 10), (iii) **Sham control Group** 'sham-operated'; wild-type mice that were administered the IgG1 isotype control (10 μg/day i.p.) 24 h and 1 h prior to sham ischemia, and were subjected to the surgical procedures described below, but were not subjected to renal I/R (*n* = 4), (iv) **Sham IL-6 MAb Group**; wild-type mice that were administered the IL-6 MAb (10 μg/day i.p.) 24 h and 1 h prior to sham ischemia, and were subjected to the surgical procedures described below, but were not subjected to renal I/R (*n* = 4). Mice were maintained under anesthesia for the duration of ischemia (i.e. 30 min). After performing a midline laparotomy, mice from the I/R groups were subjected to bilateral renal ischemia for 30 min, during which the renal arteries and veins were occluded using microaneurysm clamps (Chatterjee *et al.*, 2003). The time of ischemia chosen was based on that found to maximize reproducibility of renal functional impairment, while minimizing mortality in these animals (Chatterjee *et al.*, 2003). After the renal clamps were removed, the kidneys were observed for a further 5 min to ensure reflow after which 1 ml saline at 37^{o}C was injected into the abdomen and the incision was sutured in two layers. Mice were then returned to their cages where they were allowed to recover from anesthesia and observed for 24 h. Sham-operated mice

underwent identical surgical procedures to I/R mice except that microaneurysm clamps were not applied.

Measurement of biochemical parameters

At the end of the reperfusion period, 1 ml blood samples were collected from anesthetized mice via cardiac puncture. The samples were centrifuged (6,000 *g* for 3 min) to separate plasma. All plasma samples were analyzed for biochemical parameters within 24 h after collection or stored at -80°C. Plasma urea and creatinine concentrations were used as indicators of renal function (Chatterjee *et al.*, 2003).

Histological evaluation

Kidneys were removed from mice at the end of the experimental period after tying the renal pedicle and cut in a sagital section into two halves. These tissue samples were fixed by immersion in 10 % (wt/vol) formaldehyde in phosphate-buffered saline (PBS; 0.01 M; pH 7.4) at room temperature for 1-3 days. After dehydration using graded ethanol, the tissue was embedded in Paraplast (Sherwood Medical, Mahwah, NJ) and cut in fine (8 µm) sections and mounted on glass slides. Sections were then deparaffinized with xylene, counterstained with hematoxylin and eosin, and viewed under a light microscope (Dialux 22, Leitz, Milan, Italy).

Polymorphonuclear Leukocyte Influx into Renal Tissues

As it has become apparent that the myeloperoxidase and naphthol-AS-D-chloracetatesterase assays can crossreact with monocytes and macrophages (Ysebaert *et al.*, 2000), standard hematoxylin-eosin staining was performed to estimate the presence of PMNs, based on the morphology of the nucleus. The total number of infiltrating leukocytes (e.g. neutrophils and mononuclear cells) in cortical interstitial spaces was assessed quantitatively by counting the number of PMNs in 20 high-powered fields.

Malondialdehyde measurement

Malondialdehyde (MDA) levels in kidney samples were determined as an indicator of lipid peroxidation, as previously described (Chatterjee *et al.*, 2003). Tissues were homogenized in a 1.15% KCl solution. An aliquot of the homogenate was added to a reaction mixture containing 200 µL of 8.1% sodium dodecyl sulfate, 1500 µL of 20% acetic acid (pH 3.5), 1500 µL of 0.8% thiobarbituric acid, and 700 µL of distilled water. The mixture was then boiled for 1 h at 95ºC and centrifuged at 3,000 *g* for 10 min. The absorbency of the supernatant was measured by spectrophotometry at 650 nm.

Immunohistochemical localization of nitrotyrosine

Tyrosine nitration was detected in kidney sections by immunohistochemistry as previously described (Chatterjee *et al.*, 2003). Briefly, tissues were fixed in 10% buffered formalin and 8 µm sections were prepared from paraffin embedded tissues. After deparaffination, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the sections in 2% (vol/vol) normal goat serum in PBS for 20 min. Endogenous avidin and biotin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (DBA, Milan, Italy). The sections were then incubated overnight with a 1:1000 dilution of primary anti-nitrotyrosine antibody (DBA) or with control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA).

Immunohistochemical analysis of ICAM-1 and P-selectin

Localization of ICAM-1 and P-selectin in kidney sections was determined as previously described (Cockerill *et al.*, 2001). Briefly, sections were incubated overnight at 4°C with primary anti-ICAM-1 (CD54) or anti-P-selectin antibody (1:500 [vol/vol] in PBS) (DBA, Milan, Italy).

Controls included kidney sections incubated with buffer alone or nonspecific purified IgG (DBA). After blocking endogenous avidin and biotin, specific labeling of antigen-antibody complex was visualized using chromogen diaminobenzidine.

Measurement of cytokines

TNF-α and IL-1β levels were evaluated in kidney samples following reperfusion. The assay was carried out by using a colorimetric commercial kit (Calbiochem-Novabiochem Corp., CA, USA). The enzyme-linked immunosorbant assay has a lower detection limit of 30 pg/mL.

Materials

Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd. (Milan, Italy). All solutions used *in vivo* were prepared using non-pyrogenic saline (0.9 % [wt/vol] NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, UK).

Statistical analysis

All values described in the text and figures are expressed as mean ± standard error of the mean (SEM) for *n* observations. Each data point represents biochemical measurements obtained from four to seven separate animals. For histological scoring, each data point represents analysis of kidneys taken from four to seven individual animals. Statistical analysis was carried out using GraphPad Prism/Instat 1.1 (GraphPad Software, San Diego, California, USA). Data were analyzed using one-way ANOVA followed by Dunnett's *post hoc* test and a *P* value of less than 0.05 was considered to be significant.

RESULTS

Renal dysfunction in wild-type mice treated with an IL-6 MAb and in IL-6$^{-/-}$ mice: Plasma urea and creatinine

When compared to sham-operated mice, I/R caused a significant increase in the plasma levels of urea and creatinine in wild-type (control) mice (Figures 1A, 1B, 1C and 1D), suggesting a significant degree of renal dysfunction in both studies. The increases in the plasma levels of urea and creatinine seen in IL-6$^{-/-}$ mice subjected to I/R were significantly smaller than those seen in their wild-type littermates (Figures 1A and 1B), suggesting a marked reduction in the renal dysfunction associated with renal I/R. However, the degree of protection from renal dysfunction observed with the IL-6 MAb in wild-type mice (urea: 41 % attenuation; creatinine: 44 % attenuation) (Figures 1C and 1D) was not as substantial as that seen in IL-6$^{-/-}$ mice (urea: 58 % attenuation; creatinine: 86 % attenuation) (Figures 1A and 1B).

Renal injury in wild-type mice treated with an IL-6 MAb and in IL-6$^{-/-}$ mice: Histological assessment

When compared to sham-operated mice (Figures 2A and 3A), histological examination of kidneys obtained from wild-type (control) mice subjected to I/R demonstrated a significant degree of renal injury (Figures 2B and 3B). Specifically, kidneys obtained from these animals exhibited degeneration of tubular structure, tubular dilatation, swelling and necrosis, luminal congestion, and eosinophilia. In contrast, renal sections obtained from IL-6$^{-/-}$ mice which underwent I/R demonstrated a marked reduction in the severity of these histological features of renal injury (Figure 2B), when compared with kidneys obtained from IL-6 wild-type mice subjected to I/R (Figure 2C). In addition, renal sections obtained from mice treated with 10 μg/day IL-6 MAb which underwent I/R also demonstrated a marked reduction in the severity of the above histological features of renal injury (Figure 3B), when compared with kidneys obtained from control mice subjected to I/R (Figure 3C).

Renal inflammation in wild-type mice treated with an IL-6 MAb and in IL-6$^{-/-}$ mice: PMN counts and MDA levels

Quantitation of infiltrating PMNs into renal tissues showed that there was only a minimal number of PMNs in non-ischemic kidneys obtained from sham-operated mice in both studies (Figures 4A and 4B). However, a significant number of infiltrating PMNs were observed in the renal cortex of mice subjected to I/R of the kidney in both studies (Figures 4A and 4B). The number of PMNs infiltrating into renal tissues of IL-6$^{-/-}$ mice (Figure 4A) and mice treated with the IL-6 MAb (Figure 4B) was significantly attenuated by approximately 59 % and 50 %, respectively. When compared to sham-operated mice, the kidneys obtained from wild-type mice subjected to I/R demonstrated a significant increase in MDA levels (Figure 5), thus suggesting increased lipid peroxidation in renal tissues. The increase in the tissue level of MDA seen in IL-6$^{-/-}$ mice subjected to I/R was significantly smaller than those seen in their wild-type littermates (Figure 5).

Renal inflammation caused by I/R in IL-6$^{-/-}$ mice: Nitrotyrosine formation

When compared to sham-operated mice (Figure 6A), immunohistochemical analysis of kidney sections obtained from wild-type mice subjected to I/R demonstrated a positive staining for nitrotyrosine (Figure 6B). In contrast, renal sections obtained from IL-6$^{-/-}$ mice that underwent I/R demonstrated no positive staining for nitrotyrosine when compared with kidneys obtained from wild-type mice subjected to I/R (Figure 6C). This suggests reduced nitration of proteins during I/R in mice lacking IL-6.

Renal inflammation caused by I/R in IL-6$^{-/-}$ mice: ICAM-1 and P-selectin expression

When compared with kidneys obtained from sham-operated mice (Figures 7A and 8A), kidneys obtained from wild-type mice demonstrated marked staining for ICAM-1 (Figure 7B) and P-selectin (Figure 8B), suggesting adhesion molecule expression during reperfusion. A marked

reduction in the staining for both ICAM-1 and P-selectin was observed in kidneys obtained from IL-$6^{-/-}$ mice subjected to renal I/R (Figures 7C and 8C) when compared with kidneys from their wild-type littermates. This suggests a reduction in the expression of these adhesion molecules during reperfusion in mice lacking IL-6.

Renal inflammation caused by I/R in IL-$6^{-/-}$ mice: Kidney TNF-α and IL-1β

To determine whether endogenous IL-6 may modulate the inflammatory process through the regulation of the secretion of other cytokines, we analyzed the tissue levels of TNF-α and IL-1β in IL-$6^{-/-}$ mice and their wild-type littermates. When compared to sham-operated mice, I/R caused a significant increase in the tissue levels of TNF-α and IL-1β in wild-type mice (Figures 9A and 9B). The increases in the tissue levels of TNF-α and IL-1β seen in IL-$6^{-/-}$ mice subjected to I/R were significantly smaller than those seen in their wild-type littermates (Figures 9A and 9B).

There were no differences in any of the above parameters measured between sham-operated groups (see Figures 1, 4, 5 and 9).

DISCUSSION

We demonstrate here that the renal injury, dysfunction and inflammation caused by bilateral occlusion (30 min) and reperfusion (24 h) is significantly smaller in mice in which the gene for IL-6 was deleted (IL-$6^{-/-}$ mice) than in their wild-type littermates. Specifically, we found that I/R in wild-type mice caused (i) significant renal dysfunction; (ii) characteristic histological signs of marked tubular injury; (iii) significant PMN accumulation and lipid peroxidation; (iv) increased nitration of proteins; (v) a marked expression/upregulation of the adhesion molecules, ICAM-1 and P-selectin; and (vi) a significant increase in the renal tissue levels of the pro-inflammatory cytokines, TNF-α and IL-1β. All this data confirmed a well-known pattern of renal injury, dysfunction and inflammation caused by I/R of the kidney (Paller, 1994a;Sheridan and Bonventre, 2001). Moreover, our findings are in agreement with the notion that renal I/R causes tubular dysfunction (Paller, 1994b), as well as secondary inflammation (Dragun *et al.*, 2000) We report here for the first time that all of the above signs of renal injury, dysfunction and inflammation were significantly reduced in IL-$6^{-/-}$ mice and in wild-type mice in which endogenous IL-6 was blocked. This finding supports the view that endogenous IL-6 enhances the renal injury, dysfunction and inflammation caused by I/R in mice. Although, plasma/kidney levels of IL-6 were not measured in IL-$6^{-/-}$ mice, a previous study using this strain of knock-out mouse demonstrated that IL-6 levels were undetectable in the plasma of these mice before or after I/R (Cuzzocrea *et al.*, 1999). Additionally, although mortality was not measured or analysed statistically in this study, less IL-$6^{-/-}$ mice died during renal I/R than wild-type mice which were subjected to I/R.

There is now good evidence that I/R and/or inflammation leads to a significant increase in the expression of IL-6 in many organs including the brain (Maeda *et al.*, 1994), myocardium (Kukielka *et al.*, 1995), hind limb (Yassin *et al.*, 2002) and gut (Yao *et al.*, 1997). Our conclusion that endogenous IL-6 contributes to tissue injury and inflammation is supported by a number of other studies: In IL-$6^{-/-}$ mice, the induction of acute-phase proteins, weight loss, and

hyperglycemia caused by injection of turpentine were dramatically reduced (Alonzi *et al.*, 1998a). The recruitment of PMNs caused by injection of carrageenan into a subcutaneous air pouch was also substantially reduced in IL-$6^{-/-}$ mice when compared with their wild-type littermates (Romano *et al.*, 1997). The arthritis (accumulation of PMNs in the knee joint and related tissue damage) caused by collagen was substantially attenuated in IL-$6^{-/-}$ mice (Alonzi *et al.*, 1998b). Most notably, the tissue injury and inflammation caused by splanchnic artery occlusion and reperfusion is significantly reduced in IL-$6^{-/-}$ mice (Cuzzocrea *et al.*, 1999). All of the above studies support the hypothesis that endogenous IL-6 enhances the degree of inflammation and tissue injury caused by a number of different stimuli, including I/R.

What, then, is(are) the mechanism(s) by which endogenous IL-6 amplifies the inflammatory response and ultimately the tissue injury and dysfunction caused by renal ischemia and reperfusion? It is well known that the release of a cascade of potent inflammatory mediators into the systemic circulation is central to any severe illness. There is evidence that the pro-inflammatory cytokines TNF-α and IL-1β help to propagate the extension of a local or systemic inflammatory process (Alonzi et al., 1998a;Utsunomiya et al., 1991), especially during sepsis and have been used to predict mortality in patients. We confirm here that the inflammatory process caused by renal I/R leads to a substantial increase in the levels of both TNF-α and IL-1β in the kidney. Interestingly, the levels of these two pro-inflammatory cytokines were significantly lower in kidneys obtained from animals that are unable to produce endogenous IL-6 (IL-$6^{-/-}$ mice). This finding suggests that in the presence of endogenous IL-6, the degree of inflammation and, hence, the formation of TNF-α and IL-1β, caused by renal I/R are significantly enhanced. In addition, Simmons *et. al.* (2004) have recently reported that IL-6 and IL-8 are significant predictors of mortality in end-stage renal disease patients (Simmons *et al.*, 2004), and this is supported by previous studies demonstrating that IL-6 and mortality is highly associated in patients with chronic kidney disease (Bologa *et al.*, 1998;Pecoits-Filho *et al.*, 2002). It would appear that there were no compensatory mechanisms for IL-6 depletion in these mice, however, it

is possible that gp130 gene expression may have been higher in these mice. Further investigation of this possibility is warranted.

There is evidence that renal I/R leads to an increase in the expression of P-selectin and ICAM-1 in rat (Dragun *et al.*, 2000) and man (Koo *et al.*, 1998) and, hence, an excessive accumulation of PMNs within the kidney. IL-6 enhances PMN accumulation within the renal tissue by causing amplification (positive feed-back) of the formation of the pro-inflammatory cytokines TNF-α and IL-1β (see above) and/or of the expression of the adhesion molecules ICAM-1 and P-selectin. We document here that the degree of staining for ICAM-1 and P-selectin was significantly reduced in IL-$6^{-/-}$ mice suggesting that endogenous IL-6 enhances the expression of ICAM-1 and P-selectin. It is possible that the reduced expression of TNF-α and IL-1β observed in IL-$6^{-/-}$ mice subjected to renal I/R may contribute to the reduced expression of ICAM-1 and P-selectin in these animals. It should, however, also be noted that the expression of ICAM-1 caused by renal I/R is not abolished in mice lacking TNF-receptor-1 and IL-1-receptor-1, suggesting that TNF-α and IL-1β are not essential for the expression of ICAM-1 in the kidney (Burne *et al.*, 2001).

STAT3 is the main mediator of IL-6 cytokine signalling; it modulates cellular responses to IL-6 and mediates IL-10 function in macrophages (Maritano *et al.*, 2004). STAT3 has been implicated in conditions of inflammation and IL-6 has been shown to activate STAT3 during both ischemia and reperfusion (Hierholzer *et al.*, 1998). IL-6 related cytokines have now been demonstrated as the main activators of STAT3. In a recent study, STAT3 activation was reduced in IL-$6^{-/-}$ mice subjected to colitis (Suzuki *et al.*, 2001) and that STAT3 is most likely related to the progression or development of the disease rather than the initiation, suggesting STAT3 is most probably involved in the process of controlled inflammation rather than the disease itself. The role of STAT3 (and its modulation by IL-6) in the development of renal I/R injury is not known and certainly warrants further detailed investigation.

Recent studies have postulated a role of adaptive immunity in renal I/R injury. IL-6 was originally identified as a lymphokine inducing final maturation of B lymphocytes into antibody-secreting cells, thus the depletion of IL-6 would prevent this final maturation. Burne-Taney *et. al.* (2003) demonstrated a pathogenic role for B lymphocytes in ischemic acute renal failure by way of using mice deficient of B cells (Burne-Taney et al., 2003). The absence of IL-6 may have resulted in a reduction of B cells, an effect which may have contributed to the observed reduction in I/R injury in IL-$6^{-/-}$ mice.

I/R of the kidney leads to an enhanced formation of ROS and peroxynitrite (see Introduction). The biological activity and decomposition of peroxynitrite are very much dependent on the cellular or chemical environment and these factors influence its toxic potential (Beckman et al., 1990;Rubbo et al., 1994). We demonstrate here that renal I/R of wild-type mice leads to a subsequent increase in the degree of nitrosylation of proteins in the kidney. In contrast, the degree of staining for nitrotyrosine was markedly reduced in IL-$6^{-/-}$ mice. There is recent evidence that certain reactions can also induce tyrosine nitration; e.g., the reaction of nitrate with hypochlorous acid and the reaction of MPO with hydrogen peroxide can lead to the formation of nitrotyrosine (Eiserich *et al.*, 1998). Increased nitrotyrosine staining is considered, therefore, as an indication of increased nitrosative stress. Thus, our results suggest that the degree of nitrosative stress caused by renal I/R is reduced in kidneys from animals that are unable to produce endogenous IL-6. The enhanced generation of ROS during renal I/R may not only promote the generation of peroxynitrite, but also cause tissue injury secondary to protein denaturation, DNA damage and peroxidation of membrane lipids (Szabo *et al.*, 1997). We demonstrate here that the degree of lipid peroxidation (determined as MDA formation within the kidney) is reduced in IL-$6^{-/-}$ mice. This finding supports the view that endogenous IL-6 enhances the degree of oxidative stress.

In conclusion, this study demonstrates for the first time, that endogenous IL-6 exacerbates the degree of renal injury, dysfunction and inflammation caused by I/R injury. This finding

indicates that (i) IL-6 can act as a pro-inflammatory cytokine in renal disorders associated with I/R, and (ii) that strategies aimed at reducing the formation and/or the effects of IL-6 may be useful in conditions associated with renal ischemia and inflammation.

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FOOTNOTES

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FIGURE LEGENDS

***Figure 1*. Renal dysfunction in wild-type mice treated with an IL-6 MAb and in IL-6$^{-/-}$ mice.** Plasma urea (**A**) and creatinine (**B**) levels were measured as biochemical markers of renal dysfunction subsequent to sham-operation (Sham WT (wild-type), n = 4; Sham IL-6$^{-/-}$, n = 4) or renal I/R (I/R WT, n = 6; I/R IL-6$^{-/-}$, n = 7). Plasma urea (**C**) and creatinine (**D**) levels were measured as biochemical markers of renal dysfunction subsequent to sham-operation (Sham IgG1 (10 μg/day), n = 4; Sham IL-6 MAb (10 μg/day), n = 4) or renal I/R (I/R IgG1 (10 μg/day), n = 8; I/R IL-6 MAb (10 μg/day), n = 10). Data represent mean ± SEM for n observations, ★P<0.05 *vs*. I/R WT or I/R IgG1 group.

***Figure 2*. Renal injury in IL-6$^{-/-}$ mice: Histological examination.** Renal sections taken from (A) a sham-operated mouse, (B) a mouse subjected to renal I/R, and (C) an IL-6$^{-/-}$ mouse subjected to renal I/R. Hemotoxylin and eosin, original magnification x 150, figures are representative of at least 3 experiments performed on different days (n = 4 – 7 for all groups).

***Figure 3*. Renal injury in mice treated with an IL-6 MAb: Histological examination.** Renal sections taken from (A) a sham-operated mouse administered 10 μg/day IgG1 isotype control, (B) a mouse subjected to renal I/R administered 10 μg/day IgG1 isotype control, and (C) a mouse subjected to renal I/R administered 10 μg/day IL-6 MAb. Hemotoxylin and eosin, figures are representative of at least 3 experiments performed on different days (n = 4 – 10 for all groups).

Figure 4. **Renal inflammation in wild-type mice treated with an IL-6 MAb and in IL-6$^{-/-}$ mice: PMN infiltration.** The total number of infiltrating leukocytes (e.g. neutrophils and mononuclear cells) in cortical interstitial spaces was assessed quantitatively by counting the number of PMNs in 20 high power fields. PMN counts (**A**) were measured subsequent to sham-operation (Sham WT (wild-type), n = 4; Sham IL-6$^{-/-}$, n = 4) or renal I/R (I/R WT, n = 6; I/R IL-6$^{-/-}$, n = 7). PMN counts **(B)** were measured subsequent to sham-operation (Sham IgG1 (10 µg/day), n = 4; Sham IL-6 MAb (10 µg/day), n = 4) or renal I/R (I/R IgG1 (10 µg/day), n = 8; I/R IL-6 MAb (10 µg/day), n = 10). Data represent mean ± SEM for n observations, ★P<0.05 *vs.* I/R WT or I/R IgG1 group.

Figure 5. **Renal inflammation caused by I/R in IL-6$^{-/-}$ mice: MDA levels (lipid peroxidation).** MDA levels were measured as a marker of lipid peroxidation subsequent to sham-operation (Sham WT (wild-type), n = 4; Sham IL-6$^{-/-}$, n = 4) or renal I/R (I/R WT, n = 6; I/R IL-6$^{-/-}$, n = 7). Data represent mean ± SEM for n observations, ★P<0.05 *vs.* I/R WT or I/R IgG1 group.

Figure 6. **Renal inflammation caused by I/R in IL-6$^{-/-}$ mice: Nitrotyrosine formation;** (A) sham-operated group, (B) wild-type group subjected to renal I/R and (C) IL-6$^{-/-}$ group subjected to renal I/R. Primary anti-nitrotyrosine antibody, original magnification x 125, figures are representative of at least 3 experiments performed on different experimental days (n = 4 - 7 for all groups).

Figure 7. **Renal inflammation caused by I/R in IL-6$^{-/-}$ mice: ICAM-1 expression;** (A) sham-operated group, (B) wild-type group subjected to renal I/R and (C) IL-6$^{-/-}$ group subjected to renal I/R. Anti-ICAM-1 antibody, original magnification x 125, figures are representative of at least 3 experiments performed on different experimental days (n = 4 - 7 for all groups).

Figure 8. **Renal inflammation caused by I/R in IL-6$^{-/-}$ mice: P-selectin expression;** (A) sham-operated group, (B) wild-type group subjected to renal I/R and (C) IL-6$^{-/-}$ group subjected to renal I/R. Anti-P-selectin antibody, original magnification x 125, figures are representative of at least 3 experiments performed on different experimental days (n = 4 - 7 for all groups).

Figure 9. **Renal inflammation caused by I/R in IL-6$^{-/-}$ mice: Kidney TNF-α and IL-1β;** Kidney TNF-α (**A**) and IL-1β (**B**) levels were measured subsequent to sham-operation (Sham WT (wild-type), n = 4; Sham IL-6$^{-/-}$, n = 4) or renal I/R (I/R WT, n = 6; I/R IL-6$^{-/-}$, n = 7). Data represent mean ± SEM for n observations, ★$P < 0.05$ *vs.* I/R WT group.

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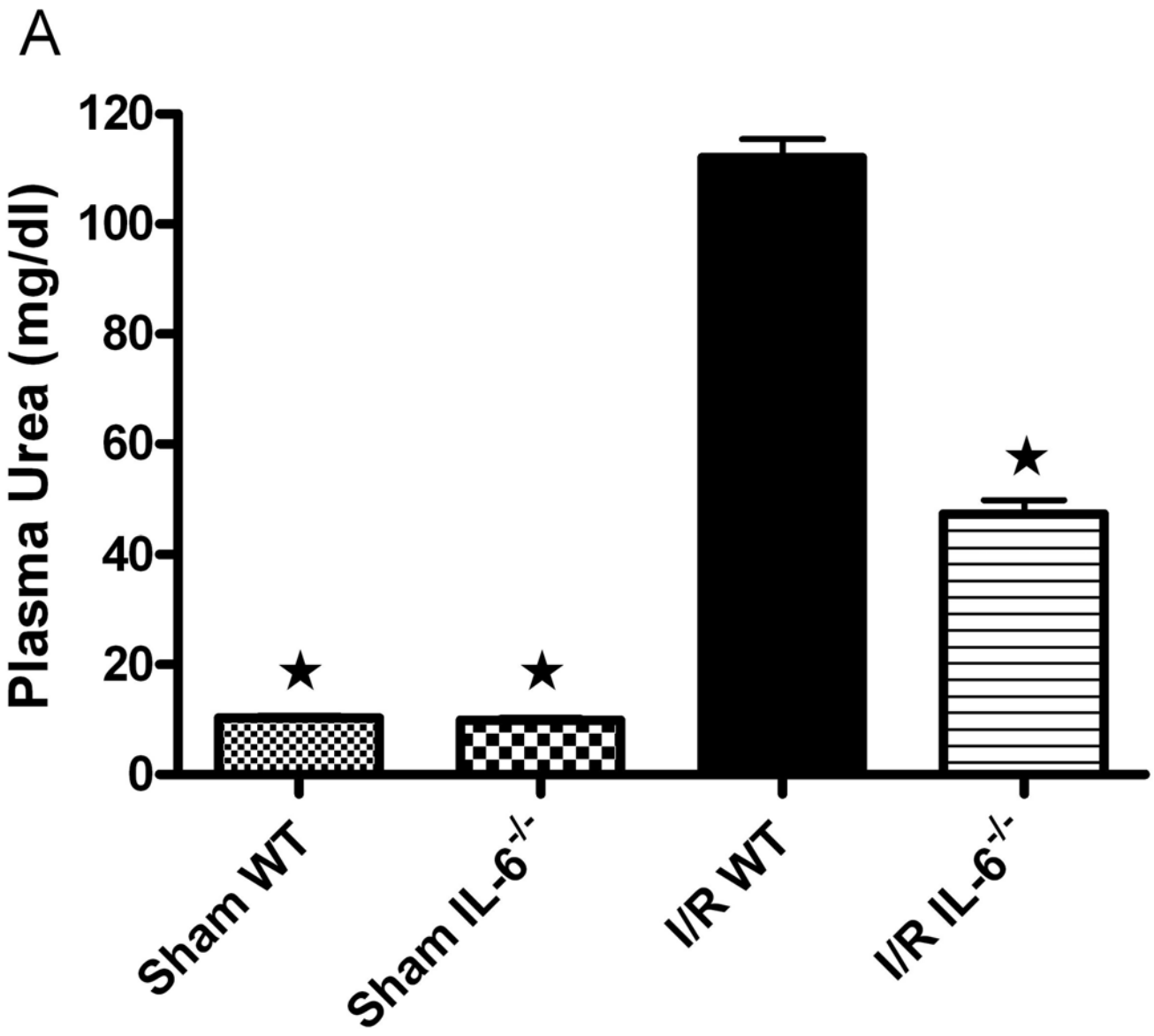

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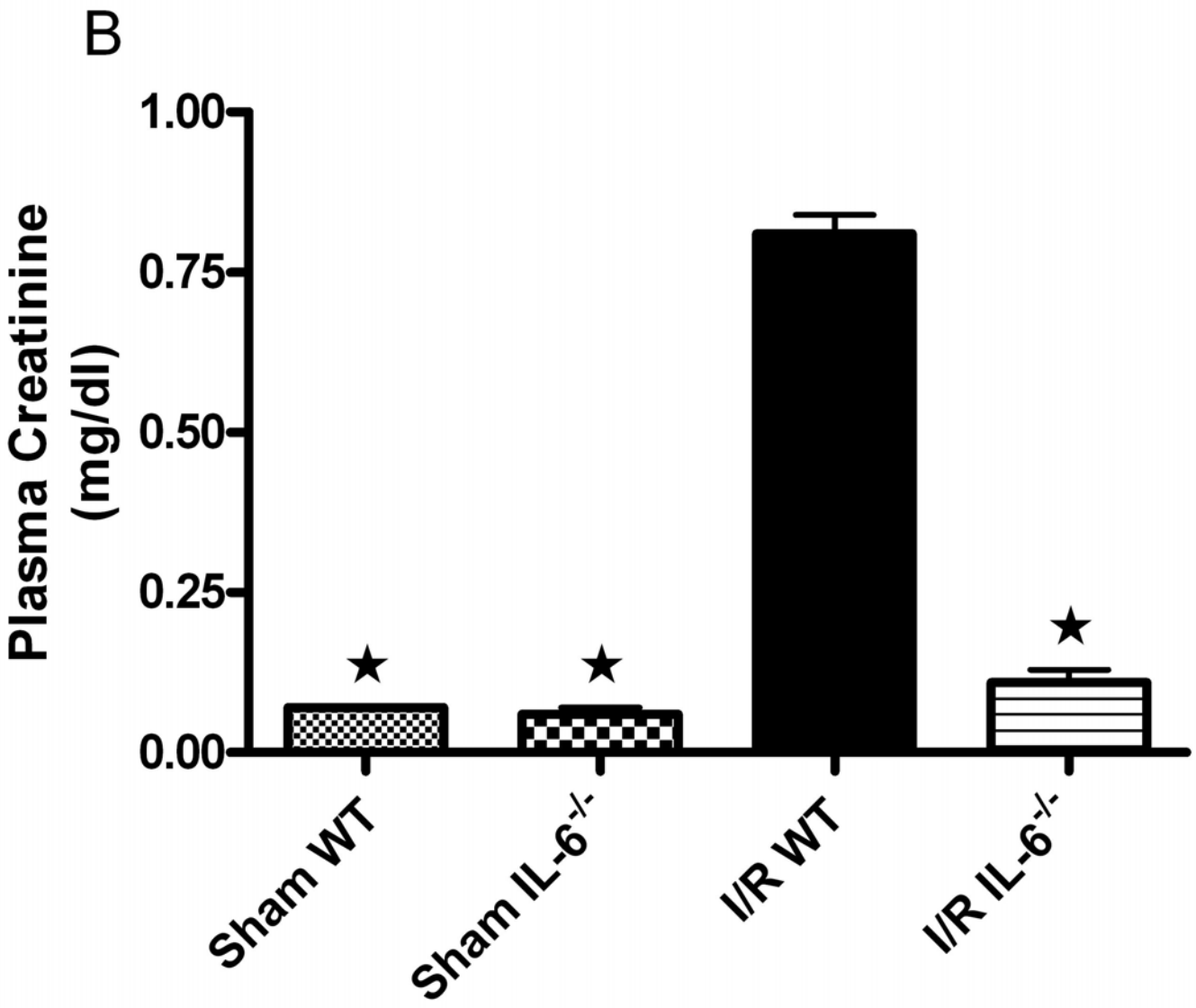

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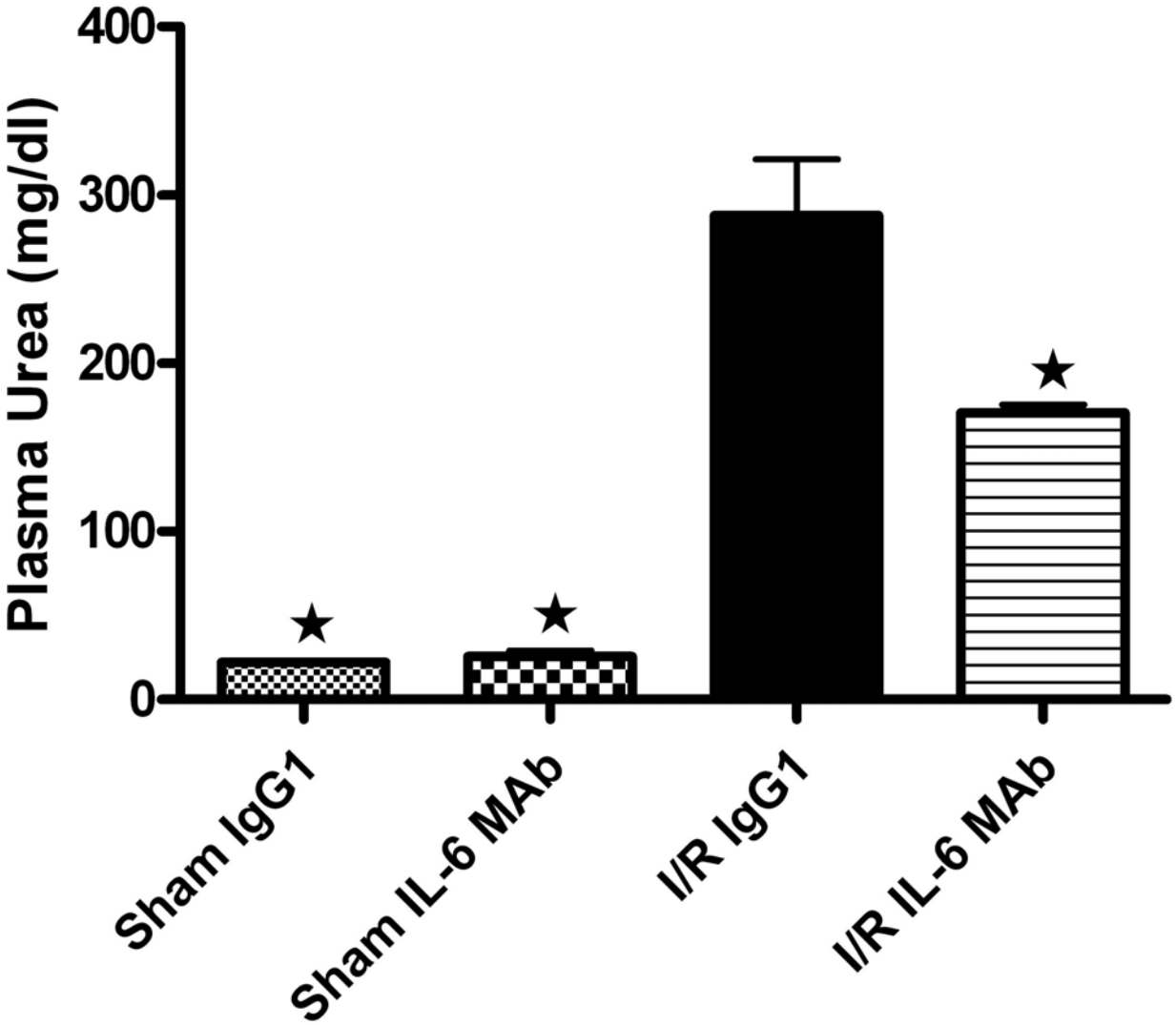

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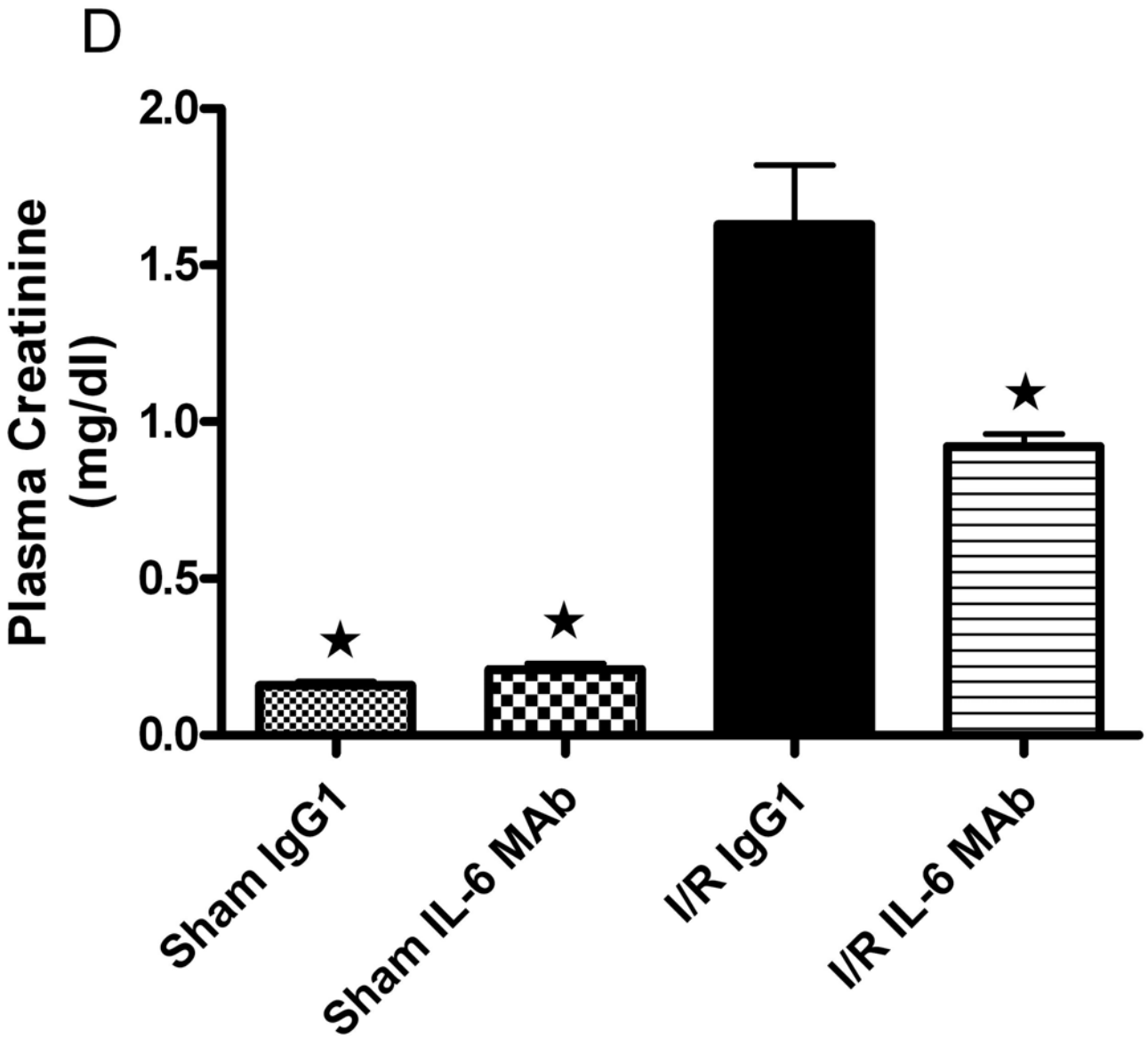

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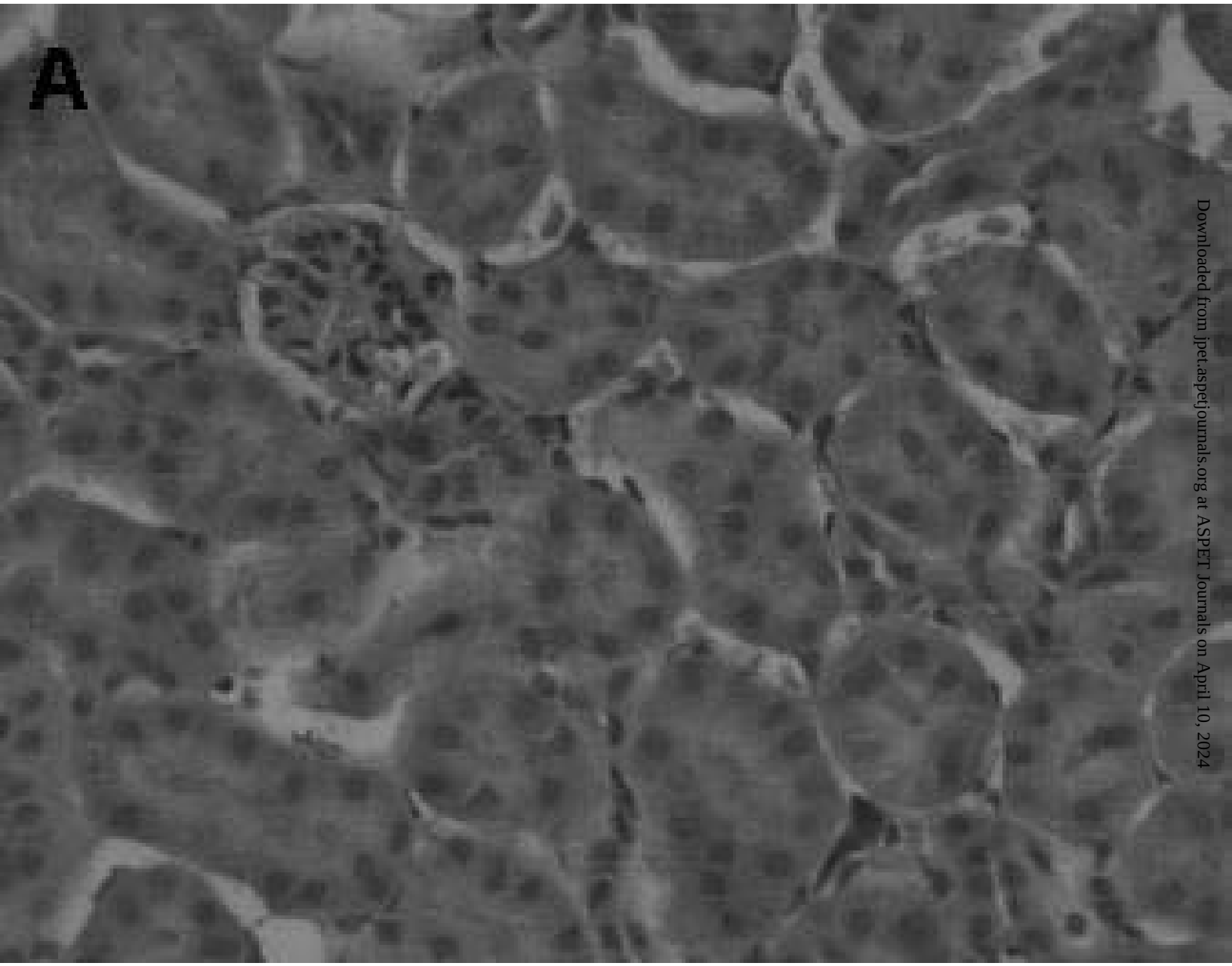

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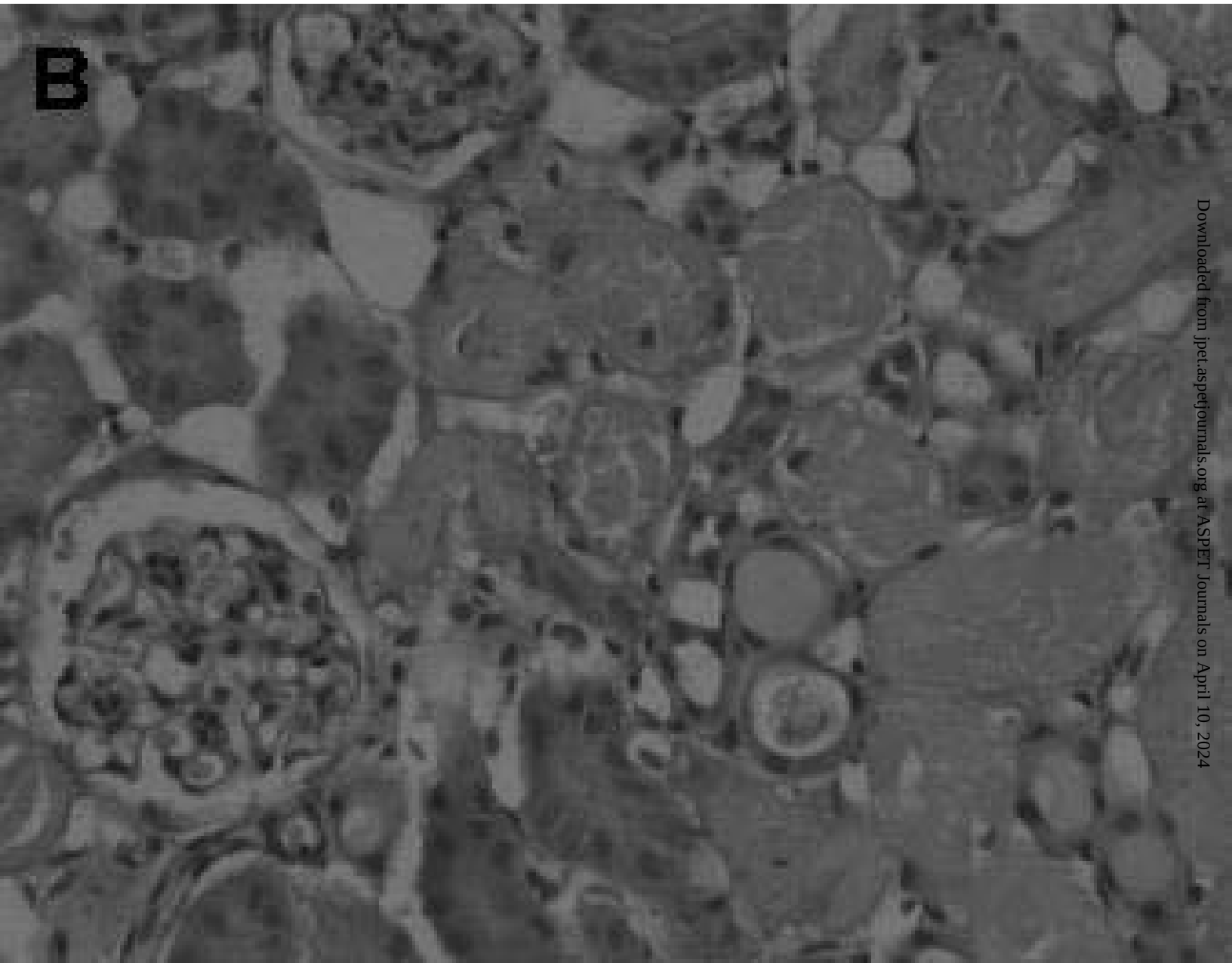

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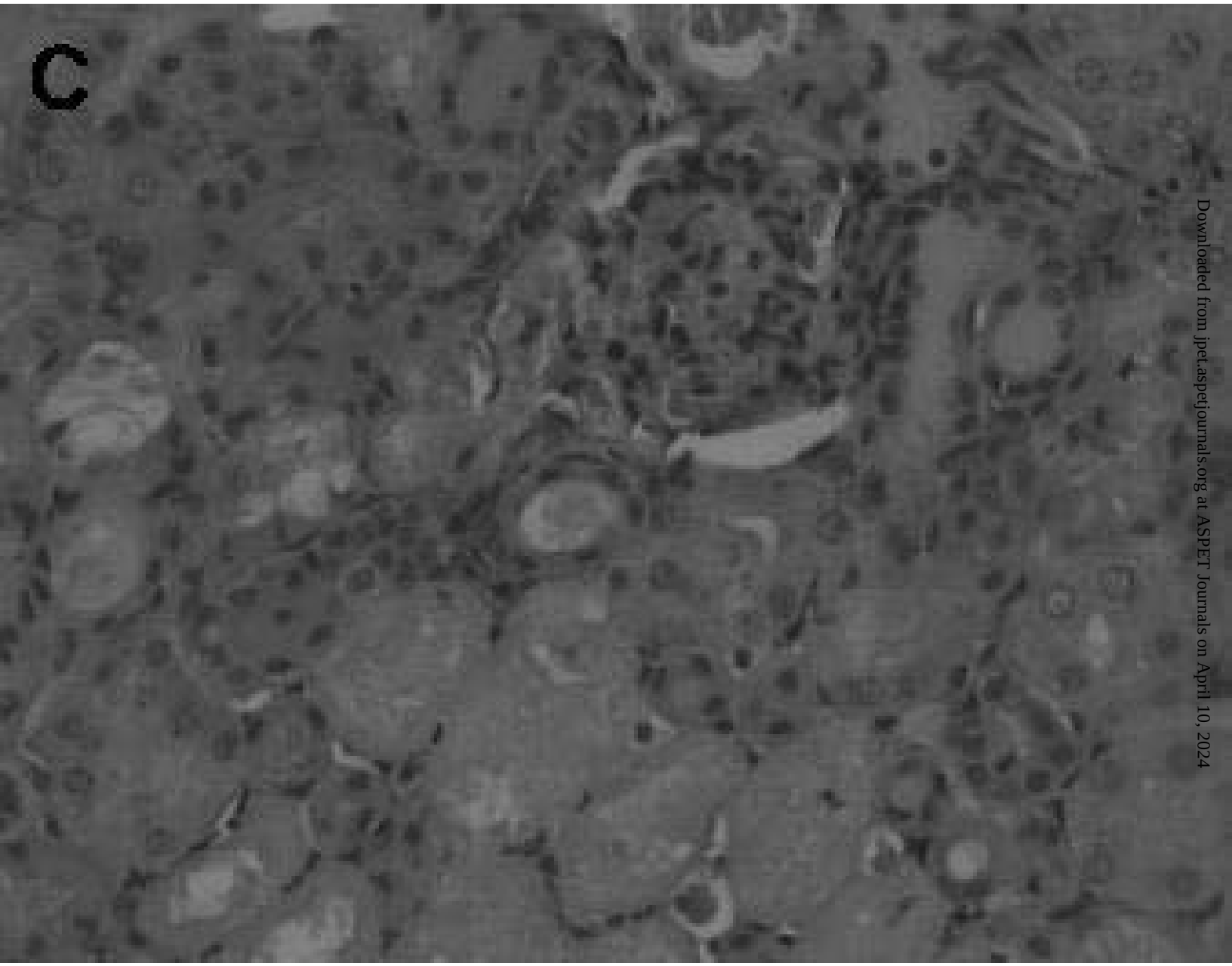

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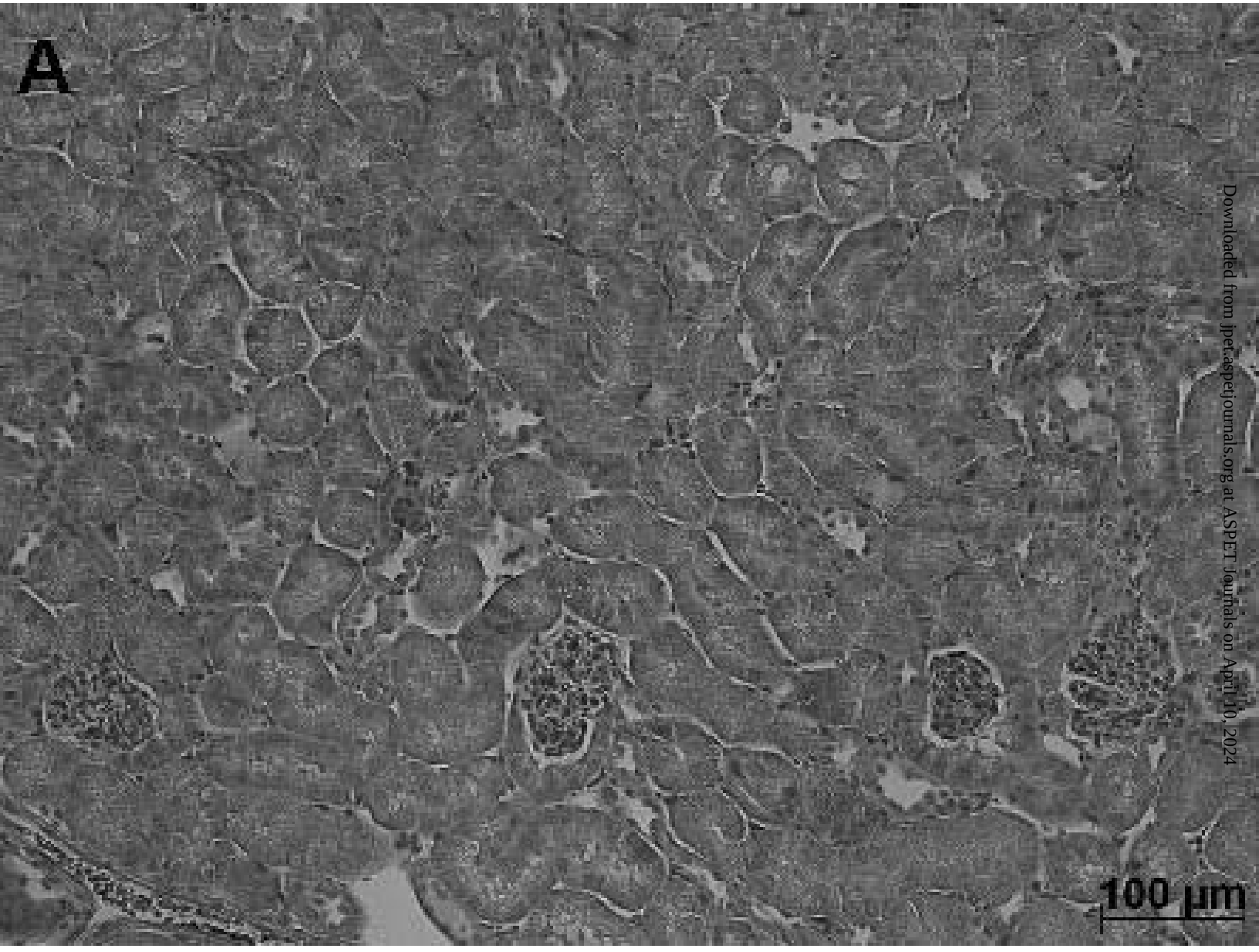

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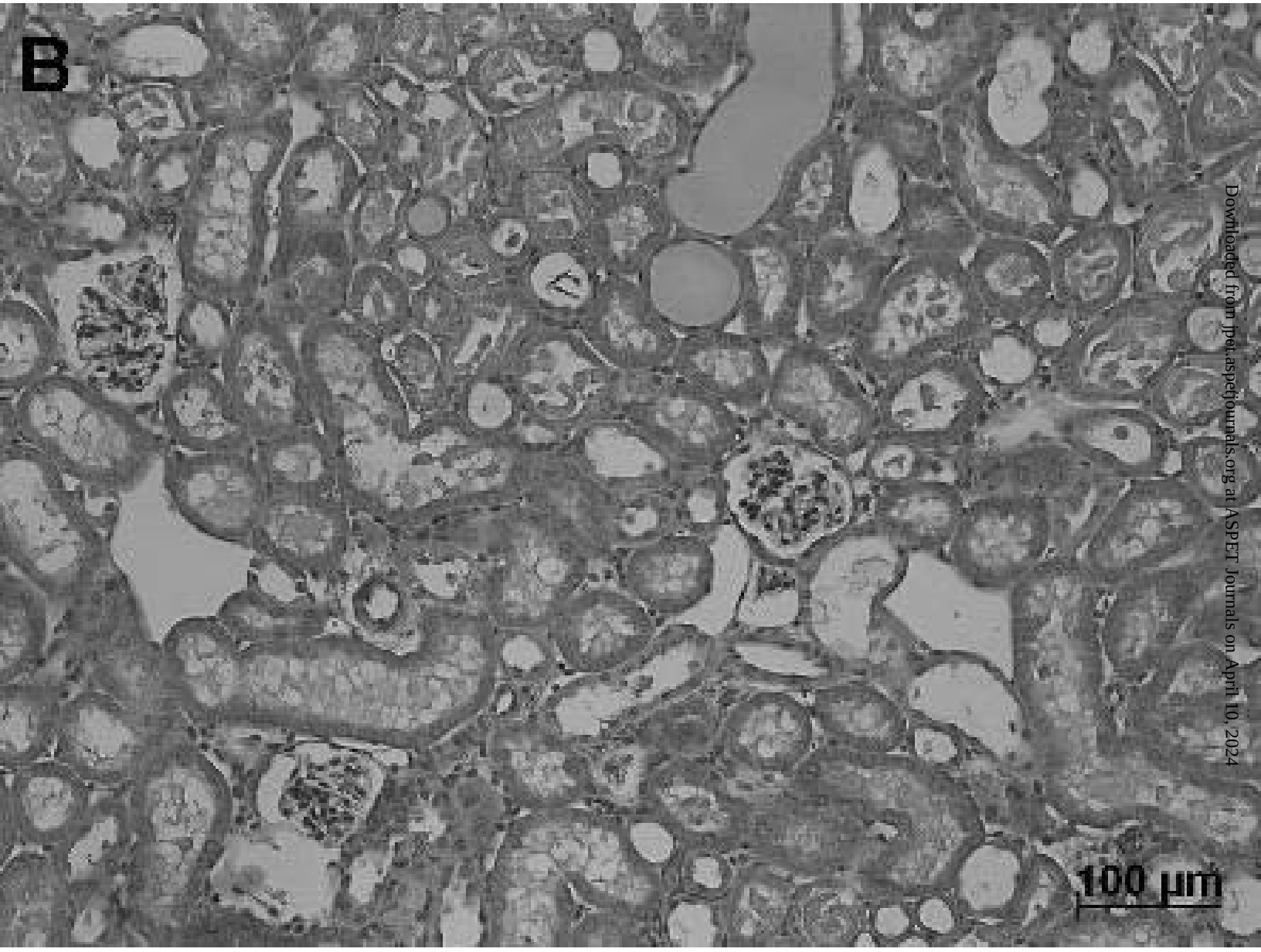

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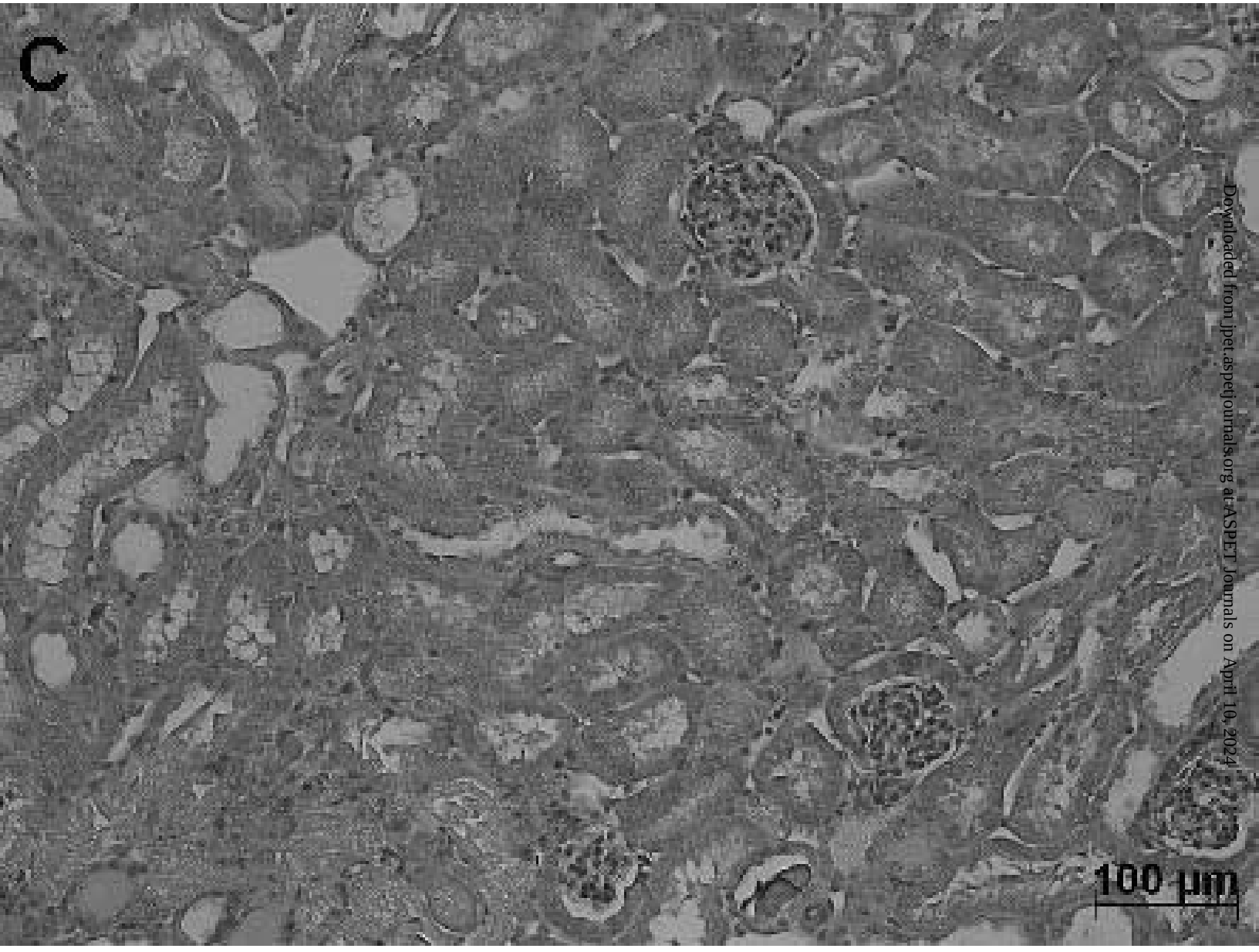

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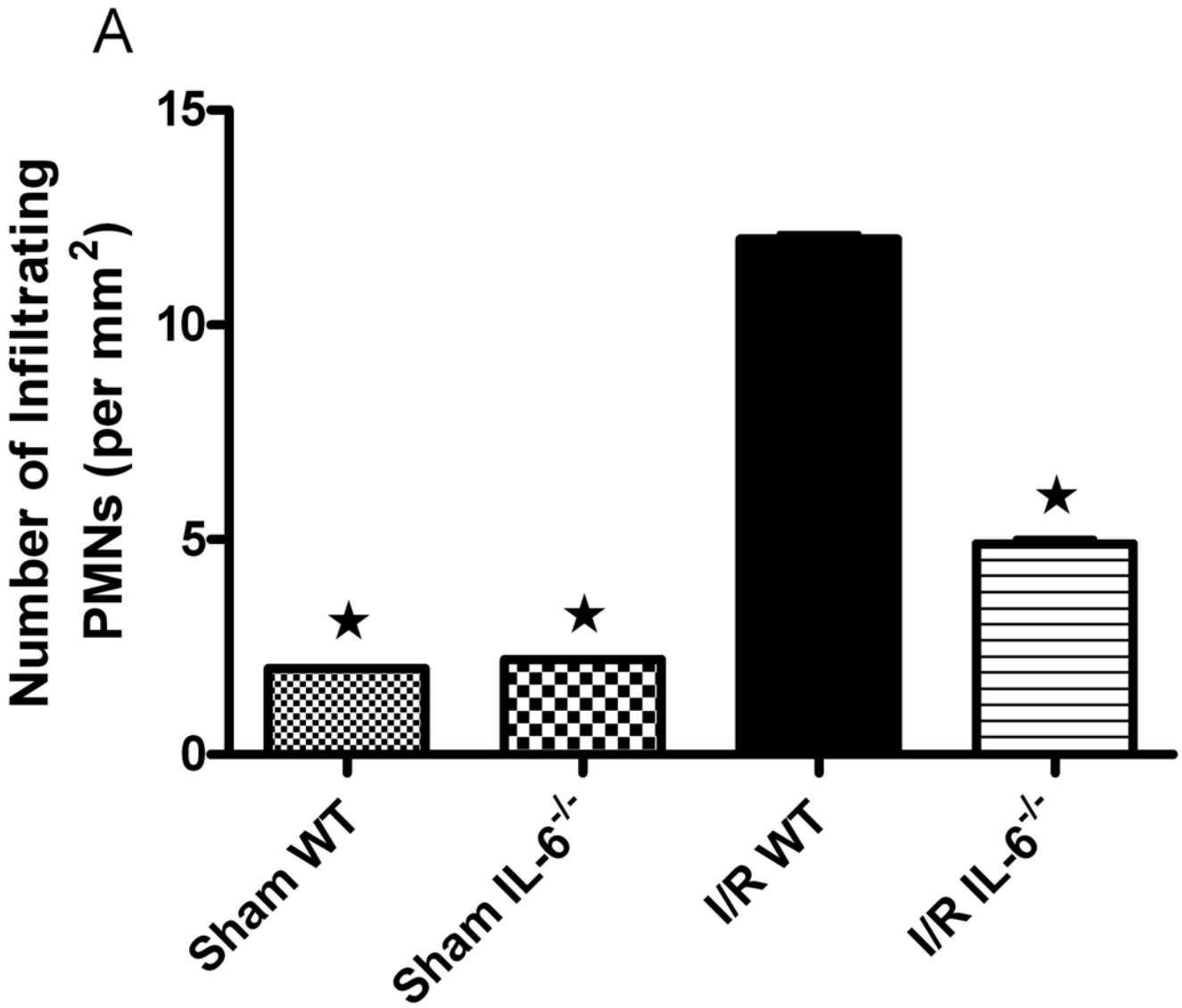

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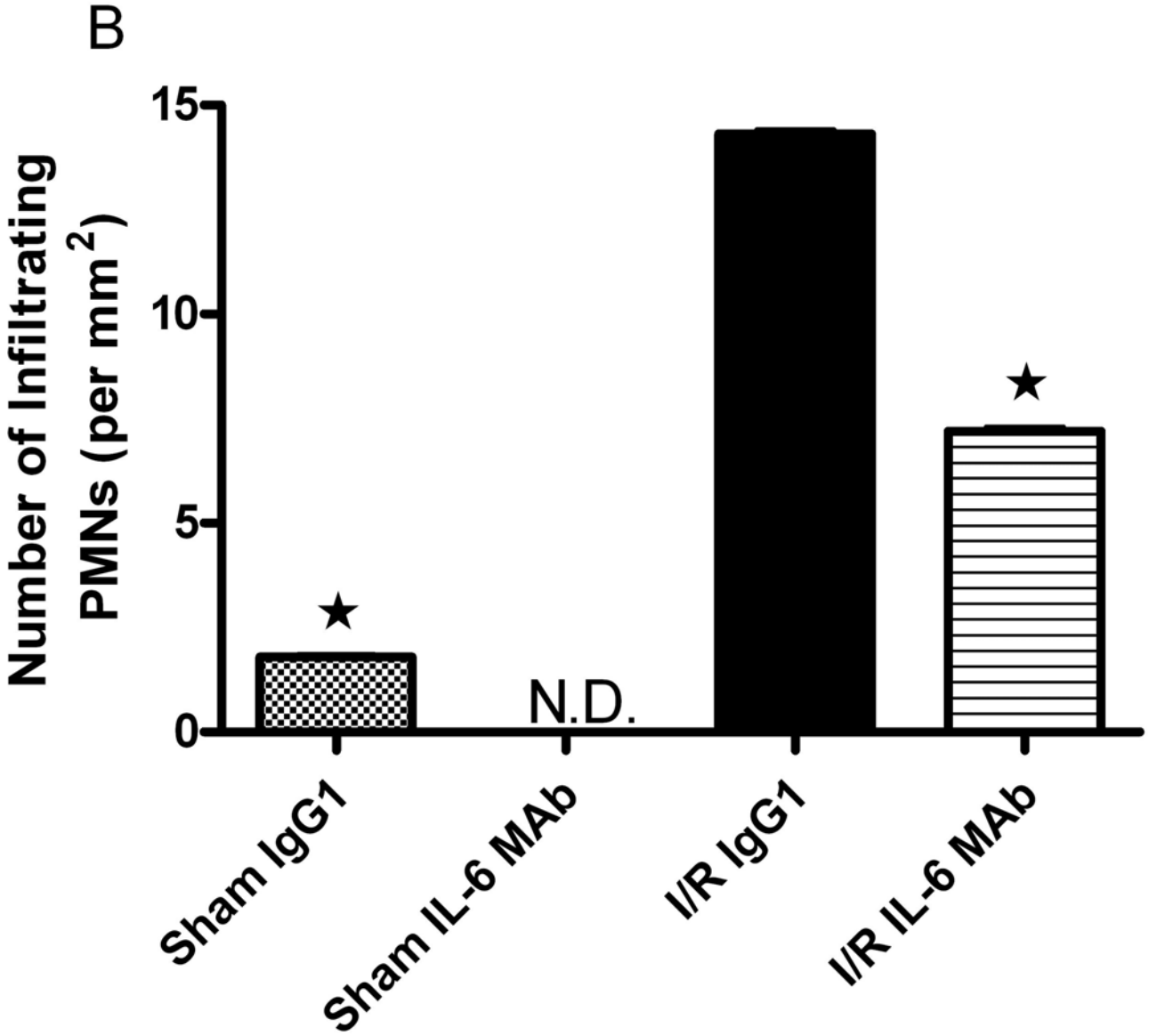

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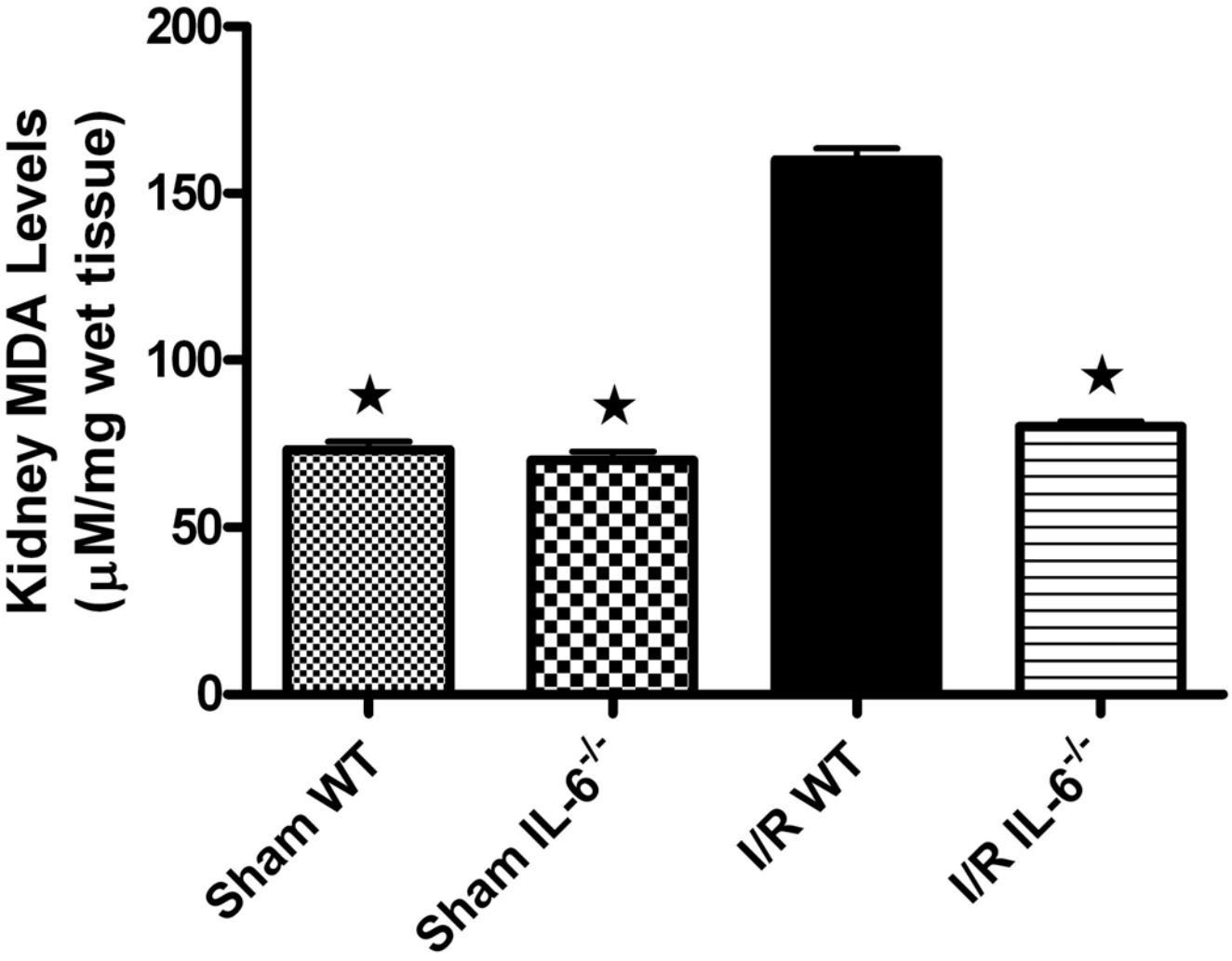

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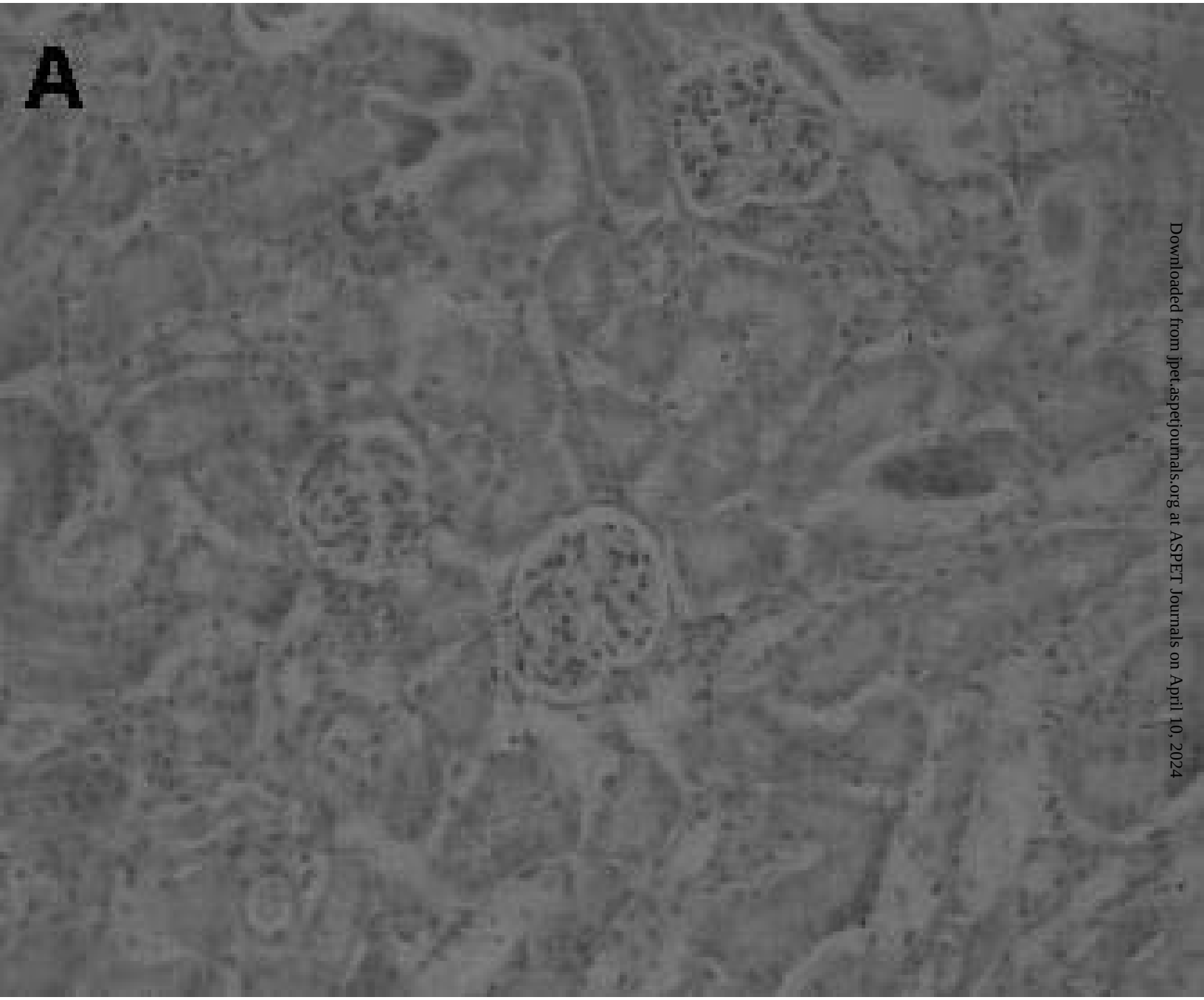

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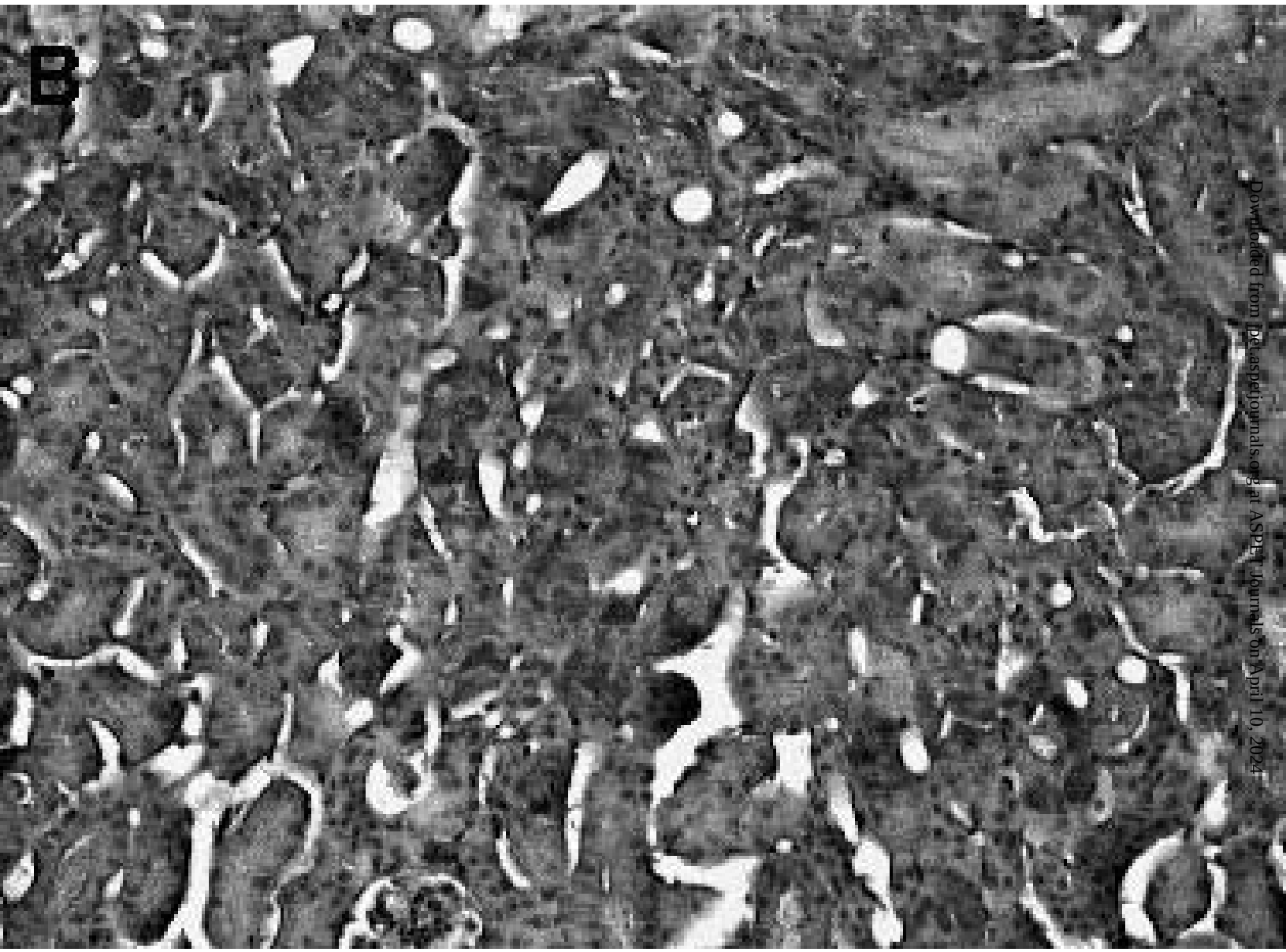

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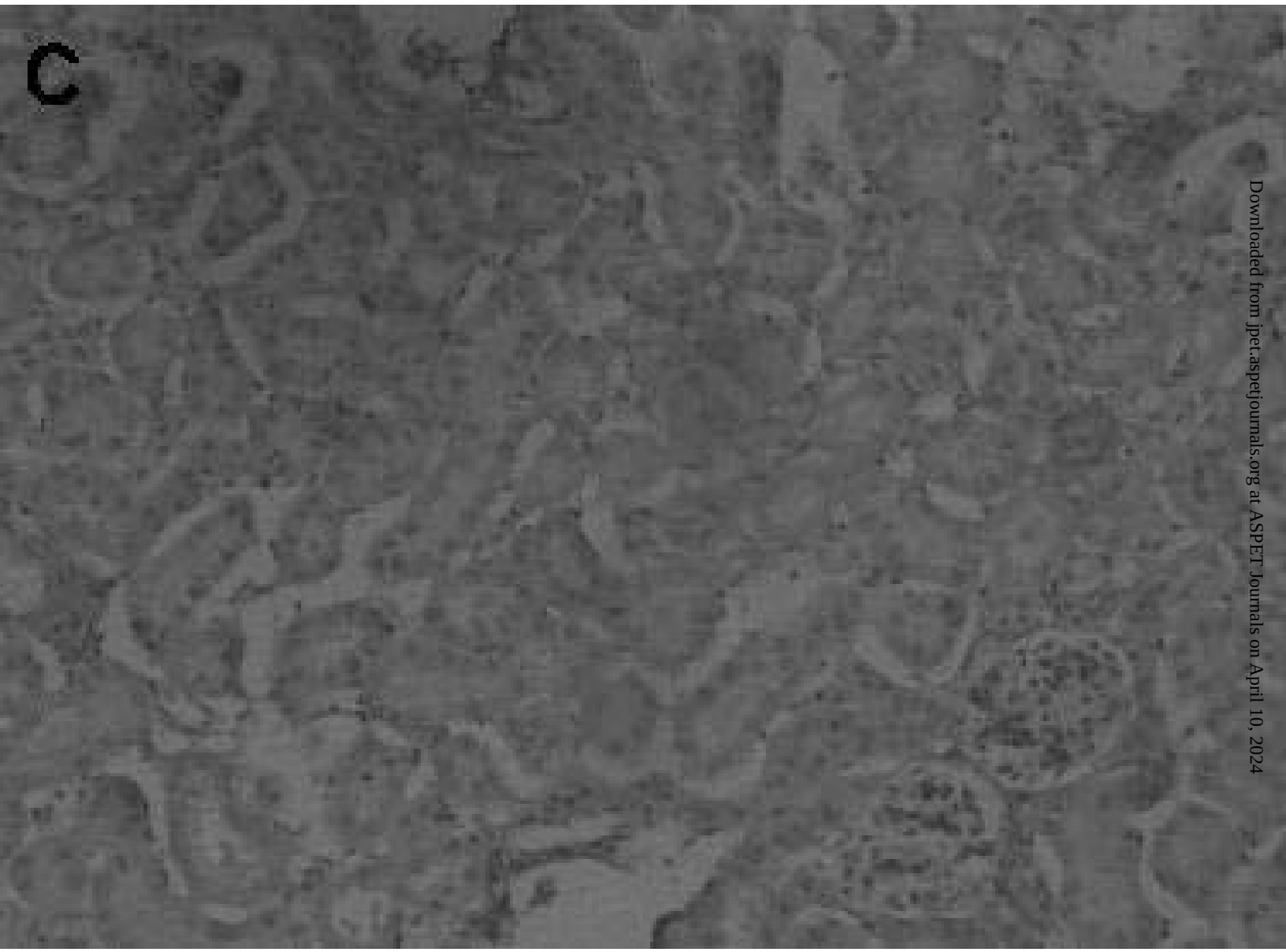

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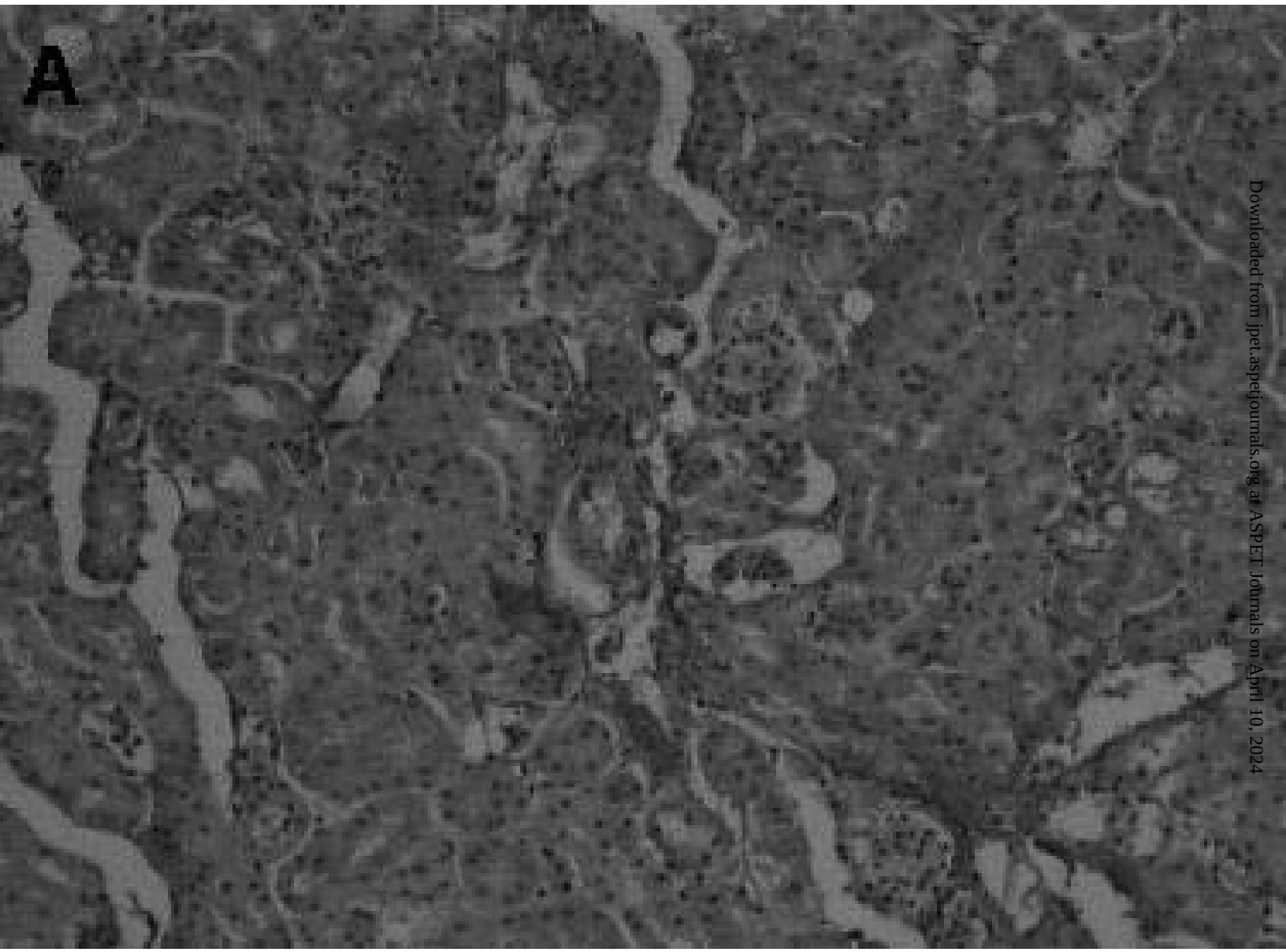

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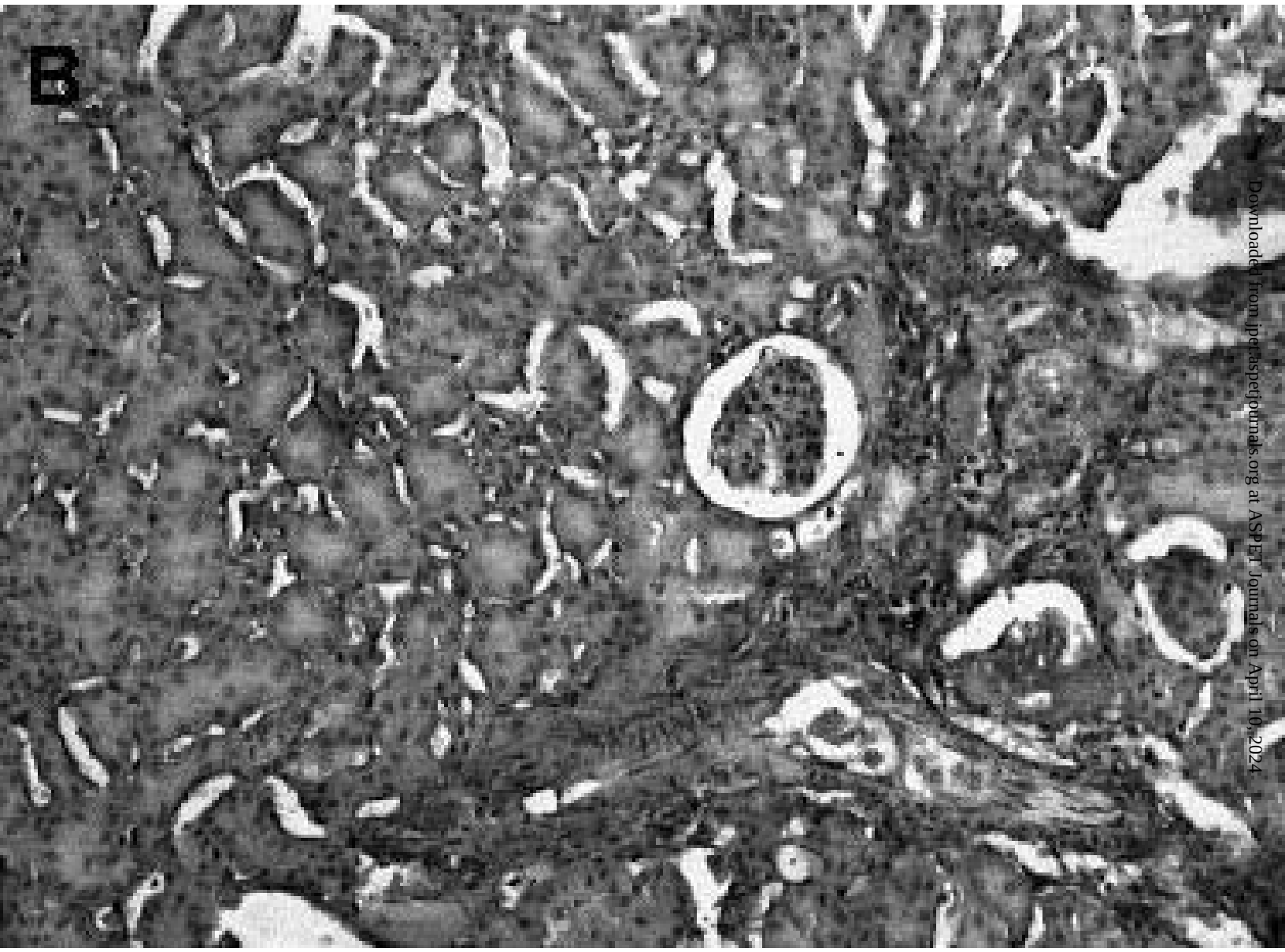

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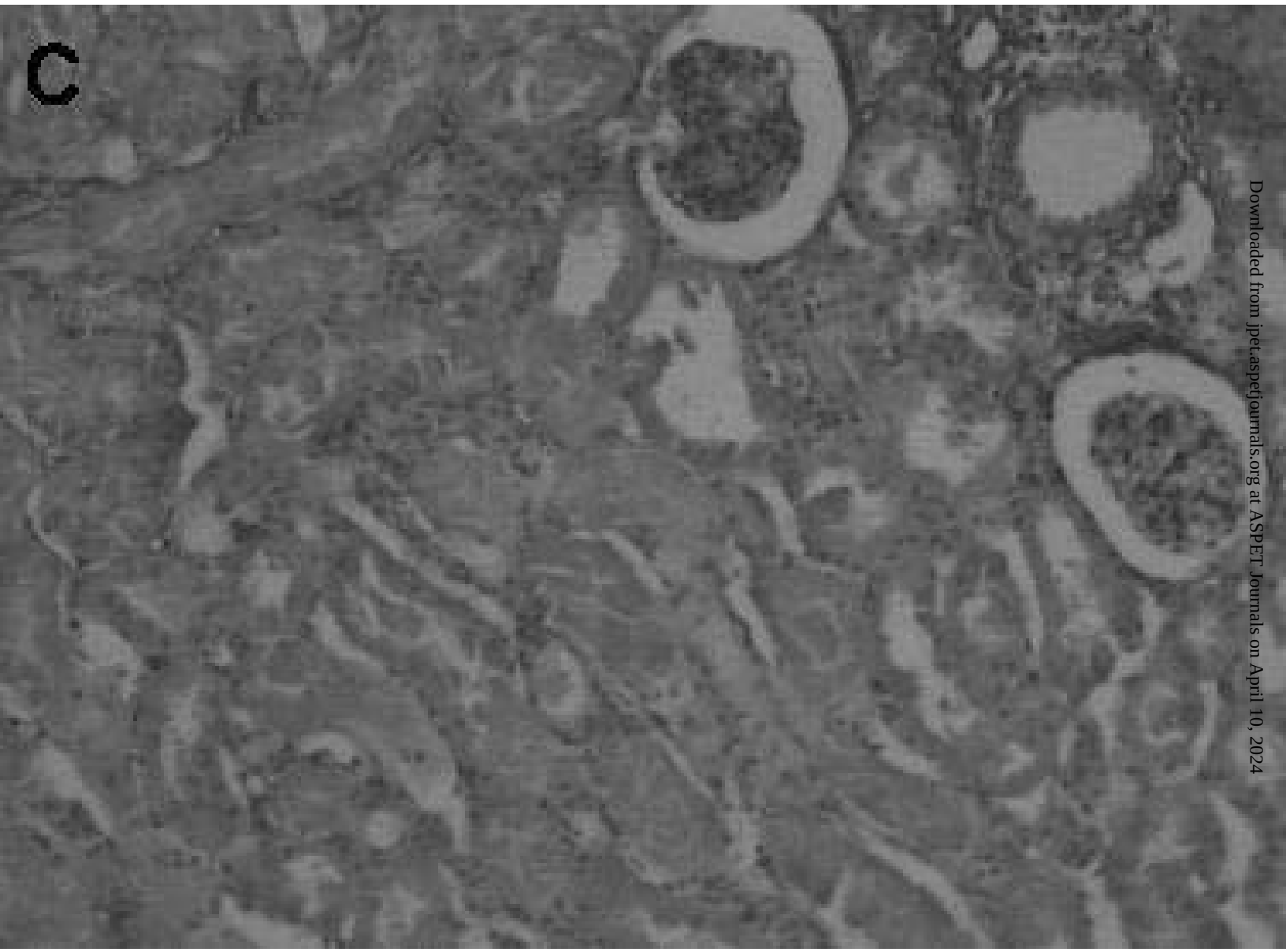

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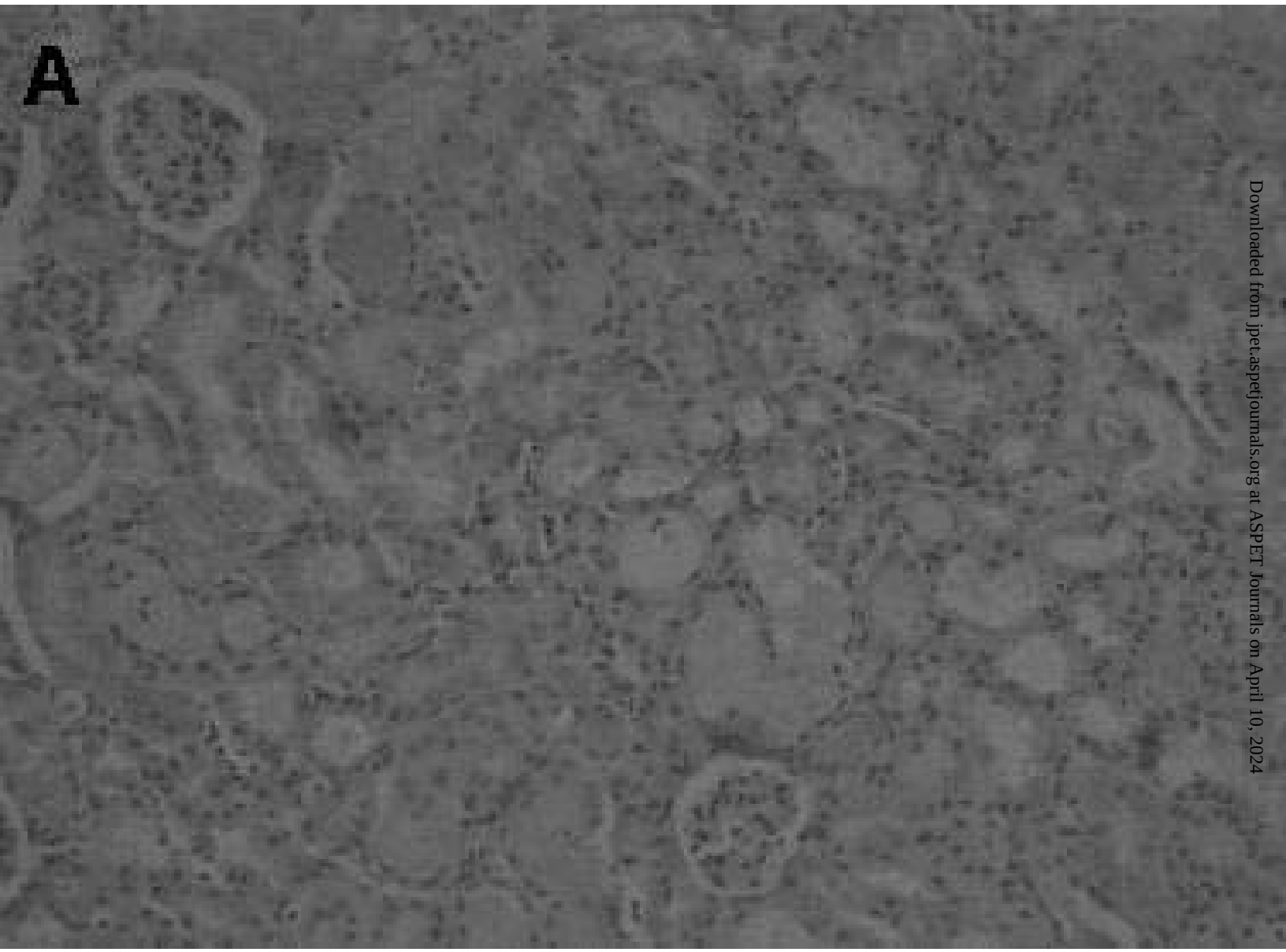

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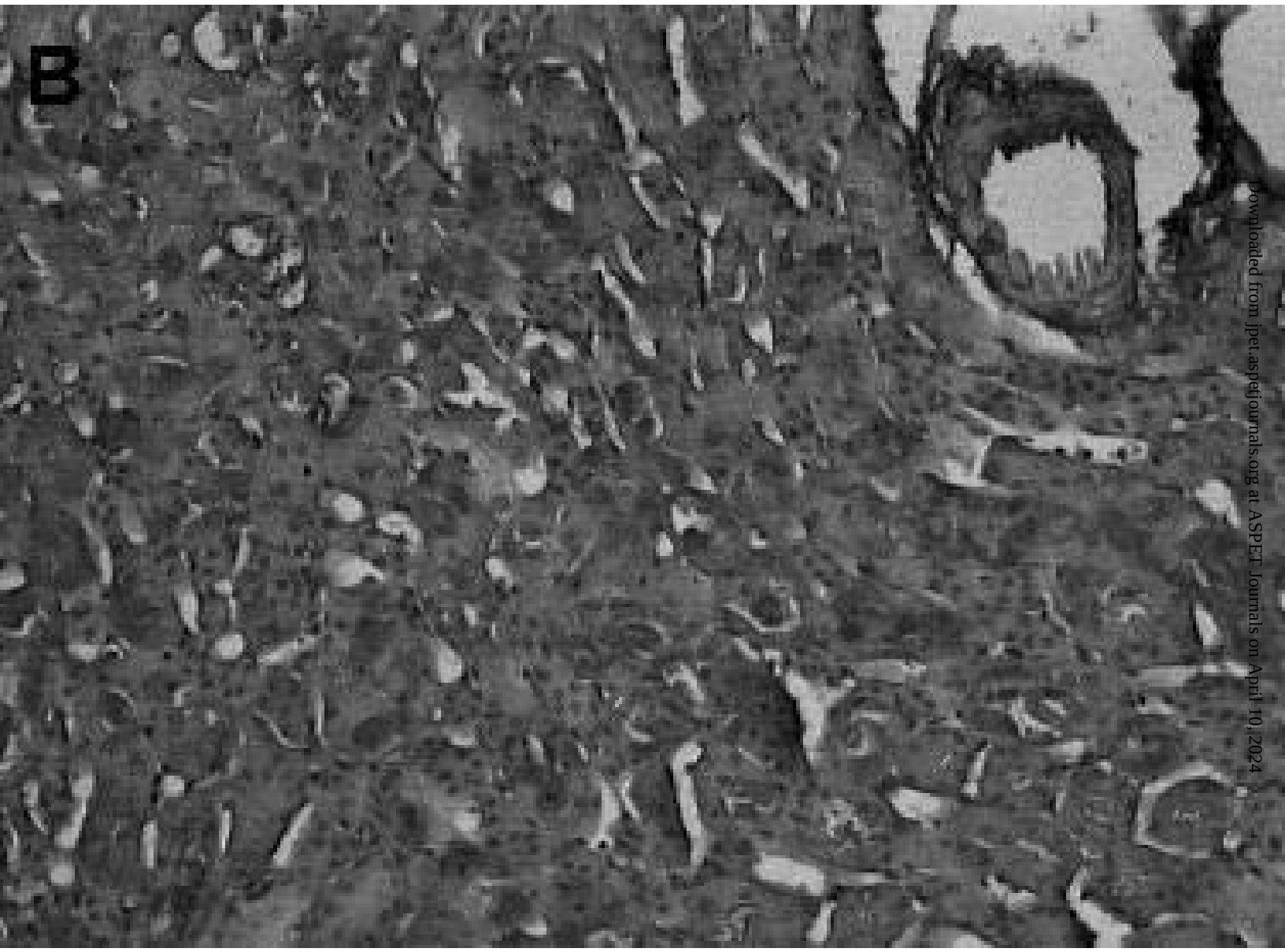

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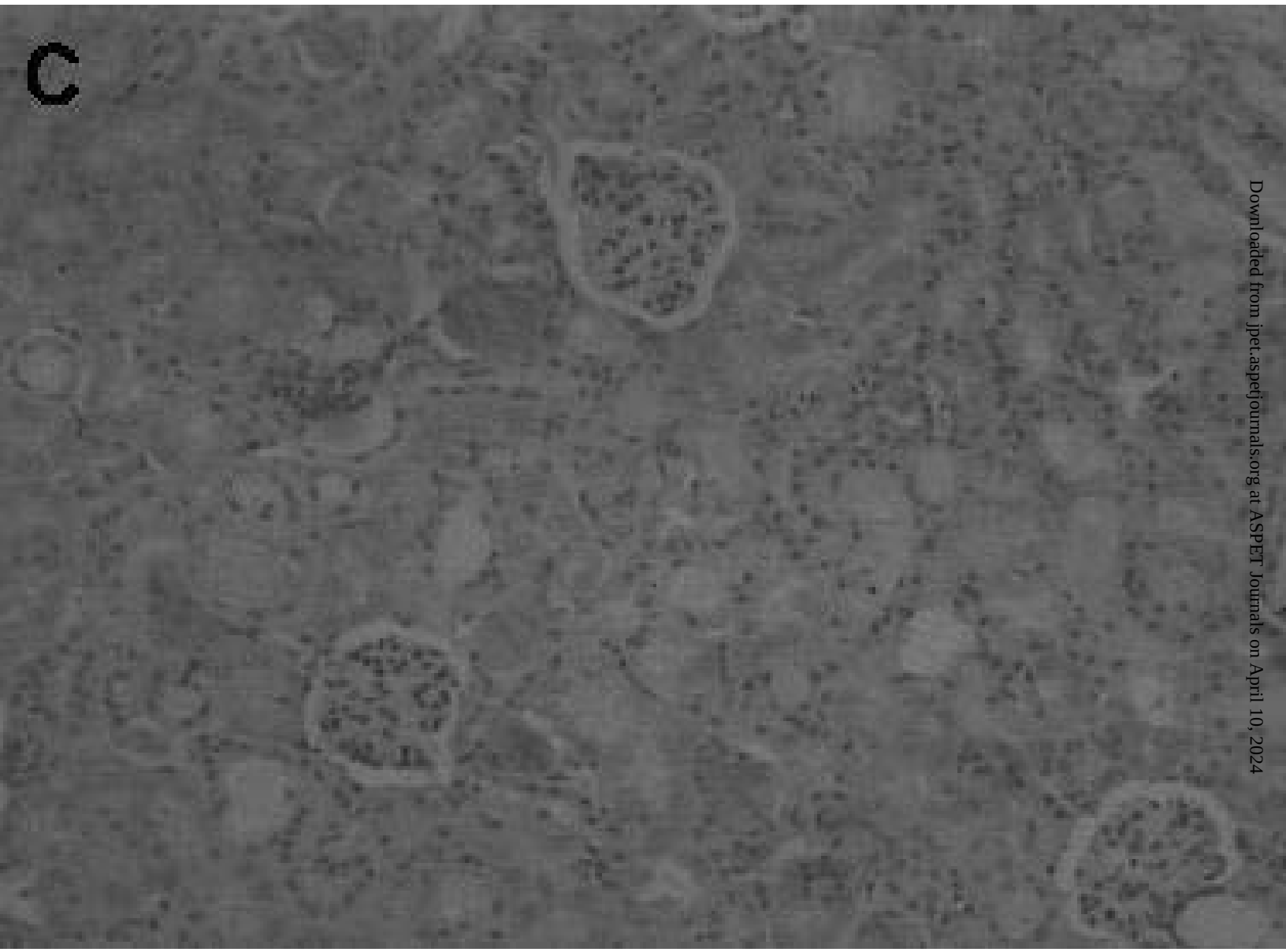

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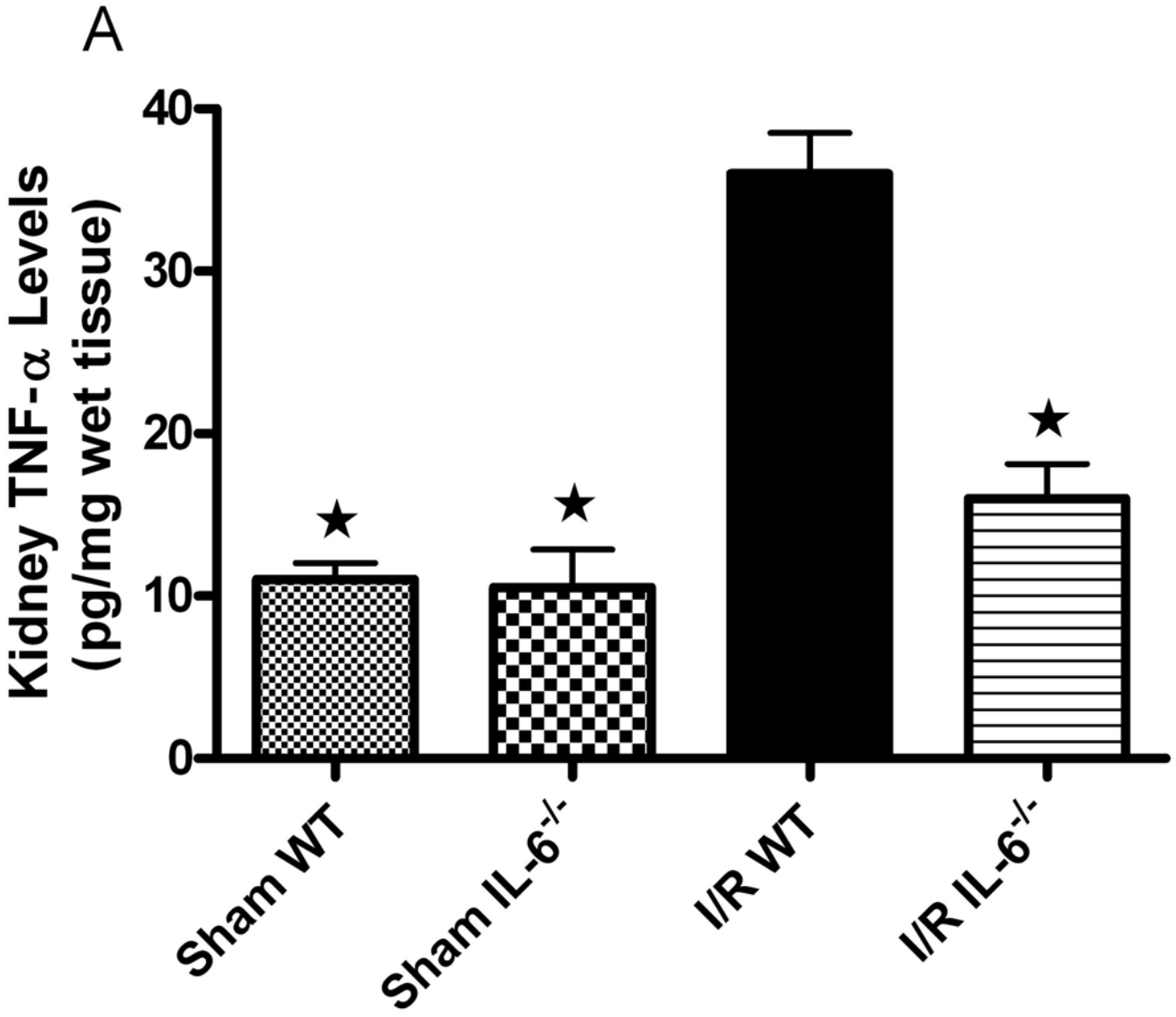

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