Research in Pharmaceutical Sciences, June 2018; 13(3): 239-249
Received: October 2017
Accepted: January 2018

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

A study on OPG/RANK/RANKL axis in osteoporotic bile duct-ligated rats and the involvement of nitrergic and opioidergic systems

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Abstract

Chronic liver disease (CLD) affects millions of people and its impact on bone loss has become a subject of interest. Nitric oxide and endogenous opioids are suggested to increase during cholestasis/cirrhosis and may impact bone resorption by different mechanisms. The receptor activator of nuclear factor-κB (RANK)/RANK-ligand (RANKL)/osteoprotegerin (OPG) signaling pathway regulates bone resorption, but its role in metabolic bone disease subsequent to CLD is unknown. We aimed to investigate the involvement of nitrergic and opioidergic systems in bone loss relative to the RANK/RANKL/OPG pathway, in bile duct-ligated (BDL) rats. Eighty BDL/sham-operated (SO) rats received injections of 3 mg/kg/day Nω-Nitro-L-arginine methyl ester ± naltrexone (10 mg/kg/day) or saline for 28 days. Plasma bone turnover markers, OPG, RANK, and RANKL along with mRNA expression levels of the latter three were assessed. Plasma bone turnover markers and OPG level increased, but RANKL decreased in the BDL group compared with their SO controls (both: $P \leq 0.05$). Administration of naltrexone reduced bone turnover markers and OPG level while increased RANKL content in comparison to BDL rats ($P \leq 0.05$). As compared to untreated BDL rats, nitric oxide inhibition showed no effect on bone turnover marker *i.e.* OPG, RANK, and RANKL levels. BDL significantly increased RANK mRNA, but had no significant effect on RANKL and OPG mRNA expression. The lack of association between plasma levels and quantitative gene expression of RANKL and OPG suggests an indirect function of these markers in BDL rats. Considering that opioid receptor blockage by naltrexone in BDL animals caused a significant decrease in OPG and an increase in RANKL plasma contents, it could be postulated that the opioidergic system may have a regulatory effect on these bone markers.

Keywords: Cirrhosis; Bone loss; Nitrergic system; Opioidergic system; BDL rats; RANK/RANKL/OPG axis.

INTRODUCTION

In chronic liver diseases (CLD), metabolic disturbance of bone is a frequent complication with the prevalence of 12% to 55% and can result in spontaneous or low-trauma fracture (1,2). However, the pathogenesis of hepatic osteodystrophy is multifactorial and remains unclear in some aspects. The malabsorption of vitamin D and calcium, genetic factors, low insulin-like growth factor 1, reduced osteoblast function, increased osteoclastic activity and receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin (OPG) system may contribute to the imbalance between bone formation and resorption (3-5).Bone remodeling is mediated by bone-forming osteoblasts and bone-resorbing osteoclasts and this process converges at the RANKL/OPG axis (6).

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Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/1735-5362. 228954

Osteoclast development requires an interaction between receptor activator of nuclear factor κB (RANK) (a surface receptor on osteoclasts) and RANKL (a secreted protein from osteoblasts). On the other hand, osteoblasts secrete an osteoclastogenesis-inhibitory factor or decoy receptor; OPG, that specifically binds to RANKL and inhibits RANK-RANKL interaction (7-9). Following this inhibition, osteoclastogenesis is interrupted by OPG; subsequently osteoclasts activity and bone resorption are reduced (9). Currently, the role of both OPG and RANKL in metabolic bone disease in CLD is unknown.

Several events occur during cholestasis and cirrhosis of which is the over production of nitric oxide (NO) through inducible nitric oxide synthase (iNOS) in the inflammatory stage of the disease which can affect various cell types and processes in the tissues (10). Furthermore, some studies showed that NO production via iNOS induces bone loss (11). Also it is proven that NO affects osteoclastogenesis through its effect on the local RANKL/OPG equilibrium (12).

Another important phenomenon in CLD is an increase in the level of endogenous opioids such as methionine enkephalin and leucine enkephalin (13). These substances have anti-inflammatory and immunomodulatory properties, associate with changes in bone mass, can be considered as a risk factor for the development of osteoporosis and demonstrating a role during skeletal ontogeny (14). Osteoblastic cells synthesize high levels of proenkephalin (an endogenous opioid) and proenkephalin-derived peptides, which exert inhibitory effects on osteoblastic alkaline phosphatase activity (15). There is limited research on the effect of endogenous opioids on osseous tissues. After bone fracture, some selective agonists of opioid receptors in the bone regeneration process accelerate the development of newly-synthesized spongy bone tissue (16). On the other hand, some studies have shown that administration of methionine enkephalin inhibits osteoblast cell growth in culture, and is reversed by naltrexone (17). Considering the enhancement of endogenous opioids and NO levels, and unknown roles of OPG and RANKL in CLD, we, for the first time, aimed to investigate the involvement of NO and the opioid system on OPG/RANK/RANKL axis in bile duct-ligation (BDL) model of cirrhosis. BDL which is a classical model for the study of chronic liver disorders and cirrhosis-induced osteoporosis was used in this study (18).

MATERIALS AND METHODS

Reagents

Naltrexone hydrochloride (NTX HCl, a non-selective opioid receptors antagonist) and Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME HCl, a non-selective inhibitor of nitric oxide synthase (NOS)) were purchased from Sigma (St Louis, USA) and were dissolved in sterile isotonic saline solution and administered intraperitoneally (i.p.) in a volume of 2.5 mL/kg of the rat body weight.

Animal manipulation

Male Sprague–Dawley rats (200-250 g) with free access to food and water were housed in groups in an environment with a temperature of 23 ± 2 °C, 50 ± 5% humidity, and a 12 h light/dark cycle. All animal procedures were in accordance with the 'Guide for the Care and Use of Laboratory Animals' (NIH US publication No. 85-23 revised 1985).

Animal model of cholestasis

Eighty animals were divided into eight groups of 10 animals each. (1) BDL animals treated with normal saline, (2) BDL animals treated with L-NAME (3 mg/kg), (3) BDL animals treated with NTX (10 mg/kg), (4) BDL animals treated with NTX (10 mg/kg) + L-NAME (3 mg/kg). For each group equal number of sham-operated (SO) animals were prepared. BDL was induced in rats as described previously (13). The animals were given intraperitoneal injections of L-NAME (3 mg/kg), NTX (10 mg/kg) or saline, once daily for 28 days after BDL or sham surgery according to previous studies (19,20). L-NAME or NTX treatments were begun at the day of BDL or SO surgery. On day 28, animals were sacrificed by exsanguination (cardiac puncture) under general anesthesia, and their femur and blood were removed. The right femur tissue was fixed in 10% formalin; also the left femur samples were stored in liquid nitrogen until use.

Nitric oxide metabolites

Nitrate and nitrite as an index of NO production were measured in plasma samples using Griess method (21).

Bone turnover markers

For the measurement of cross linked C-telopeptide of type I collagen (CTX-I) and procollagen I N-terminal peptide (PINP), blood was drawn from animals into 5 mL vacuum tubes with added sodium heparin as anti-coagulant. Blood was centrifuged at 3000 rpm for 10 min at 4 °C and the plasma was collected in 2 mL plastic tubes. Samples were stored at -70 °C until measurement. Plasma CTX-I and PINP level were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit in accordance with the protocol of the manufacturer (MyBiosource, San Diego, CA, USA).

Plasma OPG, RANK and RANKL determinations

The plasma samples were used for OPG, RANK and RANKL measurements. These proteins were also measured by a commercially ELISA kit (MyBiosource, San Diego, CA, USA).

TRAP staining (enzyme histochemistry)

After fixation, all right femora were decalcified in 15% (w/v) ethylenediamine tetraacetic acid (EDTA, pH 7.4) for 3 weeks and embedded in paraffin. Five-μ-thick sections were cut and stained with tartrate-resistant acid phosphatase (TRAP, Sigma Aldrich, St. Louis, MO, USA) which is regarded as a biochemical marker relatively specific for osteoclasts (22). $TRAP^+$ osteoclasts were counted on the trabecular bone surface in a 2.4 mm^2 area immediately below the chondro-osseous junction, under a double-headed light microscope (Olympus BX51) by two observers, blind to the experimental groups. Any disagreements were resolved by consensus.

RNA isolation and real-time PCR

The left femur bones were pulverized in liquid nitrogen with a mortar and pestle that had been pre-chilled in dry ice (23). Powdered bone was homogenized in One Step-RNA reagent (BioBasic, Canada) and RNA extraction was performed. The quality and quantity of the RNA samples were assayed spectrophotometrically, with the ratios of absorbance at 260 nm and 280 nm ranging from 1.8 to 2.0, and the integrity of the RNA preparations was examined by agarose gel electrophoresis. Complementary DNA (cDNA) was synthesized using 500 ng of RNA through a reverse transcription reaction (Takara Bio Inc., Tokyo, Japan) in proportion to the manufacturer's instructions. Real-time PCR was performed using a RotorGene 3000 instrument (Corbett Research, Australia). Primers used for OPG, RANK, RANKL, iNOS and β-actin are listed in Table 1. The mRNA levels of OPG, RANK, RANKL and iNOS were normalized to β- actin mRNA levels. PCR was run as 40 cycles at 95 °C for 15 s, 62 °C for 30 s, and 72 °C for 30 s. The negative controls for each target showed an absence of carryover. Data of target mRNA copies were calculated relative to β- actin using the $2^{-\Delta Ct}$ method.

Table 1. Primer sequences used in this study

Primer name		Sequence (5'-3')
OPG	Forward	TCTGCCTCTGATAGTCTATG
	Reverse	TAACGGTCAACTTGCCTA
RANK	Forward	ACGGAATCAGATGTGGTC
	Reverse	AGACTGGGCAAGTAAACC
RANKL	Forward	ACCAGCATCAAAATCCCAAG
	Reverse	ACGCTAATTTCCTCACCAG
iNOS	Forward	ACACAGTGTCGCTGGTTTGA
	Reverse	AACTCTGCTGTTCTCCGTGG
β-Actin	Forward	GCAGGAGTACGATGAGTCCG
	Reverse	ACGCAGCTCAGTAACAGTCC

(OPG) osteoprotegerin; (RANK) receptor activator of nuclear factor-κB; (RANKL) RANK-ligand; and (iNOS) inducible nitric oxide synthase.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM) for at least three repeats of the assays. Statistical differences between BDL and SO related groups were analyzed by Student's T test and Mann-Whitney followed by Tukey post-hoc test. Statistical significance between BDL groups was assessed by two-way ANOVA, followed by Bonferroni post-hoc test. Comparative C_T method was used for analysis of the gene expression and associations between variables were calculated by Pearson correlation. A *p*-value of 0.05 or less was considered as statistically significant.

RESULTS

Induction of cholestatic state

No abnormal histopathological changes could be found in the livers of SO animals, in contrast to the BDL rats that showed different degrees of liver damage. A mild decrease in liver alterations was observed in rats receiving L-NAME and NTX when compared with the BDL animals. Also, in accordance with histopathological changes, alkaline phosphatase (ALP) activity and total bilirubin levels showed an increase in BDL group, suggesting the occurrence of cholestasis (data not shown).

Markers of bone turnover

As can be seen in Table 2, plasma CTX-I (bone resorption marker) and PINP (bone formation marker) significantly increased in the BDL, BDL + L-NAME and BDL + L-NAME + NTX groups as compared with related SO control ($P \leq 0.05$). However, administration of NTX caused a significant decrease in CTX-I and PINP content as compared to BDL, BDL + L-NAME and BDL + L-NAME + NTX groups ($P \leq 0.05$).

Trap staining

TRAP assay showed that the number of osteoclasts in the BDL group were significantly higher than that in the relevant SO control group ($P \leq 0.05$). As shown in Table 2, administration of NTX and L-NAME caused a significant decrease in $TRAP^+$ osteoclasts as compared to the BDL group ($P \leq 0.05$).

OPG/RANK/RANKL system

As shown in Fig. 1A, plasma OPG levels significantly increased in the BDL, BDL + L-NAME and BDL + L-NAME + NTX groups as compared to their relevant SO control groups ($P \leq 0.05$). Nevertheless, the administration of NTX caused a significant decrease in OPG content as compared to BDL, BDL + L-NAME and BDL + L-NAME + NTX groups ($P \leq 0.05$). The results also, showed that the plasma RANK levels (Fig. 1B) significantly decreased in the BDL + L-NAME + NTX group as compared with BDL and BDL + NTX groups ($P \leq 0.05$). As shown in Fig. 1C, plasma RANKL levels significantly decreased in the BDL and BDL + L-NAME groups as compared with their relevant SO control groups.

Table 2. Markers of bone turnover in BDL and SO rats

	Saline		L-NAME		NTX		L-NAME + NTX	
Parameter	**SO**	**BDL**	**SO**	**BDL**	**SO**	**BDL**	**SO**	**BDL**
CTX-I (ng/mL)	2.17 ± 0.18	8.64 ± 1.57$^{*\#3}$	1.95 ± 0.27	8.83 ± 0.83$^{*\#3}$	1.92 ± 0.32	1.71 ± 0.21$^{\#1,2,4}$	2.19 ± 0.33	9.58 ± 1.33$^{*\#3}$
PINP (µg/mL)	0.68 ± 0.04	2.86 ± 0.17$^{*\#3}$	0.50 ± 0.04	2.52 ± 0.27$^{*\#3}$	0.51 ± 0.05	0.68 ± 0.13$^{\#1,2,4}$	0.58 ± 0.09	2.87 ± 0.18$^{*\#3}$
$TRAP^+$ osteoclast number	0.40 ± 0.24	6.20 ± 2.05$^{*\#2,3}$	0.00	0.00$^{\#1}$	2.00 ± 0.44	0.60 ± 0.40$^{*\#1}$	1.71 ± 0.52	1.40 ± 0.40

(CTX-I) cross linked C-telopeptide of type I collagen; (PINP) procollagen I N-terminal peptide; (TRAP) tartrate-resistant acid phosphatase; (SO) sham-operated; (NTX) naltrexone; (L-NAME) Nω-nitro-L-arginine methyl ester hydrochloride; and (BDL) bile-duct-ligated. CTX-I, PINP, and $TRAP^+$ osteoclast cell count of the study using SO, NTX, L-NAME, and NTX + L-NAME treated BDL cirrhotic rats (test). Each value represents mean ± SEM, n = 7, for the assays which performed in duplicate. *Significantly different from related SO, *P*-value ≤ 0.05. #Significantly different from BDL cirrhotic rats (test) group, [1]BDL saline; [2]BDL + L-NAME; [3]BDL + NTX; and [4]BDL + L-NAME + NTX, *P*-value ≤ 0.05.

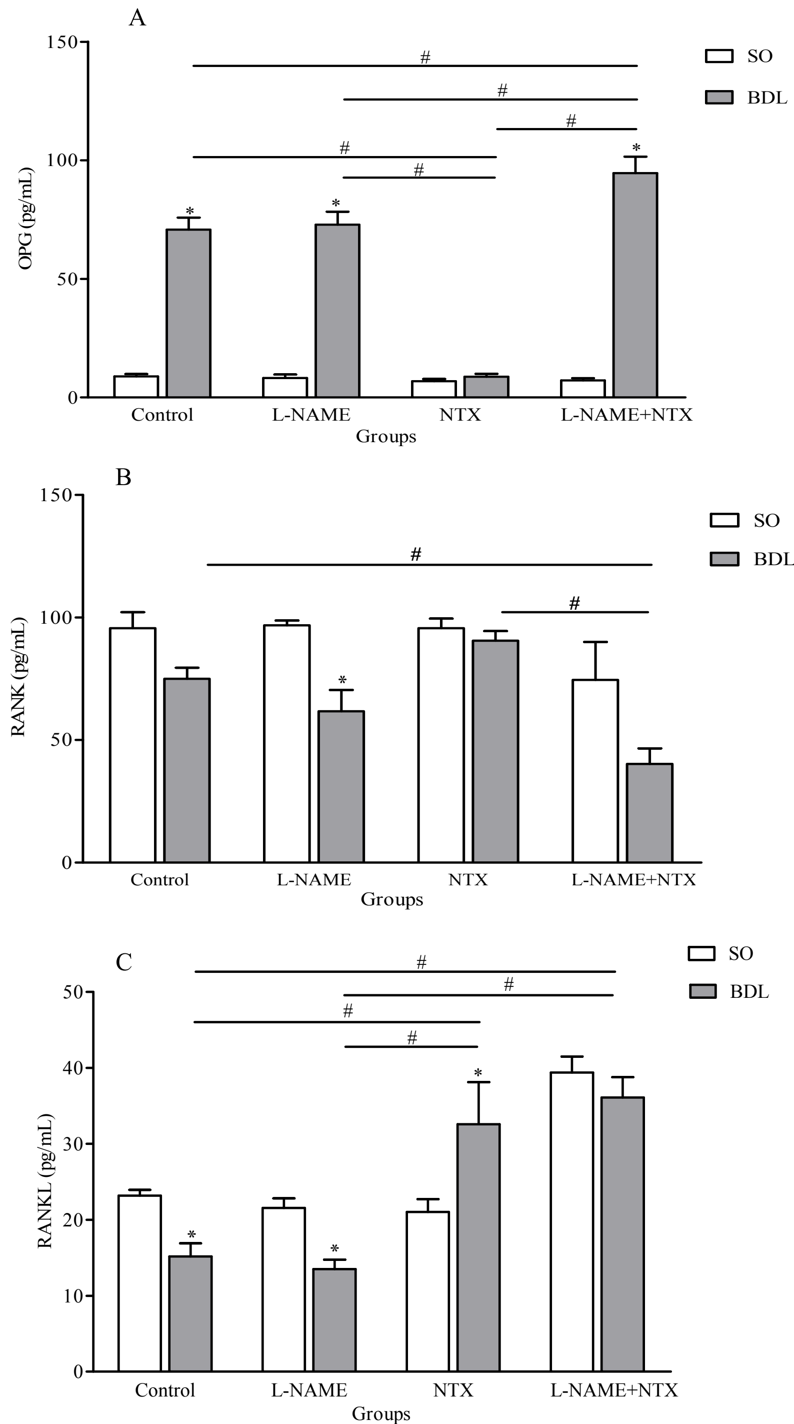

Fig. 1. OPG, RANK and RANKL protein levels of the study using SO, NTX, L-NAME, and NTX + L-NAME treated BDL cirrhotic rats (test). Each value represents mean ± SEM (n = 7) and the assays performed in douplicate. (A) OPG; (B) RANK; and (C) RANKL. *Significantly different from related SO, p-value ≤ 0.05. #Significantly different from BDL cirrhotic rats (test) group, p-value ≤ 0.05. (OPG) osteoprotegerin; (RANK) receptor activator of nuclear factor-Kb; (RANKL) RANK-ligand; (SO) sham-operated; (NTX) naltrexone; (L-NAME) Nω-nitro-L-arginine methyl ester hydrochloride; and (BDL) bile-duct-ligated.

In contrast, this factor significantly increased in the BDL + NTX group as compared with its relevant SO control rats ($P \leq 0.05$). Administration of NTX and L-NAME + NTX caused a significant increase in RANKL content as compared to BDL and BDL + L-NAME groups ($P \leq 0.05$). Bile duct ligation significantly increased transcript abundance of

RANK as compared with relevant SO control groups ($P \leq 0.05$); and however, had no significant effect on RANKL and OPG. Treatment with NTX, L-NAME or L-NAME + NTX had no effect on OPG and RANK as compared to untreated BDL and relevant SO control groups ($P \leq 0.05$) (Figs. 2A-C).

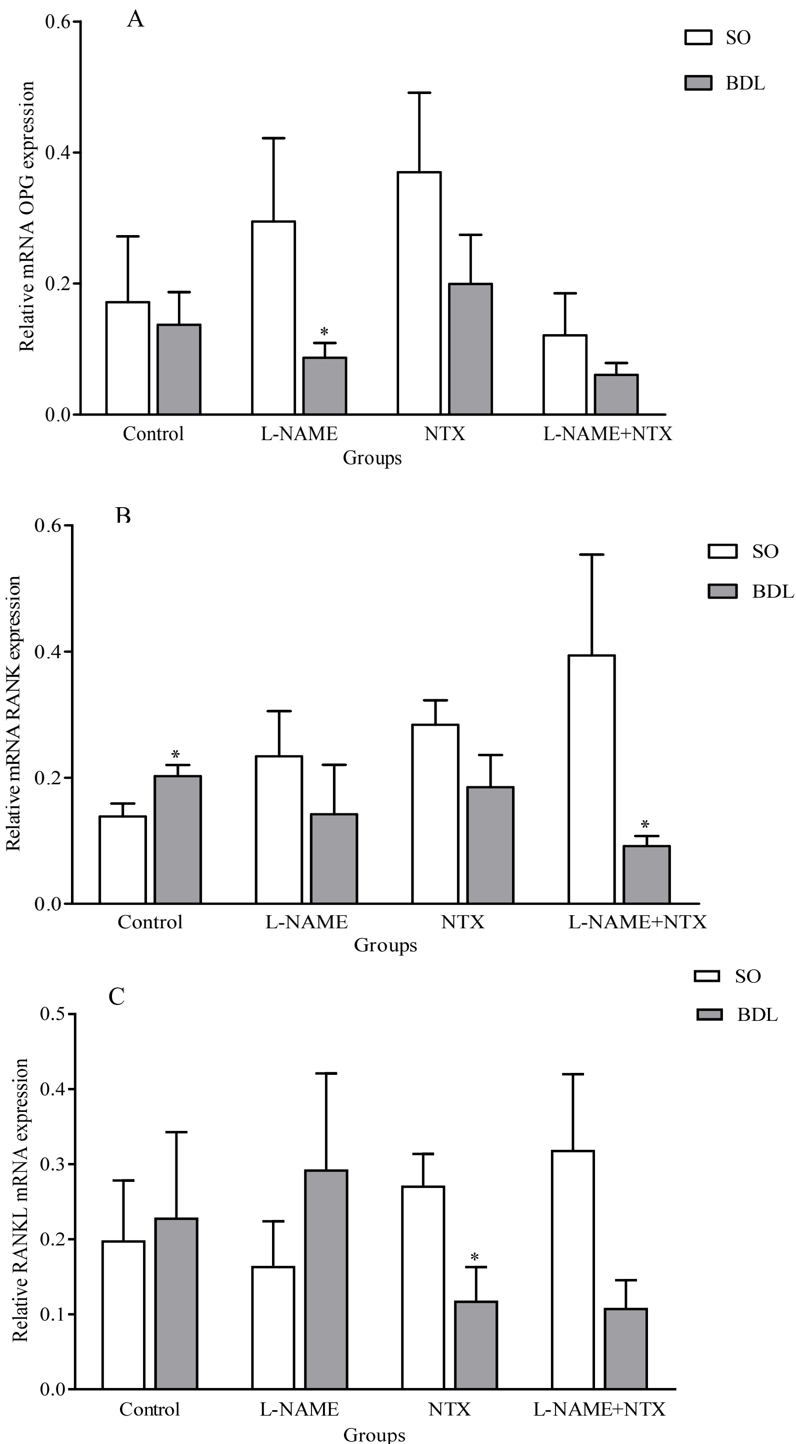

Fig. 2. OPG, RANK, and RANKL expression in the left femur bones of rats of the study using SO, NTX, L-NAME and NTX + L-NAME treated BDL cirrhotic rats (test) were detected at mRNA levels by RT-PCR. Each value represents mean ± SEM (n = 5) and the assays performed in duplicate. (A) OPG; (B) RANK; and (C) RANKL. * Significantly different from related SO, p-value ≤ 0.05. # Significantly different from BDL cirrhotic rats (test) group, p-value ≤ 0.05. (OPG) osteoprotegerin; (RANK) receptor activator of nuclear factor-Kb; (RANKL) RANK-ligand; (SO) sham-operated; (NTX) naltrexone; (L-NAME) Nω-nitro-L-arginine methyl ester hydrochloride; and (BDL) bile-duct-ligated.

NO metabolites

As can be seen in Fig. 3A, plasma NO metabolites level significantly increased in the BDL group as compared with a related SO control group ($P \leq 0.05$). Administration of L-NAME in the BDL group caused a significant decrease in NO metabolites as compared to untreated BDL and BDL + L-NAME + NTX groups ($P \leq 0.05$). Transcript abundance of iNOS was not significantly increased in the untreated BDL group as compared to the related SO control group (Fig. 3B).

Analysis of correlations between studied parameters

We found statistically significant correlations between plasma OPG and ALP activity and total bilirubin content. Also, positive correlations were observed between bone turnover markers (PINP and CTX-I) and OPG plasma levels. However, a negative association was found between OPG plasma levels and its mRNA expression in femoral bone tissue (Table 3).

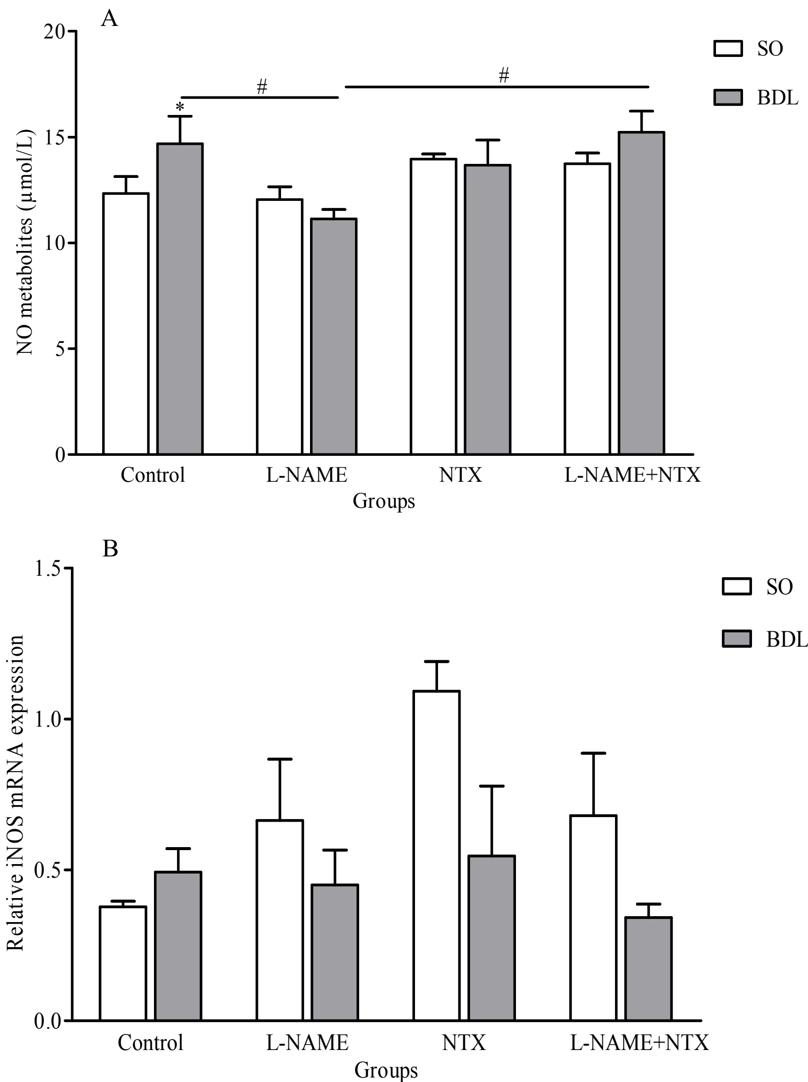

Fig. 3. NO metabolites and iNOS expression in the plasma and left femur bones of the study using SO, NTX, L-NAME and NTX + L-NAME treated BDL cirrhotic rats (test). Each value represents mean ± SEM (n = 7) and the assays performed in duplicate. (A) NO metabolites and (B) iNOS expression *Significantly different from related SO, *p*-value ≤ 0.05. #Significantly different from BDL cirrhotic rats (test) group, *p*-value ≤ 0.05. (OPG) osteoprotegerin; (RANK) receptor activator of nuclear factor-Kb; (RANKL) RANK-ligand; (SO) sham-operated; (NTX) naltrexone; (L-NAME) Nω-nitro-L-arginine methyl ester hydrochloride; (BDL) bile-duct-ligated; and (iNOS) inducible nitric oxide synthase.

Table 3. Statistical significant correlations between studied parameters

Correlations	Correlation coefficient	*P* value
OPG plasma levels *vs.* PINP	0.860	0.000
OPG plasma levels *vs.* CTX-I	0.757	0.000
OPG plasma levels *vs.* OPG mRNA expression in femur tissue	-0.414	0.019
RANK plasma levels *vs.* RANK mRNA expression in femur tissue	0.212	NS[a]
RANKL plasma levels *vs.* RANKL mRNA expression in femur tissue	-0.116	NS

(OPG) osteoprotegerin; (PINP) procollagen I N-terminal peptide; (CTX-I) cross linked C-telopeptide of type I collagen; (RANK) receptor activator of nuclear factor-κB; (RANKL) RANK-ligand; and (NS) [a]non-significant.

DISCUSSION

Several studies have demonstrated that cirrhotic patients are at a higher risk of fractures and liver cirrhosis is associated with metabolic bone disorders (24,25). Optical density increases with decreased bone mineral density (26). Regarding this fact in the skull and alteration of histomorphometric indices in the tibia bones, we showed that BDL leads to bone loss in cirrhotic animals (27). The function of OPG/RANK/RANKL axis as a regulator of bone remodeling and osteoclastogenesis in the development of osteoporosis in cirrhosis and chronic liver diseases is uncertain. Nitric oxide and endogenous opioids increased in the plasma of animals with cholestatic liver disease, but, their effects on OPG/RANK/RANKL system have not been studied. In the current investigation, NTX and L-NAME were used to investigate the role of endogenous opioids and NO on OPG/RANK/RANKL system in BDL cirrhotic rats.

The CTX-I and PINP levels, which represent the activity of bone turnover, were significantly increased in the BDL group. In fact, liver fibrogenesis due to increased collagen metabolism may influence the levels of bone markers, mainly those related to collagen synthesis and degradation (28). Bone turnover markers showed a trend towards normal levels in the NTX receiving BDL group. Moreover, we found a positive correlation between bone turnover markers and OPG plasma levels.

TRAP is a recognized marker for identification of osteoclasts and pre-osteoclasts (29). A fewer number of these cells were observed after treatment of the BDL rats with L-NAME and NTX, but not L-NAME + NTX. Bone resorption activity could be estimated using CTX-I levels and numbers of osteoclasts. The significant decrease in $TRAP^+$ cells following administration of NTX to BDL animals was confirmed by the reduction in CTX-1 plasma levels.

In this study, we demonstrated high OPG and low RANKL in the plasma of BDL rats as compared to the SO control group. Previous studies have shown high serum OPG and low, normal or high RANKL levels in patients with chronic liver diseases such as primary biliary cirrhosis (PBC), cirrhotic patients and alcoholic cirrhosis (3,4,30-33).

The evidence for the osteoclastogenic activity of RANKL and the inhibitory nature of OPG, the decoy receptor for RANKL is convincing (34,35). Since osteoblast function is reduced in PBC and OPG is produced mostly by osteoblast cells, we would expect lower OPG levels in BDL rats than in a SO control group. However, the high levels of OPG found in our study may be the result of one of the following states: (a) production by other cells or tissues for instance by fibroblasts (3) and (b) osteoblasts dysfunction, or reflection of a compensatory response to increased osteoclast activity which results in increased levels of tumor necrosis factor related apoptosis-inducing ligand (TRAIL) which is a blocking agent of OPG's anti-osteoclastogenic activity (36). Other reasons of increased OPG levels may be attributed to different procedures used to assay circulating OPG in which the monomeric or homodimeric forms are measured (37).This is a consequence of metabolism of OPG in the liver and by way, of augmented serum level of pro-inflammatory cytokines such as TNF-α, IL-6, IL-I, and TGF-β that reported in cholestatic/cirrhosis (3). In a previous study it was reported that since TNF-α and IL-6 enhance bone resorption, their relation with OPG suggests a protective effect of raised OPG on bone loss (33).

Low levels of osteoclastogenic RANKL in BDL rats are unexpected and this may represent a role for the RANKL system in the development of bone loss in CLD. Low levels of RANKL may be a compensatory reaction to increased bone loss due to a yet unrevealed mechanism.

According to our findings, opioid receptor blockade caused a significant decrease in OPG content, a significant increase in RANKL and a slight increase in RANK as compared to untreated BDL rats. However, NO inhibition did not exhibit any significant effect on OPG, RANKL, and RANK as compared to untreated BDL rats.

Our results showed that L-NAME had no effect on CTX-I, PINP, and OPG level while NTX caused significant decrease in these parameters as compared to untreated BDL animals. Furthermore, L-NAME + NTX had significant increase in these parameters as compared to NTX group; according to these results L-NAME may have opposed the beneficial effects of NTX in osteogenic markers of BDL rats.

Also, OPG, RANK and RANKL gene expression are determined in femoral bone tissues of cirrhosis liver diseases. No significant differences were observed in the quantitative OPG and RANKL gene expression in BDL samples and related SO rats.

However, the lack of association between plasma levels and the quantitative gene expression in the bones of BDL rats support an indirect mechanism for the increased plasma OPG levels in BDL rats. Simonet *et al*. (38), stated that, since OPG is a secreted protein, the place of its expression does not essentially foresee the site (s) at which its biological function is exerted. Guanabens *et al.* (30) showed that OPG serum levels were not related to bone mineral density and to bone remodeling indices when patients were analyzed in accordance with bone formation and resorption marker levels, apart from osteocalcin, which was significantly lower in patients with normal OPG levels.

Our results showed that NO inhibition did not show any effect on bone turnover markers as compared to untreated BDL rats. In addition, L-NMAE had no significant effect on OPG, RANKL and RANK in both plasma and femoral bone transcripts as compared to BDL rats. Xian *et al*. showed that RANKL protein decreased and OPG protein elevated in bone marrow stromal cells after treatment with NO (12), however, in our study increasing plasma NO levels in BDL rats had no effect on OPG and RANKL.

CONCLUSION

In summary, considering that NO inhibition by L-NMAE in BDL animals had no significant effect on OPG and RANKL plasma contents, it could be postulated that the effect of the nitrergic system on these bone markers is limited. On the other hand, injection of the opioid-receptors blocker, naltrexone, caused a significant decrease in OPG and a significant increase in RANKL plasma contents in BDL rats, which could suggest a regulatory effect of the opioidergic system on these bone markers.

ACKNOWLEDGMENTS

This project was financially supported via the Grant No. 27249 by the Research Council of Tehran University of Medical Sciences, Tehran, I.R. Iran.

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