Nitrergic and opioidergic systems affect radiographic density and histomorphometric indices in bile-duct-ligated cirrhotic rats

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Summary. Metabolic bone disease is a major issue in chronic liver disease. Increased production of nitric oxide (NO) and elevation of endogenous opioids have been suggested to occur during cholestasis/cirrhosis.

We aimed to investigate the involvement of nitrergic and opioidergic systems in bone loss after bile-duct-ligation (BDL) in rats using optical density (OD) evaluation and histomorphometric analysis.

BDL- and sham-operated (SO) rats received injections of 3 mg/kg No-Nitro-L-arginine methyl-ester-hydrochloride (L-NAME) as an NO-synthase inhibitor, 10 mg/kg naltrexone (NTX) as an opioid-receptors antagonist or saline once daily for 28 days. Lateral cephalometric radiographs were taken on days 0 and 28 and histomorphometric and biochemical indices were measured.

Plasma levels of total bilirubin and alkaline-phosphate were markedly increased in BDL compared with SO rats (p≤0.05). Among the studied variables, osteoclast number/mm trabecular surface showed significant increase in BDL animals compared to controls, which was significantly reduced following NO-synthase inhibition (p≤0.05). Similarly, cortical area slightly decreased in BDL animals in comparison to controls, whereas both L-NAME and NTX significantly increased this variable. Following BDL, optical density increased in the skulls of cirrhotic animals and showed a significant decrease after blocking opioid-receptors (p≤0.05).

Inhibition of NO-synthase and/or opioid receptors caused significant changes in OD and histomorphometric parameters in BDL rats, both in favor of reducing bone loss. If confirmed by further studies, it seems that manipulation of these systems might be able to improve bone problems in subjects with cholestasis/cirrhosis.

Key words: Cirrhosis, Bone loss, Nitrergic system, Opioidergic system, Rat

Introduction

Metabolic bone disease presenting as osteopenia/osteoporosis is one of the most important complications of cholestasis and cirrhosis (López-Larramona et al., 2015), which leads to reduced bone mass and increased fracture risk (Derakhshanian et al., 2013). The mechanisms resulting in osteoporosis in liver disease have not been clarified; however, reduced bone formation because of increased osteoblast function, increased bone resorption and imbalances in bone turnover might be responsible (Dresner-Pollak et al., 2008; Pereira et al., 2009).

Several events take place during cholestasis and cirrhosis, one of which is increased production of nitric oxide (NO) through inducible (inflammatory) NOS (nitric oxide synthase) mediation. NO over-production in cholestasis depends on the inflammatory stage of the disease which can affect various cell types and processes in the tissue (Mohammed et al., 2003; Tarsitano et al., 2007; Mahmoud et al., 2015), for example it has been shown to impact the bone remodeling cycle via cGMP
(Cyclic Guanosine Monophosphate) augmentation (Nilforoushan et al., 2009).

Another important phenomenon in cholestasis and cirrhosis is an increase in the level of endogenous opioids (Bergasa and Jones, 1992; Bergasa et al., 1994). These substances have anti-inflammatory properties in addition to demonstrating immunomodulatory characteristics (Queiroz-Junior et al., 2013). There is limited research on the effect of endogenous opioids on osseous tissues. An example of previous work in this regard is a study by Queiroz-Junior et al. (2013) who showed that inhibition of opioid receptors by an antagonist can result in alveolar bone loss in rats.

Considering the important osseous outcomes of cholestatic liver disease/cirrhosis including risk of fractures and severe bone loss (Ackerman et al., 2002; Dresner-Pollak et al., 2008) we aimed to investigate two of the main pathways involved in this disease including NO- and the opioid-systems. Bile duct ligation (BDL) is a classical model for the study of chronic liver disorders which leads to cirrhosis and was used in the present investigation to develop bone disease as performed in former studies (Ackerman et al., 2002; Dresner-Pollak et al., 2008; Pereira et al., 2009).

Materials and methods

Reagents

The opioid-receptors-antagonist naltrexone (NTX HCl) and the NOS inhibitor No-Nitro-L-arginine methyl ester hydrochloride (L-NAME HCl) were obtained from Sigma (St Louis, MO, USA). All other materials and reagents were purchased from Merck (Darmstadt, Germany) unless otherwise specified.

Animal manipulation

Male Sprague-Dawley rats (200-250 g) were housed in a temperature-controlled vivarium with a 12:12 h light-dark cycle and free access to food and water. All animal procedures were in agreement with the ‘Guide for the Care and Use of Laboratory animals’ (NIH US publication no. 85-23 revised 1985). The protocol for this research was approved by the Ethics Committee of our University.

Animal model of cholestasis

Rats were evaluated in eight experimental groups, with approximately 10 animals in each group: (a) sham-operated (SO) controls treated with saline, (b) BDL animals treated with saline, (c) SO controls treated with 3 mg/kg L-NAME, (d) BDL animals treated with 3 mg/kg L-NAME, (e) SO controls treated with 10 mg/kg NTX, (f) BDL animals treated with 10 mg/kg NTX, (g) SO controls treated with 10 mg/kg NTX + 3 mg/kg L-NAME, (h) BDL animals treated with 10 mg/kg NTX + 3 mg/kg L-NAME.

Bile duct ligation was performed in rats as described previously (Dresner-Pollak et al., 2008; Ostadhadi et al., 2015). In brief, animals were laparotomized under general anesthesia induced by ketamine HCl (50 mg kg\textsuperscript{-1}) and xylazine HCl (10 mg kg\textsuperscript{-1}). The common bile duct was exposed through a midline abdominal incision and was then ligated in two loci with a silk thread and sectioned between the ligatures. Sham operated age-matched rats served as controls. Sham operation consisted of laparotomy and bile duct identification and manipulation without ligation. The animals received intraperitoneal injections of L-NAME (3 mg/kg), NTX (10 mg/kg) or saline once daily for 28 days after BDL or sham surgery. On day 28, animals were sacrificed by exsanguination (cardiac puncture) under general anesthesia. This timeline has been shown to induce bone disease in BDL rats and was selected based on previous studies (Ackerman et al., 2002; Dresner-Pollak et al., 2008; Pereira et al., 2009). Right tibia samples were collected and immediately fixed in 10% formalin for further studies.

Liver histology

Liver tissue from each animal was fixed in 10% formalin and subjected to routine histologic processing. 6-μ sections were stained with hematoxylin and eosin and evaluated by light microscopy. Histologic scoring of liver damage induced by BDL was graded as follows based on previous studies (Dresner-Pollak et al., 2008):

- Portal inflammation: none, 0; mild, 1; moderate, 2; marked/severe, 3.
- Bile duct proliferation: none, 0; mild, 1; moderate, 2; marked/severe, 3.
- Fibrosis: none, 0; portal expansion, 1; bridging fibrosis, 2; cirrhosis, 3.

Biochemical analysis

Total bilirubin and alkaline phosphatase (ALP) were measured using relevant diagnostic kits (Pars Azmoon Inc., Tehran, Iran).

Radiographic density evaluation

According to previous studies, optical density (OD) is reversely correlated with bone density (Hernandez-Vaquero et al., 2005) and has been widely used to assess bone defects induced by different diseases (Shirazi et al., 2001, 2014). We chose this method because of its lower cost and simplicity regarding animal manipulation. Lateral cephalometric radiographs were taken on days 0 and 28 in a custom-made cephalostat under anesthesia induced by ketamine 50 mg kg\textsuperscript{-1} HCl and 10 mg kg\textsuperscript{-1} xylazine HCl according to the method developed by Gaegauf-Zollinge et al. (1982). Care was taken during cephalostat controlled positioning and projection geometry so that it did not interfere with the reading of the graphic optical densities. A standard radiographic...
machine (Trophy, Vincennes, France) with 50 kVp (peak kilovoltage), 10 mA and an exposure time of 0.25 sec was used for all radiographs in this study. The focus-film distance was constant at 50 cm, and the film used was periapical number 2 Kodak E film “31x41 mm” (size no. 4) Ultra speed (Eastman Kodak, Rochester, NY). The radiographic films were processed by an automatic film processor (Velopex Extrax, Medivance, UK) in a standard darkroom. OD readings using a digital densitometer (Tobias TBX, Ivyland, PA, USA) were made in triplicate in 1 mm perimeter around each of the points as described below:

1. Point 1 (on the skull): the most posterior point on the cranium.
2. Point 2 (on the mandibular bone): the intersection between the Go-Mn (Gonion to Menton) line and the vertical to the Go-Mn line through GN (Gnathion).

Histomorphometric studies

Bone histomorphometry was assessed in 7 animals of each group. At the end of the experimental period, the right tibia was carefully dissected and immediately fixed in 10% formalin, decalcified, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Sections (6-µ thick) were cut on a microtome (RM2125 RT; Leica, Nussloch, Heidelberg, Germany), and followed by hematoxylin-eosin staining. Measurements were performed on the cancellous bone of the right proximal tibial metaphysis, at a distance of 195 µ from the epiphyseal growth plate, in a total of 20 fields using an Olympus BX51 light microscope equipped with a digital camera (DP25, Olympus) and analysis software (DP2-BSW, Olympus). The histomorphometric indices were selected based on previous studies (Derakhshanian et al., 2013) and are listed as follows: trabecular bone thickness (Tb.Th), cortical bone thickness (Ct.Th), cortical area (Ct.a), trabecular number and osteoclast number (N.Oc) per mm trabecular surface. All animal data were obtained with blind measurements by 2 pathologists using a double-headed microscope. Disagreements were resolved by consensus. Histomorphometric variables were expressed according to the guidelines of the American Society of Bone and Mineral Research nomenclature committee (Parfitt et al., 1987).

Statistical analysis

Results are presented as mean±SEM for at least two repeats of the experiments. Statistical significance was assessed by one-way ANOVA, followed by Tukey and LSD’s post-hoc tests and paired sample T-test. A p value of 0.05 or less was considered as statistically significant.

Results

Histologic analysis of rat livers

The liver of SO animals showed no abnormal histologic changes. Portal inflammation, moderate fibrosis and bile duct proliferation were observed in the BDL group. Meanwhile, portal inflammation and bile duct proliferation were mildly reduced in the L-NAME and NTX-treated rats as compared with the BDL animals.

Biochemical parameters

As shown in Fig. 1, plasma levels of total bilirubin (5.73±0.571 mg/dl) and alkaline phosphatase (396.93±41.29 IU/liter) were markedly increased in BDL rats as compared to that of the SO group, suggesting the presence of biliary cirrhosis as a result of BDL. The results showed that NTX could significantly
reverse the increased ALP activity (258.16±23.38 IU/liter) and the total bilirubin content (0.93±0.06 mg/dl) in BDL animals (p≤0.05); revealing a somewhat protective and to some extent, curative effect of NTX against the damage caused by BDL. After 4 weeks of intervention, total bilirubin and alkaline phosphatase were significantly higher in BDL+L-NAME and BDL+L-NAME+NTX groups as compared to related SO groups. There was no evidence of systemic toxicity attributable to the doses of NTX, L-NAME and NTX+L-NAME used in this study.

Effect of BDL on radiographic density

Following BDL, the optical density was increased in the skulls and mandibles of cirrhotic animals; however, the results were only significant in the skulls (p≤0.05). The mean radiographic OD in the rat skulls between days 0 and 28 was significantly different in BDL, BDL+NTX and BDL+L-NAME+NTX groups (p≤0.05). In the mandible, a significant difference was found in the SO+L-NAME and BDL+NTX groups during the study timeline (Fig. 2).

Bone histomorphometry

Figure 3 demonstrates the histomorphometric indices analyzed in this study. Bile-duct-ligated rats showed reduction in tibial trabecular thickness, cortical thickness and cortical area as compared to SO controls. NTX increased these parameters by 8%, 17% and 64%, respectively; when compared with the BDL group and the difference in cortical area between BDL and BDL+NTX animals was statistically significant (p≤0.05). The number of osteoclasts per mm trabecular surface, significantly increased in the BDL group as compared to the controls (p≤0.05). Interestingly, treatment with L-NAME caused a significant decrease in this parameter (p≤0.05). Tibial trabecular numbers were 37% lower in BDL rats as compared to SO animals (p≤0.05). Administration of L-NAME and NTX increased trabecular number by 10% and 22%, respectively.

Discussion

Levels of endogenous opioids (Bergasa and Jones, 1992; Bergasa et al., 1994) and NO (Mani et al., 2001; Shafaroodi et al., 2010) have been shown to increase in the plasma of subjects with cholestatic liver disease, however, their bone-formative or -resorptive effects have not been adequately studied. In the current investigation, NTX (an opioid receptors blocker) and L-NAME (a non-specific inhibitor of NOS isozymes) were used to manipulate endogenous opioids and NO in BDL cirrhotic rats (Bernátova et al., 1999; Javadi-Paydar et al., 2013). According to our findings, ALP activity and total bilirubin level significantly increased in the BDL group suggesting the establishment of cholestasis. Administration of NTX in the BDL group significantly decreased ALP activity and total bilirubin level in the plasma.

Bone histomorphometry showed a significant increase in osteoclast number per mm trabecular surface and a slight decrease in cortical thickness, cortical area and trabecular thickness in the tibia of BDL rats. Treatment of the BDL animals with NTX, significantly increased cortical area and insignificantly decreased osteoclast numbers per mm trabecular surface as compared to untreated BDL animals. In a previous study we (Nilforoushan et al., 2002) found increased orthodontic tooth movement (OTM) in cholestatic rats.
which was impeded following NTX subcutaneous injection. The increase in OTM was attributed to higher opioid amounts in cholestatic subjects possibly by acting on osteoblasts or osteoblast-like cell receptors. Similarly, following repetitive administration of NTX in rats, Arnsten et al. (2007) suggested a crucial role for the endogenous opioid system in osteoblastic growth. It has been shown that opioid receptor genes are expressed in human osteoblast-like cells (Perez-Castrillon et al., 2000). According to these reports it seems that NTX, as an opioid receptor blocker, may be able to affect osteoblasts and therefore its impact on osteoclasts observed in the present study might have been secondary, justifying the insignificant decrease in the number of these cell types. We observed a significant decrease in total ALP activity following NTX administration. Despite the use of total ALP as an indicator of osteoblast activity and bone formation in previous studies (Oni et al., 1989; Leung et al., 1993), we cannot attribute the bone promoting effect of NTX to osteoblastic activity with certainty, because total ALP reflects several isoenzymes and originates from various tissues. Further studies using bone-specific ALP, immunohistochemistry and etc., are suggested to help

Fig. 3. Histomorphometric indices of trabecular and cortical bone after the completion of the study using sham-operated (SO), NTX, and L-NAME treated BDL cirrhotic rats (Test). A. Trabecular Thickness (Tb.Th). B. Cortical Thickness (Ct.Th). C. Cortical area (Ct.a). D. Trabecular Number. E. Osteoclast Number (N.Oc) per mm trabecular surface. *Significantly different from relevant SO, p-value≤0.05. ^Significantly different from BDL cirrhotic rats (Test) group, p-value≤0.05.
clarify the exact role of NTX in bone augmentation.

Our results showed that treatment of BDL rats with L-NAME, significantly increased cortical area and significantly decreased osteoclast number per mm trabecular surface as compared to the untreated BDL group. It has been demonstrated that cholestatic liver disease leads to excessive production of NO through NOS which can be inhibited by L-NAME. NO has been shown to influence the events that take place in osseous tissues. Regardless of its biphasic effect on osteoclastic bone resorbing activity, NO has been suggested to promote osteoclast formation (Nilforoushan et al., 2009). The significant reduction in osteoclast number per mm trabecular surface following L-NAME administration in the present study can be attributed to inhibiting this function of NO in BDL rats. In a previous study we (Shirazi et al., 2002) reported increased bone remodeling and OTM following L-arginine (NO precursor) local subperiosteal injection in rats, which decreased after local subperiosteal injection of L-NAME. In that study we observed an increase in the number of osteoclasts adjacent to the teeth undergoing OTM in L-arginine-treated animals while a reduction in these cells was found after treatment with L-NAME, which is in line with our recent findings. In the present study, we did not find any increase in the effects of simultaneous administration of opioid receptor blocking agents and NOS inhibition on the histologic parameters.

Optical density increases in cases with reduced bone mineral density (Hernandez-Vaquero et al., 2005) and has been used as a method for evaluating changes in bone density (Shirazi et al., 2001, 2014). In this study, the effect of the opioid and NO systems on bone structure of BDL rats was evaluated by an optical density method using conventional radiographs. The density change between 0 and 28 days in the skull, but not the mandible, was significant in BDL rats, demonstrating that the skull is more vulnerable to density changes than the mandible. We also found that optical density increased in skulls of cirrhotic animals which may be in line with previous reports indicating that opioids could reduce bone density in animals and humans (Rico et al., 1990; Perez-Castrillon et al., 2000). We observed a significant reduction in OD of the skull following NTX administration to BDL rats which may be considered to show an increased amount of bone density as a result of this substance. Accordingly, it seems that NTX may have a positive effect on osseous tissues, by accommodating bone restoration in BDL-induced osteoporosis.

The pathogenesis of osteoporosis in cholestatic and cirrhotic human subjects has not been clearly demonstrated (Rosen, 1995; Boone et al., 2006). These individuals have been reported to have an approximately 2-fold increased risk of fracture (any type), osteomalacia and low and high bone turnover osteoporosis (Dresner-Pollak et al., 2008; Pereira et al., 2009) Several events take place in cholestatic/cirrhotic patients, for example the nitrergic and opioidergic systems are affected and there is an increase in NO and endogenous opioids (Thornton and Losowsky, 1988; Mohammed et al., 2003). Regarding the increased morbidity associated with bone problems in subjects with liver cirrhosis and the lack of an efficient treatment in these cases, nitrergic and opioidergic pathways may be possible options to consider in future treatment developments.

Conclusion

In conclusion according to the results obtained in the present study, nitrergic and opioidergic systems have various impacts on bile-duct-ligated rats. At the microscopic level, NO suppression and opioid-receptors-blockage augmented cortical bone area. Further studies are required to confirm our assumption that the former possibly acts through inhibiting osteoclastogenesis while the latter exerts this effect by increasing osteoblastic activity. On radiographs, optical density decreased in animal skulls and mandibles following blocking of opioid receptors.

Acknowledgements. This study was part of a PhD dissertation supported by Tehran University of Medical Sciences (Grant No. 27249).

References


Accepted October 26, 2016