RESEARCH PAPER | Biochemistry | Volume : 4 | Issue : 4 | Apr 2014 | ISSN - 2249-555X

Bone Marrow Derived Mesenchymal Stem Cells As A Therapy for Renal Injury.

KEYWORDS: Mesenchymal stem cell; obstruction; epithelial-mesenchymal transition; fibrosis; kidney; collagen

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ABSTRACT *Purpose - Mesenchymal stem cells (MSCs) hold apromise for the treatment of renal disease, While MSCs have been shown to accelerate renal recovery and prevent acute renal failure in multiple disease models, the effect of MSC therapy on chronic obstruction-induced renal fibrosis has not previously been evaluated. Materials and Methods - 60 C57Bl/6 male mice underwent injection of bone marrow-derived stem cells (MSCs) immediately prior to sham operation or induction of left ureteral obstruction (UUO). One or 2 weeks later, the kidneys were harvested, fixed in 10% buffered formalin, and embedded in paraffin for morphological studies. Total renal collagen was measured biochemically in all mice involved in this study. Results - there was a significant decrease in renal collagen in all MSC groups compared to all control groups ($p < 0.001$). Kidney specimens obtained from mice treated with MSC before operation showed regeneration of the renal tubular cells, less tubular atrophy, very mild interstitial fibrosis and normal blood vessels. While kidney specimens obtained from mice treated with MSC (1Week)and (2 Weeks) after induction of UUO showed mild shrinkage of vascular tuft with normal basement membrane and cellularity, marked tubular atrophy with cast formation, mild interstitial fibrosis and normal blood vessels. Conclusions - Bone marrow-derived MSCs provide protection against renal tubulointerstitial injury induced by ureteral obstruction.*

Introduction:
Obstructive uropathy with resultant hydronephrosis is the eventual outcome of many urological disorders. Apart from accidental ligation or ureteric calculus, obstruction is nearly always chronic or partial (Shehab and El Helali et al., 2013). The renal consequences of chronic urinary tract obstruction are very complex, and lead to renal injury and renal insufficiency (Chevalier, 2006).

Obstruction induced renal injury is a gene-directed process that is dependent on the interaction of a variety of different biological mediators and cell signaling cascades (Zhao and Santivanez et al., 2014).

The molecular and cellular changes that occur in urinary tract obstruction, including;

i) Tubulointerstitial Inflammation
ii) Tubulointerstitial Fibrosis
iii) Apoptotic Renal Cell Death

Stem cells are characterized by their self-renewal properties and capacity to generate differentiated cell lineages (Kumar et al., 2010). They are found in adult and embryonic tissues and have potential uses in therapies designed to repair and regenerate organs .Self-renewal property aims to maintain stem cells during life (Ricardo and Deane, 2005).

Bone marrow contains multipotent marrow stromal cells called mesenchymal stem cells (MSCs) in addition to hematopoietic stem cells. They have diverse functions. They provide extracellular matrix, cytokines, and growth factors to support the growth and differentiation of hematopoietic cells in vitro culture (Lee and Shah et al., 2010). They can give rise to multiple mesenchymal lineages (Prockop et al., 2003).

(Imberti et al.,2007) suggest that this humoral function of MSCS results from insulin like growth factor 1 (IGF 1), whereas (Bi et al.,2007) attributed it to a combination of hepatic growth factor (HGF), epidermal growth factor (EGF) and insulin like growth factor 1 (IGF 1). (Togel et al., 2008) suggest that angiogenic vascular endothelial growth factor (VEGF) is the critical factor in the renoprotection afforded by MSCs. Bone morphogenetic protein 7 (BMP7) has also been shown to protect against fibrosis (Zeisberg and Kalluri, 2008).

There are endogenous sources of all these growth factors in the kidney. So why doesn't renal repair occur spontaneously in some cases?

The answer may be due to the inflammatory environment after injury (Togel et al., 2005). suggested that MSCs exert their renal protection through inhibition of proinflammatory cytokines. In fact, the reparative role of MSCs may be multifunctional and include the secretion of anti-inflammatory cytokines like transforming growth factor β1 to limit apoptosis, enhance proliferation and dampen the inflammatory response (Hopkins et al., 2009).

3 .Action of MSCs on endogenous renal cells:
Mesenchymal stem cells may act also on an existing endogenous cell population, potentially a renal stem cell population, to repair renal architecture and function (Little and Bertram, 2009). Once injury has occurred, repair of the tubule requires the dedifferentiation, migration, and proliferation of the surviving tubular cells. In addition, it has been proposed that resident renal stem cells participate in the reparative phase by migrating into the tubule and differentiating into epithelial cells (Takashima and Paul et al., 2013).

- Bones were flushed with 3-5ml of complete media from one end, the marrow plugs were expelled from the opposite end of bone into sterile 15ml tube.
- The marrow plugs were cultured in 20 ml complete media.

Culturing of bone marrow (Mcfarlin et al., 2006):
- The cells were cultured in 75cm2 tissue culture flask containing 10-15 ml complete media in humidified incubator at 37oC in 5% CO2 and 95% air (by volume).
- The cultured cells were examined daily using the inverted microscope to follow up the growth of the cells.
- After 24h the old media were removed by aspiration using sterile pipette, the cells were then washed with 5ml PBS, then 15ml complete media was added to the flask,

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MSCs were distinguished from other bone marrow cells by their ability to adhere to tissue culture polystyrene flask.
- The second exchange for media was done after 3-4 days.
- The cells take 4 weeks to be confluent and be ready for Passaging.
- Passaging was done for the cells till passage 3 were we had a suitable number of cells.
- The media changed twice a week.

Counting cells
Stem cells were resuspensed in 1 ml of appropriate media then from this cell suspension, 10 µl was removed for counting depending on the (using a microscope) cell number, a dilution factor between two and ten was used to count cells, test the cell viability 10µl of cells was add to 10 µl of Trypan blue 0.4% (lonza, USA) and mix them well and take 10µl of the mixture and put it on hemocytometer (Neubauer, Germany) and count cell under Ordinary microscope (Olympus CX31, USA). Then use this equation. NO of cells / ml. = average of count cells x dilution factor x 104 (Takahashi, Tanabe et al., 2007).

Experimental model (UUO):
Unilateral ureteral obstruction (UUO) was done as follow: with the mice under pentobarbital anesthesia (12 mg/100 gm Body Weight), then the abdomen was entered through midline laparotomy and the left ureter was ligated with 4-0 silk at two locations and cut between the ligatures to prevent retrograde urinary tract infection at the ureteropelvic junction. The abdominal incision was sutured by 4/0 silk sutures (Satoh et al., 2001).

Sham operation
Sham operation was done as follow: with the mice under pentobarbital anesthesia (12 mg/100 gm body weight), then the abdomen was entered through midline laparotomy and the left ureter was manipulated then the abdominal incision was sutured by 4/0 silk sutures (Satoh et al., 2001).

Investigations Provided to Measure Renal Injury:
Mice Sacrifice and Kidney Removal: Mice will be sacrificed to evaluate the severity of injury in each kidney, at the end point all mice will be sacrificed under anesthesia induced with phentobarbital sodium injection (50 mg/kg body weight intraperitoneal) then Kidneys will be removed, cut transversely and will be fixed in 10% buffered formalin, and embedded in paraffin for morphological study (Yamagishi H et al., 2001).

Kidney specimens obtained from mice in positive control groups treated with saline 1 and 2 weeks after induction of UUO showed marked disturbed kidney architecture in the form of shrinkage of vascular tuft, thrombosis of glomerular capillaries, disrupted glomerular basement membrane (glomerular necrosis), marked tubular atrophy with tubular necrosis, interstitial haemorhage, fibrosis and inflammation with normal blood vessels. Fig.3a and b are representive samples from positive control group (1Week), while Fig.4a and b are representive samples from positive control group (2Weeks).

Kidney specimens obtained from mice treated with MSC before operation showed regeneration of the renal tubular cells, less tubular atrophy, very mild interstitial fibrosis and normal blood vessels (fig. 5). While kidney specimens obtained from mice treated with MSC 1 Week and 2 Weeks after induction of UUO showed mild shrinkage of vascular tuft with normal basement membrane and cellularity, marked tubular atrophy with cast formation, mild interstitial fibrosis and normal blood vessels. Fig.6 is a representive sample from MSC group (1Week), while Fig.7 is a representive sample from MSC group (2Weeks).

Bone marrow-derived stem cells (BMSC) such as haematopoietic stem cells (HSC) possess a high degree of plasticity, indicated by the fact that they contribute to the restoration of injured peripheral tissue (Grove et al., 2004; Lakshmipathy and Verfaillie, 2005). The supposed mechanisms underlying this regenerative response are that BMSC transdifferentiate into the principal cells of the injured tissue and fuse with it (Rodic et al., 2004; Leri et al., 2005). Although there are also indications that a more paracrine fashion of support may arise by providing relevant growth factors (Togel et al., 2005; Hess et al., 2003). Among the non-haematopoietic tissues targeted by BMSC also the kidney can be found (Lin et al., 2003; Kale et al., 2003). Moreover, BM-derived cells have the capability to migrate through the glomerular basement membrane and can thus end up in the luminary space of Bowman' capsule, thereby crossing the obstacle set up by the basement membrane. So, the present study investigated the effect of therapy with BM derived stem cells on the regenerative process in a mice model of UUO. (Sugimoto et al., 2006).

Complete ureteric obstruction is characterized by an interstitial infiltration of mononuclear cells, release of cytokines, fibroblast activation, tubular proliferation, death and atrophy, and imbalance of extracellular matrix synthesis, and degradation (Hewitson et al., 2010).

Also, UUO is associated with progressive renal fibrosis and scarring and a decline in renal function. Inflammatory cell infiltration occurs in renal interstitium shortly after ureteral obstruction, releasing cytokines and TGF, including relatively well- known TGF-β1 and Tumor necrosis factor alpha (TNF-α), which promote extracellular matrix synthesis and proliferation of fibroblast (Chevalier et al., 2009).

The observed up-regulation of MMP at d 9 after UUO is most likely a compensatory response, with the increase in MMP potentially being a reaction to increased TGF- (Wick et al., 2001). and/or collagen (Olaso et al., 2001). production. Nevertheless, this seems to have been overwhelmed by the rapid fibrogenesis. However, it was noted that degradation of basement membranes may also promote epithelial-mesenchymal transition in kidney disease (Hewitson et al., 2007).

Extensive studies on MSC therapy in various acute and chronic renal diseases, mostly with a rodent animal model and different degrees of therapeutic effects, could be found at present so, the aim of this study was to investigate the effect of treatment with stem cells before and 1 week and 2 weeks after induction of unilateral ureteral obstruction. It was found in this study high significant improvement in hydroxy proline marker in mice exposed to UUO and treated with MSC before induction of obstruction also, mice exposed to UUO and treated with MSC 1 week and 2 week after induction of UUO showed significant reduction in hydroxy proline marker. However, the effect of treatment before induction of UUO was marked than treatment after induction by 1 Week and 2 Week.

These findings are in agreement with several studies demonstrated that the administration of bone marrow-derived MSC may protect or reverse both acute kidney injury and chronic kidney disease, as well as in other experimental models (Striker, 2011; Alexandre et al., 2009; Lindoso et al., 2011; Ninichuk et al., 2006; Perico et al., 2011; . Tögel and Westenfelder, 2010; Tögel et al., 2009; Herrera et al., 2007; Behr et al., 2009; Zhi-ming et al., 2013). Demonstration in a mice model of unilateral ureteral obstruction (UUO) that MSCs had intensified signals in left kidney region on the 3rd day after administration of it. (Zhi-ming et al., 2013).

Renal myofibroblasts are reported to be derived from different sources: from proliferating interstitial fibroblasts, from the transition of tubular epithelial cells (TEC) into myofibroblasts and from the bone marrow (BM) (Kalluri et al., 2003). Fibrocytes are circulating blood-borne cells displaying leukocyte surface markers and which produce extracellular matrix pro-

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teins. Fibrocytes are involved in wound repair, upon TGFb1 exposure may express alpha-SMA (Quan et al., 2004).

Fibrocytes are thought to be important in mediating pulmonary fibrosis (Phillips et al., 2004) and it recently have been implicated in renal fibrosis (Sakai et al., 2006).

(Stokman et al., 2008) found that the total number of alpha smooth muscle actin (alpha-SMA) expressing cells after UUO did not differ between both treatments groups, suggesting that if fibrocytes are the potential source of BM-derived myofibroblasts in the kidney, their contribution to fibrosis was not altered by cytokine induced mobilization in our study.

In accordance, collagen type I deposition by BM derived (myo) fibroblasts in UUO injury was found to be insignificant compared to that of fibroblasts of renal origin (Roufosse et al., 2006).

Table (1): Effect of mesenchymal stem cell therapy on hydroxyproline content (measure of fibrosis) in kidney tissues in mice model of unilateral ureteral obstruction (UUO) at day 14.

	Hydroxyproline content (ug/mg kidney tissue)
Sham group	17.24 ± 1.639
Control group (1 W)	66.79 ± 2.379 a
Control group (2 W)	75.67 ± 1.974 ab
UUO + SC (Before OP)	42.59 ± 1.906 abc
UUO + SC (1W)	48.10 ± 1.570 abcd
UUO + SC (2 W)	56.40 ± 1.756 abcde

All data are expressed as Mean ± SD. One way ANOVA test with posthoc Tukey's test. a significant vs sham group, b significant vs control group (1 W), c significant vs control group (2 W), d significant vs UUO (before OP), e significant vs UUO (1 W) ($p \leq 0.05$).

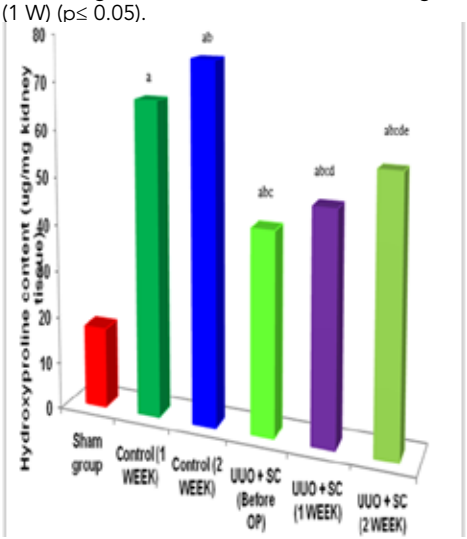

Fig. (1): Effect of mesenchymal stem cell therapy on hydroxyproline content (ug/mg kidney tissue) in unilateral ureteral obstruction mice model. a significant vs sham group, b significant vs control group (1 W), c significant vs control group (2 W), d significant vs UUO (before OP), e significant vs UUO (1 W) ($p \leq 0.05$).

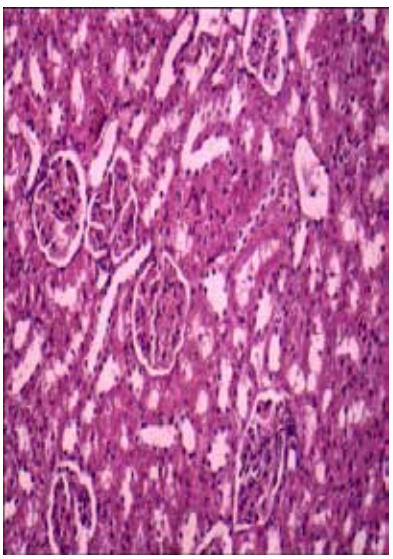

Fig. (2a): kidney specimens with normal architecture (normal glomeruli and renal tubules (sham group) (magnification 100x).

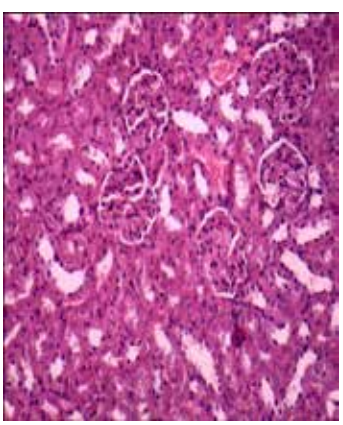

Fig. (2b): kidney specimens with normal architecture (normal glomeruli and renal tubules (sham group) (magnification 100x).

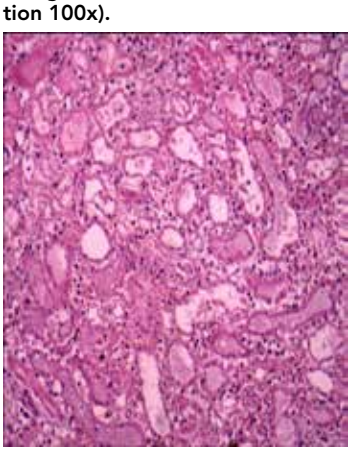

Fig. (3a): kidney specimens showing shrinkage of vascular tuft with thrombosis of glomerular capillaries, marked tubular atrophy with tubular necrosis, interstitial haemorhage and normal blood vessels. Control group (1 Week) Magnification a= 100x.

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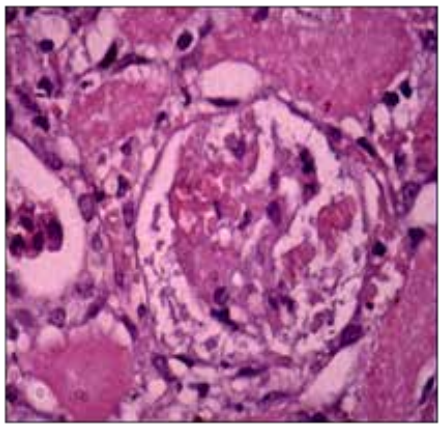

Fig. (3b): kidney specimens showing shrinkage of vascular tuft with thrombosis of glomerular capillaries, marked tubular atrophy with tubular necrosis, interstitial haemorhage and normal blood vessels. Control group (1 Week) Magnification b= 400x.

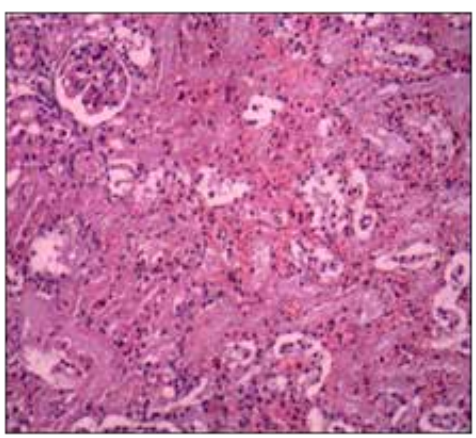

Fig. (4a): kidney specimens showing marked thrombosis in glomerular capillaries with disrupted glomerular basement membrane, Leukocytic infiltration (glomerular necrosis), marked tubular necrosis, interstitial haemorhage and inflammation and normal blood vessels. Control group (2 W) Magnification a= 100x.

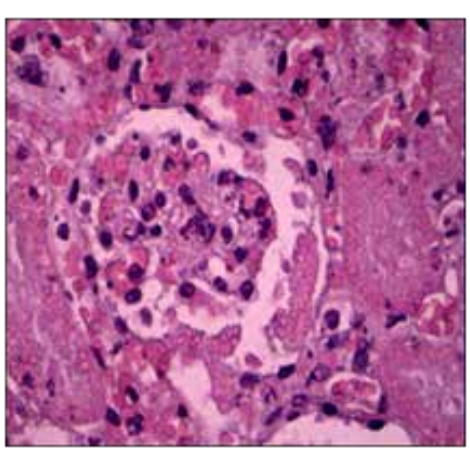

Fig. (4b): kidney specimens showing marked thrombosis in glomerular capillaries with disrupted glomerular basement membrane, Leukocytic infiltration (glomerular necrosis), marked tubular necrosis, interstitial haemorhage and inflammation and normal blood vessels. Control group (2 W) Magnification b= 400x.

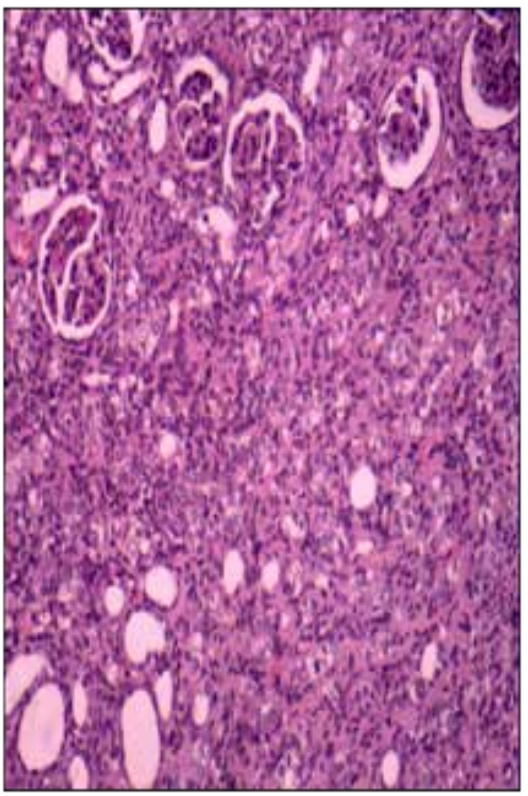

Fig. (5): kidney specimens showing regeneration of the lumen, less tubular atrophy, very mild interstitial fibrosis and normal blood vessels. MSC group (before OP) Magnification a= 100x.

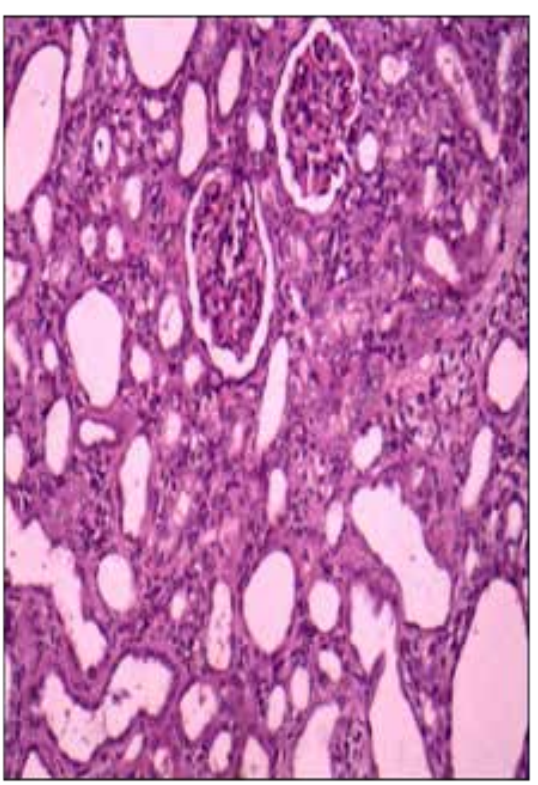

Fig. (6): kidney specimens showing normal regarding messangium and vascular tuft, mild interstitial fibrosis and normal blood vessels. MSC group (1W) Magnification a= 100x

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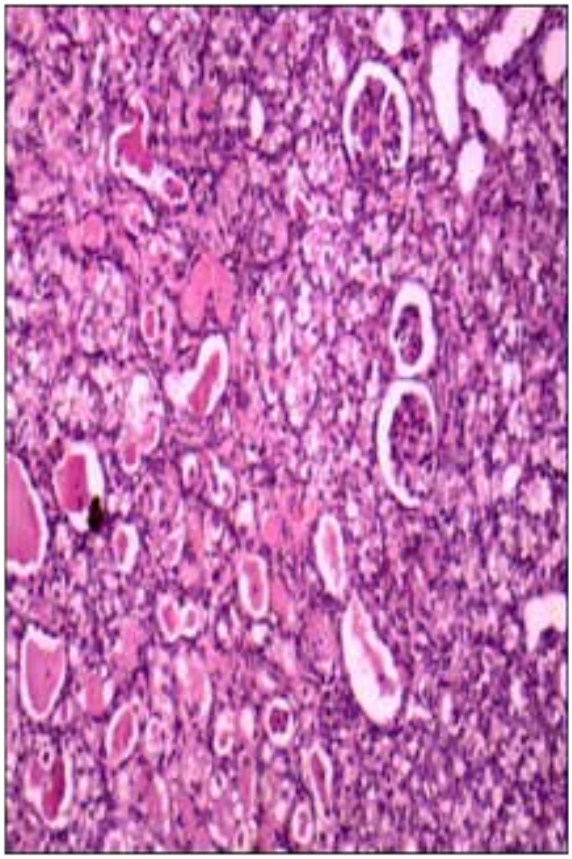

Fig. (7): kidney specimens showing showed mild shrinkage of vascular tuft with normal basement membrane and cellularity, marked tubular atrophy with cast formation, mild interstitial fibrosis and normal blood vessels. MSC group (2W) Magnification a= 100x.

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