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Mobilization of endogenous bone marrow-derived stem cells in a thioacetamide-induced mouse model of liver fibrosis

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ABSTRACT

The clinical significance of enhancing endogenous circulating haematopoietic stem cells is becoming increasingly recognized, and the augmentation of circulating stem cells using granulocyte-colony stimulating factor (G-CSF) has led to promising preclinical and clinical results for several liver fibrotic conditions. However, this approach is largely limited by cost and the infeasibility of maintaining long-term administration. Preclinical studies have reported that StemEnhance, a mild haematopoietic stem cell mobilizer, promotes cardiac muscle regeneration and remedies the manifestation of diabetes. However, the effectiveness of StemEnhance in ameliorating liver cirrhosis has not been studied. This study is the first to evaluate the beneficial effect of StemEnhance administration in a thioacetamide-induced mouse model of liver fibrosis. StemEnhance augmented the number of peripheral CD34-positive cells, reduced hepatic fibrosis, improved histopathological changes, and induced endogenous liver proliferation. In addition, VEGF expression was up-regulated, while TNF-α expression was down-regulated in thioacetamide-induced fibrotic livers after StemEnhance intake. These data suggest that StemEnhance may be useful as a potential therapeutic candidate for liver fibrosis by inducing reparative effects via mobilization of haematopoietic stem cells.

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1. Introduction

Liver fibrosis occurs as a result of chronic injury leading to the excessive accumulation of extracellular matrix and scar tissue formation. If not efficiently treated, liver fibrosis may lead to cirrhosis, inducing permanent and irreversible damage to liver structure and function with fatal consequences (Friedman et al., 2013). The most common causes of liver fibrosis are infection with hepatitis B or C virus, which represents a major public health problem that affects millions of people worldwide. Studies on the epidemiology of hepatitis C virus (HCV) infections have suggested that Egypt has one of the highest prevalence rates of HCV in the world, with seroprevalence rates of 30–40% in villagers over the age of 30 (Lehman and Wilson, 2009). With the development of new antiviral strategies, viral eradication and treatment of hepatitis can be anticipated in some patients, even in those with chronic viral hepatitis. However, liver transplantation is still the only current radical treatment in patients in which decompensated liver cirrhosis has already occurred (Dhingra et al., 2014). However, several problems, such as organ shortage, surgical complications, and expensive costs, underlie the need to develop new therapeutic strategies to attenuate liver scarring and enhance liver regeneration (Saito et al., 2013).

Stem cell therapeutic strategies are being evaluated as an attractive promising approach for liver repair. Several studies have reported the ability of various types of stem cells to improve the pathological outcome of liver cirrhosis and to attenuate the clinical symptoms of the disease (El-Ansary et al., 2012; Takami et al., 2012; Ali and Masoud, 2012; Zhang et al., 2012; Wang et al., 2012; Agaev et al., 2014). However, exogenous stem cell therapeutic strategies carry several potential risks that may limit their wider clinical application. For instance, stem cell therapy is an invasive technique that requires repeated injections often in the portal vein or hepatic artery (Kharaziha et al., 2009; Salama et al., 2010; Wang et al., 2012). Another limitation is that stem cells are exposed to several manipulations during their expansion in vitro before being transplanted, these manipulations lead to their contamination and/or cause deleterious changes in their intrinsic characteristics due to several intracellular and extracellular influences, adding additional burden on the diseased liver (Herberts et al., 2011). Based on the fact that bone marrow-derived stem cells have the ability to migrate to sites of tissue damage and participate in tissue regeneration, stimulating the mobilization of endogenous bone marrow-derived stem cells may provide a promising non-invasive alterative to exogenous stem cell transplantation.

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Many different soluble factors have the ability to mobilize bone marrow-derived haematopoietic stem cells (BM-HSCs) from the bone marrow to the peripheral circulation and hence increase their total number (Weissman et al., 2001). Granulocyte-colony stimulating factor (G-CSF) was the first factor described to have this feature. However, investigating the therapeutic potential of G-CSF has been largely limited due to the significant risks accompanied with its use for long periods of time (Bensinger et al., 1996; D'Souza et al., 2008; Barnes et al., 2014). StemEnhance (SE) is a natural stem cell mobilizer that can trigger a much milder mobilization of BM-HSCs, and its considerable safety allows for a sustained oral daily intake over long periods of time. SE is a natural water-soluble extract of the cyanophyta *Aphanizomenon flos-aquae* (AFA), which was recently shown to increase the number of circulating BM-HSCs by approximately 25% within 60 min after oral consumption (Jensen et al., 2007). Previous experimental studies reported that mobilization of BM-HSCs with SE promoted muscle regeneration in cardiotoxin-induced muscle injury (Drapeau et al., 2010) and ameliorated manifestations of diabetes in rats (Ismail et al., 2013). However, the potential effectiveness of SE in ameliorating liver cirrhosis has not been investigated.

In the current study, we sought to evaluate the effect of SE administration in thioacetamide-induced liver fibrosis in mice. In all experimental groups, the percentage of CD34-positive cells in the peripheral circulation was assessed using flow cytometry 7 days after starting SE administration in SE-treated groups. At the end of the experiment, liver function and histological assessments were conducted to investigate the potential reparative effect of the endogenously increased haematopoietic CD34-positive cells and the possible mechanisms underlying this effect.

2. Methodology

2.1. Animals

Male C57Bl/6 mice aged 7–8 weeks old were purchased from the Theodor Bilharz Research Institute, Imbaba, Egypt, and maintained in the animal house of Research Institute of Ophthalmology, Giza, Egypt. The mice were subjected to a 12: 12-h daylight/darkness and allowed unlimited access to chow and water. All of the ethical protocols for animal treatment were followed and supervised by the animal facilities at Research Institute of Ophthalmology, Giza, Egypt. All procedures involving the use of the mice were approved by The Animal Care and Use Committee.

2.2. Experimental design

The mice were randomly divided into four groups; control, SE-treated (SE group), thioacetamide-treated (TAA group), and thioacetamide plus SE-treated (TAA + SE group) ($n = 10$ per group). Liver fibrosis was induced in the TAA and TAA + SE groups by intraperitoneal injection (i.p.) of thioacetamide (TAA, Sigma, St. Louis, MO, USA) at a dose of 200 mg/kg b.w. twice a week. After 8 weeks of TAA treatment, the TAA + SE mice were orally administered StemEnhance (SE; StemTech Health Sciences, Inc., UK) at a dose of 300 mg/kg b.w. daily for an additional 4 weeks. In this group, TAA was continuously administered during the additional 4 weeks. The mice in TAA group were given TAA (200 mg/kg b.w. twice a week) for 12 weeks and SE-treated mice were given SE (300 mg/kg b.w. daily) for 4 weeks. With the exception of the flow cytometry data, there was no statistically significant difference between the liver function or histological or immunohistological outcomes of the control and SE groups; therefore, for these measured outcomes, the SE and control groups were pooled into one group (control).

2.3. Biochemical analysis

At the end of the experiment, blood samples were collected from the orbital sinus and incubated for 1 h at room temperature (RT) to allow clotting. Then, the sera were collected by centrifugation at 2400 rounds per min (rpm) for 5 min and stored at −20 °C until use. The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed using the Advia Chemical System (Siemens, Germany).

2.4. Flow cytometry

To measure the percentage of CD34-positive cells in peripheral blood, blood samples were collected from mice of all experimental groups in heparin tubes 7 days after SE administration in SE-treated group (SE and TAA + SE). 100 μl of blood was incubated with PE-conjugated rat anti-mouse CD34 (clone RAM34; BD Pharmingen, USA) for 30 min at 4 °C. After red-cell lysis (Versalyse Lysing Solution, Beckman Coulter), the samples were centrifuged, washed twice with phosphate buffer saline (PBS), and fixed with 1% paraformaldehyde (Sigma). Isotype-identical antibody served as controls. The cells were analyzed using a Beckman Coulter EPICS XL flow cytometer.

2.5. Histological and immunohistological (IHC) assessments

At the end of the experiment, each mouse was deeply anaesthetized using ketamine (90 mg/kg) and xylazine (15 mg/kg) (i.p.) and decapitated. Livers were dissected and fixed in 10% neutral-buffered formalin and embedded in paraffin wax. For histological examination, 5-μm sections were deparaffinised and rehydrated using a graded ethanol (100%, 90%, and 70%) series and stained with haematoxylin & eosin (H&E) or with Mallory Trichrome (MT) stain, for collagen fibres.

For immunohistological staining, deparaffinised and rehydrated 5-μm sections were rinsed with PBS and blocked for 30 min in 0.1% H_2O_2, as an inhibitor of endogenous peroxidase activity. After rinsing in PBS, the sections were incubated for 60 min in blocking solution (10% normal goat serum) at RT. The sections were then incubated with the primary antibody (Transforming growth factor beta (TGF-β), 1:100, ThermoScientific; Vascular endothelial growth factor (VEGF), 1:100, Cell Mark; Tumour necrosis factor alpha (TNF-α), 1:500, ThermoScientific; Ki67, 1:500, Dako) at RT for an hour. The sections were rinsed with PBS, followed by 20 min of incubation at RT with the secondary biotinylated antibody. After rinsing the sections in PBS, the enzyme conjugate "Streptavidin-Horseradish peroxidase" solution was applied to the sections for 10 min. The secondary antibody binding was visualized using 3,3-diaminobenzoic acid (DAB) dissolved in PBS with the addition of H_2O_2 to a concentration of 0.03% immediately before use. Finally, the sections were rinsed with PBS and the slides were counterstained of using two drops or 100 μl of haematoxylin. The slides were then washed in distilled water until the sections turned blue. Finally, the slides were dehydrated in ascending grades of ethanol (70%, 95%, and 100%) for 5 min each, cleared in xylene, followed by mounting with Histomount and a coverslip.

For immunohistological quantitative assessment, five non-overlapping fields per section were randomly taken using a Leica DML B2/11888111 microscope equipped with a Leica DFC450 camera. The number of immunopositive cells in fields taken from at least three sections/animal was counted using ImageJ software and averaged per field for each animal. The numbers calculated for at least five animals/experimental group were considered for comparison and statistical analyses.

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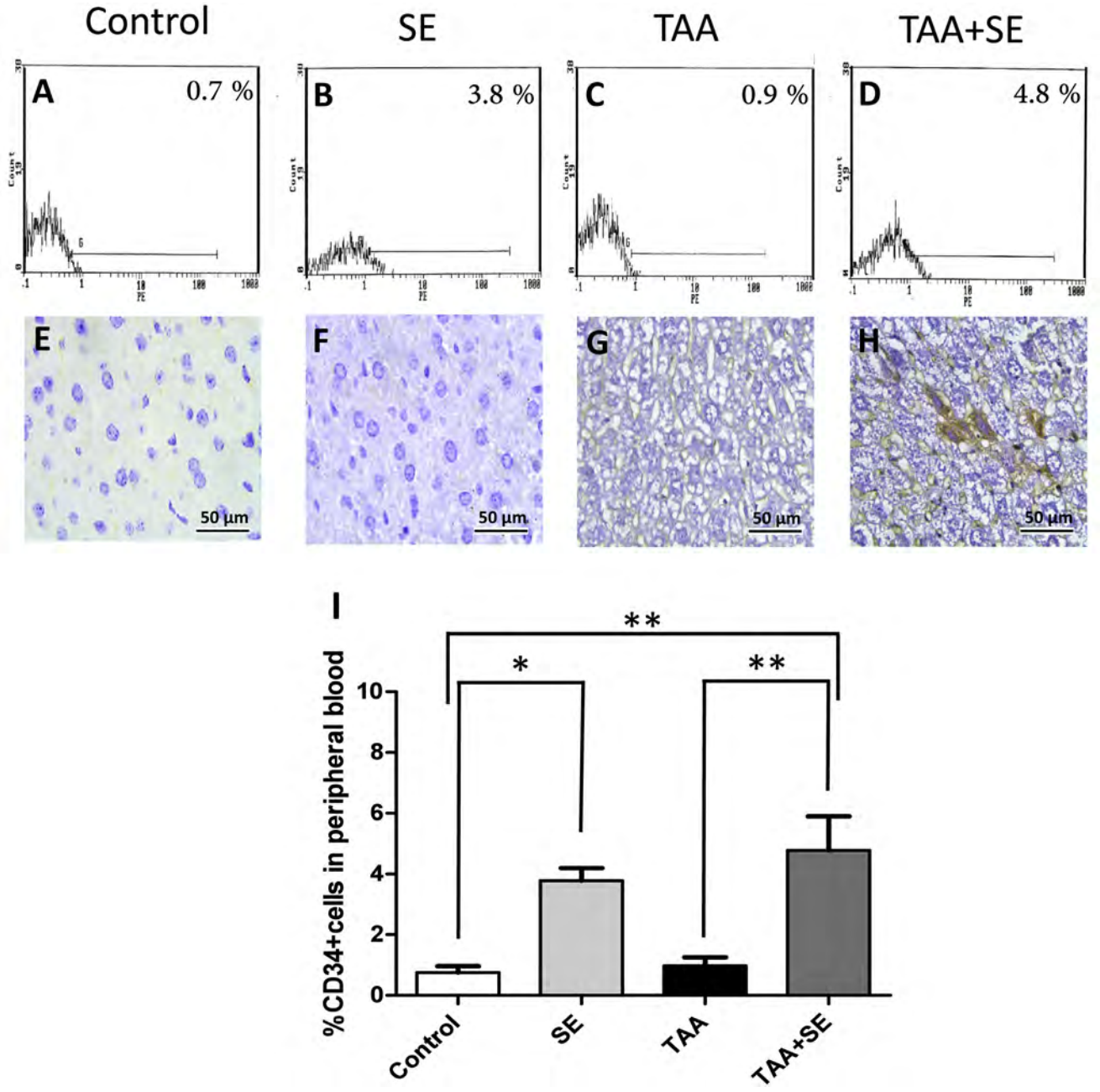

Fig. 1. Mobilization of CD34-positive cells by StemEnhance treatment. Representative flow cytometric histograms of CD34-positive cells assessment in the peripheral circulation in control, StemEnhance (SE), thioacetamide (TAA), and thioacetamide plus StemEnhance (TAA + SE)-treated mice 7 days after starting SE administration for the SE-treated groups (SE and TAA + SE) are displayed (A–D). The percentage of CD34-positive cells was significantly higher in the SE group compared with the control and in the TAA + SE group compared with both the control and TAA groups (I). A few spindle-shaped, CD34-expressing cells were detected in the centrilobular area of the TAA + SE livers, while no positive cells were detected in this area in other groups (E–H). $^{*}P < 0.05$ and $^{**}P < 0.01$.

2.6. Statistical analysis

The results are expressed as the mean ± SEM, and significant differences between groups were evaluated using one way-ANOVA followed by a post hoc Bonferroni test. The level of significance of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. SE increased the percentage of CD34-positive cells in the peripheral blood and a few were detected in TAA + SE-treated livers

In all experimental groups, the percentage of CD34-positive cells in the peripheral circulation was assessed using flow cytometry; this was performed 7 days after SE administration in groups treated with SE (SE and TAA + SE). The percentage of CD34-positive cells significantly increased in the SE group compared with the control group (3.8 ± 0.4 vs. 0.7 ± 0.2; $P < 0.05$; Fig. 1A, B and I). In addition, their percentage was significantly higher in the TAA + SE group compared with both the control and TAA groups (4.8 ± 1.1 vs. 0.7 ± 0.2; $P < 0.01$ and 4.8 ± 1.1 vs. 0.9 ± 0.3; $P < 0.01$, respectively; Fig. 1A, C, D and I). These results suggest that daily administration of SE induces the mobilization of BM-HSCs into the peripheral blood.

CD34 is not usually expressed in the normal liver; however, in chronic pathological liver conditions, its expression can be detected in the endothelial lining of capillarized sinusoids confined to the periportal area (Cui et al., 1996; Pusztaszeri et al., 2006). Using immunohistochemistry, a few spindle-shaped CD34-positive cells

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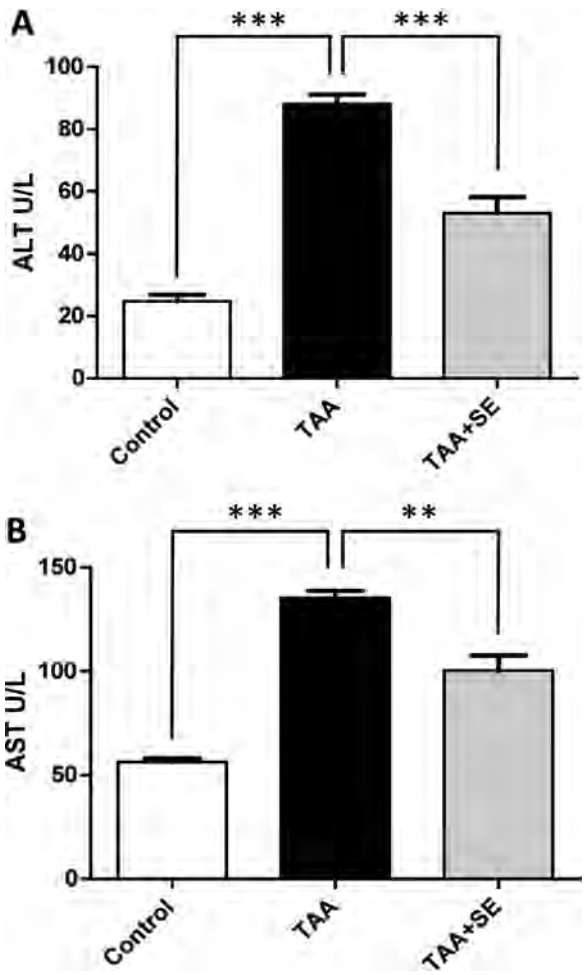

Fig. 2. Effect of StemEnhance on serum biomarker expression in thioacetamide-injured liver. The ALT (A) and AST (B) levels in control, thioacetamide (TAA), and thioacetamide plus StemEnhance (TAA + SE)-treated mice were measured at the end of the experiment. StemEnhance (SE) intake significantly decreased the elevated serum ALT and AST levels. $^{**}P < 0.01$ and $^{***}P < 0.001$.

were detected only in livers of the TAA + SE group (Fig. 1E–H). These cells were observed among hepatocytes of the centrilobular area. The morphology of these cells and their presence in the centrilobular area suggest that they were most likely mobilized BM-HSCs homed to the injured liver.

3.2. Protective effect of SE on liver function in TAA-treated livers

In the TAA group, serum ALT and AST levels increased significantly compared with the control group (ALT; 88.0 ± 3.1 vs. 24.8 ± 2.1; $P < 0.001$, AST; 135.3 ± 3.4 vs. 56.2 ± 1.8; $P < 0.001$; Fig. 2), while their levels decreased significantly in the TAA + SE group (ALT; 53.0 ± 5.2 vs. 88.0 ± 3.1; $P < 0.001$, AST; 100.3 ± 7.2 vs. 135.3 ± 3.4; $P < 0.01$; Fig. 2). These results indicate that SE exerts a protective effect on the serum markers for cirrhosis, including ALT and AST, in TAA-treated mice.

3.3. Antifibrogenic effect of SE in TAA-treated livers

TAA treatment significantly increased the extent of fibrosis compared with the control group, as determined by MT staining. In TAA mice, neighbouring central veins and portal tracts were bridged by fibrous septa, with the formation of pseudo-lobules (Fig. 3A and B). In the TAA + SE group, fibrosis was dramatically suppressed, and the fibrotic area of the livers was significantly less than that of the TAA group (4.8 ± 0.4 vs. 12.8 ± 1; $P < 0.001$; Fig. 3B, C and G). TGF-β1 is a major fibrogenic cytokine that plays a critical role in activating fibrogenic myofibroblasts. In TAA-treated livers, TGF-β1 expression was dramatically increased compared with the control (25.5 ± 2.1 vs. 1.2 ± 0.6; $P < 0.001$; Fig. 3D, E and H), while its expression significantly decreased in the TAA + SE group (5.1 ± 0.9 vs. 25.5 ± 2.1; $P < 0.001$; Fig. 3E, F and H). These results suggest that SE exerts an antifibrogenic effect on TAA-induced fibrosis.

3.4. SE ameliorated the histopathological alterations in TAA-treated livers

The results of H&E staining showed that control mice exhibited normal typical liver lobules and orderly hepatic cords (Fig. 4A and E) and that hepatocytes had granular acidophilic cytoplasm and vesicular nuclei (Fig. 4E; inset). In contrast, livers from TAA group mice exhibited noticeable fibrosis (Fig. 4B), disorganization of the hepatic cords (Fig. 4B and F), remarkable expansions of some veins (Fig. 4C and G), and apparent focal inflammatory cell infiltration around the portal area (Fig. 4B; inset and G). In addition, hydropic degeneration (ballooning) (Fig. 4F; inset *) and centrilobular hepatocyte necrosis, as indicated by karyorrhexis and karyolysis of the hepatocyte nuclei (Fig. 4F; insets ** and ***; respectively), were observed in these livers. In TAA + SE group, organized hepatic cords, almost absence of fibrosis (Fig. 4D and H), and pronounced regenerative activity with the presence of mitoses (Fig. 4H; insets * and **) were observed. In addition, the hepatocytes were nearly similar to those in normal control mice with limited centrilobular necrosis and hydropic degeneration (Fig. 4H; inset ***). These results indicate that SE attenuates liver histopathological changes in TAA-treated livers.

3.5. SE enhanced endogenous hepatocyte proliferation in TAA-treated livers

To address the underlying recovery mechanisms of damaged livers after SE intake, immunohistochemistry for Ki67 was performed to evaluate the proliferative status of the liver. The percentage of proliferating cells (Ki67-positive cells) were estimated in liver sections from control, TAA, and TAA + SE-group mice. The livers of the control group contained few Ki67-positive cells, reflecting normal hepatic homeostatic turnover (Fig. 5A), while the livers of the TAA + SE group exhibited significantly increased numbers of Ki67-positive cells compared to those of the control and TAA groups (11.7 ± 0.6 vs. 1.8 ± 0.5 and 11.7 ± 0.6 vs. 0.3 ± 0.3; $P < 0.001$; Fig. 5A–C and J). These results suggest that SE-mobilized bone marrow stem cells enhance the endogenous regenerative capacity of TAA-injured livers.

3.6. SE up-regulated VEGF and down-regulated TNF-α expression in TAA-treated livers

To further address whether SE-mobilized bone marrow stem cells exert their beneficial effect via paracrine action through secreting growth factors, the expression of VEGF was investigated in the livers of the different experimental groups. VEGF expression was significantly up-regulated in TAA + SE livers compared with that of control and TAA livers (10 ± 1.6 vs. 0.5 ± 0.2 and 10 ± 1.6 vs. 2.5 ± 0.6; $P < 0.001$ and $P < 0.01$, respectively; Fig. 5D–F and K). The immunomodulatory properties of mobilized bone marrow stem cells can also play a significant role in ameliorating liver injury. To address this issue, the expression of TNF-α, a pro-inflammatory cytokine known to act as a promoter of liver fibrosis and as an inducer of hepatocyte apoptosis, was evaluated in the different experimental groups. TNF-α expression was dramatically increased

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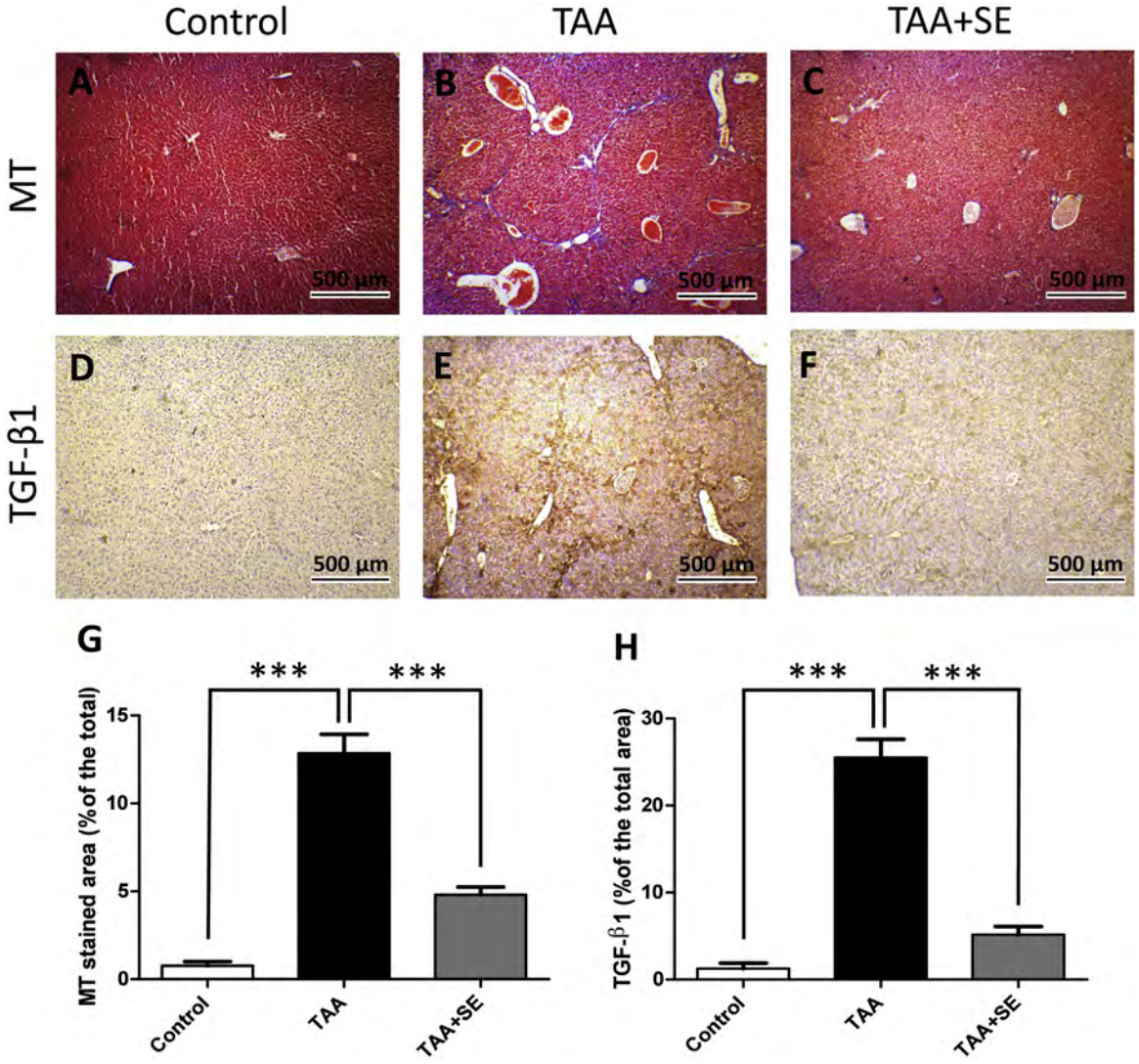

Fig. 3. Antifibrogenic effect of StemEnhance in thioacetamide-induced fibrosis. Representative images of Mallory Trichrome (MT) (A–C) and TGF-β1 (D–F), a major fibrogenic cytokine, staining in control, thioacetamide (TAA), and thioacetamide plus StemEnhance (TAA + SE)-treated mice are displayed. SE administration dramatically reduced the area of fibrosis, as indicated by MT staining (G), and significantly down-regulated the number of TGF-β1-positive cells (H) compared to the TAA-treated group. ***$P < 0.001$.

in TAA-treated livers compared with those of the control group (28.5 ± 3.0 vs. 0.3 ± 0.3; $P < 0.001$; Fig. 5G, H and L), while this increase was significantly down-regulated in TAA + SE-treated livers (4.3 ± 0.5 vs. 28.5 ± 3.0; $P < 0.001$; Fig. 5H, I and L). These results suggest that mobilized bone marrow stem cells ameliorate TAA-induced liver injury by both secreting growth factors and exerting immunomodulatory effects.

4. Discussion

This study provides a proof-of-principle that StemEnhance (SE) administration has beneficial effects on thioacetamide (TAA)-induced liver injury. This effect is mediated by the mobilization of bone marrow-derived CD34-positive cells that possibly exert paracrine effects on hepatic inflammatory and fibrotic damage and induce endogenous hepatic restoration.

In our present study, SE administration improved liver function, reduced fibrosis, and ameliorated histological alterations in TAA-injured livers. In accordance with our findings, G-CSF treatment, a common BM-HSC mobilizer, significantly improved survival and liver histology in chemically injured animals (Yannaki et al., 2005; Quintana-Bustamante et al., 2006; Mark et al., 2010; Tsolaki et al., 2014). The feasibility and safety of mobilizing bone marrow-derived cells following G-CSF administration were demonstrated in eight patients with alcoholic steatohepatitis. In that study, G-CSF was demonstrated to have a positive histological effect. Furthermore, the study reported an improved model for end-stage liver disease (MELD) scores and did not detect any development of hepatocellular carcinoma or increase in alpha fetoprotein up to 8 months after G-CSF administration (Gaia et al., 2006). A favourable effect of G-CSF administration on survival and clinical parameters in patients with liver failure has also been reported in other studies (Garg et al., 2012; Xing et al., 2013; Wan et al., 2013; Duan et al., 2013; Salama et al., 2014; Singh et al., 2014). Although the magnitude of mobilization induced by SE is milder compared with that triggered by G-CSF (Jensen et al., 2007), our results provide evidence that SE has similar capacity compared to G-SCF and is able to improve liver function and to remedy the histological changes in a TAA mouse model of liver cirrhosis. Similar capabilities were also demonstrated in different target organs. The ability of SE to mobilize bone marrow cells and to repair degeneration in cardiotoxin-induced injury was demonstrated (Drapeau et al., 2010). Furthermore, SE-mobilized BM-HSCs increased the number

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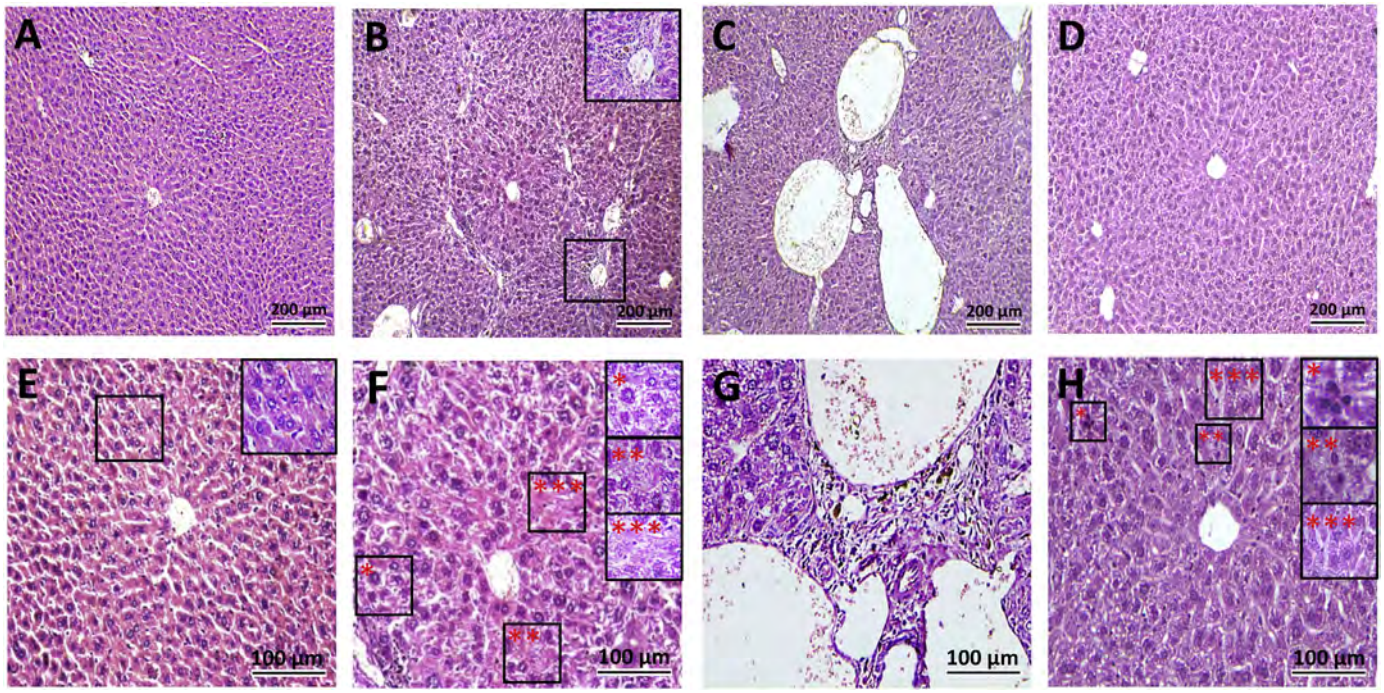

Fig. 4. StemEnhance ameliorated the histopathological alterations in thioacetamide-injured liver. Representative H&E images of control, thioacetamide (TAA), and thioacetamide plus StemEnhance (TAA + SE)-treated mice are displayed. Control livers exhibited normal liver architecture, with cords of hepatocytes radiating from the central vein and separated by blood sinusoids (A and E). The hepatocytes had granular acidophilic cytoplasm and vesicular nuclei (E; inset). TAA livers displayed noticeable fibrosis (B), areas of normal architecture loss (B and F), severe vascular dilatation (C and G), inflammatory cells around fibrotic areas and portal tracts (B; inset and G), hydropic (ballooning) degeneration (F; inset *), and severe centrilobular necrosis, as indicated by both karyorrhexis (F; inset **) and karyolysis (F; inset ***) of the hepatocyte nuclei. In the TAA + SE group, nearly normal liver structure with the absence of fibrosis (D and H) was observed. Pronounced regenerative activity with the presence of mitoses was observed (H; insets * and **). In addition, there was limited centrilobular necrosis and hydropic degeneration (H; inset ***). The central areas of A–D appear at a higher magnification in E–H; respectively. The boxed areas in B, E, F, G, and H appear at a higher magnification in the insets.

of insulin-producing cells in islets of Langerhans and reduced blood glucose levels in diabetic rats (Ismail et al., 2013).

Several studies have demonstrated the capability of stem cells to exert a paracrine proliferative effect on endogenous hepatocytes. In fibrotic liver, hepatocytes reach replicative senescence after many cycles of injury and repair, which reduces their proliferative capacity (Trak-Smayra et al., 2004; Aini et al., 2014). Bone marrow stem cell mobilization may enhance the intrinsic capability of hepatocytes to proliferate by releasing of proliferative cytokines and/or reducing fibrosis, thereby removing the block in the way of hepatocyte proliferation (Wang et al., 2010). G-SCF treatment significantly increased the number of Ki67-positive hepatocytes in different models of liver injury (Zhang et al., 2011; Inderbitzin et al., 2007). The expression of growth factors, including HGF (hepatocyte growth factor) and VEGF, enhanced liver regeneration and hepatocyte proliferation (Majka et al., 2001; Yuan et al., 2013). The ability of primary cytokines, including HGF, VEGF, and NGF (Nerve growth factor), to increase the intrinsic proliferative capability of hepatocytes or to help in the removal of scar tissue has been demonstrated (Hsiao et al., 2012; Nejak-Bowen et al., 2013). A recent study compared the antifibrotic potential of G-SCF with that of another haematopoietic stem cell mobilizer, Plerixafor, in CCl4-injured liver. This study revealed that both agents significantly mobilized HSCs and reduced fibrosis. Interestingly, Plerixafor-mobilized HSCs exhibited reduced liver homing potential and their beneficial effect was mainly attributed to paracrine mechanisms such as increasing VEGF expression (Tsolaki et al., 2014). In our study, the expression level of VEGF was significantly up-regulated in the TAA + SE group. In addition, the number of Ki67-positive hepatocytes was significantly higher in the TAA + SE group compared with the other groups, suggesting that mobilized cells exert a paracrine proliferative action on endogenous hepatocytes in TAA-induced liver injury. Indeed, it is plausible to conclude that the improvement of liver function in TAA + SE-treated mice was partially due to enhanced endogenous hepatocyte proliferation.

There is a substantial body of evidence demonstrating that bone marrow-derived stem cells have a potent paracrine effect, even with low engraftment (Gnecchi et al., 2008). Our results showed a potent expression of VEGF in TAA + SE-treated livers, although a few CD34-positive cells were detected. Several explanations can be proposed to interpret this phenomenon. First, we have focused our study on CD34-positive mobilized cells, however, it cannot be excluded that SE treatment not only mobilized HSCs, but also other types of bone marrow stem cells, such as mesenchymal stem cells, were mobilized and homed to the injured liver, augmenting the paracrine effect (Ripa et al., 2007). Second, several studies have reported that bone marrow mobilizers such as G-CSF have a direct paracrine effect on damaged tissue (Kurdi and Booz, 2007). Finally, mobilized cell engraftment was examined after 4 weeks of SE intake; however, good homing of CD34-positive cells could have occurred at earlier stages after SE intake.

Amelioration of hepatic inflammatory and fibrotic injury via bone marrow stem cell therapy can promote the proliferation of residual hepatocytes. Down-regulation of pro-inflammatory cytokines, such as TNF-α, has been described in kidney, lung injury, and fulminant hepatic failure models after bone marrow stem cells transplantation (Togel et al., 2005; Ortiz et al., 2007). Furthermore, TNF-α signal is important for regulating the improvement of liver fibrosis after bone marrow cell infusion (Hisanaga et al., 2011). G-SCF treatment significantly down-regulated TNF-α expression in CCl4-injured liver (Cho et al., 2011; Tsolaki et al., 2014); moreover, controlling the production of cytokines, such as TGF-β and TNF-α, via mesenchymal stem cell infusion improved liver fibrosis (Mizunaga et al., 2012). Indeed, in vivo inhibition of TGF-β

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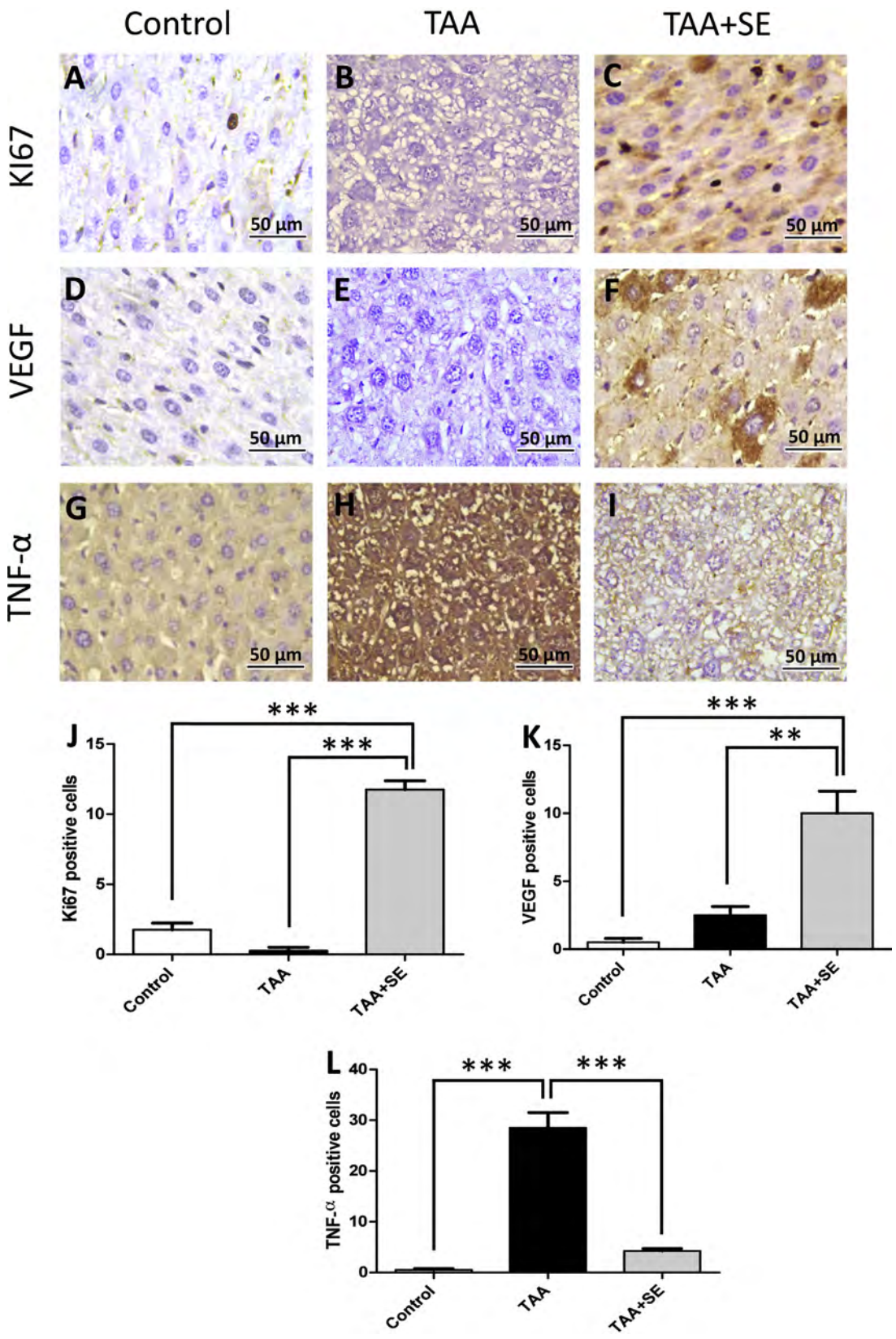

Fig. 5. StemEnhance enhanced endogenous hepatocyte proliferation and exerted immunomodulatory actions in thioacetamide-injured liver. Representative images of Ki67 (A–C), VEGF (D–F), and TNF-α (G–I) staining in control, thioacetamide (TAA), and thioacetamide plus StemEnhance (TAA + SE)-treated mice are displayed. SE significantly increased the number of Ki67-positive cells (J) and up-regulated VEGF expression (K), while down-regulated TNF-α expression (L). $^{**}P < 0.01$ and $^{***}P < 0.001$.

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signalling, using adenovirus-mediated dominant-negative type II TGF-β receptor gene transfer, prevented liver fibrosis, enhanced cell proliferation, and reduced hepatocyte apoptosis in toxin-induced liver injury (Nakamura et al., 2000, 2004). These results, along with our present findings, suggest that mobilized bone marrow stem cells down-regulate pro-inflammatory cytokines, such as TGF-β and TNF-α, in TAA-treated livers.

Several studies have demonstrated the ability of mobilized CD34-positive cells to home to the site of liver injury. In our study, a few spindle-shaped CD34-positive cells were detected among hepatocytes in the centrilobular area of livers of the TAA + SE-treated group. This observation suggests that these cells are most likely bone marrow-mobilized HSCs, rather than due to a pathological increase in CD34 expression due to cirrhosis, which is usually confined to the endothelial lining of periportal sinusoids (Cui et al., 1996; Pusztaszeri et al., 2006).

Although flow cytometric assessment of CD34-positive cells in the peripheral blood showed an increased percentage of CD34-positive cells in both the SE and TAA + SE groups compared with the control group, CD34-positive cells, albeit at limited numbers, were detected only in TAA + SE-treated liver, and no CD34-positive cells were detected in the SE group. Indeed, several studies have been conducted to elucidate the mechanism(s) underlying homing of bone marrow stem cells to injured livers (Dalakas et al., 2005). These studies have demonstrated the involvement of several chemokines in the augmentation of stem cell engraftment into injured areas (Joshi et al., 2015). It is likely that TAA-injured livers enhance the mobilized CD34-positive cells to be specifically recruited to the site of injury, properly by secreting chemokines. We acknowledge that our study has some methodological limitations and that determining the fate of mobilized bone marrow cells was challenging. However, this study sheds light, for the first time, on SE as a new potential therapeutic candidate for liver fibrosis. Indeed, further studies are needed to address whether transdifferentiation of mobilized HSCs into functional hepatocytes was one of the potential underlying mechanism that could have led to the beneficial effects reported in this study.

Authors' contribution

Gehan El-Akabawy conceived the study; designed and conducted all experiments; acquired, statistically analyzed, and interpreted all data; wrote and revised the manuscript; and financially supported the study. *Abeer El-Mehi* conceived the study, contributed to the examination of H&E slides, and financially supported the study.

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