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RESEARCH ARTICLE

THE ROLE OF STEM CELL INJECTION IN AMELIORATION OF DIABETIC NEPHROPATHY IN EXPERIMENTALLY INDUCED DIABETIC RATS.

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Abstract

Background:- Stem cell therapy holds a great promise for the repair of injured tissues and organs, including the kidney.The aim of the present study was to study the effect of human adipose derived mesenchymal stem cells (HADMSCs) on experimental diabetic nephropathy (DN) in a rat model.

Materials and methods:- Rats were divided into controls, DN group, early stage DN rats receiving HADMSCs and late stages DN rats received HADMSCs. The HADMSCs were injected once in rat tail vein as a single dose of $1X10^6$ cells in 1 ml PBS. At the end of the experiment, each group was subjected to 24 hour urine collection for urinary albumin,urinary N-acetyl B-D glucosaminidase (NAG),creatinine clearance measurements and blood sampling through retro-orbital vein for blood glucose, serum urea, and serum creatinine estimation. This was followed by scarification of all groups to obtain the kidneys for histopathological examination and DNA extraction.

Results:- HADMSCs therapy significantly improved 24 hour urine collection for urinary albumin, urinary NAG, blood glucose,serum ureaand serum creatinine levels in HADMSCs recipient rats when compared to DN group. Histopathological examination showed minimal degeneration in renal tubules and normal tufts of glomeruli after injection of HADMSCs in DN rats. Interestingly, treated rats at early DN stages showed better results than treated rats at late DN stages. Lastly, the HADMSCs were engrafted into the rat renal tissue.

Conclusion:- The current study suggests that intravenous injection of HADMSCs into rats with DN improved renal function and regenerating kidney tissues. HADMSCs accomplish this function by decreasing blood glucose levels or by direct effect due to their homing into the affected kidney.Furthermore, treated rats at early DN stages showed better results than treated rats at late DN stages.

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Introduction:-

Diabetic nephropathy (DN) is a major complication of diabetes and represents the leading cause of end–stage renal disease worldwide. It has been considered that about 25%–40% of patients with type 1 or type 2 diabetes develop nephropathy within 20–25 years of the onset of diabetes. To date, there is no cure for DN. Drugs that decrease blood glucose, lower blood pressure, or inhibit the actions of the hormone angiotensin can delay, but not eliminate, the onset of DN. Thus, the development of novel therapeutic strategies that could specifically target DN is necessary **[1].**

The basic underlying mechanisms of DN involvemetabolic and hemodynamic alterations**:** Such as hyperglycemia,augmented oxidative stress,accumulation of advanced glycation end products "AGEs"**[2,3].**

Stem cell therapy holds a great promise for the repair of injured tissues and organs, including the kidney.Indeed, MSCs have been used in experimental acute renal failure, which could lower renal injury, accelerate tubular proliferation and improve renal function **[4].** MSCs are attractive candidates for renal repair, because nephrons are of mesenchymal origin and stromal cells are of crucial importance for signaling, leading to differentiation of both nephrons and collecting ducts**[5].** Several studies have shown that mesenchymal stem cell (MSCs) therapy improves microalbuminuria and preserves the normal renal histology of diabetic mice. The exact mechanisms of stem cell therapeutic effects on microalbuminuria have not been clearly defined. It remains unknown **[6].**

Adipose derived-MSCs (ADMSCs); the multipotent stem cells within adipose tissue, are one of the most promising stem cell population identified thus far, since human adipose tissue is ubiquitous and easily obtained in large quantities with little donor site morbidity or patient discomfort**[7].** Therefore, the use of autologous ADMSCs as both research tools and as cellular therapeutics is feasible and has been shown to be both safe and efficacious in preclinical and clinical studies of injury and disease **[8].**The proliferation capacity of ADMSCs seems to be greater than that of bone marrow-derived MSCs. Previous reports have shown that the doubling times of ADMSCs during the logarithmic phase of growth range from 40 to 120 hours **[9].**The younger the donor, the greater theproliferation and cell adhesion of the ADMSCs, while cells gradually lose their proliferative capacity with passaging. Based on b-galactosidase activity, senescence in ADMSCs is similar to that in bone marrow-derived MSCs**[10].**ADMSCs are generally considered to be stable throughout long-term culture, as it was reported that even ADMSCs that had passed more than 100 population doublings had a normal diploid karyotype**[11].**

The aim of the present study was to investigate the role of human ADMSCs (HADMSCs) injection in amelioration of DN in experimentally induced diabetic rats.

Materials&Methods:-

Isolation of adipose derived MSCs (ADMSCs) from human lipoaspirate:-

Once lipoaspirate was obtained; through liposuction, it was placed in sterile, ice-filled plastic bags, human fat were obtained from 3 patients under surgery of liposuction after taking their consent. The lipoaspirate sample was diluted with an equal volume of PBS and centrifuged at 430×g for 10 minutes. After centrifugation, the target cell–containing lipid phase from the top was aspirated and applied to another clean tube, then washed a further two times with an equal volume of PBS.Washed aspirates were treated with 0.075% collagenase (typeI; Sigma-Aldrich, St. Louis, MO) in PBS for 30 min at 37°C with gentle agitation. The collagenase was inactivated with an equal volume of DMEM/10% fetal bovine serum (FBS) and the mixture centrifuged for 10 min at low speed. The cellular pellet was re-suspended in DMEM/10% FBS and filtered through a 100-μm mesh filter to remove debris. The filtrate was centrifuged at 600×g for 10 minutes and the pellet was re-suspended in complete culture medium. Morphologically, ASCs were adherent to plastic and demonstrated fibroblastic spindle shape. Flow-cytometric analysis of cell surface markers in ASCs expressed CD105 but did not express CD34.

Experimental animals:-

The study was carried on 78 adult healthy male albino rats of an average weight 150-220 gm. They were obtained from the animal house from Faculty of Veterinary medicine of Zagazig University. The animals were kept in steel wire cages (4-6 / cage) in the Physiology animal house in Faculty of Medicine of Zagazig University under hygienic conditions. The 78 male rats were randomly divided as follow: group I: normal control group (n=10) and diabetic nephropathy groups (n=68).

Induction of type I diabetes

Sixty-eight rats were injected intraperitoneally with streptozotocin (STZ) dissolved in saline at a dose of 60 mg/kg for 5 consecutive days**[12].** The development of hyperglycemia in rats was confirmed by blood glucose estimation after 11 days of first STZ injection. Rats were considered diabetic if random blood glucose levels were above 250 mg/dL, on 3 consecutive determinations **[13]**and the diabetic rats were selected for further studies.

Diabetic nephropathy was confirmed after 4 weeks from first STZ injection by follow up the urinary albumin execration. Every week, 2 rats were scarified to evaluate the histopathological changes. Early diabetic nephropathy was defined as there is only hyperglycemia, glucosuria and microalbuminuria, but still without renal histopathological changes. Late diabetic nephropathy was considered when rats developed macroalbuminuria and there was an evidence of histopathological changes. In our experiment, the histopathological changes have been shown from day 50 after the 1st STZ injection. Then, the 60 rats were divided into: **Group II** (Diabetic nephropathy rats contained 20 diabetic un-treated rats): Ten rats of this group received 1 ml of PBS at 4th week from STZ injection and the other 10 received 1 ml of PBS at 50th day from STZ injection.**Group III** (Early diabetic nephropathy rats treated with HADMSCs (n=20). Rats in this group had hyperglycemia, glucosuria and microalbuminuria, but still without renal histopathological changes. They were injected once with ADMSCs in the tail vein as single dose of 1X10^6 cells in 1 ml PBS per rat at 4th week from STZ injection (at the onset of diabetic nephropathy). **Group IV**(Late diabetic nephropathy group treated with ADMSCs (n=20). Rats in this group developed macroalbuminuria and there was an evidence of histopathological changes. They were injected once with ADMSCs in the tail vein as single dose of 1X10^6 cells in 1 ml PBS per rat at day 50th from STZ injection (at late stages of diabetic nephropathy).

Sampling:-

At day 90 post STZ injection, each group was subjected to 24 hour urine collection for urinary albumin and creatinine clearanc measurementsand urinary NAG levels, blood sampling through retro-orbital vein for blood glucose, serum urea, serum creatinine estimation. This was followed by scarification of all groups to obtain the kidneys. Kidneys were removed immediately, after authorizing, cleaned with physiological salt buffers (0.9%NaCl). The right kidney fixed in 10% neutral-formalin immediately for 2 days, then the specimens were dehydrated, cleared, and embedded in paraffin for histopathological analysis. The left kidney used for DNA extraction.

Homing of HADMSCs in the rat renal tissue by detection of the human GAPDH gene:-

The presence of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene in the HADMSCs recipient rats was determined by PCR analysis. DNA was extracted from renal tissue using DNA extraction kits according to their protocol. Amplification of the human GAPDH; the housekeeping gene was performed by conventional PCR using forward primers: 5'- TGA AGG TCG GAG TCA ACG GAT TTG GT-3' and reverse primer 5'- CAT GTG GGC CAT GAG GTC CAC CAC-3'. PCR was carried out in a final volume of 20 µL containing 3 µL extracted DNA, 10 pmol of each primer, and 10 µL of Taq PCR Master Mix (BIORON). The amplification protocol was as follows: initial denaturation of 8 minutes at 94°C; followed by 40 cycles of 94°C for 1 minute, 65°C for 1minute, and 72°C for 1 minute; concluding with 7 minutes at 72°C. PCR products (983 bp) were separated with the use of 2% agarose gel electrophoresis.

Statistical analysis:-

Statistical analyses were carried out using the Statistical Package for the Social Sciences for Windows (version 17.0; SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation and were analyzed using ANOVA and LSD test,*P*-values were considered significant if <0.05.

Results:-

HADMSCs improve the kidney function:-

The result of the present study showed a significant improvement in kidney function; serum urea and creatinine were decreased in DN/HADMSCs groups when compared to DN group. There was a significant decrease of urinary NAG levels in both DN treated groups when compared to un-treated rats ($p<0.001$). Interestingly, the plasma glucose levels were significantly decreased in HADMSCs treated groups. Lastly, the improvement of kidney function in rats received HADMSCs in early stage group showed better results than in rats received HADMSCs in late DN stages (Table 1).

Table 1:-Mean ±SD of different laboratory parameters in the studied groups.

spuorG	Blood Glucose (mg/dl)	Serum Urea (mg/dl)	Urinary Albumin (mg/24h)	Serum Creatinine (mg/dl)	Creatinine Clearance (ml/min)	Urinary NAG (U/l)
slortnoC	82.7±7.13	32.02±6.95	29.5±2.5	0.34±0.11	1.47±0.12	2.9±1.2
ND	270.4±50.76	78.33±6.39	335.2±20.4	1.13±0.19	1.94±0.06	47.4±12.1
ND ylraE	147.5±17.29	45.17±10.8	41.3±10.4	0.39±0.10	1.38±0.14	31.2 ±7.4
ND etaL	186.3±21.79	55.95±4.58	136.4±12.3	.55±0.09	1.61±0.09	22.5 ± 4.3
eulaV P	0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Homing of human adipose derived-MSCs in the rat renal tissue by detection of the human GAPDH gene:-
The PCR analysis of the renal tissue homogenate revealed that detection of human GAPDH gene in both HADMSCs treated groups (Fig 1).

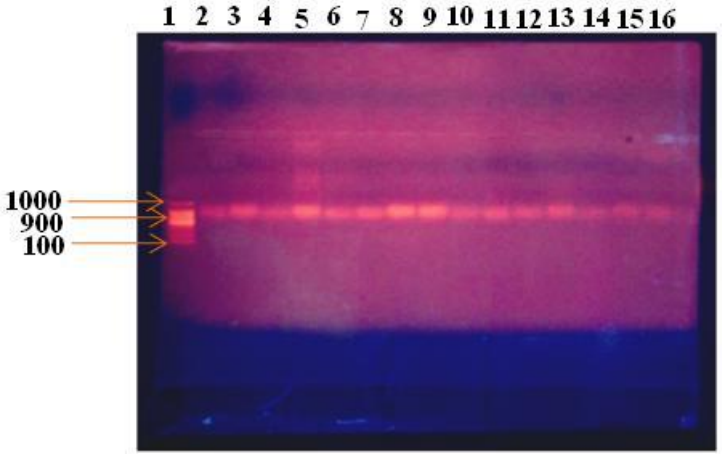

Figure 1:- Agarose gel electrophoresis of PCR product (983 bp) of human GAPDH gene showing lane 1; marker (100bp), positive results for rats received ADMSCs at early DN (lanes 2-8), positive results for rats received ADMSCs at late DN (lanes 9-16).

Histopathological results:-
Data obtained from normal rat (group 1) Fig2(A, B), Early DN rats (C,D),Late DN rats (E,F,G,H).

Rats which received HADMSCs (group 3, 4) at early DN Fig2(I,J) and rats received HADMSCsat late DN (K,L).

Improvement of histopathological picture after the administration of MSCs into DN rats (group3, 4) was demonstrated in (Figure I, J, K and L); with minimal reversible cell damage in the form of mild mesangial sclerosis, normal tufts of glomeruli and normal blood vessels wall.

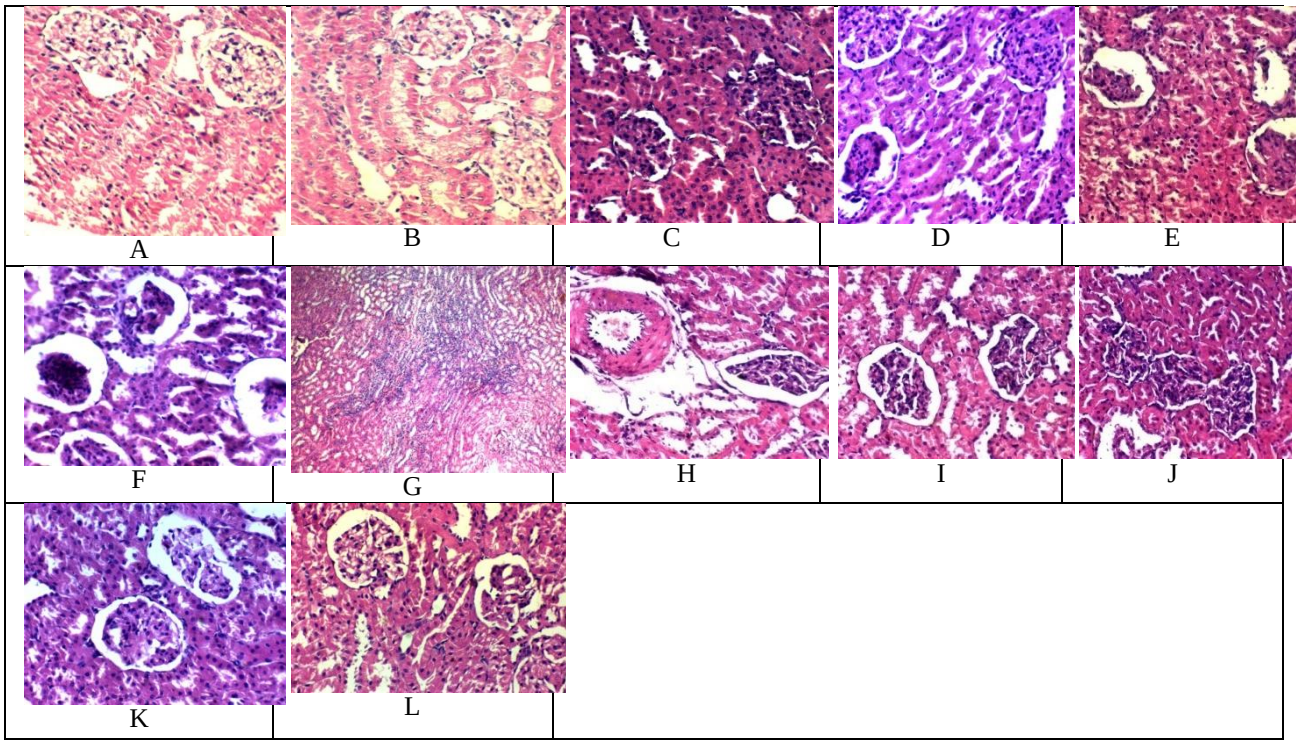

Figure 2:- (A and B) Hematoxylin and eosin (H&E) stained sections in Kidneyof albino rat from the control group, showing normal glomeruli (original magnification x 400) **(C):** H&E stained section in the kidney of albino rat with early diabetic nephropathy; the glomerular tufts shows mesangial cell proliferation (original magnification x 400). **(D):** H&E stained section in the kidney of albino rat with early DN; the glomerular tufts shows mesangial cell proliferation, one tuft shows sclerosis (original magnification x 400). **(E):** H&E stained section in the kidney of albino rat with advanced DN the glomerular tufts shows nodular glomerulonephrosis (original magnification x 400).**(F):**H&E stained section in the kidney of albino rat with advanced DN; the glomerular tufts are collapsed (original magnification x 400**).(G):**H&E stained section in the kidney of albino rat with advanced diabetic nephropathy showing interstitial inflammation (original magnification x 400).**(H):**H&E stained section in the kidney of albino rat with advanced diabetic nephropathy showing thick walled blood vessel (original magnification x 400).**(I,J):** H&E stained section in the kidney of albino rat treated with MSCs for early diabetic nephropathy showing nearly normal glomeruli (original magnification x 400).**(k):**H&E stained section in the kidney of albino rat treated with MSCs for advanced diabetic nephropathy showing mild mesangial sclerosis (original magnification x 400).**(L):**H&E stained section in the kidney of albino rat treated with MSCs for advanced diabetic nephropathy showing mild mesangial sclerosis. One glomerulus shows normal tuft (original magnification x 400).

Discussion:-

DN is the most common cause of end-stage renal disease in the world, and could account for disability and high mortality rate in patients with diabetes. DN affects approximately one third of people who suffer with type 1 or type 2 diabetes**[14].** Although the time of clinical debut of DN varies between patients with T1DM and T2DM, clinical and histological progressions in both conditions are quite similar **[15].**Changes in the filtration unit begin soon after DM onset, and take place "silently" for a long time before the appearance of the first clinical signs of the disease. In susceptible patients DN follows a well-known physiopathological course micro albuminuria is the earliest clinically detectable sign of kidney damage. It is associated with histological changes that include extracellular matrix deposition, glomerular basement membrane thickening and glomerular mesangial expansion. In later stages patients develop macro albuminuria, followed by a progressive decline in the glomerular filtration rate. At this stage, histological changes include glomerulosclerosis, tubulointerstitial fibrosis and arteriolar hyalinosis were appeared [**16].**

To date, there is no cure for DN. Drugs that decrease blood glucose, lower blood pressure, or inhibit the actions of the hormone angiotensin can delay, but not eliminate, the onset of DN. Thus, the development of novel therapeutic strategies that could specifically target DN is necessary**[17].**

Stem cell therapy is a novel strategy for various diseases and has the potential to be more effective than traditional therapies**.**Mesenchymal stem cells (MSCs) are one of the mostimportant multipotent adult stem cells. Owing to theircapacities to differentiate into replacement cells in damagedtissues, modulate their local environment, activateendogenous progenitor cells, and secrete various factors **[18].** MSCs hold great promise for treating pathophysiology, as well as tissue engineering and regeneration for treatment of several diseases [**19].**
MSCs can be isolated from multiple sources, including adipose tissue, bone marrow and other tissues [**20].** Much attention has been paid to adipose-derived MSCs (ADMSCs) because adipose tissue is an abundant, easily accessible, and appealing source of donor tissue for autologous cell transplantation. Moreover, large amounts of cells can be isolated from the adipose tissue, yielding 100–500 fold higher stem cells per tissue volume than bone marrow **[21].**

In the present study, we estimated the levels of urinary NAG; as an indicator of renal tubular affection. Changes in urinary NAG activity can reflect the activity of the disease as well as the residual functional capacity of the kidney **[22]**. **[23]**. Stated that NAG changes occur prior to microalbuminuria, probably because the tubular cells can reabsorb the increase of albumin load that result from glomerular affection but the increased NAG will be lost from damaged cells. **[24].** showed that urine NAG increased surprising 9-folds in normoalbuminuric patients with diabetes compared to control group. It increased further with the development and the progress of microalbuminuria. In the present study, we found that increase of urinary NAG in DN group compared to the normal control group. After injection of HADMSCs, there were significant decreases of urinary NAG levels in the HADMSCs/DN groups compared to DN rats.

Our results showed a significant improvement in kidney function after HADMSCs administration. Serum urea and creatinine were decreased in the HADMSCs recipient groups compared to the DN group as well as 24 h urinary albumin. In the present study, histopathological results showed also preservation of normal renal histology.The amelioration of the kidney function and the regeneration of the kidney tissues in HADMSCs recipient DN rats shown in the present study are similar to the results of other studies. Data obtained from studies using NOD/SCID mice transplanted with human MSCs (hMSCs) and C57Bl/6 mice that received murine MSCs indicate that injected MSCs engraft in damaged kidneys, differentiate into renal cells, and regulate the immune response resulting in an efficient treatment of DN **[25].**

The precise mechanisms of action of MSCs as a therapeutic agent in pre-clinical models of DN has been studied, but not fully elaborated**.** In the present study, we measured blood glucose levels in all groups and found that significant decrease of glucose levels in rats receiving HADMSCs. We could explain that improvement of the kidney function in stem cells recipient groups as that the ability of HADMSCs to ameliorate hyperglycemia; then the reduction of glucose levels subsequently improve kidney function. Similar results have been showed.

In mice with T1DM induced by the administration of five low doses of streptozotocin, **Ezquer et al. [28]** showed that the intravenous administration of bone marrow-derived MSCs ($\approx 20x10^6$/kg body weight) results in the reduction of microalbuminuria and the preservation of normal renal histology. They showed also reversion of hyperglycemia and glycosuria and beta-pancreatic islets regeneration. They suggested that bone marrow-derived MSC transplantation as a cell therapy strategy to treat type 1 diabetes and prevent diabetic nephropathy **[28].** In rats with diabetes induced by the administration of a single high dose of streptozotocin, the intracardiac infusion of allogeneic MSCs ($\approx 10x10^6$/kg body weight) along with cyclosporine resulted in a transient amelioration of renal function and structure associated with an improvement in the diabetes condition **[29].**

In contrast to these results, **Ezquer et al. [30]**administered syngeneic MSCs in a mouse model that develops severe diabetes. Despite not sharing the etiology of either T1DM or T2DM, these animals showed a rapid progression of renal failure and developed most of the pathognomonic signs of DN. In these diabetic mice, MSC administration did not result in hyperglycemia correction; however, renal failure did not progress. Interestingly, at least up to three months after MSC administration donor cells were found in the kidney of severe diabetic mice. Their results indicated that the renoprotective effect of MSCs may not due to indirect effect i.e. hyperglycemia correction but direct, i.e. due to protection/regeneration of renal tissue. In addition, **Wang et al. [26]** provided clear evidence that

although the injected MSCs prevented development of albuminuria and loss of podocytes, there was no improvement in blood sugar levels.

These controversies can be attributed to many factors such as ratio of MSCs to renal cells, nature of diabetic case and stage of DN, integrity of immune system, number of stem cell passages and site of injection; all can affect the outcome of MSCs used in condition. Therefore, the "lack of reproducibility" is at least partially due to large experimental differences **[27].**

Data supporting the contribution of MSCs to the management of DN have been generated in animal models of DN. Unfortunately; those models only reproduce the earlier stages of DN. Streptozotocin-induced diabetic mice progress to proteinuria and hyper filtration. They also present variable degrees of mesangial matrix expansion and glomerular capillary basement membrane thickening, but infrequently develop nodular glomerulosclerosis, a pathognomonic sign of advanced human DN. Hence the impact of MSC transplantation in advanced DN remains unproved. The novelty of the present study was that we also studied the therapeutic effect of the HAMSCs in the late stages of DN. We found there were improvements of DN rats receiving HADMSCs either biochemically or histopathologically. In the present study, we compared the efficacy of HADMSCs in early stages DN to late stages. We found that HADMSCs administration at early DN showed better results than when administrated at late stage DN. It means that early stem therapy could be more effective in DN individuals.

In conclusion, HADMSCs are capable of improving the kidney function and regenerating kidney tissues in DN rat most probably through decreasing blood glucose levels or by direct effect due to their homing into the affected kidney**.**

Conflict of interist:-

The authors declared that there is no conflict of interest.

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