Effect of combined mesenchymal stem cell and derived exosomes in control of STZ induced diabetic hepatopathy in Wistar rat model

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1. Abstract

Diabetes mellitus (DM) is a chronic widespread metabolic disorder, involving a high blood glucose level which causes overtime multiple serious complications. Due to the unwanted side effects of the anti-diabetic medicines and high dose required of the herbal plants which used for a long time for DM treatment. The current studies directed to the use stem cells and exosomes as regenerative medicine to overcome these limitations of traditional therapy.

We aimed to investigate the antidiabetic effect of combined Bone marrow mesenchymal stem cells and exosomes against Diabetes induced by streptozotocin (STZ) in Wistar rats through hindering the inflammatory reactions and the hypolipidemic effect by preventing lipid accumulation.

Our study was conducted on 21 male Wistar rats divided into three groups (control non diabetic group, control diabetic group and combined Stem cell and exosomes treated group). Blood glucose level and liver function enzymes were assayed in addition to a lipid profile test. Moreover, the enzymatic and non-enzymatic antioxidants were assayed in addition to lipid peroxidation products in the liver. It was found that the injection combination of both stem cells and exosomes into diabetic rats improved the destructive effects that happened as a result of STZ injection and reinstated the biochemical functions in addition to levels of hepatic antioxidants to normal.

Keywords: Hyperglycemia, Oxidative Stress, GSH, Malondialdehyde, MDA.

2. Introduction

Based on previous studies confirmed the **dangerous** metabolic illness called Diabetes mellitus (DM), different causes can lead to diabetes development as decreased insulin secretion (as shown in Type one Diabetes mellitus [T1DM]) or inability of pancreatic β cells to secret insulin (as shown in Type two Diabetes mellitus [T2DM]) and defect of distribution of insulin in peripheral regions. In clinical courses, the most happened types: T1DM, T2DM, and gestational type of diabetes (GDM) seen during pregnancy and characterized by decreased insulin sensitivity and considered a temporary condition[1-4]

Recent techniques for the treatment of diabetes via using stem cells as regenerative medicine, among types of stem cells, we focus on the efficacy of bone marrow mesenchymal stem cells (BMMSCs) for the treatment of diabetic liver damage, BMMSCs are considered the most commonly used in the clinical experiments [5, 6], they are progenitor cells in BM which capable for proliferation, differentiation in some cell lines, self-renewal, regulation of the immune system and support hematopoietic cells.

Another novel technique we used to take exosomes derived from mesenchymal stem cells. Exosomes are small membranes extracellular vesicles [7]**.** Exosomes transfer nucleic acids, proteins, and lipids between cells; these vesicles of Nano size diameter ranging from (30) to (150) found in urine, blood, cerebrospinal fluid and saliva [8] those used as a biomarker for diagnosis of diseases. Exosomes derived from stem cells were studied for the regenerative properties particularly the vesicles derived from BMMSCs[9] and had the interest in treatment of various diseases including diabetes.

Furthermore, exosomes can ameliorate damaged pancreatic β cells [10], affect glycolytic enzymes, inhibit insulin secretion, and encourage glucose metabolism [11, 12]

Use of BMMSCs derived exosomes had beneficial effects in a variety of animal models of liver disease, including drug-induced acute liver injury [13]**,** diabetic hepatopathy [14] and hepatocellular carcinoma [15]. They showed that these exosomes reduced liver injury and fibrosis progression, suggesting a potential therapeutic role of exosomes in diabetes-related liver complications.

The current study aimed to investigate the antidiabetic effect of combined Bone marrow mesenchymal stem cells and exosomes against Diabetes induced by streptozotocin (STZ) in Wistar rats as a model.

3. Materials and Methods

3.1. IACUC approval:

The experimental processes, including animal anesthesia, sampling and euthanasia were approved ethically from the Veterinary Medicine Cairo University Institutional Animal Care

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2024.342340.1042

and Use Committee (Vet- CU- IACUC) with approval No.: Vet CU 25/12/2023 879and following the UK guidelines.

3.2. Experimental animals' preparation:

Twenty-one adult male albino rats, weighing between 200-250 gm, chosen for the experimental study were obtained from the Egyptian organization of Vaccines (Cairo, Egypt), the animal house at the Department of Toxicology and Forensic Medicine Faculty of Veterinary Medicine Cairo University-Egypt.

The selected rats were kept in plastic cages at 45–55% humidity, 25 C, and 12/12 h light/dark under a completely controlled environment for 7 days for acclimatization. All Rats were fed with standard pellets and water was delivered ad libitum.

3.3. Experimental design (grouping of the study):

The study compared three groups of male albino rats (21 rats). Group I (control negative) normal rats (n = 7) received no induction or treatment for 4 weeks. Diabetes was induced using STZ that obtained from Sigma Aldrich Chemicals Co., St. Louis, USA. in group II (control positive) (n = 7) and group III (diabetic rats treated with combined stem cells and derived exosomes) (n = 7) by a dose of (60 mg/kg STZ in 0.01 M citrate buffer, pH 4.5 one shot only[16,17]. Groups I and II received just saline for 4 weeks. Group III animals injected IV (10^7 BMMSCs cells) and (5×10^9 exosome particle/rat) through tail vein twice per week for one month. Presented this data at table (1). The rats provoked hyperglycemia after 72 hours of STZ administration. Rats with fasting blood glucose ≥ 250 mg/dl were selected for the experimental study[18].

3.4. Preparations and extraction of stem cells and exosomes:

3.4.1. Preparation and culturing of BMMSCs from the rat

Three normal rats were sacrificed and bone marrows were flushed from long bones (femur and tibia) using Dulbecco's Modified Eagle Medium (DMEM) by a needle and syringe under sterile condition. The aspirate was filtered by 70 μm filter to get rid of bone fragments. The filtered bone marrow for 10 min at 1000 rpm and supernatant was discarded. The pellets were resuspended in 0.83% ammonium chloride to destroy RBCs and recentrifuged for 10 min at 3000 rpm. The new pellets were seeded in a culture dish containing a suitable medium (DMEM). After that, dishes are incubated at 37°C with 5% CO_2 [19] for 72 hrs. The non-adherent cells were flushed and discarded. The culture media was replaced and BMMSCs adherent cells left to grow. On the 7^{th} day, completely adherent cells were detached by trypsin, centrifuged at 3000 rpm, and the pellet were cultured till reach 80–90% confluence[20].

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2024.342340.1042

3.4.2. Isolation and confirmation of exosomes gained from BMMSCs

Rat BMMSCs (2 x 10^5) cells were implanted in 150 cm^2 culture plates. After cells reached 80% confluence, the supernatant of the cultured BMMSCs was collected and subjected to sequences of centrifugation steps (Ultracentrifugation method at Faculty of Science, Alexandria University). The supernatant is centrifuged for 10 min at 500 *x g* to remove pelleted cells, followed by 20 min at 2000 *xg* to remove pelleted cell debris, then 30 min at 10000 x g to remove the large vesicles, and finally 70 min at 100000 *xg* to pellet the exosomes. The pellet is washed with Phosphate Buffer Saline (PBS) and exposed to another round of ultracentrifugation to purify the exosomes[21]**.** Resuspend the collected purified exosomes in PBS that were preserved at -80°C for long-term preservation[22,23]**.**

3.4.3. Characterization of BMMSCS

One of the important suggested criteria of BMMSCS by the International Society for Cellular Therapy (ISCT) is expression surface markers positive CD34, CD73, CD105, and CD90 while negative through CD44 and CD45. In addition to the ability of stem cells to adhere to plastic surfaces and differentiation into multiple cell lines according to in vitro additional items [24-26] as shown in figure (1).

3.4.4. Characterization of BMMSCs exosomes

To identify the exosomes, first, measuring the exosomes using Nanoparticle tracking analysis (NTA). Secondly by using surface markers, CD9, CD63, CD81, and expression was estimated by western blot. Then finally by TEM morphology[23,27,28]**.**

3.4.5. Estimation of particle size of exosomes using nanoparticle tracking analysis (NTA)

The NTA 2.2 Analytical Software was used to detect and process particle-size distributions. After diluting exosomes in PBS with 1:100, the solution was inoculated into the Particle Sizing System, Santa Barbra, USA at National Research Center, Egypt. Exosomes sizes were 30 to 150 nm in size, and peaked at 134 nm.[28, 29]**.** **(**Figure 2/A).

3.4.6. Surface markers detection of exosomes

The purified rat BMMSC exosomes revealed the presence of typical exosomal proteins; CD63, CD81, and CD9 that were devoid of contamination with cellular proteins. Zhao et al. [28] have used anti-CD63, anti-CD81, and anti-CD 9 antibodies diluted 1:100 dilution. The sample was evaluated using flowcytometry (Becton-Dickinson, Canada) as in figure 2/B.

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2024.342340.1042

3.4.7. The detection of exosome morphology using transmission electron microscopy

Exosomes were resuspended in PBS and fixed for 30 minutes at room temperature with 3% glutaraldehyde solution. Exosomes stained with uranyl acetate stain at room temperature for 30 seconds after pouring them onto a carbon-coated copper grid (CCG). At Cairo University, Faculty of Agriculture, the dried grids examined using transmission electron microscope (JEOL GEM-1010) at 80 kV[28]. Exosomes are generally 103 nm in size (Figure 2/C).

3.5. Evaluation:

On the day thirty of the experiment, rats were humanely anesthetized with a combination of ketamine (Ketamar ® 5% Sol. Amoun Co., A.R.E) and xylazine (Xyla-Ject ® 2%, ADWIA Co., A.R.E.) administrated by I/M injection of xylazine (5 mg/kg) and I/M ketamine (100 mg/kg) [30]. The blood was gathered from retro-orbital venous plexus and centrifuged at 3500 RPM for 15 min to separate the serum. The serum transferred to Eppendorf's tubes for estimation serum blood glucose, lipid profile and liver function enzymes. After collecting blood samples, all Rats were euthanized through cervical dislocation. The liver specimens were kept at -80 °C for oxidative stress analysis.

3.5.1. The clinical biochemical parameters

3.5.1.1. Blood glucose levels

Serum blood samples from overnight fasting rats have been taken from retroorbital venous plexus then directly blood glucose levels were measured by colorimetric glucose kit obtained from Spectrum Co., Egypt [31].

3.5.1.2. Liver function enzymes

For assessment of hepatic function, the activities of Aspartate Transaminase Test (AST), Alkaline Phosphatase (ALP) and Alanine aminotransferase (ALT) [32] were measured following the manufacturer protocol of the kits (Spectrum Co., Egypt) in a semi-automated spectrophotometer.

3.5.1.3. Lipid profile assessment

The total cholesterol was assessed by the method of Young et al. [33] and triglycerides (TG) was estimated according to the procedures of Bucolo and David [34] following the kits instruction (Bio diagnostic Co., Egypt).

3.5.2. Oxidative stress measurement

Liver tissue sample were evaluated for determining oxidative stress markers level of reduced glutathione (GSH) [35] and malondialdehyde (MDA) [36] formation. Liver tissues were perfused with PBS solution pH 7.4 containing 0.16 mg / ml heparin to get rid of any RBCs or blood clots before the dissection, then liver sample were homogenized in cold buffer (10 ml per

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2024.342340.1042

gram of tissue, 50 mM potassium phosphate, 1 mM EDTA) pH equal 7.5, then take the supernatant after centrifugation at 4000 RPM for 15 min at 4 °C and used for assessment of GSH and MDA (expressed as Thio barbituric acid reactive substance [TBARS]) following the kits instructions (Biodiagnostic Co., Egypt).

3.5.3. Statistical analysis:

All results of the oxidative stress, and chemical tests, were analyzed in one-way analysis of variance (ANOVA) then by using GraphPad Prism version 8.4.3. Differences considered significant when $P \leq 0.05$. Significance shown by different superscript letters[37].

4. Results

4.1. Biochemical results:

4.1.1. Blood glucose level

The blood glucose levels in the STZ injected group were significantly ($p < 0.05$) greater (507 ± 0.01) than those in the control group (90 ± 0.01), On the other hand, the blood glucose levels were significantly lower (179 ± 0.05) in the rats co-administered with combination of exosome and stem cell.as shown at figure (3)

4.1.2. Liver function enzymes

Injection intravenous of BMMSCs (10^7) with (5×10^9 particle/rat) of derived exosomes through tail vein twice per week for a month caused a significant decrease in ALT, AST, and ALP levels (40.44 ± 0.009, 59.5 ± 0.023 and 199 ± 0.01 respectively) compared to rats of the STZ injected group, which showed a significant increase in ALT, AST, and ALP levels (263.8 ± 0.02, 71.2 ± 0.05, and 595 ± 0.02, respectively) at $p < 0.05$. as shown at figures (4 and 6)

4.1.3. Lipid profile

Results showed that combination of exosomes and stem cell caused a significant decrease in cholesterol and triglyceride levels (58.4 ± 0.03 and 105.4 ± 0.03 respectively) compared to those in STZ-injected rats (103.4 ± 0.02 and 53.2 ± 0.08, respectively) ($P < 0.05$) Furthermore, combination of exosomes and stem cell reverted triglyceride levels to normal levels. as shown at figure (7,8)

4.2. Oxidative stress:

The rats exposed to STZ (group II) showed an increase in oxidative stress markers, as indicated by a significant increase in the level of MDA and a significant decrease in GSH in liver tissue compared to the control negative group. On the other side, administration mix of (BMMSCs and exosomes) to rats (group II) protected hepatic tissue from STZ - induced oxidative burst through enhancement of the GSH level and lowering of the MDA level (table 2 and figures 9 and 10).

5. Discussion

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2024.342340.1042

The field of stem cell research is continuously evolving, there were ongoing studies investigating the potential antidiabetic activity of stem cells. Mesenchymal stem cells have been extensively studied for their potential therapeutic effects in diabetes. These cells have shown promise in improving glucose control, reducing inflammation, and promoting pancreatic beta-cell regeneration in preclinical studies. Some studies have demonstrated that MSCs can enhance insulin production and secretion, protect beta cells from damage, and improve insulin sensitivity[38].

The combination of BMMSCs and exosomes may serve as a promising therapy for addressing DM and its complications in the immediate future [39].

These biochemical results were supported by the remarkable improvements in blood glucose level, liver function (AST, ALT, and ALP) and lipid profile (cholesterol and triglycrides). Similar to the influence of cell therapy, BMMSCs injections successfully upturned hepatocyte damage, as shown by recovery of ALT and AST levels [40].

The remarkable reductions in blood glucose levels, suggesting enhanced glucose metabolism and possible restoration of pancreatic β-cell function [41].

Elevated levels of liver enzymes markers of hepatocyte damage, that occurs in diabetes due to oxidative stress and chronic inflammation. The combination therapy normalized these levels, indicating hepatoprotective effects. BMMSCs contribute to liver regeneration through direct differentiation, while exosomes enhance these benefits by delivering growth factors and anti-inflammatory agents directly to damaged liver cells [42].

Dyslipidemia, characterized by elevated cholesterol and triglycerides, is a hallmark of diabetes. The combination therapy significantly improved lipid profiles, likely due to the anti-inflammatory and lipid metabolism-regulating roles of exosomes [43].

In the present work revealed, the hepatoprotective efficacy of BMMSCs and derived Exosomes against STZ-induced hepatopathy, these combination efficiently reduced hyperglycemia, improved the liver function enzymes and lipid profile, attenuated oxidative stress, and enhanced the antioxidant activity of GSH.

STZ induced disruption of cell membrane oxidative phosphorylation can be made by free oxygen species, which negatively affect the integrity of the junctional complex The accumulation of STZ in the mitochondria leads to excess reactive oxygen species (ROS) production, and disturbance in the mitochondria respiratory chain activates oxidative stress and apoptotic progressions in liver tissues [44]**.**

Excessive production of ROS cause lipid peroxidation, protein damage and DNA strand breaks,

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2024.342340.1042

resulting hepatocyte damage and leakage of liver enzymes into the blood stream. STZ induced adverse effects on the antioxidant defense mechanism, as indicated by a significant decrease in GSH and an increase in MDA. These results are in accordance with the previous reports[45]**.**

Administration of BMMSCs and derived exosomes in the current study presented significant protection hepatocyte from oxidative stress induced by STZ, as determined by a significant enhancement in GSH level with decline of MDA level in comparison to STZ induced group, may attribute to the anti-apoptotic and tissue regeneration effects [46] and the anti-oxidants ability of the stem cells via ROS-scavenging properties, these results are compatible with the previous reports [47].

6. Conclusion

This combination therapy represents a promising way for innovative diabetes treatment strategies by addressing not only hyperglycemia but also systemic complications like liver dysfunction and dyslipidemia as well as restoring the levels of hepatic antioxidants to normal.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank Egyptian Knowledge Bank (EKB) as well as all technicians in Veterinary Teaching Hospital, Cairo University, for their help during the work.

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Table (1): Explanation of the experimental design of the study

No	**(Group I)**	**(Group II)**	**(Group III)**
Name	Control negative	Control positive (Diabetic)	Diabetic rats treated with stem cell and exosome
First week	Acclimatization		
Second Week: Diabetes Induction	Saline only	Single Intraperitoneal (IP) injection of STZ (60 mg/kg) (One shot)	
Treatment for one month	Saline only	Saline only	Injected IV (10^7 BMMSCs) +(5×10^9 particle/rat) through tail vein 2 times per week

Table (2): Oxidative stress markers levels in all groups

Oxidative stress markers	Control negative	Control positive (Diabetic)	Diabetic rats treated with stem cell and exosome
MDA(nmol/g tissue)	(12 ± 0.001)	(42 ± 0.08)	(17.4± 1.4)
GSH(µmol/g tissue)	(2.9 ± 0.002)	(0.6 ± 0.02)	(1.98 ± 0.17)

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2024.319718.1039

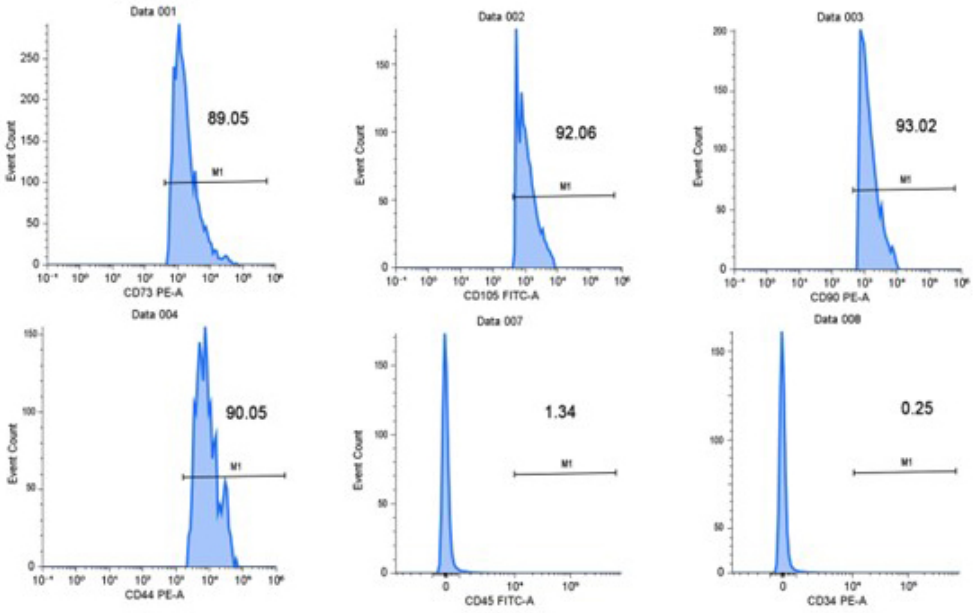

Fig. 1. the main represented BMMSCs surface markers are considered positive for CD34, CD73, CD105, and CD90 but CD44 and CD45 were negative.

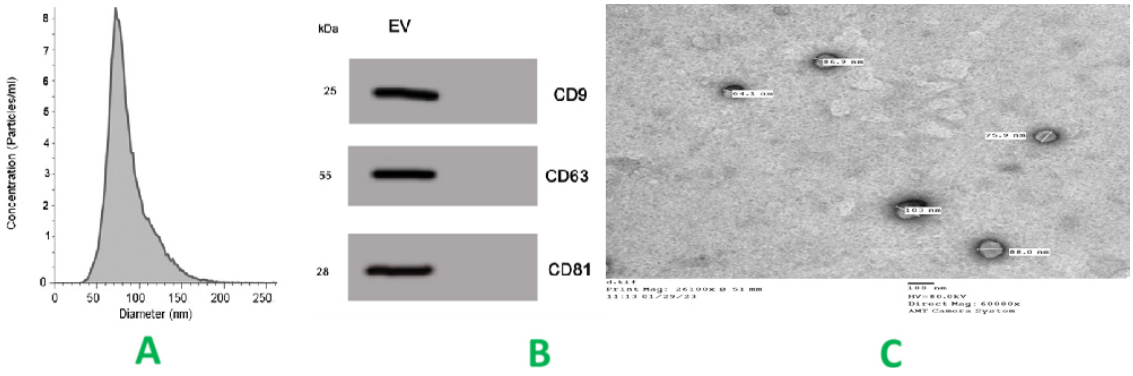

Fig. 2. Showing the characterization of BMMSCs derived exosomes. **A:** distribution of Particle size assessed by (NTA). **B**: the analysis of exosome surface markers. **C**: The morphology of the Exosome exposed by transmission electron microscope (TEM).

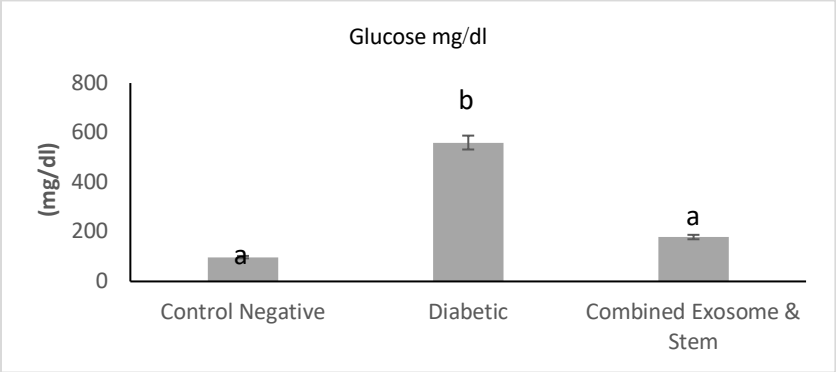

Fig. 3. The effect of injection of the combined exosome and stem cells on blood glucose level. Values are presented as mean ± SEM (n = 7 rats/group). Different superscript letters indicate a significant difference at $P \leq 0.05$.

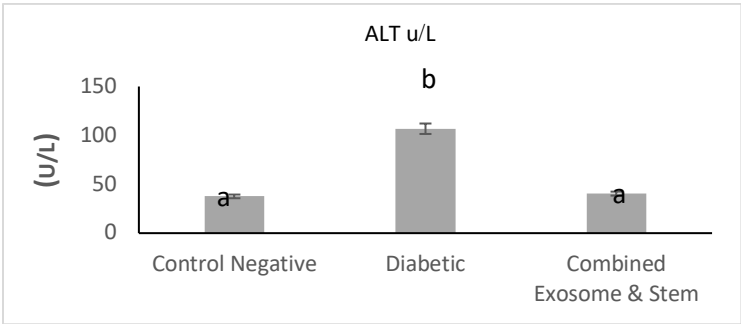

Fig. 4. Explain the effect of injection of the combined exosome and stem cells on liver enzyme: alanine amino transaminase (ALT). Values are presented as mean ± SEM (n = 7 rats/group). Different superscript letters indicate a significant difference at $P \leq 0.05$.

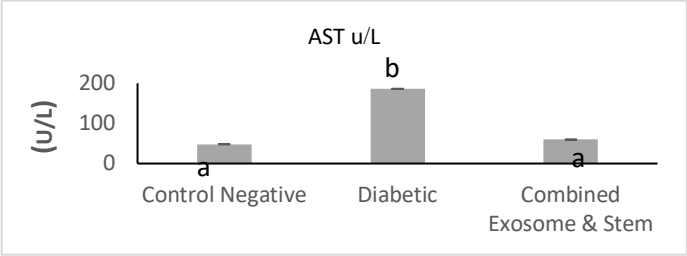

Fig. 5. Explain the effect of injection of the combined exosome and stem cells on liver enzyme: aspratate aminotransferase (AST). Values are presented as mean ± SEM (n = 7 rats/group). Different superscript letters indicate a significant difference at $P \leq 0.05$.

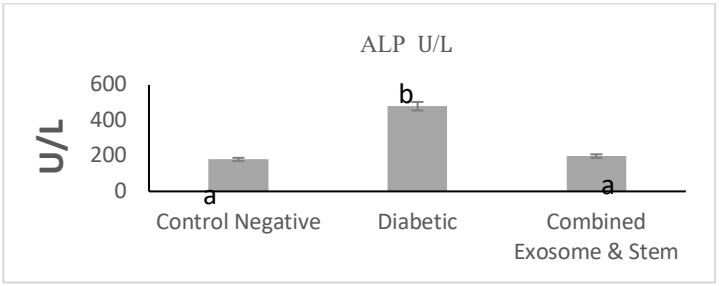

Fig. 6. Explain the effect of injection of the combined exosome and stem cells on liver enzyme: alkaline phosphatase (ALP). Values are presented as mean ± SEM (n = 7 rats/group). Different superscript letters indicate a significant difference at $P \leq 0.05$.

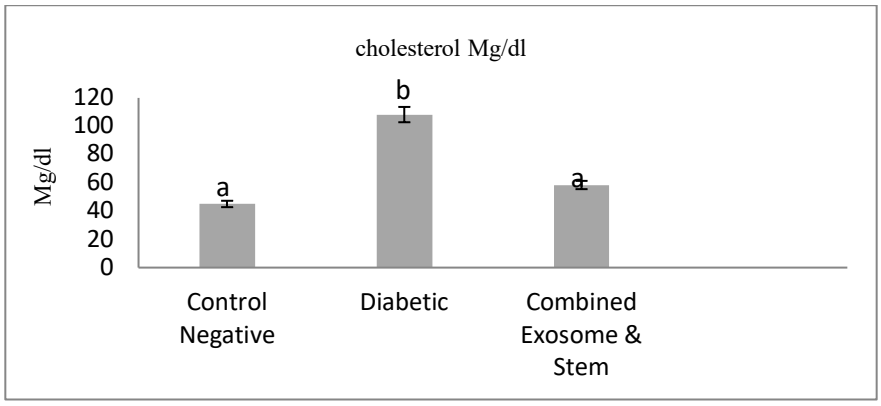

Fig. 7. Effect of the injection of exosomes and stem cell on the serum activity of cholesterol in different groups. Values are presented as mean ± SEM (n =7 rats/group). Different superscript letters indicate a significant difference at $P \leq 0.05$.

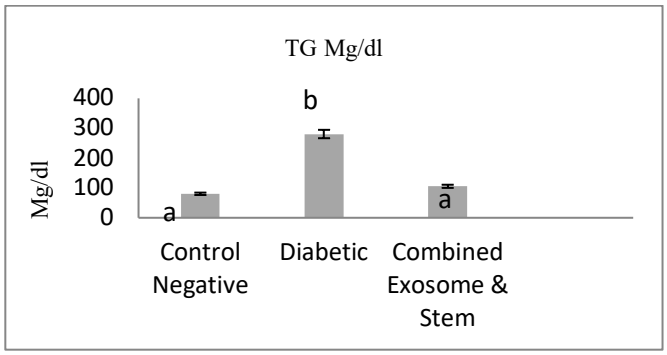

Fig. 8. Effect of the injection of exosomes and stem cell on the serum activity of triglycride in different groups. Values are presented as mean ± SEM (n =7 rats/group). Different superscript letters indicate a significant difference at $P \leq 0.05$.

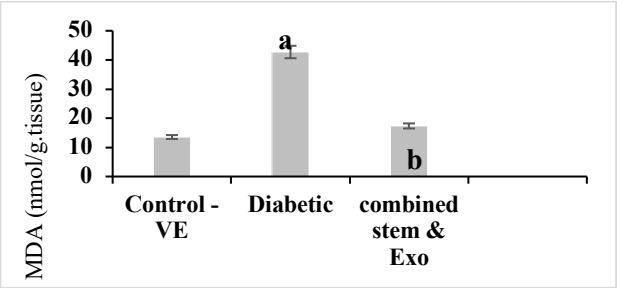

Fig. 9. Liver oxidative stress markers MDA level in different groups. Values are presented as mean ± SEM (n = 7 rats/group). Different superscript letters indicate a significant difference at $P \leq 0.05$.

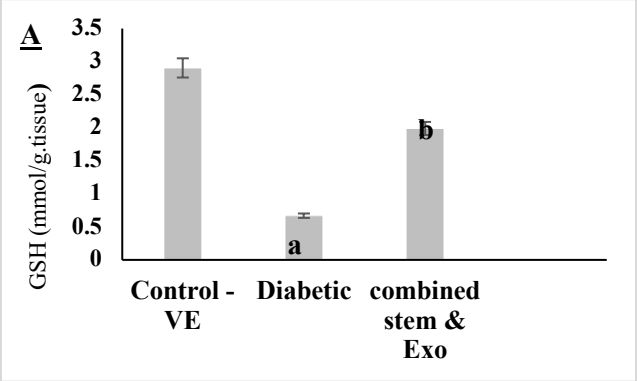

Fig. 10. Liver oxidative stress markers GSH level in different groups. Values are presented as mean ± SEM (n = 7 rats/group). Different superscript letters indicate a significant difference at $P \leq 0.05$.