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Adverse effects of energy drink on rat pancreas and the therapeutic role of each of bone marrow mesenchymal stem cells and Nigella Sativa oil

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Background: *Energy drinks have been observed to threaten public health leading to many medical problems. Bone marrow-derived mesenchymal stem cells (BMSCs) have broad prospects in tissue regeneration. Nigella Sativa (NS) possess great therapeutic properties for the treatment of a wide range of diseases.*
Materials and methods: *Forty adult male albino rats were divided into: control group and treated group. The treated group was further subdivided into: energy drink subgroup 2a, BMSCs-injected subgroup 2b, NS-injected subgroup 2c. Histological, immunohistochemical and biochemical assessment was performed.*
Results: *Administration of energy drink revealed that it adversely affected the pancreatic cytoarchitecture. BMSCs and NS have been similarly observed to significantly ameliorate the histological, biochemical and immunohistochemical changes induced by energy drink.*
Conclusions: *The extent of pancreatic regeneration, exerted by each of BMSCs and NS oil, is nearly similar but the effect of BMSCs is more superior; however, NS could be privileged to BMSCs as a line of treatment being easily accessible and of lower cost.* (Folia Morphol 2020; 79, 2: 272–279)

Key words: energy drink, pancreas, bone marrow derived mesenchymal stem cells, Nigella Sativa

INTRODUCTION

Energy drinks are types of stimulants-containing beverages which are marketed to improve mental and physical performance [18]. These beverages contain large concentrations of caffeine and other additives such as taurine, creatine, herbal supplements, sugar and gurana [3]. The adverse reactions and toxicity of energy drinks stem primarily from their caffeine content [41]. Energy drinks have been mentioned to have negative impact on different body organs [25] including hepatotoxicity [15], derangement of secretory glands [32], nephrotoxicity [26], haematopoietic disorders [34], overweight/obesity risk and type 2 diabetes mellitus [2]. However, reviewing the literature has displayed few studies on the effects of these drinks on the pancreas.

Mesenchymal stem cells (MSCs) are multipotent undifferentiated stromal cells that are capable of self-renewal and multidirectional differentiation [24]. Administration of stem cells can regulate the activity of native stem cells and modify the inhibitory regenerative environment through secretion of soluble trophic factors that can exert potent paracrine effects on other cell types [16].

Nigella Sativa (NS) is an annual herb belonging to the plant family Ranunculaceae [9]. Studies have

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shown that NS seeds possess great therapeutic properties as an antidiabetic [8], anti-apoptotic [44] and antioxidant agent [17].

Thus, this study was designed to detect the adverse changes that occur in both exocrine and endocrine parts of pancreas of rat and to determine the possible therapeutic role of each of bone marrow mesenchymal stem cells (BMSCs) and NS oil (NSO).

MATERIALS AND METHODS

Chemicals

Energy drink which is known in the Egyptian market by the name Red Bull [Red Bull GmbH, 5330 Fuschl am See, Austria]. It is available in the form of cans of 250 mL. Each 100 mL of the drink contains a mixture of water, sucrose, glucose, sodium citrate, citric acid, carbon dioxide, taurine (0.4%), caffeine (0.03%), inositol (0.02%), niacin (8 mg), pantothenic acid (2 mg), vitamin B6 (2 mg), B12 (0.002 mg), riboflavin, caramel, natural and artificial flavouring and colouring agents. It was used in this study in a dose 10 mg/kg/day (equivalent to 5 mL) by gastric tube [3].

Fluorescent labelled BMSCs were obtained from Biochemistry Department, Faculty of Medicine, Cairo University. Each rat was given single injection in the tail vein at a concentration of 1 million units/mL [28]. NSO was purchased in the form of a bottle of 30 mL (Cap Pharma Company, Egypt) given by intraperitoneal injection at a dose of 0.2 mL/kg [1].

Flow cytometry for bone marrow derived mesenchymal stem cells

The isolated cells were washed and re-suspended in phosphate buffered saline (PBS). Cells were incubated with fluorescein isothiocyanate-conjugated mouse monoclonal antibodies against rat CD90 (Becton Dickinson, Franklin Lakes, NJ), fluorescein isothiocyanate-conjugated hamster anti-rat CD29 monoclonal antibody (Becton Dickinson), phycoerythrin-conjugated mouse monoclonal antibodies against rat CD34 (Santa Cruz Biotechnology, Santa Cruz, CA), and were characterised as MSCs by fluorescence-activated cell sorting FACS Caliber; Becton Dickinson). Isotype-identical antibodies served as controls.

Animals

This experimental study was approved by Cairo University Institutional Animal Care and Use Committee (CU-IACUC). Forty adult male albino (Sprague Dawley) rats, aged 3 to 5 months and weighing 150––200 g were obtained from the Animal House, Faculty of Medicine, Cairo University. They were housed under standard laboratory and environmental conditions. The rats were divided into two main groups: group1 (control group; n = 10) and group 2 (treated group; n = 30). Control group rats were kept on normal diet and water and received no medication. Group 2 (treated group) in this group rats received energy drink for 4 weeks and they were further divided into three subgroups:

— subgroup 2a (energy drink) — rats were sacrificed 4 weeks after ingestion of energy drink at a dose of 10 mg/kg/day (equivalent to 5 mL) by gastric tube [3];
— subgroup 2b (BMSCs) — energy drink-administered rats were further injected, into the tail vein, with single injection of fluorescent labelled BMSCs at a concentration of 1 million units/mL. The rats were sacrificed 4 weeks following this injection [28];
— subgroup 2c (NSO) — energy drink-administered rats were intraperitoneally injected with NSO at a dose of 0.2 mL/kg for 6 days a week for 4 weeks [1]. Rats were sacrificed 6 h after the last meal by cervical dislocation. A midline ventral abdominal incision was performed and the pancreas was dissected. The splenic part of the pancreas was chosen in all rats [35].

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Histological study

The pancreatic tissue was fixed in 10% formalin overnight and processed for paraffin blocks and then serial sections of 5 μm thick were obtained. Sections were subjected to haematoxylin and eosin and Masson's trichrome staining. Unstained sections were used to detect fluorescent labelled BMSCs under the fluorescent microscope (Leica-Germany) at a magnification of $\times$100 to detect the PKH26-labelled cells. PKH26 is the cell linker dye of choice for in vitro and in vivo cell tracking studies.

Biochemical study

Blood samples were collected through retro-orbital puncture, 6 h after the last meal. After centrifugation, serum was collected and kept at –80°C.

Serum insulin was determined by enzyme-linked immunosorbent assay (ELISA) method, serum glucose level was determined using an oxidase-peroxidase system supplied as kits by "Diamond Diagnostics, Egypt". The serum level of tumour necrosis factor--alpha (TNF-α) was measured using ELISA kits supplied by "My Biosource-USA". Serum level of nitric oxide (NO) was measured using NO assay kit for quantitative determination of nitrite and nitrate supplied by "Biodiagnostics, Egypt" according to the manufacturer's instructions. Levels of malondialdhyde (MDA), reduced glutathione (GSH) and superoxide dismutase (SOD) were measured in pancreatic tissue homogenate as previously described [37] using kits supplied by "Biodiagnostics, Egypt" according to the manufacturer's instructions.

Immunohistochemical study

Immunohistochemistry was carried out using the peroxidase-labelled Streptavidin-Biotin Technique [38]. The sections were blocked with 1.5% normal goat serum in PBS. Anti-caspase-3 rabbit monoclonal antibody (Abcam, Cairo, Egypt, Catalogue No. ab184787 at a dilution 1/1000) was used for detection of apoptosis (active) [6]. Anti-proliferating cell nuclear antigen (PCNA) rabbit monoclonal antibody (Abcam, Cairo, Egypt, Catalogue No. ab92552 at a dilution 1/1000) was used for detection of proliferating cells. Slides were rinsed well in PBS and then incubated with biotinylated secondary antibody. Substrate chromagen (diaminobenzidine [DAB]) mixture was applied then rinsed well. Slides were counterstained with haematoxylin, dehydrated and mounted [42].

Histomorphometric assessment

Quantitative data were obtained using "Leica Qwin 500 C" image analyser computer system Ltd. (Cambridge, England). At magnification of 400, 10 non overlapping fields from ten slides of each animal in the different groups were randomly chosen for assessment of the area percentage for collagen in Masson's trichrome sections, the area percentage of caspase-3 immuno-expression and the number of PCNA-positive nuclei in both pancreatic acinar and islets cells.

Statistical analysis

Numerical data of the histomorphometric measurements and the biochemical levels were analysed using Statistical Package for Social Science (SPSS) version 21 using one-way analysis of variance (ANOVA) followed by Bonferroni pairwise comparisons. Results were presented as mean ± standard deviation (SD). Significance was considered when the p-value was ≤ 0.05.

RESULTS

Light microscopic results

Haematoxylin and eosin stained sections. In energy drink-administered rats (subgroup 2a), the pancreatic acini as well as the islets of Langerhans were distorted with loss of the normal structural pattern. In BMSCs-injected rats (subgroup 2b), there was a restoration of the acinar and islet cytoarchitecture. In NSO-injected rats (subgroup 2c), the pancreatic acini and islets of Langerhans showed regaining of their normal structure. However, some vacuolated islet cells were noticed with areas of wide intercellular spaces (Fig. 1A–D).

Masson's trichrome stained sections. Energy drink-administered rats showed a significant increase in the pancreatic mean area percentage of collagen fibres. There was a significant decrease in the mean area percentage of collagen fibres in each of subgroups 2b and 2c when compared to subgroup 2a. However, there was an insignificant difference on comparing subgroup 2b with subgroup 2c (Fig. 1E–H; 2A).

Caspase-3 immunoexpression sections. Subgroup 2a showed a significant increase in the mean area percentage of caspase-3 immuno-expression in both the exocrine and the endocrine parts. However, subgroups 2b and 2c displayed significant decrease in the area percentage of caspase-3 immuno-expression in both pancreatic parts when compared to subgroup 2a. The mean area percentage of caspase-3 immuno-expression in the pancreatic acinar cells versus that of the islet cells displayed a significant decrease in all of the control group and subgroups 2a, 2b, and 2c (Fig. 1I–L; 2B, C).

PCNA-immunostained sections. The mean number of PCNA-positive nuclei in the pancreatic acinar and islet cells was significantly increased in the energy drink-administered rats when compared to the control group and the subgroups 2b and 2c. There was a significant decrease in these values in subgroup 2b when compared to subgroup 2c. The number of PCNA-positive nuclei of pancreatic acinar cells versus that of the islet cells showed a significant increase in all the studied groups (Fig. 1M–P; 2D, E).

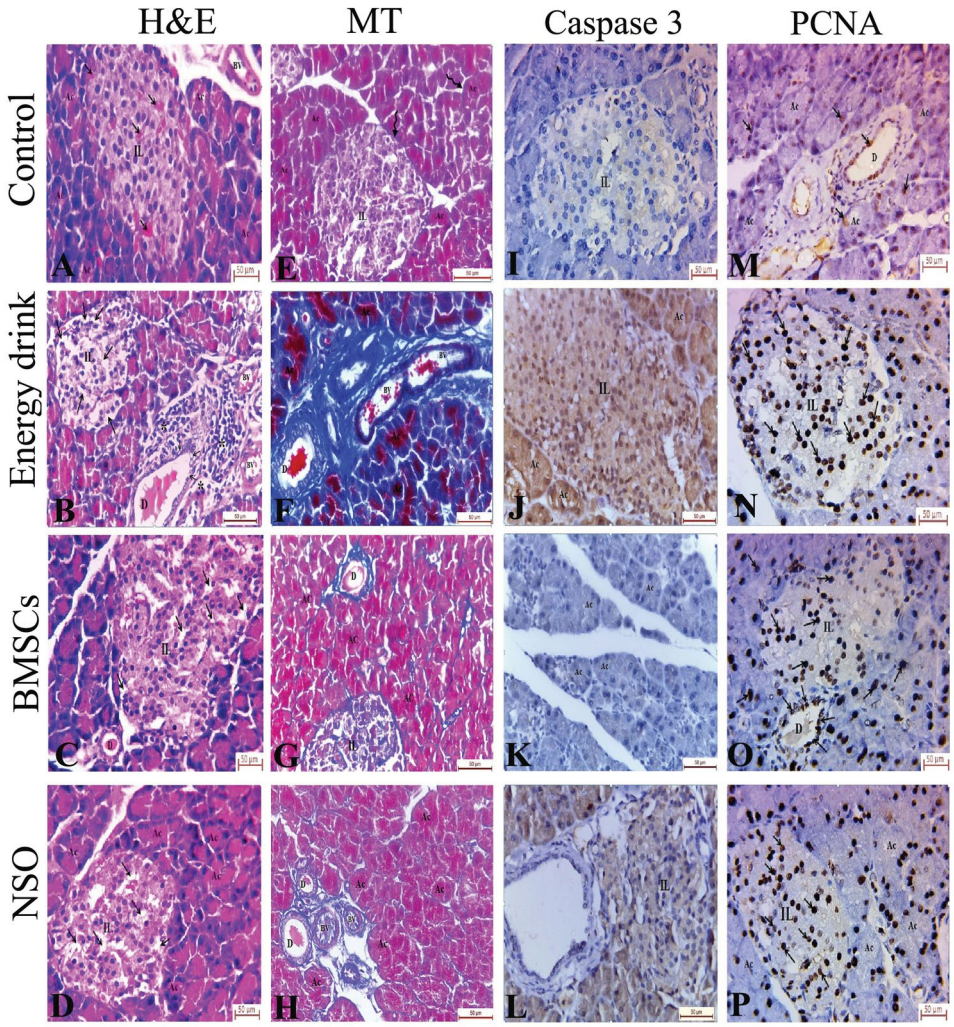

Figure 1. A. Haematoxylin and eosin stained section of control group showing islet of Langerhans with oval, darkly-stained nuclei and pancreatic acini with apical acidophilia (**A**); subgroup 2a showing lost architecture of islet cells, pyknosis, wide intercellular spaces, vacuolations and massive inflammatory cell infiltrate (**B**); subgroup 2b showing relatively normal cytoarchitecture of the pancreatic acini and islets (**C**); subgroup 2c showing some areas of wide intercellular spaces (asterisks) (**D**). Masson's trichrome stained section of control group (**E**); subgroup 2a showing extensive collagen deposition around the pancreatic acini, the interlobular duct and blood vessels (**F**); subgroup 2b showing a moderate amount of collagen (**G**); subgroup 2c showing considerable amount of collagen between the acini as well as around the interlobular ducts and the blood vessels (**H**). Caspase-3 immuno-expression in control group (**I**); subgroup 2a showing strong caspase-3 immuno-expression in the islets and surrounding acinar cells (J); subgroup 2b showing weak caspase-3 immuno-expression (**K**); subgroup 2c showing weak caspase-3 immuno-expression in the cytoplasm of the acinar and islet cells (**L**). Anti-proliferating cell nuclear antigen (PCNA)-immunoreactivity in control group (**M**); subgroup 2a showing strong PCNA-positive immunoreactivity (**N**); subgroup 2b showing PCNA-positive nuclei immunoreactivity (arrows) in the pancreatic acini, the islet of Langerhans and the intralobular duct (**O**); subgroup 2c showing PCNA-positive nuclei immunoreactivity (arrows) in the cells of islet of Langerhans and in the surrounding pancreatic acinar cells (**P**); IL — islet of Langerhans; Ac — acinar cells; D — duct; BV — blood vessels.

Biochemical assay results

Effect on serum insulin and glucose levels (Fig. 2F, G). Energy drink administered rats (subgroup 2a) showed a significant decrease in serum insulin level with a significant increase in serum glucose level when compared to the control group. However, subgroup 2b displayed a significant increase in serum insulin level when compared to subgroup 2a and insignificant difference when compared to control group as well as a significant decrease in serum glucose level when compared to subgroup 2a. However, subgroup 2c showed an insignificant increase in serum insulin level and a significant decrease in serum glucose level as compared to subgroup 2a. On

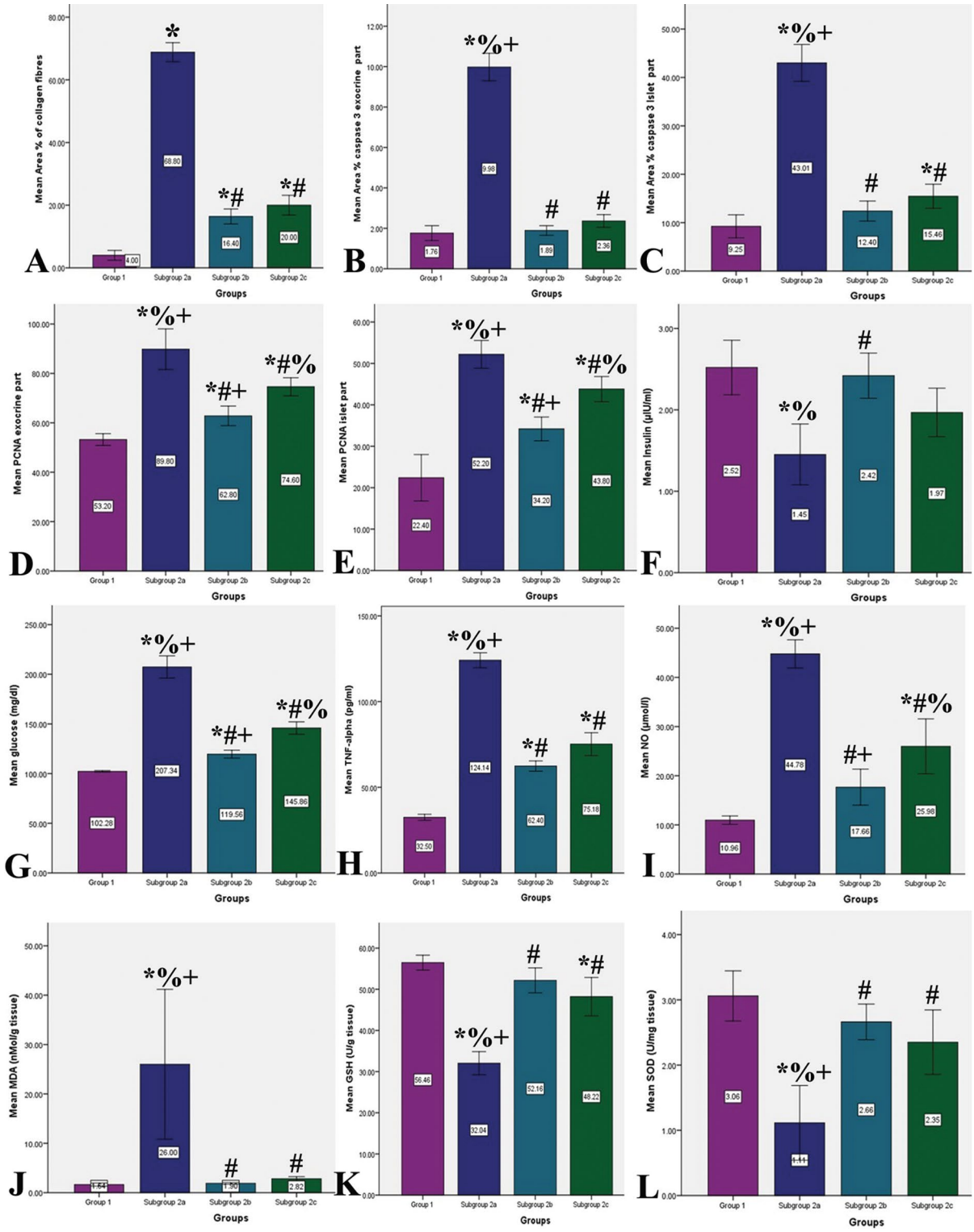

Figure 2. Mean values of area percentage of collagen (**A**), area percentage of caspase-3 immuno-expression of the pancreatic acinar cells (**B**), area percentage of caspase-3 immuno-expression in islets of Langerhans (**C**), number of PCNA-positive nuclei in the acinar cells (**D**), number of anti-proliferating cell nuclear antigen (PCNA)-positive nuclei in the islets of Langerhans (**E**), serum insulin level (**F**), serum glucose level (**G**), serum level of tumour necrosis factor alpha (TNF-α) (**H**), serum level of nitric oxide (NO) (**I**), malondialdhyde (MDA) level (**J**), reduced glutathione (GSH) level (**K**), superoxide dismutase (SOD) level (**L**); *statistically significant compared to control group, #statistically significant compared to subgroup 2a, %statistically significant compared to subgroup 2b, @statistically significant compared to subgroup 2c; +statistically significant compared to subgroup 2c.

comparing subgroup 2b with subgroup 2c, there was an insignificant increase in serum level of insulin and a significant decrease in serum glucose level.

Effect on serum levels of TNF-α and NO (Fig. 2H, I). Subgroup 2a showed a significant increase in serum levels of TNF-α and NO as compared to control group. In subgroups 2b and 2c, there was a significant decrease in both values in each subgroup as compared to subgroup 2a.

Effect on pancreatic tissue levels of MDA, GSH, and SOD (Fig. 2J–L). Subgroup 2a displayed a significant increase in MDA level and a significant decrease in GSH and SOD levels as compared to control group. Each of subgroups 2b and 2c showed a significant decrease in MDA level and a significant increase in GSH and SOD levels. Insignificant differences were detected on comparing subgroup 2b with subgroup 2c regarding these parameters.

DISCUSSION

Increased consumption of energy drinks among adolescents and young adults has raised attention [39]. In the present study, histological examination of pancreas of rats ingesting energy drink revealed marked distortion of the pancreatic cytoarchitecture with pyknotic nuclei and cytoplasmic vacuolations. Those findings are similar to those reported by Ayoub and ElBeshbeishy [6] in rat pancreas. Manifestations of acute pancreatitis in patients ingesting energy drinks have also been reported [14, 45]. These findings were attributed to the high caffeine-content of these drinks and the interaction between it and taurine as an ingredient in the energy drinks [6, 52]. The cytoplasmic vacuolations of injured cells, observed in the present work, could be explained by fatty degeneration of the affected cells [32]. The addition of sodium benzoate as a preservative could also be the cause of those nuclear changes [32].

The BMSCs-injected rats, has revealed restoration of pancreatic cytoarchitecture. This therapeutic effect could be attributed to the differentiation ability and immunoregulatory functions of BMSCs [20, 52]. In the present work, fluorescent labelled-BMSCs homing was identified in the pancreatic lobules and in the interlobular spaces. This is similar to previous observation [31].

The NS-injected rats have demonstrated obvious islet and acinar improvement. These findings are consistent with previous findings [21, 31]. The protective effect of NS could be attributed to its role in reduction of inflammatory cytokines and lipid peroxidation [12, 48]. The increased area percentage of collagen fibres in the energy drink-treated group could be attributed to the toxic effect of caffeine [32]. The ameliorating effect of BMSCs on fibrosis runs in accordance to the previous reports which related it to inhibition of tissue stellate cells activity [23, 36, 46, 53]. The potent antifibrotic effect of NS has been previously attributed to downregulation of mRNA expression of fibrosis-related genes [4, 33]. The increase in immunoreactivity of caspase-3 with energy drink could be attributed to the effect of the high caffeine content of energy drink on oxidative stress-induced apoptosis [6]. The BMSCs- and NSO-injected subgroups have showed significant decrease in caspase-3 immuno-expression. Decreased apoptosis in response to BMSCs therapy in different tissues has been reported [27, 51]. The literature is deficient in observations comparing the anti-apoptotic effect of BMSCs and NSO on pancreas of animals.

In the present work, there is a significant increase in the area percentage of caspase-3 immuno-expression in the cytoplasm of islets cells as compared to that in the cytoplasm of the pancreatic acini, in all investigated subgroups. This endocrine versus exocrine difference in mean caspase-3 immuno-expression could be explained by the fact that the pancreatic islet cells are more vulnerable to apoptosis than the exocrine cells [19] and that the pancreatic β-cells possess diminished content of antioxidant enzymes than other tissues [22].

The regenerative effect of MSCs is reported to be due to either reduction of inflammation in-situ or paracrine action. On the other hand, the diminished rate of proliferation observed with NS is attributed to the antioxidant activity of NS [5, 49].

The mean number of PCNA-positive cells in the exocrine part of pancreas of different rat subgroups has been significantly higher than in the endocrine part of the pancreas. The apoptotic β-cells release chemokines that stimulate pancreatic duct gland; the latter was considered as a pancreatic stem cell niche that results in increased proliferation in response to pancreatic inflammation [47].

The energy drink-induced hyperglycaemia noticed in the current study is attributed to the synergistic action of glucose and caffeine [6, 10]. The caffeine content of energy drink leads to decreased tissue sensitivity to insulin and increased secretion of stress hormones [43]. In current BMSCs-injected rats, the

serum glucose is significantly decreased whereas the serum insulin is significantly increased. These findings confirm previous studies [29–31].

The decreased serum glucose level in NSO-injected rats confirms previous findings [12]. NS extract resulted in direct stimulation of isolated pancreatic islets and release of insulin [40].

In the current investigation, the energy drink has caused significant increase in the serum level of TNF-α. This finding is in agreement with previous reports [6, 11].

The decrease in serum TNF-α level in the BMSCs- and NSO-injected subgroups could be attributed to the immunomodulatory effect of BMSCs [52, 54]. The current decreasing effect of NSO on the serum level of TNF-α is concordant with previous findings [7, 13].

In the current work, there is a significant increase in the serum level of NO in with energy drink. This could be attributed to the effect of energy drink on activation of inducible nitric oxide synthase and increase in NO concentration [6, 11]. The BMSCs- and NSO-injected subgroups have shown increase in the serum level of NO. Similar findings have been reported previously [7, 50, 54].

Tissue levels of MDA, GSH and SOD levels have been significantly increased in energy drink-administered rats in comparison to control rats and significantly decreased in BMSCs- and NSO-injected rats in comparison to energy drink-administered rats. This anti-oxidant effect explains the ability of MSCs and NSO to repair the injured pancreatic tissue in the current study.

CONCLUSIONS

Chronic ingestion of energy drink has been observed to have a negative impact on the integrity of exocrine and endocrine portions of rat pancreas. Islets of Langerhans are more adversely affected by energy drinks than the pancreatic acini. The extent of pancreatic regeneration exerted by each of BMSCs and NSO is nearly similar but the effect of BMSCs is more superior.

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