Somatostatin analogue, Octreotide, improves restraint stress-induced liver injury by ameliorating oxidative stress, inflammatory response, and activation of hepatic stellate cells

Neven Makram Aziz^{1,2} · Merhan Mamdouh Ragy¹ · Sabreen Mahmoud Ahmed^{1,3}

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Abstract

The aim of this study is to investigate the effect of somatostatin (SST) analogue, Octreotide, on some features of liver injury induced by immobilization stress (IS) in adult male albino rats. Eighteen adult male albino rats were randomly divided into three equal groups: control, IS, and Octreotide-treated stressed groups. Octreotide (40 µg/kg body weight, subcutaneously) was administrated twice daily for 8 days during the exposure to IS. Octreotide was found to reduce the IS significantly and induce elevations in the plasma level of corticosterone, liver transaminases, and tumor necrosis factor α (TNF- α) as compared with IS group. Furthermore, Octreotide administration has significantly elevated the decline in the total antioxidant capacities (TAC) and lowered the elevated malondialdehyde (MDA) levels observed with IS in the hepatic tissue. Additionally, Octreotide treatment provided protection against the histopathological changes in the stressed liver in the form of significant reduction in the mean number of degenerated hepatocytes, the area % of collagen fibers, and glial fibrillary acid protein (GFAP) immunostaining with a significant increase in the mean number of normal hepatocytes. In conclusion, stressed rats showed disturbed liver functions and its oxidant–antioxidant status with highly expression hepatic stellate cells (HSCs), which were all improved by Octreotide administration, SST analogue.

Keywords Somatostatin analogue · Octreotide · Immobilization stress · Oxidative stress · Tumor necrosis factor α · Glial fibrillary acid protein

Introduction

Stress response is known as behavioral and metabolic changes to maintain body homeostasis that is caused by internal or external sources like physical or psychological stimuli known as stressors (Anusha et al. 2013). Animal immobilization or restraint stress is

known to be an applicable, easy, and convenient model to induce both psychological and physical stress (Khataibeh 2016).

The liver is the target organ for stress. Extreme stimuli induce significant changes in the tissue structure (Serikov and Lyashev 2015). Kupffer cells, hepatic stellate cells (HSCs), and endothelial cells are potentially more exposed to oxidative stress. With regard to HSCs, the proliferation and collagen synthesis of HSCs are triggered by lipid peroxidation caused by oxidative stress (Li et al. 2015). HSC activity has been evaluated by measuring hepatic expression of glial fibrillary acidic protein (GFAP) to indicate the magnitude of fibrosis and necroinflammatory activity. GFAP is a more useful marker of early HSC activation, and it is a member of intermediate filaments maintaining cell's mechanical strength and structure (Hassan et al. 2014) which were detected between sinusoidal endothelial cells and hepatocytes (Stewart et al. 2014).

Somatostatin (SST) is a cyclic and regulatory peptide consisting of 14 amino acids (naturally tetra-decapeptide) that produced by neuroendocrine, immune, and inflammatory cells in response to many kinds of factors, such as ions, nutrients, neuropeptides, and neurotransmitters (Wang et al. 2013). In

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✉ Merhan Mamdouh Ragy
Merhanel@yahoo.com

¹ Present address: Department of Physiology, Faculty of Medicine, Minia University, Minia 61111, Egypt

² Deraya University, New Minia, Egypt

³ Department of Anatomy, Faculty of Medicine, Minia University, Minia, Egypt

the liver, somatostatin-containing axons are present in the space of Disse and exert paracrine effects on nearby cells, depending on the expression of the five somatostatin receptor (SSTR) subtypes. The cirrhotic liver may express all five subtypes in both hepatocytes and HSCs, indicating possible sites of action in chronic liver disease (Hessheimer et al. 2014).

Octreotide is an octa-peptide that pharmacologically mimics natural somatostatin (Seo et al. 2014). From the abovementioned, an important question to this discussion is as follows: “Can somatostatin analogue, Octreotide, improve hepatic damage induced by immobilization stress (IS)?” To clarify this question, different hepatic oxidative and inflammatory parameters were measured by liver histology and immunohistochemical examination for HSC expression in the restraint stress model in order to shed light on the possible Octreotide protective mechanisms involved against stress-induced liver damage.

Material and methods

Animals

Adult male albino (Sprague–Dawley strain) rats, of average weight 150–200 g, about 4 months old were used during the present study. Rats were bought from the National Research Center, Cairo, Egypt. All animals were housed in stainless steel cages offering individual housing. Each rat had a tag number. They were left freely wandering in their cages for 2 weeks with normal hour's dark/light cycle for acclimatization before starting the experiment. All the procedures followed with the rats were in accordance with our institutional guidelines. The ethics protocol was approved by The Laboratory Animals Maintenance and Usage Committee of Faculty of Medicine in Minia University and Canadian Council on Animal Care (CCAC) guideline. Rats were divided into the following groups (six rats each):

- Control group: rats received no treatment and were left freely wandering in their cages.
- Immobilization stress (IS) group: each rat was immobilized on a wooden board by taping the four limbs with surgical tapes to a specially prepared metal mounts 2 h once a day from 10 am to 12 am for 8 days (Qin et al. 2011) according time-dependent study (4, 8, and 16 days).
- Octreotide-treated stressed group (octreotide + IS): stressed rats were treated by Octreotide at a dose of 40 µg/kg body weight subcutaneously twice daily (one dose before stress period and other dose 12 h after first dose) for 8 days (Li et al. 2016). Dose-dependent study was performed because Octreotide action is definitely dose dependent (Klironomos et al. 2014) and may have completely opposite effects according to concentration (Tsagarakis et al. 2011).

Biochemical analysis

Twenty-four hours after the last restraint session, after an overnight fasting, the animals were sacrificed by cervical dislocation following anesthesia. Blood samples were taken from the jugular vein in a tube containing 0.5% heparin as the anticoagulant and immediately centrifuged at 5000 rpm for 10 min at 4 °C. The obtained clear plasma was stored at –80 °C until used for:

- 1- Spectrophotofluorometric measurement of corticosterone concentration according to the method of Silber et al. (1958).
- 2- Enzymatic colorimetric estimation of liver transaminases including alanine transaminase (ALT) and aspartate transaminase (AST) (Biodiagnostic, Egypt).

Analysis of liver homogenates

The liver tissue was excised and washed with ice-cold saline and was immediately immersed in liquid nitrogen and stored at –80 °C for further biochemical analysis. The liver tissue was homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4). The ratio of tissue weight to homogenization buffer was 1:10 (0.5 g from each liver was homogenized in 5 ml buffer). The homogenates were centrifuged at 5000 rpm for 10 min at 4 °C. The resulting supernatant was used for determination of:

- 1- Malondialdehyde (MDA) content according to the method of Ohkawa et al. (1979)
- 2- Total antioxidant capacity (TAC) was also determined using colorimetric assay kit according to the manufacturer's instructions (Biodiagnostic, Egypt).
- 3- TNF-α level by using rat's TNF-α ELISA kit (Lab Vision Corporation, USA) according to the manufacturer's instructions

Histological and immunohistochemical examination

For the histological preparations, the liver tissue was cut into small pieces and then fixed in 10% formol saline for 24 h, embedded in paraffin, and sectioned at 5 µm thickness with a microtome and stained with different histological and immunohistochemical stains.

Histological stains used in this study were hematoxylin and eosin (H&E) (Swisher et al. 2002) and Masson's trichrome (MTC) for the demonstration of collagen fibers (Bancroft and Gamble 2008). On the other hand, immunohistochemical staining was carried for the detection of hepatic stellate cells (HSCs) by expression of the glial fibrillary acid protein (GFAP) (Labvision, Thermo Scientific, USA) rabbit polyclonal antibody, code no. AB5804. The reaction is cytoplasmic (Xiao et al. 2014).

Morphometric analysis

Measurements were carried out in five non-overlapping fields from five different sections of five different rats, in each group at $\times 400$ magnification, using the image analyzer (Leica Imaging System, Germany) to measure:

- Area percentage (percentage proportion) of MTC-positive material in MTC stained sections
- Area percentage of immunopositive reaction of GFAP antibody
- Number of normal, binucleated, and degenerated hepatocytes

Statistical analysis

Data were represented as means $\pm$ standard errors of the mean (SEM). Statistical analysis was performed using GraphPad Prism 5 software, and significant difference between groups was done by one-way ANOVA followed by Tukey-Kramer post hoc test for multiple comparisons with a value of $P \leq 0.05$ considered statistically significant.

Result

Determination of the hepatic injury markers

As shown in Table 1, IS significantly increased the plasma levels of ALT, AST, and hepatic MDA with significantly decreased in the hepatic TAC level when compared with the control group. Treatment with Octreotide significantly reduced these levels in the

stressed group with significantly higher level of hepatic TAC; however, these levels still significantly differ from the control group.

Determination of the plasma corticosterone

Subjecting the rats to IS produced the significant highest plasma level of corticosterone (Table 2) amongst the all studied groups. Octreotide treatment significantly ameliorated the increased level of corticosterone during stress but never inhibited it completely as these levels remained significantly higher than the control group.

Determination of the hepatic TNF- α level

As shown in Table 2, IS significantly increased the hepatic TNF- α level as compared with control group. On the other hand, Octreotide treatment significantly decreased the hepatic TNF- α level when compared with IS group but still significantly higher than control group.

The results of the present study clearly demonstrated that there is a significant higher positive correlation between the hepatic TNF- α and plasma corticosterone in the studied groups (Fig. 1). Furthermore, there is also a significant positive correlation between plasma corticosterone and hepatic MDA levels in the studied group (Fig. 2).

Histological study of the hepatic tissue

In H&E sections The liver of the control group showed normal morphological structure in the form of plates of hepatocytes radiating from the central vein and separated from each other by blood sinusoids. The hepatic sinusoids lined with simple squamous endothelial cells. The hepatocyte nuclei were large,

Table 1 Effect of IS with or without Octreotide on the hepatic injury markers in stressed male rats

Parameters			Groups		
			Control	IS	Octreotide + IS
Hepatic injury parameters	In blood	• ALT (U/ml)	11.07 $\pm$ 1.3	21.2 $\pm$ 1.2 ^a	14.7 $\pm$ 0.9 ^{ab}
		• AST (U/ml)	27.61 $\pm$ 1.28	39.6 $\pm$ 1.9 ^a	32.4 $\pm$ 1.2 ^{ab}
	In hepatic tissue	• Hepatic MDA (pg/mg tissue)	20.4 $\pm$ 2.3	38.7 $\pm$ 2.6 ^a	28.3 $\pm$ 1.7 ^{ab}
		• Hepatic TAC (μ M/mg tissue)	35.6 $\pm$ 1.5	23.6 $\pm$ 1.1 ^a	29.1 $\pm$ 2.1 ^{ab}

Data are expressed as mean $\pm$ S.E.M. of six rats in each group

IS immobilization stress, MDA malondialdehyde, TAC total antioxidant capacity, ALT alanine transaminase, AST aspartate transaminase

^a Significant from control group

^b Significant from IS group, $P \leq 0.05$

Table 2 Effect of IS with or without Octreotide on the plasma corticosterone and hepatic metabolic TNF- α levels in stressed male rats

Parameters	Groups		
	Control	IS	Octreotide + IS
Plasma corticosterone ($\mu\text{g/ml}$)	62.1 $\pm$ 2.5	94.2 $\pm$ 1.4 ^a	73.03 $\pm$ 2.01 ^{ab}
Hepatic TNF- α (pg/mg tissue)	23.2 $\pm$ 1.1	49.9 $\pm$ 2.2 ^a	33.3 $\pm$ 1.5 ^{ab}

Data are expressed as mean $\pm$ S.E.M. of six rats in each group

IS immobilization stress, TNF- α tumor necrosis factor alpha

^a Significant from control group

^b Significant from IS group, $P \leq 0.05$

rounded, and vesicular. Numerous hepatocytes were bi-nucleated. The cytoplasm is acidophilic. The portal area was cornered between the hepatic lobules displaying the portal vein and the bile duct (Fig. 3).

While liver of the IS group showed disorganized hepatic architecture with marked congested dilated blood vessels and inflammatory cellular infiltration, hepatocytes displayed marked degeneration with vacuolated cytoplasm and pyknotic shrunken nuclei. The portal area displays congested dilated portal veins (Fig. 4).

On the other hand, liver of the Octreotide + IS group showed microscopic examination exhibited morphological changes as compared to the control group. Some hepatic lobules showed congested central veins. Hepatocytes with vacuolation and others with pyknotic nuclei were observed. In addition, a little inflammatory cellular infiltration was detected (Fig. 5).

In MTC sections Liver of the control group showed delicate collagen fibers around the central veins and the portal tracts (Fig. 6a, d), while liver of the IS group showed dense collagen fibers around the central veins and the portal tracts (Fig. 6b, e). Additionally, liver of the Octreotide + IS group showed fine

collagen fibers around the central veins and the portal tracts (Fig. 6c, f).

GFAP immunohistochemical stained sections

Liver of control rats showed negative immunoexpression for glial fibrillary acid protein (GFAP) (Fig. 7a), while liver of the IS group showed positive immunoexpression for GFAP in the cytoplasm of hepatic stellate cells (HSCs) (Fig. 7b). On the other hand, liver of Octreotide + IS group showed weak positive immunoexpression for GFAP (Fig. 7c).

Morphometric results

In Table 3, the mean number of normal, binucleated, and degenerated hepatocytes, the mean area percentage for collagen fiber accumulation, and the mean area percentage for glial fibrillary acid protein (GFAP) immunostaining as evaluated revealed a statistically significant variance between the different studied groups ($P < 0.05$). The scores of binucleated and degenerated hepatocytes numbers, collagen fiber accumulation, and GFAP immunostaining were significantly higher in the IS group as compared with the other groups ($P < 0.05$),

Fig. 1 Correlation between hepatic TNF- α and plasma corticosterone levels in the different studied groups ($r = -0.9$; $P \leq 0.001$). TNF- α tumor necrosis factor alpha

Fig. 2 Correlation between plasma level of corticosterone and hepatic MDA level in the different studied groups ($r = -0.9$; $P \leq 0.001$). MDA malondialdehyde

whereas the score of normal hepatocytes number was significantly lower for the IS group when compared with the other groups ($P < 0.05$).

Discussion

The influence of stress on the liver is of importance from the clinical point of view, because stress plays a potential role in aggravating liver diseases in general and hepatic inflammation

in particular, probably through the generation of reactive oxygen species (ROS). On the basis of clinical observations, a correlation between hepatic disease and psychological stress has been suggested. The authors have suggested that emotional stress worsens the symptoms of hepatic disorders and alters blood chemistry related to liver function (Kim et al. 2016).

The present investigation was an attempt to study the effect of somatostatin analogue, Octreotide, on the stress-induced hepatic changes in adult male albino rats. This was done by estimation of plasma corticosterone level and the hepatic enzymes, oxidant–antioxidant capacities, and inflammatory marker with liver histology and immunohistochemical examination of HSC activation.

Fig. 3 A photomicrograph of a rat liver from the control group showing the central vein (CV) and plates of hepatocytes (arrows) separated by blood sinusoids (S) which are lined by endothelial cells (dotted arrow). Notice that some hepatocytes are binucleated (thick arrow). Inset: a portal tract containing branches of the portal vein (PV) and bile duct (BD). H&E, $\times 400$

Fig. 4 A photomicrograph of a rat liver from the IS group showing marked dilatation of the blood vessel (B.V.) and inflammatory cellular infiltration (double arrows). Many hepatocytes are vacuolated (arrow heads). Others appear with pyknotic nuclei (arrows). Inset: The portal area displays congested dilated portal vein (PV). H&E, $\times 400$

Fig. 5 A photomicrograph of a rat liver from the Octreotide + IS group showing congested central vein (CV). Some hepatocytes are vacuolated (arrow heads). Others appear with pyknotic nuclei (arrow). Notice the inflammatory cellular infiltration (double arrows). H&E, $\times 400$

In the present study, plasma corticosterone level was increased in the stressed group. The present results are in accordance with the previous studies (Shanmugapriya et al. 2012) claiming that exposure to different types of stress, including immobilization, restraint, and psychological stressors in rats significantly increased plasma corticosterone level. The present result showed that there was a positive correlation between plasma corticosterone and hepatic MDA level in the IS group which suggests the probability that the elevation of corticosterone level in response to stress accelerates the generation of ROS that were previously confirmed by Jafari et al. (2014).

ROS may result in increasing lipid peroxidation in the liver causing hepatic tissue damage as shown in the present histological examination in the form of distorted hepatic architecture with marked congested dilated blood vessels and inflammatory cellular infiltration. Furthermore, morphometrically, results approved that IS adversely affects the hepatic morphology. This result was consistent with other studies carried out by Solin and Lyashev (2014). In addition, the density of

Fig. 6 A photomicrograph of a rat liver. **a** and **d** are from the control group showing delicate collagen fibers around the central vein and the portal tract, respectively. **b** and **e** are from the IS group showing dense collagen fibers around the central vein and the portal tract, respectively. **c**

and **f** are from Octreotide + IS group showing minimal amount of collagen fibers around the central vein and the portal tract, respectively. Masson's trichrome, $\times 400$

Fig. 7 A photomicrograph of a rat liver. **a** is from the control group showing negative immunoexpression for glial fibrillary acid protein (GFAP). **b** is from the IS group showing positive immunoexpression for glial fibrillary acid protein (GFAP) in the cytoplasm of hepatic stellate

cells (HSCs) (arrows). **c** is from Octreotide + IS group showing weak positive immunoexpression for glial fibrillary acid protein (GFAP) in the cytoplasm of HSCs (arrows). GFAP immunostaining, $\times 400$

collagen fibers increased around the central veins and the portal areas. These results were previously confirmed by Amin et al. (2017) and Ohta et al. (2007).

Savransky et al. (2007) reported that the pathogenesis of IS could be attributed to the reduction of the hepatic blood flow which may cause hypoxia or ischemia in the hepatic tissue. Repeated cycles of hypoxia and re-oxygenation result in different mitochondrial oxidative pathways (dysfunction) with generation of ROS, which may cause fibrosis and collagen deposition (Richter and Kietzmann 2016).

The present results clearly demonstrated that IS significantly elevated plasma TNF- α level as compared to control group which may be attributed to the link that exists between the activation of inflammatory cells and the generation of ROS, which further aggravates TNF- α induced cellular damage as the hepatic stellate cells (HSCs) which are proved in the present study by significant increasing in the GFAP immunoreaction. These results were previously reported by Amin et al.

(2017). Additionally, a variety of cytokines like TNF- α can be produced in Kupffer cells that induced by oxidative stress and increasing inflammation and apoptosis (Li et al. 2015).

Moreover, in the present study, there was a positive correlation between TNF- α and plasma corticosterone level. These results were previously confirmed by Ma et al. (2016) who reported that the release of TNF- α increases the levels of adrenocorticotrophic hormone (ACTH), corticotropin-releasing hormone (CRH), and GC, which has a direct effect on pituitary gland and hypothalamic cells, and upregulates the hypothalamic–pituitary–adrenal axis (HPA axis); so, this positive correlation confirmed the stimulatory effect of TNF- α on the HPA axis.

In the present study, IS was shown to cause oxidative stress in liver which was associated with significantly lowered activities of TAC. In addition, IS induction developed a significant increase in the activities of liver enzymes: AST, ALT, and hepatic MDA which indicate a liver damage and loss of functional integrity. These results were previously confirmed

Table 3 Morphological changes in rat liver in the different studied groups

Groups	Parameters				
	Normal hepatocytes (%)	Degenerated hepatocytes (%)	Bi-nucleated hepatocytes (%)	Area % of collagen fibers	Area % of GFAP
Control	84.1 $\pm$ 1.0	11.7 $\pm$ 0.9	17.1 $\pm$ 0.7	2.36 $\pm$ 0.2	0.0 $\pm$ 0.0
IS	56.5 $\pm$ 1.3 ^a	39.3 $\pm$ 1.3 ^a	20.9 $\pm$ 0.9 ^a	6.79 $\pm$ 0.8 ^a	6.21 $\pm$ 0.9 ^a
IS + Octreotide	82.0 $\pm$ 1.1 ^b	14.0 $\pm$ 0.8 ^b	18.0 $\pm$ 0.6 ^b	2.93 $\pm$ 0.7 ^b	2.59 $\pm$ 0.7 ^{ab}

Data are expressed as mean $\pm$ S.E.M. of six rats in each group

IS immobilization stress, GFAP glial fibrillary acid protein

^a Significant from control group

^b Significant from IS group, $P \leq 0.05$

by Khataibeh (2016). It could be explained by the fact that stress hormones (glucocorticoids and catecholamines) produce metabolic changes in the enzyme activities of glucose and phosphate pathways which lead to decrease anti-oxidant levels through diminished production of nicotinamide adenine dinucleotide phosphate (NADPH) (glucose-6-phosphate dehydrogenase regenerates NADPH) (Rahal et al. 2014).

The oxidative stress triggers hepatic damage by not only inducing irreversible alteration of lipids, proteins, and deoxyribonucleic acid (DNA) contents but also, more importantly, modulating pathways that control normal biological functions. Since these pathways regulate gene transcription, protein expression, cell apoptosis, and HSC activation, oxidative stress is regarded as one of the pathological mechanisms that results in initiation and progression of various hepatocyte damage that was proved in the present study by increasing its enzymes AST and ALT (Singal et al. 2011) and highly expression of GFAP indicating the HSC activation (Amin et al. 2017).

Gandhi (2012) reported that ROS and lipid peroxides act on the quiescent HSCs, which release retinoids and change their phenotype to myofibroblast-like cells, while these mediators continue to induce HSC activation and also proliferation of activated HSCs and promote their fibrogenic activity. On the other hand, TNF- α increases HSC survival, but not activation. Hepatocyte death results in the engulfment of apoptotic bodies by macrophages and HSCs. Engulfment of apoptotic bodies by HSCs increases the profibrogenic responses. HSCs also support B cell survival. In fibrotic liver, B cells increase proinflammatory cytokines, which can accelerate liver fibrosis (Yang and Seki 2015).

Octreotide, a synthetic and eight-peptide analogue of somatostatin (SST), has a strong affinity to somatostatin receptor 2 (SSTR2), which was highly expressed in the liver (Li and Low 2015). The results of the present study demonstrated that treatment of stressed group by Octreotide showed improvement in the liver function by improving liver enzymes and oxidant–anti-oxidant capacities. These results were previously confirmed by Schaalan and Nassar (2011) who reported that the anti-oxidant effects of Octreotide could be attributed to its sulfhydryl group and its capacity to induce other free radical scavenging systems.

This finding also is in agreement with Tahan et al. (2010) who reported that lanreotide, another SST analogue, decreases MDA and increases anti-oxidant enzymes in the hepatic tissue due to its hepato-protective actions through the modulation of Kupffer cell functions. In addition, Du et al. (2016) reported that Octreotide improved liver function by regulating the methionine cycle reaction and increased the 5'-methylthioadenosine in hepatocytes that protect hepatocytes against stress injury.

In the present study, the treatment of the stressed rats by Octreotide significantly reduced the plasma TNF- α level. This could be explained by the fact that Octreotide decreased the expression of nuclear factor-kappa B which increases the expression inflammatory genes, including TNF- α , thus affording

protection against stress-induced damage (Schaalan and Nassar 2011). Additionally, in the present study, the plasma corticosterone level significantly reduced in the stressed rat's treated with Octreotide as compared to the stressed rats secondary to their highly positive correlation with TNF- α level as proved in the present study and others (Ma et al. 2016).

Finally, in the present study, pretreatment of stressed rats by Octreotide provided some protection against the histopathological changes in the stressed livers, as most of the hepatic lobules showed apparently normal structure. However, congested central veins and vacuolated hepatocytes were still observed. These findings were consistent with another study carried by Du et al. (2016) and Guo et al. (2015) using Octreotide. Additionally, an improvement was observed in the form of significant reduction in the mean number of degenerated hepatocytes, the area percentage of collagen fibers, and GFAP immunostaining in stressed group treated by Octreotide. These findings were previously reported by Tahan et al. (2010) who used another somatostatin analogue, lanreotide.

Furthermore, Pan et al. (2004) added that the somatostatin receptors have been detected on HSCs, and Octreotide exerts inhibitory effect on HSCs by downregulating the expression of growth factors in HSCs Song et al. (2004). Moreover, Valatas et al. (2004) demonstrated that Octreotide can regulate the chemokine secretions by Kupffer cells and regulate liver macrophages. So, in many hepatic injuries, Octreotide has a protective effect against fibrotic and inflammatory changes.

In conclusion, we have shown that Octreotide treatment with IS (i) limited the IS induced-deterioration in liver functions, (ii) associated with reduced inflammatory response by acting primarily through a decrease in pro-inflammatory cytokines as TNF- α , (iii) decreased the oxidative stress response in the hepatic tissue, and (iv) has a protective effect against fibrotic and inflammatory changes in many hepatic injuries. This finding suggests that Octreotide could efficiently protect rats against stress-induced hepatic changes which may accompany different diseases. Further studies may be needed to identify the molecular mechanisms underlying the etiology of this Octreotide protection.

Compliance with ethical standards

The ethics protocol was approved by The Laboratory Animals Maintenance and Usage Committee of Faculty of Medicine in Minia University and Canadian Council on Animal Care (CCAC) guideline.

Conflict of interest The authors declare that there is no conflict of interest.

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