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The influence of TRAIL, adiponectin and sclerostin alterations on bone loss in BDL-induced cirrhotic rats and the effect of opioid system blockade

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ABSTRACT

Aims: Osteoporosis is a common extra-hepatic complication in patients with chronic liver disease. Tumor necrosis factor related apoptosis-inducing ligand (TRAIL), sex hormones, adiponectin, and sclerostin are involved in the regulation of bone turnover but little is known about their role in the promotion of hepatic osteodystrophy. Endogenous opioids are reported to increase during cholestasis and may influence bone resorption. The purpose of this study was to investigate the circulating levels of these factors and their expression in the femur of bile duct ligated (BDL) rats, to evaluate the biomechanical bone strength, and the effect of naltrexone (NTX).

Materials and methods: BDL and sham-operated (SO) rats received 10 mg/kg NTX as an opioid-receptors antagonist or saline once daily for 28 days intraperitoneally. Three-point bending test was performed on the right femurs and, plasma bone alkaline phosphatase (BALP), sex hormones, TRAIL, adiponectin, sclerostin, as well as the mRNA expression levels of the latter three proteins, were measured in the femur tissues.

Key findings: Plasma TRAIL, estrogen, adiponectin, sclerostin and, BALP levels increased in BDL animals when compared to the related controls, whereas testosterone level decreased and NTX reversed these effects significantly. Femur strength decreased in cirrhotic animals and interestingly, blocking opioid-receptors by NTX improved it significantly ($p \leq 0.05$).

Significance: High levels of TRAIL, adiponectin and, sclerostin after bile duct ligation, suggest that these factors may have some roles in bone loss after cirrhosis. Administration of NTX improved all the mentioned factors except for bone strength. Effect of NTX on bone loss in BDL rats needs more study to clarify.

1. Introduction

Osteoporosis is one of the most important extra-hepatic and liver disease-related complications which is associated with reduced bone mass and increased fracture risk, leading to what is termed hepatic osteodystrophy [1,2]. The exact pathophysiology of this complication is not completely understood [1]. Liver dysfunction probably causes alteration of several factors and hormones [3] which affect the bone tissue through RANK (receptor activator of nuclear factor-κB)/RANKL (RANK-ligand)/OPG (osteoprotegerin) system as a key regulator of bone turnover, resulting in increased bone resorption and decrease bone formation [4]. RANKL with binding to its receptor RANK stimulates osteoclastogenesis and activation of osteoclasts; the bone-resorbing cells. On the other hand, OPG as a decoy receptor deactivates this process [5]. However, several studies have shown elevated levels of OPG in liver diseases, contributing to bone loss [6,7].

Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is a glycoprotein, which belongs to the TNF family [8]. This protein plays a significant role in the formation and differentiation of osteoclasts [9,10] and by binding to OPG, prevents its inhibitory effect on osteoclastogenesis [8]. Also, the evidence presented has revealed the ability of sex hormones to stimulate OPG and reduce RANKL production *in vitro* and *in vivo* [11,12], and that cirrhosis is associated with impairment of the serum level of sex hormones [13]. Therefore, the observed increase levels of OPG in hepatic osteodystrophy may be related to changes in the level of TRAIL and sex hormones in this situation.

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As mentioned above, following liver diseases bone loss is developed and is associated with increased bone resorption and decreased bone formation [14]. Adiponectin as an adipocyte-secreted hormone accelerates bone turnover and enhances bone resorption through regulating OPG/RANKL expression [15]. Moreover, sclerostin; a bone formation inhibitor, suppresses bone formation by preventing the Wnt signaling pathway [16]. The effect of these two factors may be considered as the same in mechanisms involved in hepatic osteodystrophy.

Increase in the level of endogenous opioids is important in bone quality during fibrosis and cirrhosis [17]. Binding of these opioids to their receptors inhibit the secretion of osteocalcin in osteoblasts [18] and believed to reduce bone density in animals and humans [19,20].

Up to now, the expression of TRAIL, adiponectin, and Sclerostin genes have not been analyzed in the bone tissue, although increased circulating levels of these factors have been described in chronic alcoholism and in liver cirrhosis [10,16,21]. Meanwhile, the role of these factors, along with opioid receptor antagonist (NTX) has not been studied in cholestatic liver cirrhosis; a disease clearly associated with bone loss.

This study aimed to measure the circulating levels of TRAIL, sex hormones, adiponectin and sclerostin in BDL-induced cirrhotic rats and their relationship with bone strength and bone turnover marker (BALP). The mRNA levels of some factors in the bone of BDL rats and their association with their circulating levels were assessed. Also, based on the effect of endogenous opioids on bone quality and density during fibrosis and cirrhosis [19,20,22,23], the influence of NTX (a non-specific opioid receptor blocker) on the above mentioned factors was investigated to find out the eventual role of endogenous opioids in the process.

2. Methods

2.1. Chemicals

Drugs and reagents; all with analytical grade, used in this study were as follows:

Naltrexone (non-specific opioid receptor antagonist) was obtained from Sigma (St Louis, MO, USA), ketamine hydrochloride, xylazine hydrochloride, were purchased from Daroupakhsh (Tehran, Iran) and formaldehyde was prepared from Merck (Darmstadt, Germany).

2.2. Animals

Male Sprague–Dawley rats (n = 38, weight 200–250 g) were purchased from the animal facility of the Institute of Biochemistry and Biophysics, (IBB, University of Tehran, Tehran, Iran). The animals were maintained four per cage under constant temperature and humidity (23 ± 2 °C, 50 ± 5%) in 12 h light/dark cycle with free access to food and water.

Prior to commencing the study, the protocols and procedures were approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC-1396-2026) which were in accordance with the 'Guide for the Care and Use of Laboratory Animals' (NIH US publication no. 85–23 revised 1985).

2.3. Study design

In this experiment, BDL rats were used as an animal model with induced liver cirrhosis. Animals were randomly assigned into five experimental groups: (a) baseline controls, n = 6, (b) sham-operated (SO) controls treated with saline, n = 6, (c) BDL animals treated with saline, n = 10, (d) SO controls treated with 10 mg/kg NTX, n = 6, (f) BDL animals treated with 10 mg/kg NTX, n = 10.

The bile duct ligation surgery was conducted according to the procedure described previously [2]. Briefly, under anesthesia (ketamine HCl, 50 mg/kg, and xylazine10 mg/kg, *i.p.*) laparotomy was accomplished, bile duct was recognized and ligated in two locations with a silk thread and resected between the ligatures. In the SO group, the bile duct was manipulated without ligation. Finally, the abdominal wall was closed in two layers and, all animals received 0.5 ml sterile isotonic saline solution intraperitoneally after surgery. Unoperated rats served as baseline controls.

The animals received intraperitoneal injections of NTX (10 mg/kg) or saline once daily for 28 days after BDL or sham surgery. Animals' body weight was measured at the day of surgery and the next weeks until the end of the experiment. The dose of Naltrexone was selected based on previous studies [6,24].

Twenty-eight days after operation, animals were sacrificed by exsanguination (cardiac puncture) under general anesthesia. This timeline has been shown to induce bone disease in BDL rats [2]. Blood samples were collected and plasma aliquots stored at −80 °C until use. The femurs and liver samples were removed rapidly and snap-froze in liquid nitrogen or were immersed in 10% buffered formalin for further studies and histological assessment.

2.4. Histology

Liver tissues were fixed in 10% formaldehyde and embedded in paraffin blocks. The blocks were sectioned and stained with Masson's Trichrome for structural and fibrosis detection and observed under a light microscope (Olympus CX31, Japan).

2.5. Biochemical analysis

Total bilirubin, alanine aminotransferase) ALT(, aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (γ-GT) as biochemical markers of liver injury were measured in plasma samples using specific related kits (Pars Azmoon Co., Tehran, Iran).

2.6. Biomechanical strength testing

Three-point bending test was performed at the right femurs midpoint using a material testing machine (Zwick/Roell 0.5 KN, Ulm, Germany). Femurs were positioned so as their lateral surface was placed on two base supports of the bending apparatus and fixed (Fig. 2A). With constant speed of 1 mm/s of the moving edge, force applied to the femur midpoint until fracture occurred and the breaking strength (maximal load) was reported.

2.7. ELISA assay

Estrogen hormone, TRAIL, adiponectin, sclerostin and bone alkaline phosphatase (BALP) were measured in the plasma samples from the BDL rats as well as the SO groups using enzyme-linked immunosorbent (ELISA) assay kit (MyBioSource, USA) according to the instructions. The intra-assay coefficient of variation (CV) was below 8%, and inter-assay CV was below 10% for all ELISA results. Testosterone was assessed by rat specific ELISA kit (CUSABIO, USA).

2.8. Real-time PCR

Expression of TRAIL, adiponectin, and sclerostin genes was evaluated by real time PCR in left femurs prepared from BDL or sham operated rats using a RotorGene 3000 instrument (Corbett Research, Australia). Briefly, total RNA was isolated from the tissue homogenate using the ONE STEP-RNA Reagent (BioBasic, Canada). After RNA extraction, DNA elimination and RNA purification was achieved by adding DNase and Riboclear™ plus kit (Gene all, Korea). A reverse transcription reaction was performed using PrimeScrip™ RT Reagent Kit (Takara Bio Inc., Tokyo, Japan) at 37 °C for 15 min, followed by 85 °C for 5 s. Real-time PCR reactions were run using SYBR Premix Ex Taq II

Table 1
Oligonucleotide primers for real-time-PCR.

Sequence	Primer
TRAIL	Forward: AGACAGTCTCGAAGGACGGA Reverse: AGCAGTATGGGATCGGGGTA
Adiponectin	Forward: CAAGGCCGTTCTCTTCACCT Reverse: CCCCATACACTTGGAGCCAG
Sclerostin	Forward: GTACATGCAGCCTTCGTTGC Reverse: ACTCGGACACGTCTTTGGTG
β-Actin	Forward: GCAGGAGTACGATGAGTCCG Reverse: ACGCAGCTCAGTAACAGTCC

TRAIL, tumor necrosis factor related apoptosis-inducing ligand.

kit (Takara Bio Inc., Tokyo, Japan) as per the manufacturer's instructions with the following cycle parameters: 95 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s, 40×. β-Actin was used as internal standard (housekeeping gene). Oligonucleotide primer sequences used are shown in Table 1. The ratio of transcriptional difference between cirrhotic rats and the control group was calculated relative to the level of β-actin RNA expression using the $2^{-\Delta Ct}$ method.

2.9. Statistical analyses

All data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. Differences between two groups were analyzed by Mann–Whitney *U* test. Multiple comparisons were performed by one-way ANOVA tests, and Tukey test was used for *post hoc* analysis. Comparative C_T method was used for analysis of the gene expression. Spearman rank correlation was calculated to quantify the degree of linear association between two variables. A p value < 0.05 was considered as statistically significant.

3. Results

3.1. Histological analysis

Results of histological evaluation of liver tissues by Masson's Trichrome staining revealed an elevated deposition of collagen fibers in BDL rats (Fig. 1C). Additionally, bile duct proliferation in the port space, thickening of the portal wall, and formation of communication septum known as biliary fibrosis were observed (arrow). Abnormality in liver tissues was expanded in NTX-treated BDL rats. Meanwhile, the liver of SO animals showed no abnormal histologic changes and the liver parenchyma was completely normal.

3.2. Biochemical parameters and body weight

Results of liver biochemical parameters and the animal body weight changes are shown in Table 2. After four weeks, plasma bilirubin and liver enzymes (AST, ALT, ALP, and γ-GT) were significantly elevated in BDL rats compared with sham controls. These findings supported histopathology data and confirmed liver injury. The results showed that NTX markedly reduced these parameter values in BDL rats compared to the untreated group ($p \leq 0.05$).

To test whether BDL has any effect on the animal's body weight, BDL and control groups were weighed before and post-surgery weekly until the end of the experiment. Although at the beginning of the experiment the mean body weight of rats was in the same range (200–250 g), but after 4 weeks of surgery, all BDL animals showed a significant reduction in their body weights compared with baseline and their related SO controls, $p \leq 0.05$ (Table 2).

3.3. Biomechanical properties

In three-point bending test, the mean femoral bone strength decreased following BDL, which was significant in comparison to the baseline (157.45 ± 9.35 *vs.* 178.04 ± 12.03, $p = 0.008$) (Fig. 2B). Unexpectedly, the cirrhotic animals receiving 10 mg/kg NTX showed a significant reduction in bone strength compared to the BDL group (119.54 ± 10.28, $p = 0.009$). Reduced femur strength in BDL rats was associated with loss of body weight ($r = 0.634$, $p = 0.01$), a finding that probably confirms the concomitance of osteoporosis with weight loss.

3.4. Plasma BALP level

In this study, the plasma BALP as a marker of bone formation and high bone turnover was measured. Protein levels of BALP in the untreated BDL group showed a significant increase compared to the SO group ($p = 0.008$), which indicates increased activity of osteoblasts and bone remodeling (Fig. 4A). By comparing BDL groups, NTX was able to significantly reduce the amount of BALP compared to the untreated BDL group ($p = 0.008$).

3.5. Sex hormone alterations

As shown in Fig. 3, 28 days after bile duct ligation the plasma level of total testosterone decreased in BDL rats as compared to sham-operated group (Fig. 3A) (0.89 ± 0.01 *vs.* 1.26 ± 0.06, $p = 0.004$). Treatment with NTX inhibited this process in BDL rats significantly ($p = 0.03$). Testosterone was negatively associated with BALP ($r = -0.639$, $p = 0.01$).

Bile duct ligation was also accompanied by a significant increase in plasma estrogen level ($p = 0.008$) (Fig. 3B). However, administration of NTX did not affect the estrogen levels significantly. There was a positive correlation between the values of estrogen and liver biochemical markers *i.e.,* bilirubin ($r = 0.656$, $p = 0.000$), AST ($r = 0.587$, $p = 0.002$), ALP ($r = 0.551$, $p = 0.000$) and γ-GT ($r = 0.831$, $p = 0.000$), suggesting the role of liver on metabolism of sex hormones. In addition, estrogen was positively associated with BALP ($r = 0.469$, $p = 0.018$), although, a significant negative correlation appeared between plasma estrogen levels and mechanical strength of the femur ($r = -0.564$, $p = 0.028$).

3.6. Plasma levels of TRAIL, adiponectin and sclerostin

BDL significantly increased the level of TRAIL in rats as compared to SO control group (173.89 ± 16.82 *vs.* 34.10 ± 1.48, $p = 0.008$) (Fig. 4B). Treatment with NTX counteracted the effect of BDL. There was a significant difference between BDL and BDL + NTX levels of TRAIL (173.89 ± 16.82 *vs.* 43.30 ± 7.43 respectively, $p = 0.01$).

The study demonstrated a significant positive correlation between plasma TRAIL and bilirubin ($r = 0.637$, $p = 0.001$), ALP ($r = 0.551$, $p = 0.005$), and GGT ($r = 0.632$, $p = 0.002$) levels. Moreover, TRAIL in plasma was found to be positively correlated with BALP level ($r = 0.606$, $p = 0.002$). However, no correlation was observed between TRAIL and femur strength in this regard.

Following BDL, circulating adiponectin and sclerostin were significantly increased compared to SO group (Fig. 4C and D). Treatment with NTX offsets the effect of BDL significantly when compared with the untreated BDL group. The data showed that adiponectin and sclerostin levels were correlated markedly with markers of hepatic injury *i.e.,* bilirubin and liver enzymes. Results disclosed a significant negative association between adiponectin with femoral bone strength and testosterone level ($r = -0.585$, $p = 0.04$, $r = -0.839$, $p = 0.001$ respectively). Moreover, significant association between adiponectin and sclerostin ($r = 0.680$, $p = 0.001$) were observed. Also, an inverse correlation between sclerostin and testosterone was evident ($r = -0.604$, $p = 0.017$).

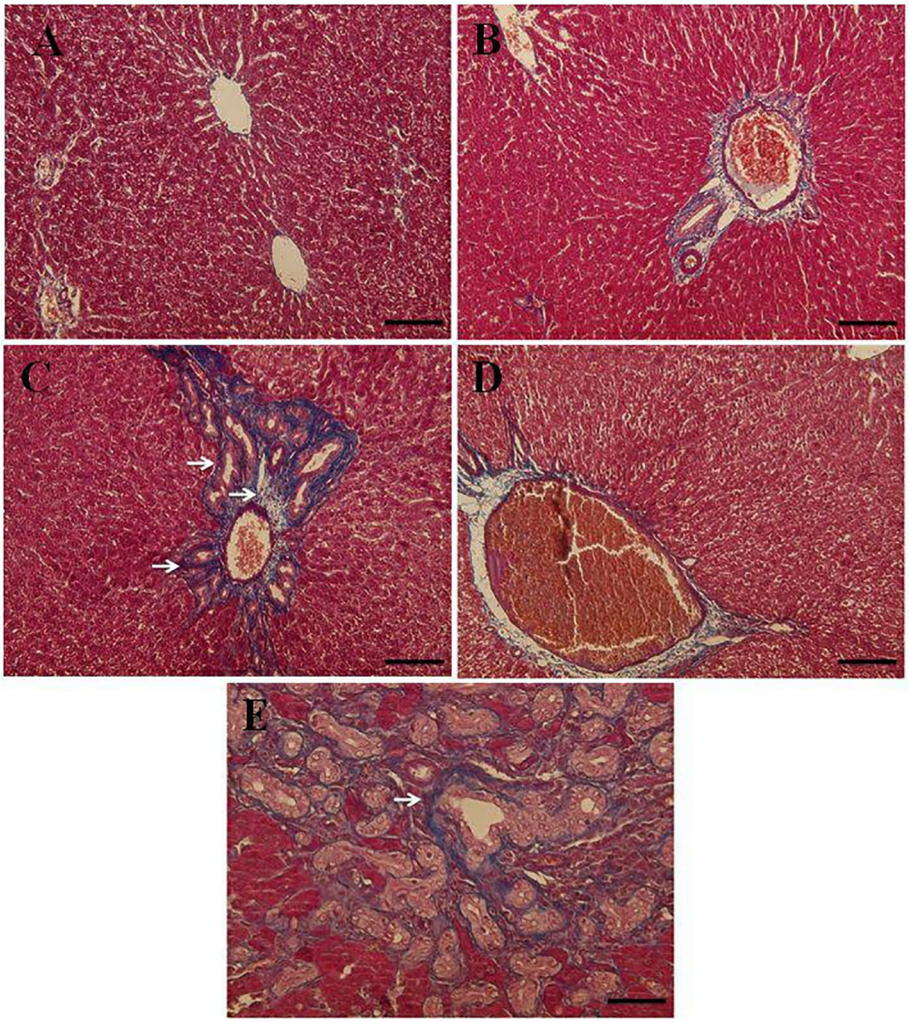

Fig. 1. Representative microscopic view of Masson's Trichrome staining of liver tissue. (A) Baseline, (B) sham-operated (SO), (C) BDL, and (D) SO + NTX and (E) BDL + NTX groups (scale bar, 50 μm).

3.7. Gene expression assessment

For the first time, the quantitative analysis of mRNA expression of TRAIL, adiponectin and sclerostin was assessed in femoral bone of BDL rats by real-time PCR (Fig. 5).

Expression of TRAIL gene in BDL groups was lower than baseline control ($p \leq 0.05$), however, no significant changes were found between BDL groups. Expression of adiponectin also decreased in BDL group when compared with the baseline ($p \leq 0.01$). Nevertheless, alteration in gene expression of adiponectin was not different in BDL rats compared to sham and BDL + NTX groups. There was no difference in expression of sclerostin in femur tissue.

Table 2
Body mass and biochemical parameters of control and test group.

Parameter	Baseline control	Saline		NTX	
		SO	BDL	SO	BDL
Initial body weight (g)	250.6 ± 0 29.81	225.96 ± 15.47	251.50 ± 15.24	226.24 ± 12.80	233.50 ± 14.32
Final body weight (g)	352 ± 47.39	265.88 ± 12.24	231.24 ± 26.24$^{\#,*}$	255.16 ± 11.66	231.08 ± 26.42$^{\#}$
Total Bilirubin (mg/dl)	0.35 ± 0.24	0.47 ± 0.10	13.56 ± 1.58$^{\#,*,\hat{}}$	0.31 ± 0.11	4.08 ± 1.12$^{\#,*,\hat{}}$
ALT (IU/L)	40.49 ± 4.55	74.04 ± 4.71	122.47 ± 1.52$^{\#,*,\hat{}}$	52.60 ± 1.47	36.52 ± 1.34$^{*,\hat{}}$
AST (IU/L)	52.80 ± 2.06	36.12 ± 1.15	239.98 ± 2.88$^{\#,*,\hat{}}$	88.31 ± 3.01	71.46 ± 3.60$^{\#,*,\hat{}}$
ALP (IU/L)	124.46 ± 4.10	140.97 ± 9.39	303.66 ± 8.63$^{\#,*,\hat{}}$	140.05 ± 3.25	221.38 ± 16.36$^{\#,*,\hat{}}$
γ-GT (IU/L)	8.45 ± 0.23	5.21 ± 0.68	27.56 ± 0.67$^{\#,*,\hat{}}$	6.71 ± 0.23	23.16 ± 1.55$^{\#,*,\hat{}}$

Biochemical measurements after completion of the study using SO and NTX treated BDL cirrhotic rats (Test). Each value represents mean ± SEM and the experiments performed in duplicate.
SO, sham-operated; BDL, bile duct- ligated; NTX, Naltrexone; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline aminotransferase; γ-GT, gamma-glutamyltransferase.
$^{\#}$ Significantly different from Baseline, *P*-value ≤ 0.05
* Significantly different from SO.
$^{\hat{}}$ Significantly different from BDL cirrhotic rats (Test) group.

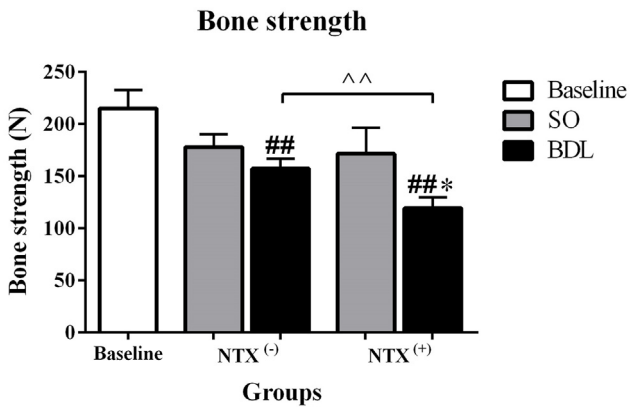

Fig. 2. (A) Three point bending test (B) The femoral breaking strength (in Newton) of rats in the experimental groups. Data are presented as the mean ± SD; # significantly different from baseline, p-value ≤ 0.05. * Significantly different from relevant SO, p-value ≤ 0.05. ^ Significantly different from BDL cirrhotic rats (Test) group, p-value ≤ 0.05.

4. Discussion

It has been proposed that accumulation of endogenous opioid peptides in cholestasis affects many organs such as bone [17,18]. However, the effects of the receptors antagonists on bone quality have not been adequately studied. Meanwhile, although there is no direct evidence about the relationship between opioids and TRAIL, adiponectin, and sclerostin in the literature but, there is some evidence about the relationship between opioid receptors and bone loss [19,23,25]. So, to address this concept, in the current study, NTX (a non-specific opioid receptor blocker) was used to investigate the effect of endogenous opioids in BDL-induced cirrhotic rats.

Different studies have shown that NTX reduces liver injury due to increased liver glutathione and improving the antioxidant capacity of the tissue [17,26]. In the present study improvement of liver injury was observed based on the reduction in plasma liver specific enzymes after treatment of BDL rats with NTX.

The data obtained showed that cholestatic disease affects bone tissue based on biomechanical strength testing and BALP measurements. Reduction of femoral bone strength in cirrhotic rats is clearly shown in the results of biomechanical examination, resembling the previous data on BDL rats [2,27,28] in which it is postulated that reduction of osteoprogenitor cells in liver cholestasis is a mechanism responsible for reducing bone mass and strength [28]. Interestingly, the study revealed a significant reduction in femur strength after treatment with NTX. These data are unexpected, while NTX exerted a positive effect on bone histomorphometry as shown in our previous study [29]. It is evident that the strength of bones is determined by the amount of bone tissue and geometry [30]. A Study by Turner et al. demonstrated that in rabbits, despite increased bone mass, treatment with fluoride did not cause increased bone strength due to alteration in mineral crystal size and shape [31]. Another explanation for the reduction observed in biomechanical bone strength following NTX administration is that, metabolic activity of trabecular and cortical bones is different and does not respond similarly to the pharmacological interventions [32]. Therefore, it can be said that the effect of NTX on femur which mainly consists of cortical bone and possesses a lower turnover, needs more time interval to be detected. However, further studies are warranted to clarify this issue.

In addition, Treatment with NTX decreased BALP levels markedly, suggesting a role of NTX in reducing bone turnover, despite decreasing bone strength.

In a previous study, we reported that BDL cirrhotic rats exhibit increased OPG levels [6]. Inhibition of bone resorption requires OPG, an inhibitor of osteoclastogenesis and bone resorption [5]. In current study to address an unexpected increase in OPG level without any positive effect on bone quality, the plasma levels of TRAIL in cholestatic rats was evaluated; TRAIL was increased after BDL, significantly. It has been reported that this cytokine prevents OPG inhibition of RANKL-induced osteoclastogenesis [8] and stimulates differentiation and activation of osteoclasts [9,10]. Also, previous studies have demonstrated the high levels of TRAIL and its expression in liver disease [33,34]. The observed elevation in the plasma level of TRAIL in the BDL rats demonstrates a probable defect in TRAIL production by the abnormal liver and its proposed effect on bone loss. This may cause continuum bone resorption by binding of TRAIL to OPG and blocking its activity. Decreased level of this factor after opioid receptor blockade with NTX was apparent. However, we could not show a significant difference in expression of TRAIL in femurs between BDL and sham-operated groups

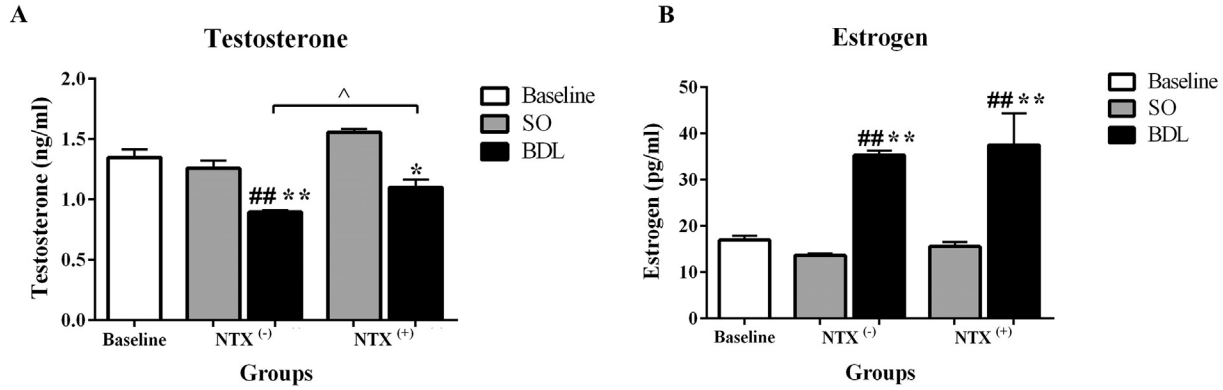

Fig. 3. (A) Testosterone (ng/ml) (B) Estrogen (pg/ml) levels of baseline, sham-operated (SO), BDL and NTX treated BDL cirrhotic rats after completion of the study. Each value represents mean ± SEM (n = 6) and experiments performed in duplicate. # significantly different from baseline, p-value ≤ 0.05. * Significantly different from relevant SO, p-value ≤ 0.05. ^ Significantly different from BDL cirrhotic rats (Test) group, p-value ≤ 0.05

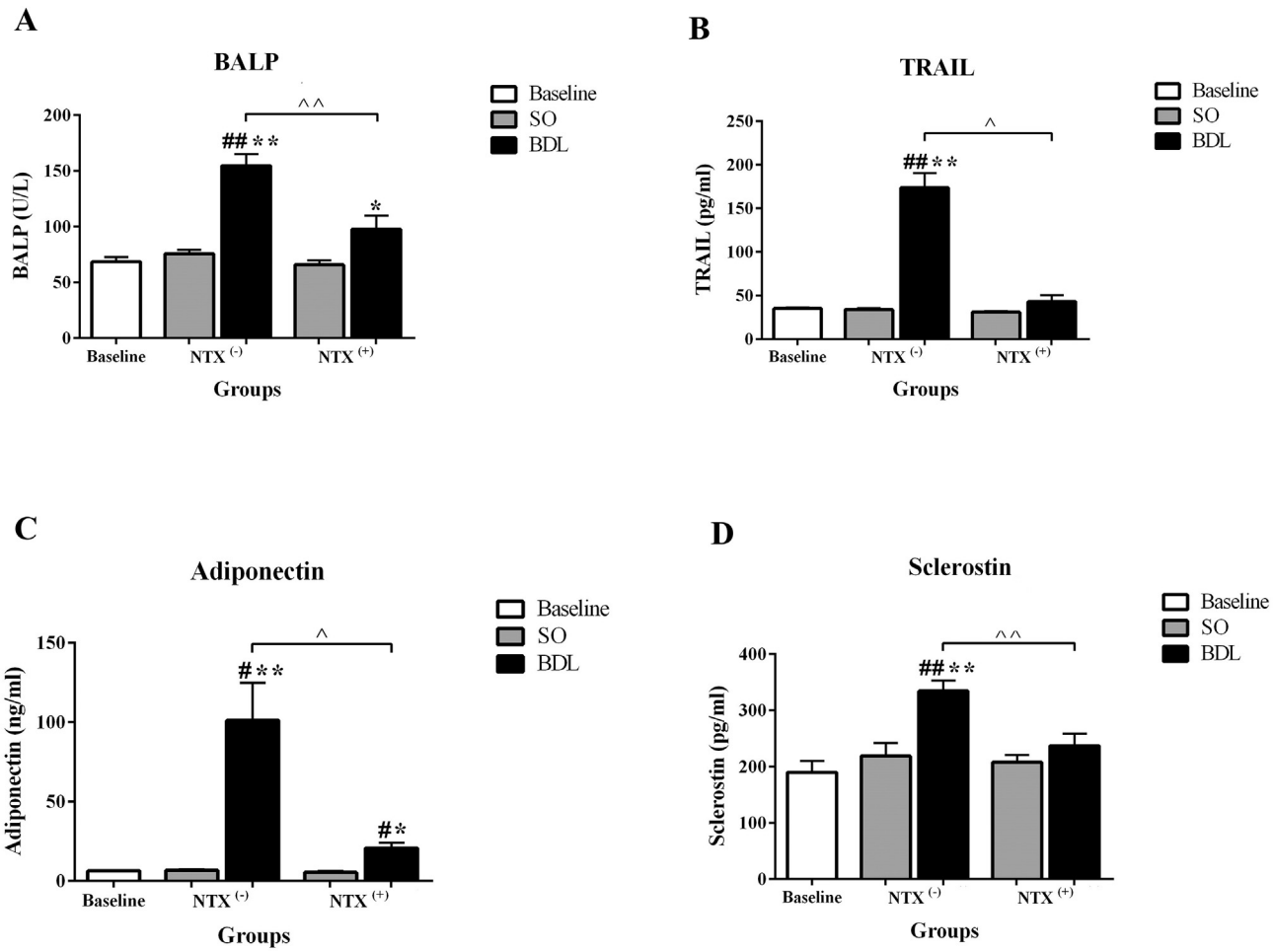

Fig. 4. Plasma (A) BALP (U/L), (B) TRAIL (pg/ml), (C) adiponectin (ng/ml), and (D) sclerostin (pg/ml) levels in different experimental animal groups. Each value represents mean ± SEM (n = 6) and experiments performed in duplicate. # Significantly different from baseline, p-value ≤ 0.05. * Significantly different from relevant SO, p-value ≤ 0.05. ^ Significantly different from BDL cirrhotic rats (Test) group, p-value ≤ 0.05.

and NTX treated rats. Statistical analysis revealed an inverse but not significant association, between plasma TRAIL and femur strength.

Moreover, sex hormones have been reported to increase OPG expression in osteoblasts [10,11]. Our hypothesis is that impairment of metabolism of sex hormones (especially estrogen) in cirrhosis may have a role in the increased OPG levels in hepatic osteodystrophy. It has been shown that reduced estrogen and testosterone lead to increased osteoclast activity and enhance bone loss [11,35]. Moreover, low serum testosterone and high estrogen levels have been reported in cirrhosis [36,37]; a finding which was confirmed in this study. The decreased level of testosterone in plasma has been improved in the cirrhotic rats as a result of NTX treatment, while the other was unchanged.

Alvaro et al. reported that reduction of estrogen receptor (ER) in hepatocytes causes the reduction of estrogen metabolism and its increase in BDL rats [37]. Moreover, low testosterone level in cirrhosis causes the raised level of estrogen because the expression of estrogen-metabolizing enzymes is androgen-dependent [38,39]. Elevated levels of estrogen in cirrhosis probably cause the level of OPG to be high. But, high estrogen and OPG levels cannot offset the liver disease-associated bone resorption [40]. The latter, may be due to an increase in the TRAIL levels.

Sclerostin which is a Wnt antagonist secreted almost exclusively by osteocytes, and adiponectin; the adipocyte-secreted hormone; were measured to further investigate of bone resorption and formation conditions in cirrhosis.

The negative association between serum adiponectin and bone mineral density (BMD) had been reported previously [15,41,42]. On the other hand, in liver cirrhosis, high levels of adiponectin in serum have been reported [21,43]. Our results are in agreement with the previous works. Further research is needed to completely understand the role of adiponectin in the development of bone disease in cirrhosis, and the present study is the first in this way.

As the results showed, plasma levels of adiponectin inversely correlated with weight loss and reduced bone strength. In addition, positive correlation between adiponectin and plasma BALP ($r = 0.665$, $p = 0.001$), suggested that adiponectin is associated with increased turnover of bone, similar to the results of Ding Peng [42].These findings suggests that high circulating level of adiponectin in cirrhosis can promote bone loss.

In this study sclerostin was assessed as a likely candidate for suppression of bone formation. The results showed that BDL rats had a higher level of sclerostin with a positive correlation with BALP (r-0.647, $p = 0.002$) and an inverse but not statistically significant correlation with bone strength. Although increased circulating levels of sclerostin have been reported in liver diseases [16] there was only one study demonstrating the role of sclerostin in reduced bone formation associated with cholestatic liver disease. A study has showed that serum sclerostin levels are significantly higher in primary biliary cirrhosis (PBC) patients and that it is positively correlated with BMD [44]. The presented data in this study confirm previous findings and contributes additional evidence which suggests that high sclerostin levels following cholestasis augmented osteoporosis and low bone formation induced by cirrhosis.

An inverse correlation between sclerostin and testosterone levels

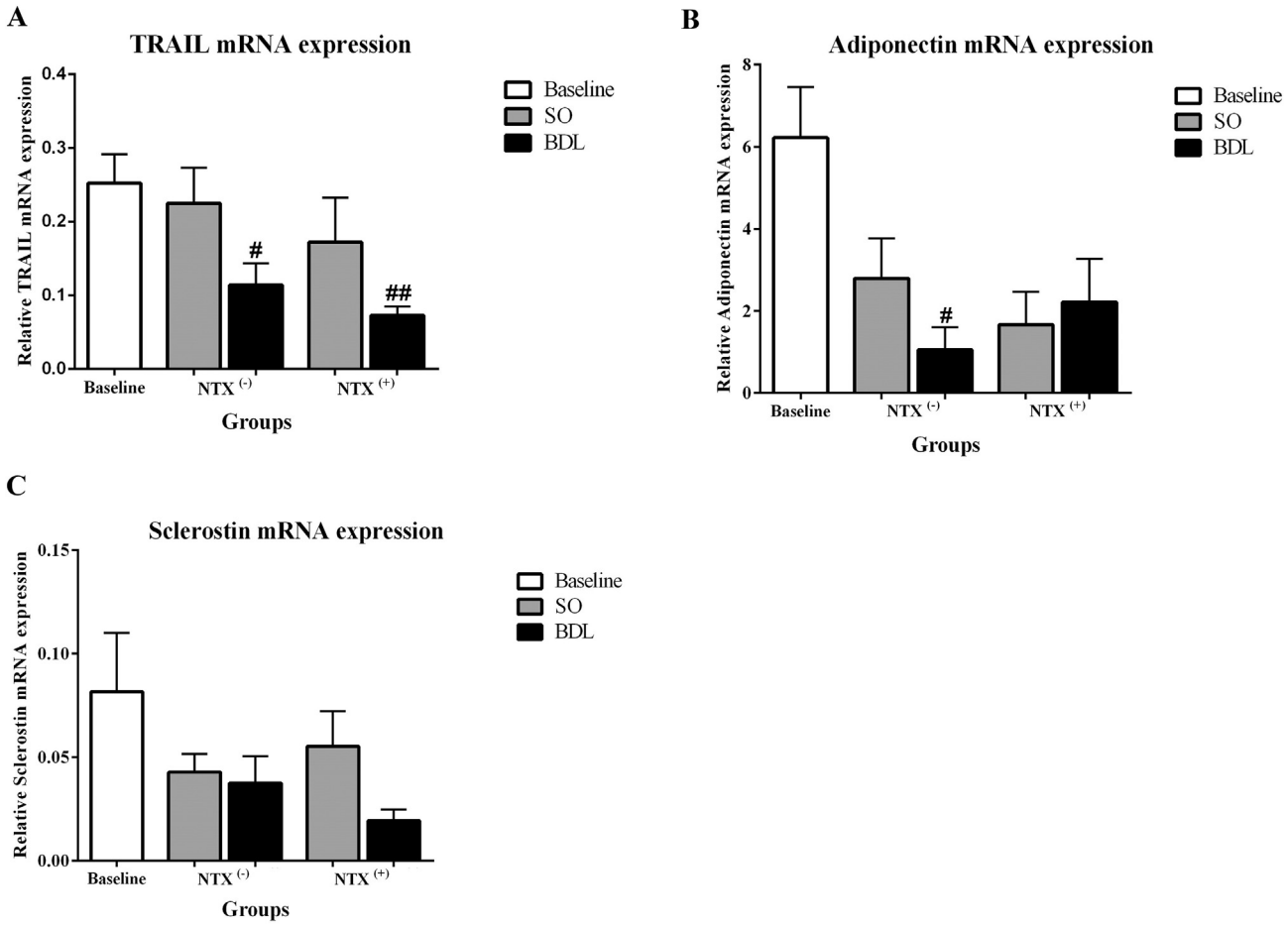

Fig. 5. mRNA expression of (A) TRAIL, (B) adiponectin and (C) sclerostin in femur of rats. Each value represents mean ± SEM (n = 6) and experiments performed in duplicate. # Significantly different from baseline, p-value ≤ 0.05.

was found in this study, as the finding which was reported in a previous study in men with idiopathic osteoporosis [45]. According to the data, probably, low testosterone level in BDL-induced cirrhotic rats contributes to high sclerostin levels which can exacerbate its negative effects on the bone formation.

Generally, the study showed that BDL-induced cirrhotic rats had higher TRAIL, adiponectin and sclerostin levels. Although the exact mechanisms of increased levels of these factors in cirrhotic rats are not well known, but there are some points. Firstly, clearly association of this elevation with liver injury markers suggests that liver dysfunction and cholestasis might have affected the metabolism and/or clearance of the mentioned parameters. Secondly, hepatic production of the factors might be another cause. Studies have demonstrated that after hurt, liver expresses a significant amount of TRAIL, adiponectin, and sclerostin mRNA [33,44,46]. However, it could not be established a significant relationship between plasma levels of these factors and expression of related genes in femur bone tissue, therefore it can be concluded that plasma levels of TRAIL, adiponectin, and sclerostin might not be representative of local expression of these factors in the bone tissue. In addition, another aptness is that these circulating factors might be derived from another multiple sources other than skeletal tissue. Thus, further investigation is necessary to understand the exact role of bone in their expression in hepatic osteodystrophy.

In depth study of association between reduced bone strength following BDL and NTX treatment with bone microarchitecture appears to be critical because such a study will provide supplementary information about the actual impact of structural abnormalities on bone strength and quality. Also, it seems plausible to assay other biomechanical properties such as the stiffness along with histological or histomorphometric analysis of the bones in future relevant studies. On the other hand, increasing the sample size may provide more reliable results. These may be considered as the main limitations of the present study.

In conclusion, we demonstrated that plasma levels of TRAIL, adiponectin and sclerostin are increased in cirrhotic rats. These may have a potential role in mediating the associated bone loss in the animals (Fig. 6). Impaired repair and remodeling of bone occurs in hepatic osteodystrophy due to increased levels of TRAIL. This is counteracted by a compensatory increased level of OPG, and high levels of adiponectin and sclerostin which accelerates bone loss. NTX improved all the mentioned factors with a positive effect on bone quality, but decreased bone strength unexpectedly. So, if it is regarded as a therapeutic agent for improvement of hepatic osteodystrophy, further rigorous studies is needed.

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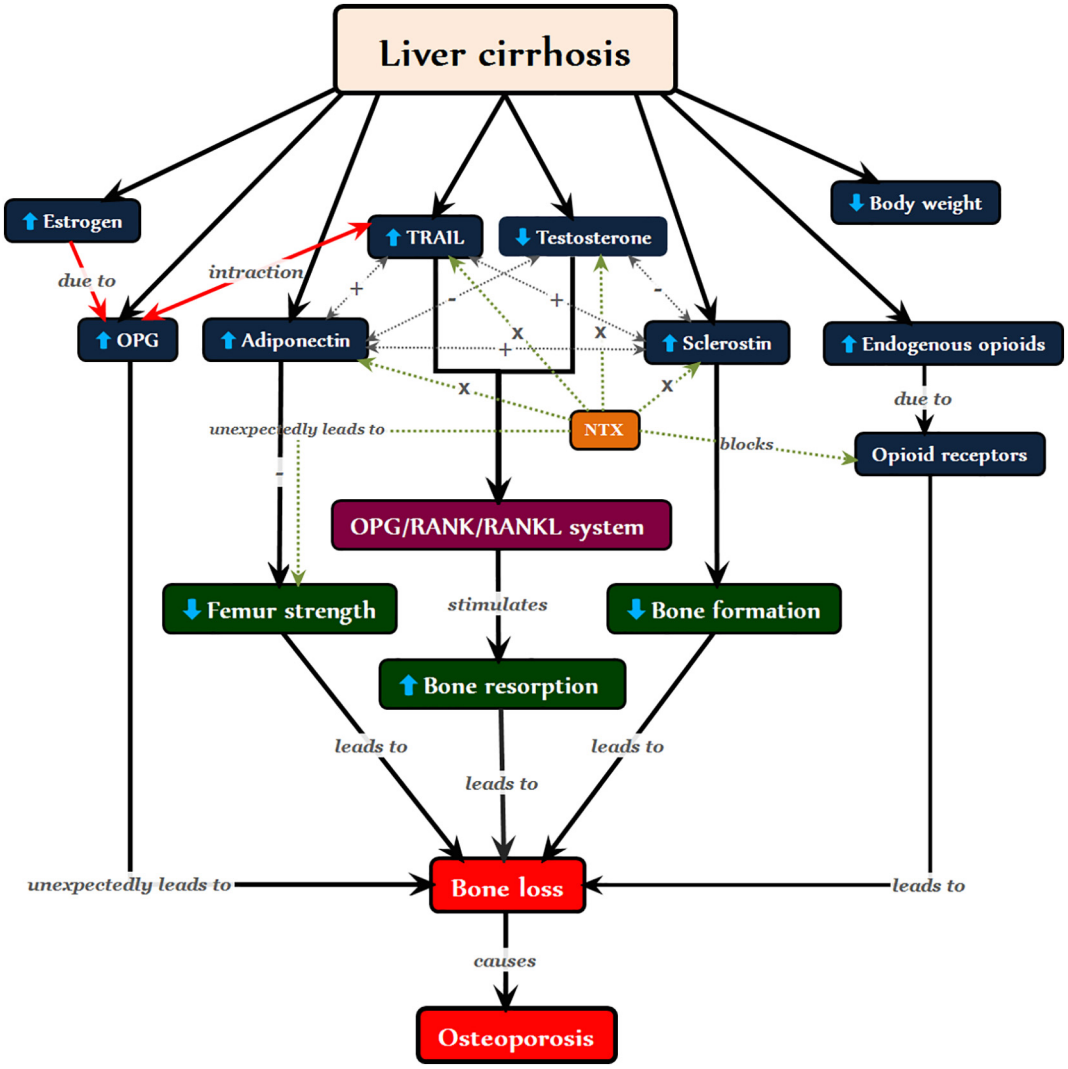

Fig. 6. Concept diagram showing the altered status of factors in BDL rats. (+) positive correlation, (−) negative correlation between parameters, (×) Inhibition.

Declaration of competing interest

The authors declare that they have no conflict of interest related to the data or interpretation of this study.

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