Role of L-carnitine Treated Mesenchymal Stem Cells on Histological Changes in Spleen of Experimentally Induced Diabetic Rats and the Active Role of Nrf2 Signaling

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Original Article

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ABSTRACT

Introduction: Diabetes mellitus induces atrophy in splenic lymphoid follicles, which may affect the body defense mechanisms. Mesenchymal stem cells could be a practical approach to treat diabetes-induced spleen damage.

Aim of work: To demonstrate the histological changes in adult albino rat's spleen after induction of diabetes and to assess the effect of injected mesenchymal stem cells (MSCs) treated with L-carnitine on alleviating these changes.

Materials and Methods: Thirty adult male albino rats were divided into 3 main groups: Group I (control), Group II (diabetic): the rats were injected once with intra-peritoneal Streptozotocin (STZ) 50mg/kg and group III (Diabetic +MSCs): the animals were injected intravenously with MSCs four weeks after diabetes confirmation. All rats were sacrificed four weeks after MSC injections (8 weeks from the beginning of the experiment) and spleen specimens were processed to be examined by light microscope. Blood specimens were collected to measure glucose and insulin levels. The mRNA expression for Nrf2, IL 10 and IL 17A was measured by Real-time Polymerase Chain Reaction (RT-PCR).

Results: Degenerative changes were observed in diabetic group in the form of reduced size of white pulp with many apoptotic nuclei. Widened congested red pulp, statistical significant reduction of insulin level, white pulp volume, area percent of CD4+ and nuclear factor erythroid 2 related factor 2 (Nrf2) immunoexpression and also in qRT-PCR relative gene expression of Nrf2 and IL 10. There was significant increase in blood glucose level and qRT-PCR relative gene expression of IL 17A. All changes were reversed in MSCs treated group.

Conclusion: We concluded that L-carnitine pretreatment of MSCs releases a novel way to enhance MSCs therapeutic potential efficacy in spleen of experimental diabetic rats with recommendation of trying further researches for their application in other vital organs.

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INTRODUCTION

Diabetes mellitus (DM) is considered to be one of the most common metabolic disorders occurring in humans and animals. Hyperglycemia and other environmental factors are claimed to be the reason of its appearance[1]. Its incidence is increasing very rapidly as in 2008; about 347 million persons were proved to be diabetic with prevalence of 9.2% in females and 9.8% in males[2].

Two forms of diabetes mellitus are known; type 1 and type 2, most of the genetic causes are linked to environmental conditions that contribute to the development of the ailment[3]. Many complications are also reported which could be fatal if not treated as diabetic ketoacidosis, while long-standing chronic complications may significantly affect organs performance[4].

Experimental models of diabetes are primarily based on streptozotocin (STZ) which is known to be an antineoplastic and antibiotic drug associated with nitrosuria. It is made up of Streptomyces achromogenes[5]. STZ can pass via the glucose transporter 2 (GLUT2) to β cell of pancreas[6]. Its work is to destroy cellular DNA by increasing pancreatic β-cell polyadenine diphosphate ribose synthetase activity and cause degenerative lesions by lowering Nicotinamide adenine dinucleotide (NAD) levels. These effects on pancreatic B cells lead to blocking the pro-insulin synthesis and occurrence of type I diabetes characterized by insulin insufficiency[7].

Formerly, diabetes treatment was depending on symptomatic treatment and complications management. Recent researches have shown that endogenous and

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exogenous mesenchymal stem cells (MSCs) can repair or enhance the biological functions of the impaired organs or tissues[8]. In addition to their capacity to proliferate and differentiate, MSCs secrete several growth factors that directly activate target cell regeneration. They also modulate cell and tissue repair by paracrine and immune modulatory approaches[9].

Migration and transfer to the injured tissue depends on the amount of cells and passages, the conditions of culture and the method of delivery[10]. The most popular process of MSC transplantation is intravenous route. It allows MSCs to be distributed mainly in the lungs, spleen, kidneys, liver, thymus, bone marrow, skin and many other organs[11]. The chemo-attractants released from damaged tissue have a positive effect on MSC homing. So, they act more perfect when injected just after injury. Their bioavailability and effectiveness inside the body depend on the host conditions[12].

Endogenous stem cells are present in many organs; however, some of these cells differentiate only to replenish the cells of their organs. Others can differentiate to replace different organs also. For example, splenic stem cells; they are able for self-renewing and also of differentiating and restoring a wide variety of tissues, such as pancreatic islet cells and salivary gland cells[13,14]. These cells erase the interpretation of the spleen as an unnecessary organ containing only hematopoietic and lymphoid elements[13].

L-Carnitine (LC) (3-hydroxy-4-N-trimethylammoniumbutyrate) is an amino acid derivative which promotes cell proliferation and the synthesis of ATP[15]. L-carnitine may overwhelm apoptosis by increasing beta oxidation of fatty acids[16]; counteracting hyperglycemic oxidative stress by increasing super oxide dismutase (SOD2) expression[17]. It also increases cell survival gene expression as myeloblastosis (myb), erb-b2 receptor tyrosine kinase (erbb2) and leukemia inhibitory factor (lif)[18]. Based on these data, our objectives were to assess the role of L-carnitine-pretreated injected MSCs in the preservation of splenic structure in diabetic experimental rats.

MATERIALS AND METHODS

Experimental animals

Thirty-six male albino rats have been used. Six rats for stem cell preparation (6 weeks old) and thirty rats (12-14 weeks) were used for experimental design. Their weight ranged from 180 g to 200 g. They were gotten from the Animal House presented in Faculty of Medicine, Zagazig University, Egypt. They were retained in cages 2 weeks for adaptation. Temperature, humidity and illumination were controlled. They were permitted to food and water freely. care provided to rats occurred according to the Ethical Committee of Zagazig University.

Experimental design

Rats were spread into three groups; each group had 10 animals:

Group I (control group): rats were similarly sectioned into two subgroups:

• **Subgroup A:** (negative control): Rats had no treatment.

• **Subgroup B:** Every rat gained a single intra-peritoneal injection of 0.5 ml phosphate buffered saline (PBS); vehicle of STZ.

Group II (Diabetic Group): injected with single dose of Streptozotocin (STZ) (50 mg / kg; powder; CAS No. 18883-66-4; purity of 95%) obtained from Sigma-Aldrich, Cairo, Egypt) intra-peritoneal. It was dissolved in phosphate buffered saline just before the injection. After injection, rats should take 10% glucose solutions for approximately 24 hours for fear of hypoglycemic shock. After 3 days, tail blood was acquired from 8 hours fasting rats to asses blood glucose levels. Diabetic rats have blood glucose above 250 mg / dL[19].

Group III (Diabetic rats received MSCs): were given STZ as Group II. 4 weeks after diabetes confirmation; rats were injected with 5×10^4 L- carnitine treated MSCs in 0.5 ml phosphate buffered saline (PBS) by intravenous injection and left for another 4 weeks[20].

At the completion of the experiment, four weeks after MSC injections[20], rats of all groups were sacrificed by injection of sodium phenobarbital (50 mg / kg) intraperitoneal[21]. Blood samples and samples from spleen have been taken for biochemical, histological and immunohistochemistry studies.

Experimental procedures

Preparation of Bone marrow derived-mesenchymal stem cells (BM-MSCs):

Bone Marrow samples were obtained from anesthetized six-week-old male albino rats by injection of Dulbecco's modified Eagle medium (DMEM) in rats´ femurs. It was laid in Petri dish, and then brought to the laminar flow to pull out the bone marrow. Nucleated cells were sequestered and relayed in a culture medium and put in 5% humidified CO2 for 12-14[22], the fusiform adherent cells are MSCs[23].

Stem cells were pre-treated with 10 mM of L-carnitine (CAS No 541151) purchased from Sigma-Aldrich, Steinheim, Germany. MSCs were centrifuged[24,25] and then labeled in dye solution with Paul Karl Horan 26 (PKH-26) dye[26].

The sections were inspected with a fluorescent microscope (Olympus BX50F4, No 7M03285, Tokyo, Japan) to trace MSCs homing to spleen by discovering and mapping PKH26-labelled cells in the Department of Biochemistry in Zagazig University of Medicine.

Biochemical analysis

Venous blood was collected by retro-orbital perforation of all rats to measure serum insulin using rat insulin C-peptide ELISA kits[27]. Blood glucose was determined using a diagnostic reagent kit (DiaSys Diagnostic Systems GmbH, Germany)[28].

Analysis of quantitative real-time polymerase chain reaction (qPCR) to gene expression:

Real-time Polymerase Chain Reaction (RT-PCR) has tested the mRNA expression for cluster of differentiation CD34, CD105, CD45, CD73, Nrf2, IL 10, and IL 17A using RNA isolation kit (RNeasy, Qiagen, UK).

Total RNA was extracted and reverse-transcribed to cDNA. It was exposed to denaturation, annealing and extension. The aimed gene was stated as a ratio to the housekeeping gene Glyceraldehyde 3- phosphate dehydrogenase (GADPH). The primers were designed according to rat sequence.

Nrf2 Forward, 5-CAC ATT CCC AAA CAA GAT GC-3 and reverse, 5-TCT TTT TCC AGCGAG GAG AT-3.

IL-10 Forward 5'-CACCTTCTTTTCCTTCATCTTTG-3' and reverse 5'-GTCGTTGCTTGTCTCTCCTTGTA-3'

IL-17A forward, 5′-ATCCCTCAAAGCTCAGCGTGTC-3′ and reverse, 5′-ATCCCTCAAAGCTCAGCGTGTC-3'

CD34Forward5′-GCTATTCCCGAAAGACTCTGATTGC-3' and reverse 5′- GACTCCAACTGCGGCGGTTCAT-3'

CD45 Forward 5'- GAGCATTCCACGGGTATTCAGCA-3'and reverse 5′- GTGGACCCTGCGTCTCCATTTATTT-3'

CD105 Forward 5'- CCGCAGAGGCTTGTTGCCAGTA-3' and reverse 5′- CACAGCAGTGCGGTGTCTTTCT-3'

CD73 Forward 5'- GATCCCCTCCTCAGAAATGTCAAA-3' and reverse 5′- CCAACAATCCCCACAACCTCAC-3'

Histological study

• Light microscope analysis

Specimens from each animal were fixed in 10% saline formalin and managed for the preparation of 5 µm-thick paraffin sections for Haematoxylin and Eosin (H&E) staining[29].

• Immunohistochemical study

Immunohistochemical staining for the location of (Nrf2) and (CD4 +) was performed using the Avidin Biotin Complex (ABC) method (Dako ARKTM, Code No. K3954, Dako, Glostrup, Denmark).

Paraffin sections were submitted to wax removal, hydration and then microwaved for antigen retrieval. Endogenous peroxidase has been removed and non-specific binding has been blocked. Sections were covered with anti Nrf2 antibody (Rabbit polyclonal antibody; No. GTX103322; dilution 1:100-1:1000; Gene Tex, Irvine, CA, USA). Also, anti CD4+ (Rabbit polyclonal antibody; No. HPA004252; dilution 1:50–1:1200; Sigma–Aldrich, Steinheim, Germany). Sections were then covered with secondary antibodies. The antigen site will appear brown.

Morphometrical study

The relative value of white spleen pulp % was measured using H&E stained sections by Digimizer.4.3.2 (MedCalc Software bvba, Belgium). From anti NrF2 and anti CD4+immunohistochemically stained sections; the area % of positive nuclear factor Nrf2 andCD4+ immuno-expression was measured in all groups using Leica Quin 500 Image Analyzer (Leica Ltd., Cambridge, UK) in the Image Analyzing Unit of the Pathology Department, Faculty of Oral and dental medicine, Cairo University (Egypt). The magnifications of all measured slides were 400[30]. They were applied in ten non-overlapping fields from each slide of each rat in each group.

Statistical analysis

Data collected from the morphometric study were identified and evaluated using IBM SPSS Statistics Software for Windows, Version 20 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) with Post Hoc least significant difference (LSD) test was used to compare the groups. The data was expressed as the mean (M), standard deviation (SD) and differences were significant at $P < 0.01$.

RESULTS

Examinations of sub-groups A and B of group I revealed similar results. Only results of sub-group A were presented.

Biochemical results

Blood glucose levels (mg/dl)

Statistical analysis of the mean values of the blood glucose levels showed a highly significant elevation in diabetic group (II) relevant to control group (I) and MSCs treated group (III). By applying LSD, there was a highly significant elevation in blood glucose level in group II when compared to the group (I) however, a non-significant increase was discovered between group (III) and control group (I) (Table 1).

Serum insulin levels (mu/L)

Statistical analysis of the mean values of the serum insulin levels displayed a highly significant reduction in diabetic group (II) relevant to both control group (I) and MSCs treated group (III). By applying LSD for comparison between the groups, there was a highly significant reduction in serum insulin level in group II when compared to the group (I) however, a non-significant reduction was detected between group (III) and control group (I) (Table 1).

Detection of stem cells homing

Spleen slides were examined with a fluorescence microscope (Olympus BX50F4, No. 7M03285, Tokyo,

Japan) to identify the cells stained with PKH26 in Biochemistry Department, Faculty of Medicine, Zagazig University. They appeared as bright red dots in sections (Figure 1).

Histological results

Examination of H&E-stained sections of the control group revealed normal spleen architecture with its two major components; white pulps and red pulp, isolated by marginal zones which contained less densely packed lymphocytes. The white pulp consisted of follicles with pale germinal center and peripherally-located central arterioles, and bounded by a cover of lymphocytes; periarterial lymphatic sheath. The red pulp was composed of splenic sinuses and cords of Billroth (Figures 2A,B). Regular smooth capsules were seen covering the surface of splenic tissues (Figure 2C).

H&E-stained sections of the diabetic group revealed small size of white pulp follicles with few lymphocytes. Thickened trabeculae and widened congested sinuses were seen in the red pulp (Figures 3A,B). focal areas of thickening with other areas with indentations were seen in capsules covering the surface of splenic tissues (Figures 3C,D). Groups of hemosiderin laden macrophages were seen. Also, the central arteries appeared hypertrophied with thick walls (Figure 3E). White pulp showed many vacuolations containing fragmented nuclei indicating apoptosis (Figure 3F). Marked congested red pulp, widened congested sinuses, large congested blood vessels with conglomerations of basophilic darkly stained cells around them were observed (Figures 3G,H). Megakaryocytes with their lobulated nuclei and groups of hemosiderin laden macrophages were seen in the red pulp (Figures 3I).

H&E-stained sections in the spleen of the stem cells treated group showed almost normal spleen architecture with white pulp, red pulp, central arteries and periarterial lymphatic sheath (Figures 4A,B). Smooth regular capsules were seen covering the surface of splenic tissue (Figure 4C).

Immunohistochemically stained slides in the spleen of different groups discovered few brown cytoplasmic immune responses for Nrf2 in the control group (Figure 5A). Decreased reaction in diabetic group (Figure 5B) and increased reaction in stem cell group especially in red pulp (Figure 5C).

Examination of CD4+ immunostained slides showed brown cytoplasmic immune responses in the PALS of the white pulp and marginal zones of the control group (Figure 6A). Decreased reaction in diabetic group (Figure 6B) and marked increased reaction in stem cell group especially in red pulp (Figure 6C).

Morphometrical results

Volume of white pulp of the spleen (%)

Statistical analysis of the mean values of the white pulp volume discovered a highly significant reduction in the white pulp volume in diabetic group (II) relevant to both control and MSCs treatment groups. By applying LSD, there was a significant reduction in white pulp volume in group II when compared to the control group. However, a non-significant decrease was detected between MSCs treatment group and control group (Table 2).

Area percentage of nuclear factor Nrf2 and CD4+

Statistical analysis of the area % of Nrf2 and CD4+ immunoexpression showed a significant reduction in diabetic group (II) relevant to both control and MSCs treatment groups. By applying LSD there was a significant reduction in group II as compared to control (group I); however, a highly-significant increase was detected between MSCs treatment group and control group (Table 2).

qRT-PCR relative gene expression BM-MSC surface markers were positive for CD105 and CD73 while were negative for CD34 and CD45.

A significant reduction in Nrf2 and IL 10 gene expression in diabetic group compared to control and stem cell group was detected, while there was significant rise in IL 17A gene expression in group II compared to group I and group III (Table 3).

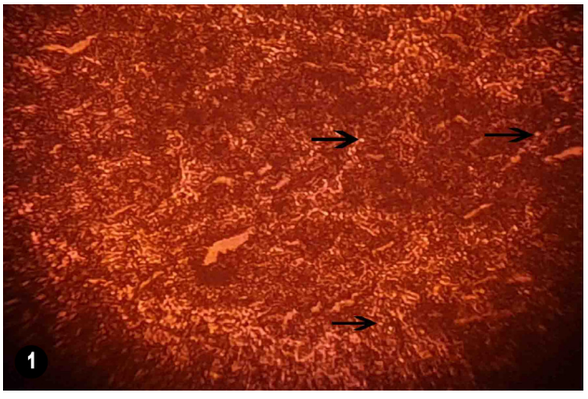

Fig. 1: A section of stem cell treated spleen showing PKH26 labeled cells appearing as bright dots within red and white pulp (arrow). (Fluorescent Microscope x 400).

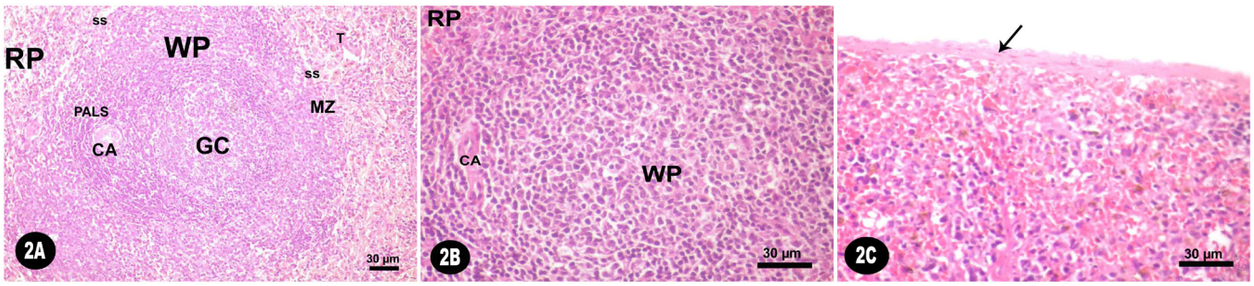

Fig. 2: H&E-stained sections in the spleen of the control group. A,B: normal spleen architecture with its two major components; white pulp (WP) and red pulp (RP), separated by marginal zone (MZ) containing loosely arranged larger lymphocytes. The white pulp has follicle with pale germinal center (GC) and a peripherally located central arteriole (CA), surrounded by periarterial lymphatic sheath (PALS). The red pulp contains splenic sinuses (ss). A part of the fibrous trabecula (T) is also seen. C: regular smooth capsule is covering the surface of splenic tissue (arrow). (H&E 2A x 200) (2B, 2C x 400) scale bar 30 µm.

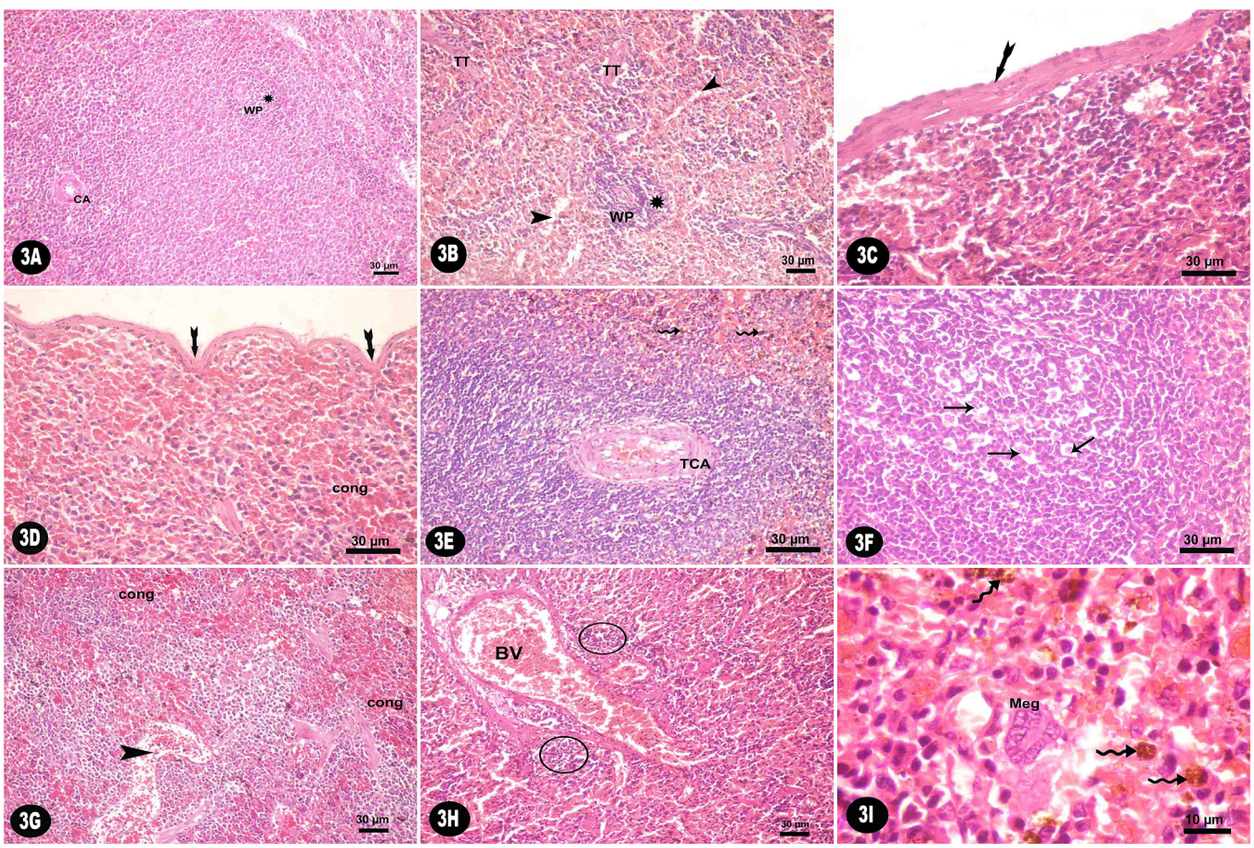

Fig. 3: H&E-stained sections in the spleen of the diabetic group. A,B: reduced size of white pulp (WP) follicles with depletion of lymphocytes (star). Thickened trabeculae (TT), central arteriol (CA) and widened congested sinuses (arrowheads) are seen in the red pulp. C,D: focal areas of thickening with other areas with indentations in capsules covering the surface of splenic tissues (tailed arrow). Congestion (cong) is also obvious E: groups of hemosiderin laden macrophages (wavy arrow) are seen. Also, the central artery appears hypertrophied with thick wall (TCA). F: white pulp show many vacuolations like cavities containing many fragmented nuclei indicating apoptosis (arrow). G,H: marked congested red pulp (cong), widened congested sinuses (arrowheads), large congested blood vessel (Bv) with Conglomerations of basophilic darkly stained cells in around it (circle). I: megakaryocytes (Meg) with their lobulated nuclei and groups of hemosiderin laden macrophages (wavy arrow) are seen. (H&E 3A, 3B, 3G, 3H x 200) (H&E 3C, 3D, 3E, 3F x 400) (3I x 1000) scale bar 30 µm and 10 µm.

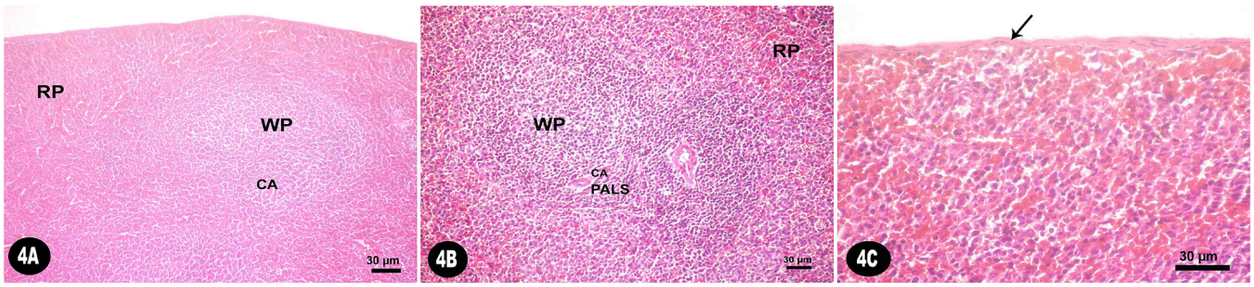

Fig. 4: H&E-stained sections in the spleen of the Stem cells treated group. A,B: nearly normal spleen architecture, white pulp (WP), red pulp (RP), central artery (CA) and periarterial lymphatic sheath (PALS). C: smooth regular capsule (arrow) is covering the surface of splenic tissue. (H&E 4A, AB x 200) (4C x 400) scale bar 30 µm.

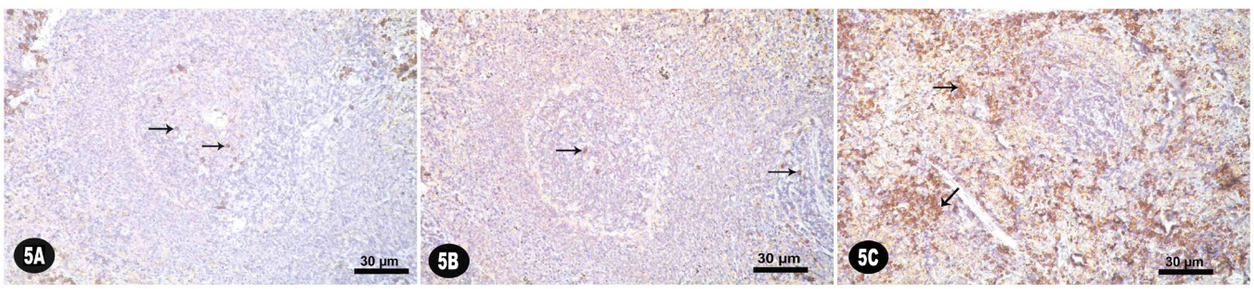

Fig. 5: Immunohistochemically stained sections in the spleen of albino rats of different groups. brown cytoplasmic immune reaction for Nrf2 (arrow) A: few reaction in control group. B: decreased reaction in diabetic group. C: increased reaction in stem cell treated group especially in red pulp. (Nrf2 5A, 5B, 5C x 400) scale bar 30 µm.

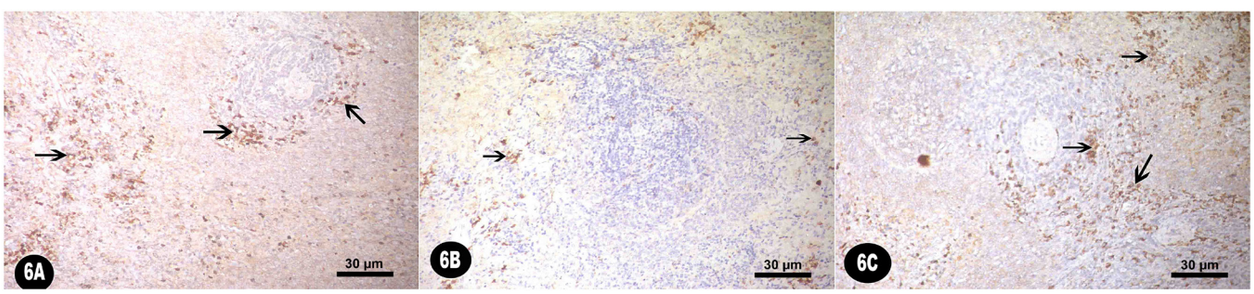

Fig. 6: Immunohistochemically stained sections in the spleen of albino rats of different groups. brown cytoplasmic immune reaction for CD4+ (arrow) A: brownish CD4+ cells were observed in the PALS of the white pulp and marginal zone in control group. B: decreased reaction in diabetic group. C: increased reaction in stem cell treated group. (CD4+ 5A, 5B, 5C x 400) scale bar 30 µm.

Table 1: Statistical analysis of the mean values of blood glucose level and serum insulin level among studied groups

	Control group I Mean±SD	Diabetes II Mean±SD	Diabetes+MSCs III Mean±SD	*P*
Blood glucose levels (mg/dl)	86.50±7.62	465.64±46.7^{++}	115.65±28.4^{NS}	0.000**
Serum insulin levels (mu/L)	15.23±2.12	6.22±1.28^{+}	13.48±1.22^{NS}	0.019*

**Highly significant difference when comparing diabetic group II with both control and diabetes+ MSCs groups (p<0.001).
* Significant difference when comparing diabetic group II with both control and diabetes+ MSCs groups (p<0.05).
++ Highly significant when comparing diabetic group II with control groups (p<0.05).
+ Significant difference when comparing diabetic group II with control group (p<0.001).
NS Non- significant difference when comparing diabetes+ MSCs group with control group (p>0.05).

Table 2: Statistical analysis of the mean volume of white pulp of the spleen (%) and area % of nuclear factor Nrf2 and CD4+ immunoexpression among studied groups

	Control group I Mean±SD	Diabetes II Mean±SD	Diabetes+MSCs III Mean±SD	*P*
Volume of white pulp of the spleen (%)	29.11 ±9.56	25.02±2.13*	27.01±3.42NS	0.014*
Area percentage of nuclear factor Nrf2	39.24±0.75	30.72±0.23*	59.63±1.02**	0.000**
Area percentage of nuclear factor CD4+	37.13±0.75	29.17±0.23*	60.60±1.02**	0.000**

* Significant difference when comparing diabetic group II with both control and diabetes+ MSCs groups (p<0.05).
**Highly significant difference when comparing diabetic group II with diabetes+ MSCs groups (p<0.001).
NS Non- significant difference when comparing diabetes+ MSCs group with control group (p>0.05).

Table 3: Real-time PCR analysis for Nrf2, IL 10 and IL 17A gene expression in the study groups

	Control group I Mean±SD	Diabetes II Mean±SD	Diabetes+MSCs III Mean±SD	*P*
Nrf2 gene expression	1± 0.05	0.7± 0.06	1.2± 0.11	0.000**
IL 10 gene expression	1.08 ± 0.08	0.61 ± 0.07	1.41 ± 0.38	0.000**
IL 17A gene expression	1.7 ± 0.22	5.8± 0.74	3.6± 0.64	0.000**

Values are expressed as mean± standard deviation (SD) of n = 10 animals/group. **Highly significant difference when comparing diabetic group II with diabetes+ MSCs groups (p<0.001).

DISCUSSION

Despite treatment with insulin is considered the most effective medical treatment of diabetes mellitus; it cannot prevent diabetic complications. Also, strict personal attention to the levels of blood glucose is irritating[31]. Thus, it is necessary to find other therapies to manage diabetes and its complications. So, many researches nowadays are directed to replacement therapy. Accordingly, we planned to estimate the role of pretreated stem cells with L-carnitine in treating diabetes and its complications especially in spleen. The main function of L-carnitine inside cells is aiding in ATP production and prevents apoptosis. It can do that by achieving inner mitochondrial membrane stability, changing membrane potential and suppressing mitochondrial fission[16,32,33].

L-carnitine also has anti-inflammatory and antioxidant characters and increases insulin sensitivity in diabetic cases[34]. Moreover, LC can alleviate DM by increasing glucose utilization, increasing muscle glycogen and decreasing muscle lactate content[35]. It aids in multiplying and differentiation of cells by promoting Ca2+ availability through depolarization of calcium channels[36].

In the present study, histological features of diabetic group revealed significant reduction in size of lymphatic nodules in splenic white pulp compared to control. Ebaid,[37] found similar results and said that diabetes decreases immune response function and leads to atrophy of immune organs. This can be explained by increasing secretion of cortisol induced by oxidative stress that causes reduction in splenic lymphoproliferation[38]. In the same context, oxidative stress from noise exposure induced a reduction in lymphatic nodules size and number especially splenic CD4+ cells and also decreased serum immunoglobulins[39].

Moreover, Ebaid *et al*.[40] mentioned that, beside the decrease in lymphocyte numbers in spleen of diabetic rats, there was increased expression of Fas gene (a cell surface death receptor) which is recruited by tumour necrosis factor (TNF) and caused activation of a signal transduction pathway that induces apoptosis[40]. These events could be the potential mechanisms underlying the immunotoxicity of hyperglyceamia and also explained vacuolations and apoptosis in the white pulp cells seen in this work. Apoptosis also may result from hyperglycemia altering mitochondrial electron transport chain with creation of reactive oxygen species (ROS)[41].

On contrary, Abou-Elghait and Galal[42] found widening of the lymphatic follicles with expansion of marginal zone after Malathion exposure for a short period (2 weeks). However, with increasing period (4 and 6 weeks); there were marked degeneration in the lymphocytic contents of the splenic white pulp. So, changes are time dependent.

One of the greatest important goals of transplanted stem cells is the ability to replace the exhausted splenic cells compartment[43]. Many researches suggested that giving MSCs in diabetic rats delayed the disease progress, decreased blood glucose concentrations, increased insulin production and reduced pancreatic inflammation[44,45]. Excess stem cells were established in lymphoid tissues 24 hours next to intravenous injection. Spleen gets 12-19% of the cells however, lymph nodes get 4-5%. Homing in the target organs is also very rapid which can occur minutes or even seconds after intravenous transplantation[46,47].

In this work, significant increase in lymphatic nodules diameters in MSCs treated rats was obvious when compared with the treated group. Hand in hand with Yaochite *et al*.[48] who mentioned that the injected MSCs increased β-cell mass and also reversed diabetes in 67 % of treated mice.

This was explained by increasing the proportion of intrasplenic CD4+ subset by MSCs leading to marked elevation of the ratio of CD4+/ CD8+ cells[49]. This was

reinforced by our immunohistochemical results for CD4+ localization which was seen in PALS in control group; however significant reduction in its area percent was seen in diabetic group which was then amplified in the group injected with stem cells.

The precise way by which MSCs increased lymphoid nodule size is that they enhance the naive T regulatory lymphocytes (CD62L/CCR7) homing to the lymphoid organs with suppression of dendritic cells in the same organs[49].

Our study suggested apoptosis occurrence in group II by appearing of some vacuolated cells with fragmented nuclei in the lymphatic nodules. This is due to hyperglycemia causing free radicals exudation by affecting Krebs cycle and electron transfer within the mitochondrial membrane[50].

Appearances of dark conglomerated cells in the red pulp and around blood vessels are seen in the diabetic group of this work. These results were clarified by Stan *et al*.[51] who found that high glucose level increased cellular Nuclear Factor Kappa NF-kB activation resulting in discharge of a great amount of inflammatory cytokines. Monocytes also are activated in these patients due to increased Tumour Necrosis Factor α (TNF-α), interleukin 1b (IL-1b)[52]. Moreover, oxidative stress occurring from hyperglycemia promotes excess pro-inflammatory cytokines production[53]. Also, the appearance of extra-medullary megakaryocytes in diabetic rats of our study was confirmed by Aula *et al*.[54] and suggested to compensate decreased platelets count.

Conglomerated, apoptotic cells and megakaryocytes were reduced in stem cells treated group of this work. Dazzi *et al*.[55] mentioned that when MSCs are attracted by Interferon- gamma (IFNγ) and TNFα (inflammatory ligands molecules) to the site of inflammation; they decrease the concentrations of these molecules and increased anti-inflammatory molecules concentrations as IL-4 and IL-10[56]. This was confirmed in this study by significant increase in qRT-PCR relative gene expression of IL17A (pro-inflammatory cytokine) in diabetic group relevant to control and stem cell groups. Also, we found significant decrease of IL10 (anti-inflammatory cytokine) in diabetic group compared to control and stem cell group. This IL-10 has many functions; it inhibits macrophage cytokine production and enhances B-cell to produce antibodies[57].

On the cellular level, MSCs directly modulate macrophage activity[58] and inhibit lymphocyte reactions by increasing the regulatory T-cell response[59].

In diabetic group, we saw many hemosiderin laden macrophages. These results were seen also by Cesta[60] and mentioned that these macrophages phagocytose damaged RBCs and any blood-borne substances However, Suttie[61] thought that this view represents increased extramedullary hematopoiesis which results from systemic anemia, infections or any insult elsewhere[61].

Reported vascular congestion in diabetic group, may be due to accumulation of nitric oxide (NO), which is known to be a potent vasodilator that increase blood flow[62]. Unfortunately, NO is released without NO synthase requirement when STZ is injected[63]. However, Fernyhough and Calcutt[64] reported micro vascular narrowing and disruption of Na+/Ca+ ionic homeostasis and related these changes to vascular congestion. The thickened vascular layers were explained by Zhao *et al*.[65] to be related to thioredoxin-interacting (cellular redox) protein[65].

Regarding splenic capsules and trabeculae; they were thickened in the diabetic group. This finding may be part of connective tissue expansion which approved by Yonemoto *et al*.[66] in diabetic nephropathy cases[66] or due to increase Runx2 transcription factor (fibrosis inducing factor) by hyperglycemia[67].

In this work, MSCs treated rats showed non congested red pulp, sinusoids and nearly normal capsular and trabecular thickening. This may occur due to trophic factors discharge from stem cells which improve tissue repair[68]. Or due to MSCs creating insulin-producing cells which reverse hyperglycemia[69]. Additionally, MSCs improve body response to insulin via increased insulin receptor substrate-1 [IRS-1] and GLUT-4 on cell membrane when insulin administered[70].

Nrf2 is a transcription factor that activates antioxidant genes as the heme-oxygenase-1 (HO-1)[71]. In this work, immunohistochemical determination of Nrf2 and qRT-PCR gene expression was made in all study groups. It revealed significant decreased expression in diabetic group relative to control and stem cell groups. This was similar to results of Tan *et al*.[72] in diabetic heart. They suggested that Nrf2 is increased at the initial stage of diabetes to overwhelm diabetic damage. However, at late stage, even the antioxidant function is deteriorated, leading to its decreased expression. Sasaki *et al*.,[73] added that long term oxidative stress overloads this protective response in DM.

Chen *et al*.[74] recorded survival of adipose tissue grafts after stem cells transplantation via enhancing (Nrf2) expression. This Nrf2 then provokes MSCs to improve its anti-inflammatory properties[75].

Nrf2 also prevent pancreatic β-cell apoptosis[76], control obesity by decreasing gluconeogenesis genes and improving insulin sensitivity in diabetic cases[77].

On the other hand, Soares *et al*.[78] reported that diabetes induced reduction in Nrf2 expression due to hyperglycemia stimulated Keap1 (Kelch like associated protein-1) dysfunction, which results in decrease cellular Nrf2[78].

CONCLUSION

So, we concluded that L-carnitine pretreatment of MSCs releases a new way to enhance MSCs therapeutic potential efficacy in spleen of experimental diabetic rats with recommendation of trying further researches for their application in other vital organs.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دور الخلايا الجذعية الميزنشيمية المعالجة بالأل كارنيتين في التغيرات الهستولوجية لطحال الفئران بعد احداث مرض البول السكري والدور النشط للعامل النووي لكريات الدم الحمراء

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المقدمة: ان البول السكري يسبب ضمور في العقيدات الليمفاوية للطحال والذي بدوره يؤثر علي مناعة الجسم. يمكن استخدام الخلايا الجذعية الميزنشيمية عمليا في علاج دمار الطحال الناتج من البول السكري.

الغرض من البحث: تهدف الدراسة الحالية لتوضيح التغيرات النسيجية في طحال الفأر الأبيض البالغ بعد احداث مرض البول السكري و لتقييم تأثير حقن الخلايا الجذعية الميزنشيمية المعالجة بالأل كارنيتين في تحسين هذه التغيرات.

المواد و الطرق: قسم ثلاثون من ذكور الفئران البيضاء البالغة الى ثلاث مجموعات: المجموعة الاولى (الضابطة) و المجموعة الثانية (المصابة بالبول السكري): حقنت الفئران مرة واحدة داخل الغشاء البريتوني ب ٥٠مجم/كجم من الستربتوزوتوسين و المجموعة الثالثة (المصابة بالبول السكري + الخلايا الميزنشيمية): حقنت الحيوانات بالخلايا الجذعية داخل الوريد بعد أربع أسابيع من تأكد اصابتها بمرض البول السكري.تم ذبح كل الفئران بعد أربع أسابيع من حقن الخلايا الجذعية (بعد ثمان أسابيع من بداية التجربة) و تمت معالجة عينات الطحال لفحصها بالمجهر الضوئي. و تم تجميع عينات الدم لقياس مستوى الجلوكوز و الانسولين بها.و تم قياس التعبير عن الحمض النووي الريبوزي الرسول الخاص ب العامل النووي للكريات الحمر ٢، انترلوكين ١٠، انترؤلوكين ١٧أ بواسطة تفاعل البلمرة المتسلسل في الوقت الحقيقي.

النتائج: لوحظت تغيرات تنكسية في المجموعة المصابة بالبول السكري في صورة نقص في حجم اللب الابيض مع وجود أنوية عديدة ميتة. و اتساع و احتقان في اللب الأحمر ، و نقص ذو دلالة احصائية في مستوى الأنسولين، حجم اللب الأبيض، و نسبة مساحة التمثيل المناعي لل سي دي ٤+ و العامل النووي للكريات الحمر ٢ و ايضا نقص في التعبير الجيني النسبي للعامل النووي للكريات الحمر ٢ و الانترلوكين ١٠ بواسطة تفاعل البلمرة المتسلسل في الوقت الحقيقي.كما وجد زيادة واضحة في مستوى الجلوكوز في الدم و التعبير الجيني النسبي للانترلوكين ١٧أ بواسطة تفاعل البلمرة المتسلسل في الوقت الحقيقي. كل هذه التغيرات قد عكست في المجموعة المعالجة بالخلايا الجذعية الميزنشيمية.

الاستنتاج: نستنتج أن المعالجة المسبقة بالأل كارنيتين للخلايا الجذعية الميزنشيمية تطلق طريقة جديدة لتعزيز الفعالية العلاجية المحتملة لهذه الخلايا في طحال الفئران المصابة بداء السكري التجريبي مع التوصية بمحاولة إجراء المزيد من الأبحاث لتطبيقها في الأعضاء الحيوية الأخرى.