



POTENTIAL THERAPEUTIC ROLE OF OSTEOPONTIN INHIBITION IN LIVER FIBROSIS -INDUCED BY THIOACETAMIDE IN RATS

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ABSTRACT

To examine the effect of osteopontin inhibition by tranilast on liver fibrosis, four groups of rats were used throughout this study. Group I (Control group): rats received the solvent. Liver fibrosis was induced in Groups II, III, and IV by thioacetamide (TAA; 200mg/kg, ip) twice weekly for 6 weeks. Group II served as (TAA group). Groups III and IV (Treatment groups): rats were given tranilast for 6 weeks after TAA discontinuation. Liver osteopontin (OPN), transforming growth factor- β (TGF- β 1), tumor necrosis factor alpha (TNF- α), alpha-smooth muscle actin (α -SMA), reduced glutathione (GSH), superoxide dismutase (SOD) and lipid peroxidation (MDA) were measured. Additionally, expression of α -smooth muscle actin (SMA) and caspase (Cas)-3 were assigned immunohistochemically. Treatment with tranilast prevented the development of hepatic fibrosis and the activation of stellate cells, and down-regulated the expression of genes for osteopontin and osteopontin-target molecules, including TGF- β and TNF- α and α -SMA. Tranilast significantly decreased MDA and increased levels of GSH, and SOD. Our findings suggest that targeting osteopontin with tranilast represents a new mode of therapy for liver fibrosis.

Keywords: antioxidant, apoptosis, liver fibrosis, Osteopontin.

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INTRODUCTION

Liver fibrosis is the final common pathological pathway of liver damage arising from various etiologies including chronic viral hepatitis, genetic deficiency, metabolic derangement, infection, autoimmunity, and exposure to physical and chemical agents. This condition yearly affects millions of patients across the world. Progressive liver fibrosis, leading to cirrhosis, is the most common cause of liver failure (Fausther *et al.* 2017). Many types of cells and cytokines are involved in the initiation and progression of liver fibrosis. Activation of hepatic stellate cells (HSCs) is a pivotal event in fibrosis (Zhou *et al.* 2014). HSCs are stimulated by fibrogenic cytokines, one of which is osteopontin, a pro-inflammatory cytokine and matrix protein. The profibrogenic effect of osteopontin is associated with an increased concentration of transforming growth

factor β 1 (TGF- β 1) (Alex *et al.* 2014). Previous research has showed that osteopontin increased the TGF- β 1 mRNA expression in the activated HSCs, and this effect was completely blocked by one of the osteopontin inhibitors, tranilast (Huang *et al.* 2014).

Osteopontin (OPN), which is also known as secreted phosphoprotein 1 (Spp1) and early T-lymphocyte activation 1 (ETA-1), is a highly phosphorylated glycoprotein that is a prominent component of the mineralized extracellular matrix of bone and expressed by a variety of cells and tissues (De Fusco *et al.* 2017). A wide variety of cell types express OPN and, thus, it plays many physiological and pathological events, such as cell migration, cell survival, regulation of inflammation and tumor metastasis (Kothari *et al.* 2016). OPN exerts its functions via binding integrin receptors. OPN has two integrin binding domains, RGD and SVVYGLR

sequences. OPN binds to RGD-recognizing integrins such as $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins via the RGD domain; and binds to $\alpha 9\beta 1$ and $\alpha 4\beta 1$ integrins via the SVVYGLR domain (Kon *et al.* 2014). OPN is extensively altered through post-translational modifications such as phosphorylation, sulfation, and glycosylation. These modifications have significant implications on the interaction with integrins (Pritchett *et al.* 2012).

Tranilast is an anti-allergic compound that has been approved for use in the human population in Japan and South Korea since 1982 for the treatment of bronchial asthma, atopic dermatitis and allergic rhinitis. Since that time, the effectiveness of tranilast as a therapeutic agent for a range of fibrotic disorders and its mechanism of action have been studied extensively both *in vitro* and *in vivo* (Swiderski *et al.* 2014).

Tranilast has been reported to act as an anti-inflammatory and anti-fibrotic compound through suppression of collagen synthesis by fibroblasts via down-regulation of cytokine release from monocytes/macrophages (Nagate *et al.* 2007). Previous studies observed decreasing expression of Osteopontin protein and macrophage infiltration as one mechanism of tranilast treatment thereby attenuating tubulointerstitial fibrosis in a chronic rat model of nephrotoxicity (Tao *et al.* 2011). Therefore, the aim of this study is to evaluate the role of osteopontin inhibition by using tranilast in treatment of liver fibrosis.

MATERIALS AND METHODS

Drugs and chemicals

Tranilast was purchased from Xiamen Kerda trade CO., LTD., Xiamen City, China. Thioacetamide (TAA) was purchased from Sigma-Aldrich, USA. All other chemicals used throughout the experiment were of the highest analytical grade available.

Preparation of drugs

All drugs and chemicals were freshly prepared prior to use. Tranilast was dissolved in 0.5% carboxymethyl cellulose (CMC) for oral administration. Thioacetamide was dissolved in 0.9% (w/v) saline solution for intra-peritoneal (*i.p.*) injection.

Experimental animals

Adult male Wistar rats weighing 200-240 g were utilized. Rats were obtained from the Animal House Colony of the National Research Centre (Dokki, Giza, Egypt). Rats were housed in stainless

steel cages under controlled conditions with room temperature or 23-26 °C and 12/12 h light/dark cycle. The animals were allowed for free access to water and a standard rodent chow diet. Rats were allowed to adapt to the laboratory environment for one week before starting the experiment. All animals received humane care in compliance with guidelines of the Ethical Committee of the National Research Centre (NRC), Egypt and followed the National Institutes of Health Guide Recommendations Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Experimental design

Twenty-eight rats were allocated into 4 groups each of 7 animals. The 1st group was kept as normal control, rats received (0.5% CMC, orally). The 2nd group was kept without treatment and served as fibrotic control, injected *i.p.* with TAA twice weekly (days 2 and 6) at a dose of 200 mg/kg for 6 successive weeks (Ftahy *et al.* 2013). The 3rd and 4th groups, rats received daily oral dose of tranilast at doses of 150 and 300 mg/kg for 6 weeks after TAA discontinuation (Abdelaziz *et al.* 2015). After the end of the experiment, rats were sacrificed by cervical dislocation and the liver tissues were rapidly removed, washed in ice-cooled saline, plotted dry and weighed. The left lobe of each liver was dissected and placed in 10% formalin in saline, to be used for biochemical and immunohistochemical examination.

A weighed part of each liver was homogenized, using a homogenizer (Medical instruments, MPW-120, Poland), with ice-cooled saline to prepare 20% w/v homogenate. The homogenate was then centrifuged at 4000 rpm for 5 min. at 4°C in a cooling centrifuge to remove cell debris (Laborzentrifugen, 2k15, Sigma, Germany). The aliquot was kept at -80 °C for estimation of transforming growth factor- β (TGF- $\beta 1$), osteopontin (OPN), tumor necrosis factor alpha (TNF- α), alpha-smooth muscle actin (α -SMA) and superoxide dismutase (SOD) by using commercial ELISA kits. In addition, lipid peroxidation (MDA) and reduced glutathione (GSH) contents were measured by using traditional chemical methods.

Thiobarbituric acid reactive species measurement

Brain lipid peroxides formation were measured as malondialdehyde (MDA), which is the end product of lipid peroxidation and reacts with thiobarbituric acid (TBA) as a TBA reactive substance (TBARS) to produce a pink colored complex which has peak absorbance at 535 nm as described previously (Ruiz-Larrea *et al.* 1994).

Determination of reduced glutathione level

The level of reduced glutathione (GSH) was determined as described previously (Ellman 1959) with modification (Bulaj et al. 1998).

Immunohistochemistry for α -SMA and caspase-3

Immunohistochemical studies were carried out for detection of caspase-3 and α -SMA expression on paraffin sections of liver of control and all treated groups using avidin-biotin peroxidase (DAB, Sigma Chemical Co.) according to method described by (Hsu et al. 1981). Tissue sections were incubated with a monoclonal antibody for caspase-3 and α -SMA (Dako Corp, Carpinteria, CA) and reagents required for the avidin-biotin peroxidase (Vectastain ABC peroxidase kit, Vector Laboratories) method for the detection of the antigen-antibody complex. Each marker expression was visualized by the chromagen 3,3 - diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.).

Statistical analysis

The results are expressed as mean \pm S.D. of seven animals, and all statistical comparisons were made by means of one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The data were analyzed with GraphPad Prism v. 5.0 (GraphPad Software, Inc., CA, USA). Difference was considered significant when *p* value is <0.05

RESULTS

Effect on reduced glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD) levels in liver homogenate

The results in Table (1) showed that TAA administration led to a significant depletion of hepatic GSH content and SOD activity, as compared with normal control rats. On the other hand; MDA level in liver homogenate was significantly increased in TAA-intoxicated group, as compared to normal control group. Treatment with tranilast (150 and 300 mg/kg) for 6 weeks after induction of liver fibrosis showed significant increase of hepatic GSH content and SOD activity as compared to TAA group. Meanwhile the elevation of MDA level in liver homogenate was significantly decreased in rat groups treated with tranilast as compared with TAA group (Table1).

Effect on osteopontin (OPN) and transforming growth factor beta1 (TGF- β 1) in liver homogenate

Significant elevation was recorded in hepatic OPN and TGF- β 1 levels of the TAA group compared to that of the normal control group (Figure 1 A and B). Meanwhile, OPN and TGF- β 1 levels were significantly decreased in groups treated with tranilast (150 and 300 mg/kg), for 6 weeks after induction of TAA as compared to TAA group (Figure 1 A and B).

Effect on tumor necrosis factor alpha (TNF- α) and alpha-smooth muscle actin (α -SMA) in liver homogenate

The results in (Figure 2 C and D) exhibited significant elevation of TNF- α and α -SMA levels in TAA-intoxicated group as compared to normal control group. The elevation of TNF- α and α -SMA levels in liver homogenate was suppressed in groups treated with tranilast (150 and 300 mg/kg) for 6 weeks after induction of liver fibrosis, as compared to TAA-intoxicated group (Figure 2 C and D).

Immunohistochemical analysis of α -SMA and caspase-3

As shown in figure 3, Microscopical examination of liver of control rats revealed normal positive expression of α -SMA around central veins and portal veins and negative expression of caspase-3 (Figure 3 A and B). While examination of livers of thioacetamide administrated rats revealed marked increased positive expression of α -SMA along the portal to portal fibrous stands and extending among the parenchyma as well as in the portal triads along the proliferated fibrous connective tissue especially pericanalicular (Figure3 C and D). Marked positive expressing of caspase-3 in the cytoplasm of the swollen hepatocytes (Figure 3 E), as well as in the apoptotic cells and apoptotic bodies (Figure 3 F) was noticed in liver of TAA administrated rats. While Livers of TAA and tranilast administrated rats showed limitation of α -SMA expression to the portal areas and scattered faint positivity in the parenchyma (Figure3 G). Many apoptotic cells and bodies were observed in those livers' sections (Figure3 H).

Table 1: Effect of treatment with tranilast (150 and 300 mg/kg) for 6 weeks on the liver GSH, MDA and SOD in control healthy and TAA induced liver fibrosis rats.

	Normal Control	TAA group	Tranilast (150 mg/kg)	Tranilast (300 mg/kg)
GSH (μ mol/g tissue)	5.37 \pm 0.26 ^b	0.89 \pm 0.03 ^a	1.42 \pm 0.03 ^a	2.31 \pm 0.05 ^{ab}
MDA (nmol/g tissue)	9.53 \pm 0.73 ^b	73.37 \pm 1.31 ^a	47.07 \pm 1.03 ^{ab}	27.86 \pm 1.00 ^{ab}
SOD (U/g tissue)	18.99 \pm 0.52 ^b	2.92 \pm 0.18 ^a	4.64 \pm 0.18 ^a	7.67 \pm 0.14 ^{ab}

N = 7; Values are expressed as mean \pm SEM
a = significant at $P < 0.05$ when compared with control group
b = significant at $P < 0.05$ when compared with TAA group

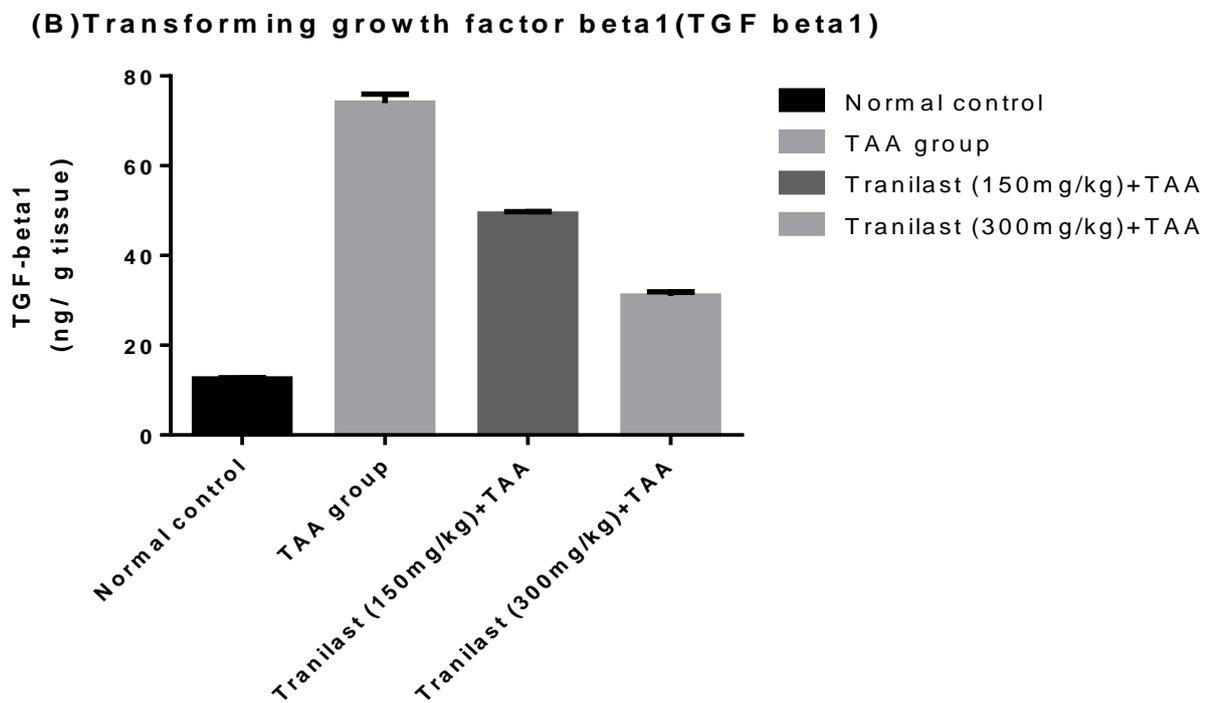
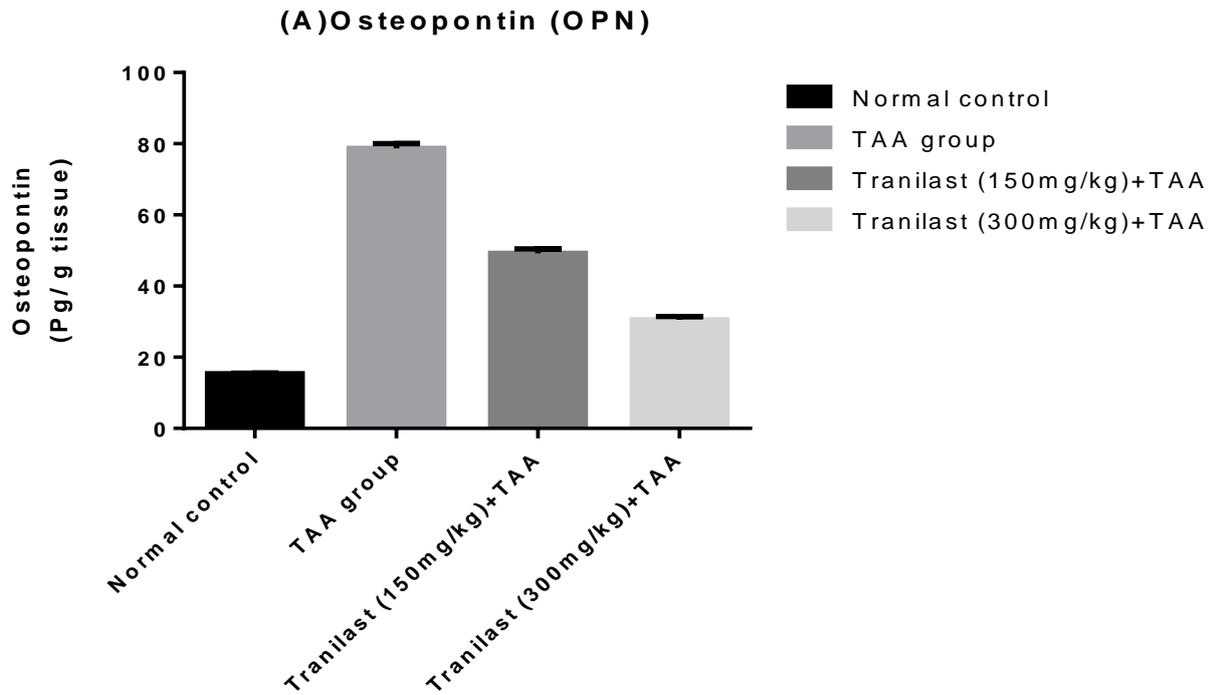


Fig. 1: Effect of oral administration of tranilast for 6 weeks after liver fibrosis induction on OPN (A) and TGF- β 1 (B) in liver tissue of TAA group.

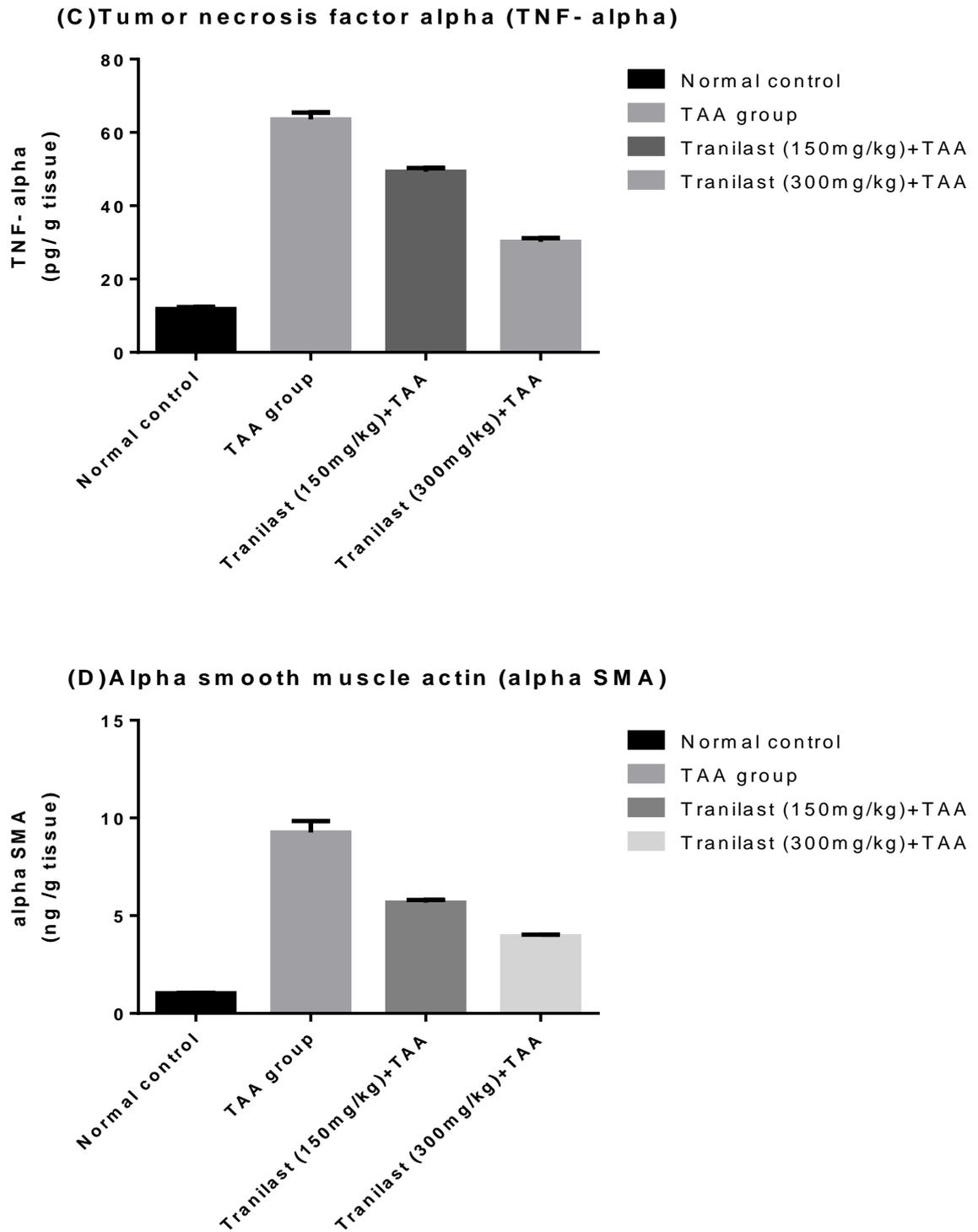
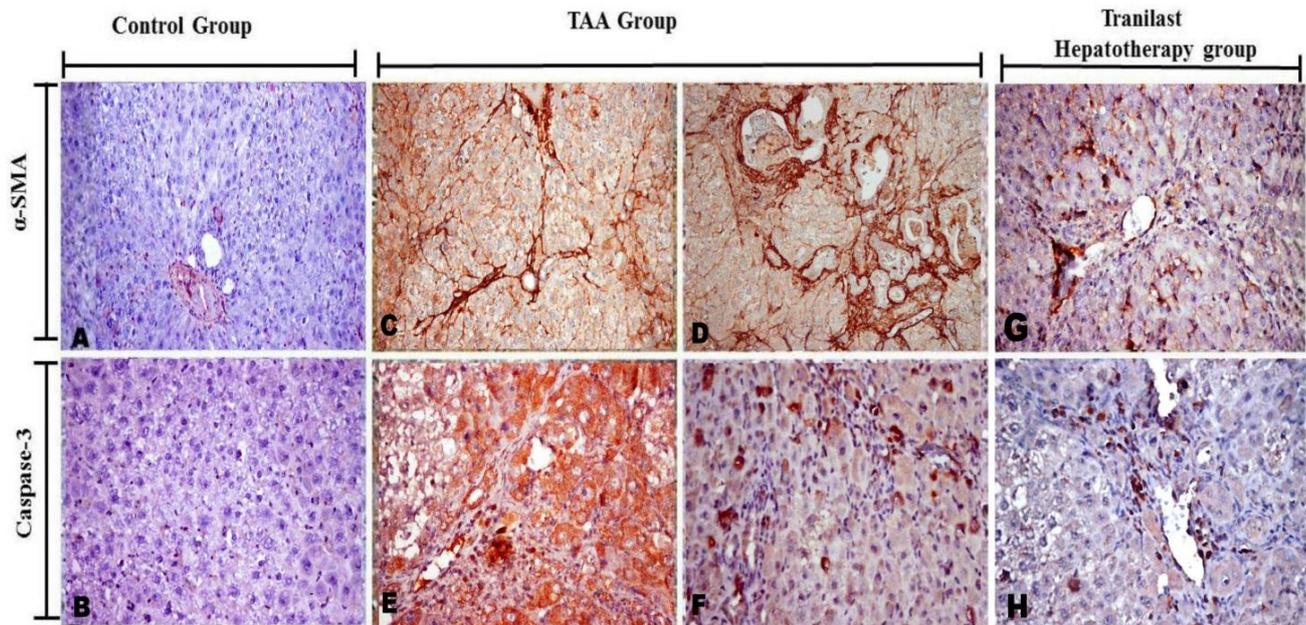


Fig. 2: Effect of oral administration of tranilast for 6 weeks after liver fibrosis induction on TNF- α (C) and α -SMA (D) in liver tissue of TAA group.



Figs. 3: A and B; liver of control rat showing mild positive expression of α -SMA around portal vein (A) and negative expression of caspase-3 (B). C-F: liver of TAA administrated rat showing marked increased positive expression of α -SMA along the portal to portal fibrous stands (C) and in portal areas along the proliferated fibrous connective tissue especially pericanalicular (D). E,F: positive expressing of caspase-3 in the cytoplasm of the swollen hepatocytes (E) and in the apoptotic cells, and apoptotic bodies (F). G, H: Livers of thioacetamide and tranilast administrated rat showing limitation of α -SMA expression (G) and many apoptotic cells and bodies (H). (X400).

DISCUSSION

Liver fibrosis results from many chronic injuries and often progresses to cirrhosis, liver failure, portal hypertension, and hepatocellular carcinoma. Liver transplantation is the alternative for patients with advanced stages of liver fibrosis. Accordingly, there is a critical need to find new approaches for anti-fibrotic therapy (Koyama *et al.* 2016). Indeed, there are many existing antifibrotic drugs with well-established safety profiles; they also have been developed for other indications (Lee *et al.* 2015).

One of the most important goals of the targeted therapy development is to determine the appropriate molecular target proteins and enzymes, hormones, peptides, genes, and specific reactions involved in the pathological processes that can result in the disease resolution/reversion (Bansal *et al.* 2016). Osteopontin (OPN) augments hepatic fibrosis, thus targeting osteopontin might be an appropriate therapeutic approach for the management of liver fibrosis. In the current study, we studied the effect of TAA on pro-inflammatory cytokines and fibrosis markers and the effect of tranilast administration for 6 weeks on liver fibrosis through down regulation of OPN expression. It was observed that there was a significant increase in hepatic OPN and TGF- β 1 expressions in TAA group as compared with the

normal control group. Similarly, (Arffa *et al.* 2016) reported that TAA injections caused hepatic fibrosis as demonstrated by up regulation of OPN and increased expression of α -SMA and TGF- β 1. This perhaps may be explained by the increase in oxidative stress and activation of pro-inflammatory cytokines caused by TAA. Oral administration of tranilast after induction of liver fibrosis significantly reversed the level of hepatic OPN and TGF- β 1 produced by TAA and caused a subsequent recovery towards normalization. Similarly, (Huang *et al.* 2014) reported that tranilast could reduce myocardial fibrosis by decreasing the number of mast cells and inhibiting the expression of osteopontin (OPN) and transforming growth factor- β (TGF- β 1).

Next, we found a significant increase in TNF- α and α -SMA levels in TAA-treated rats as compared to normal control group. These findings were parallel with findings of (Hung and Lee 2017). The increases in inflammatory and fibrogenic markers were ameliorated by treatment with tranilast as compared with TAA group which are supported by the work of (Said *et al.* 2016). These results suggest that OPN down-regulation could be an important contributing factor to the antifibrotic activity of tranilast.

Reduction in hepatic antioxidant levels is the main mechanism of TAA action. GSH and SOD are considered as the first line of the cellular antioxidant defence system. Regarding the effect of osteopontin inhibition on oxidative stress markers, TAA injections significantly elevated liver MDA content while, significantly decreased liver GSH content and SOD activity. This may be due to the lipid peroxidation and disturbance of Ca²⁺⁺ induced by toxic agents (Chen *et al.* 2012). Treatment with tranilast succeeded to antagonize the deleterious effects induced by TAA, as evidenced by the reduction in the level of MDA and maintenance of intracellular level of GSH content and SOD activity which may have contributed in amelioration of the damaging effect of ROS on biological membranes (Said *et al.* 2016). Therefore, it is suggested that osteopontin inhibition by tranilast restored impaired host oxidant/antioxidant balance.

In our work, the liver caspase-3 showed a significant down-regulation and a significant up-regulation of α -SMA expression following TAA injection for 6 week as compared to normal control group, which is consistent with a previous report of (Jing *et al.* 2015). In groups treated with tranilast, our results showed significant increase in caspase-3 level, indicating tranilast induces caspase mediated programmed cell death through inhibition of osteopontin. These findings were parallel with findings of (Huang *et al.* 2010) who reported that, OPN enhances chemoresistance in small-cell lung cancer and breast cancer by blocking caspase-3-dependent cell apoptosis. Concerning the effect on α -SMA level in liver homogenate, there was down-regulation of liver α -SMA expression in groups' treated with tranilast as compared to TAA group, which might be attributed to osteopontin inhibition. This result was also in consistent with the results of a previous study by (Lenga *et al.* 2008) who demonstrate that, OPN is required for the differentiation and activity of myofibroblasts and found that knock down of OPN reduced α -SMA expression.

CONCLUSION

The current study found that targeting osteopontin might be an appropriate therapeutic approach for the management of liver fibrosis.

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