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Research Article

Evaluation of Changes in Antioxidant Factors and Albumin Level Following the Administration of a Controlled-Releasing Drug Delivery System of Chitosan Hydrogel Loaded with Buprenorphine and Ketorolac in an Experimental Bone Defect in the Tibia of the Rat Model

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Abstract

Background: In the present study, the effectiveness of a controlled-release drug delivery system of chitosan hydrogel loaded with ketorolac and buprenorphine on oxidative stress indices and albumin changes in the experimental bone defect model was considered.
Methods: After creating an experimental defect in the right tibia of each rat, 5 groups, including (A) the control group that did not receive any pharmacological intervention, (B) the chitosan hydrogel receiving group, (C) the group receiving chitosan hydrogel loaded with buprenorphine, (D) the group receiving chitosan hydrogel loaded with ketorolac, and (E) the group receiving chitosan hydrogel loaded with ketorolac and buprenorphine, were considered. Serum concentrations of antioxidant factors and albumin levels were then measured on days 0, 3, 7, and 21 after surgery.
Results: In the control group, the maximum amount of oxidative stress and the maximum activity of antioxidant enzymes on the third and seventh days were compared between the 4 treatment groups. Moreover, the maximum amount of albumin on the third day was recorded and compared between the 4 other treatment groups. In 4 treatment groups, a significant decrease was observed in the mean of parameters related to oxidative stress compared to the control group, which was more noticeable in the group receiving ketorolac.
Conclusion: In the present study, the highest rate of control of oxidative stress conditions was observed in the group treated with the ketorolac-loaded chitosan hydrogel system, possibly due to its antioxidant properties and better control of inflammatory conditions caused by the use of chitosan and ketorolac in this treatment group.

Keywords: Bone; Fracture Healing; Oxidative Stress; Buprenorphine; Ketorolac

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Background

Bone injuries occur under various clinical conditions, such as fractures, orthopedic injuries, spinal surgery, and amputations. Bone defects and their subsequent oxidative damage are important in orthopedics and fracture management. The bone healing process is biologically intertwined with oxidative damage, acute inflammation, and the innate immune system. During surgery and bone injury, ischemic reperfusion injuries often occur, leading to the production of oxygen free radicals. Osteoblast-like cells produce significant amounts of hydrogen peroxide and superoxide during ischemic reperfusion injury (1). In addition, it has been reported that reactive oxygen species (ROS) may be involved in the pathogenesis of bone loss and may also be responsible for the progression of osteoporosis (2). Oxidative stress increases bone resorption and osteoclastogenesis (3, 4). Oxidative stress has been shown to regulate mineral tissue homeostasis and increase bone resorption (5, 6). Oxidative stress has been suggested to lead to bone pathogenesis, which ultimately leads to the development of bone diseases such as osteoporosis, the development of bone tumors, and bone complications induced by diabetes (7). Increased activity of ROS after bone injury causes overexpression of tumor necrosis factor α (TNF-α), receptor activator of nuclear factor kappa-B ligand (RANKL), and macrophage colony-stimulating factor (M-CSF), which enhance the function of osteoclasts, and ultimately, lead to the induction of bone loss (4).

Superoxide radicals can enhance bone resorption by degrading cellular matrix proteins, and weakening bone tissue that is easily digested by enzymes (8). It is stated that an imbalance between oxidative stress and antioxidant compounds enhances osteoclast activity and inhibits osteoblast activity (4). Oxidative stress and nitrogen active species can be defined as an imbalance between the presence of high amounts of ROS and reactive nitrogen species and antioxidant defense mechanisms. Activated nitrogen species, like activated oxygen species, can play a key role in the pathogenesis of many diseases (9). Low molecular weight non-enzymatic antioxidant compounds include cellular glutathione, vitamins C, E, and B, carotene, and uric acid, neutralize free radicals (10). Antioxidant enzymes include catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD), and

glutathione reductase (11). CAT, GPX, superoxide, and thioredoxin reductase dismutase are all endogenous antioxidants, which increase in the case of severe trauma by free radicals (12). ROS are reactive, short-lived, and difficult to measure directly. Indirect markers are often used to predict the amount of ROS or the amount of oxidative stress. The biochemical markers identified in this regard are glutathione S-transferase (GST), GPX, malondialdehyde (MDA), glutathione (GSH) levels, CAT, SOD, and total antioxidant capacity (TAC). It is important to note that each indicator has its own limitations in predicting oxidation in biological systems; thus, the use of at least two markers is recommended (13).

Serum albumin is the most abundant plasma protein produced by liver cells and plays an important role in regulating osmotic-colloidal blood pressure. Albumin production depends on several factors, such as the presence of amino acids, plasma oncotic pressure, inhibitory cytokine concentrations [especially interleukin (IL-) 6], and some hepatocyte activity (14). Albumin activity is a major determinant of plasma oncotic pressure and is used as a transporter of drugs, hormones, and waste products such as bilirubin. Albumin is also used as a source of amino acids for synthesizing other proteins. Inflammation has been reported to mainly enhance vascular permeability through chemicals produced by neutrophils (15, 16). The indirect antioxidant activity of albumin is its ability to transport bilirubin, which has a high affinity to bind with Lys240 molecules (17). Albumin-bound bilirubin has also been shown to act as an inhibitor of lipid peroxidation (18).

There are two important pharmacological interventions to control these injuries and pain from fractures, surgery, and inflammation. First, nonsteroidal anti-inflammatory drugs (NSAIDs) are used to inhibit cyclooxygenase and prostaglandin production, which in turn reduce the sensitivity of pain receptors. This effect even prevents the spread of hyperalgesia in humans and animals. The most common and important indication for the use of NSAIDs in bone trauma is their analgesic effects. The combination of these effects of NSAIDs reduces the need for narcotic analgesics (19). In the second drug intervention, opium is prescribed as an analgesic that can directly inhibit the sensitivity of pain receptors by permanently blocking their environmental activity (20).

Ketorolac has more advantages than other NSAIDs because it can be administered both orally and by injection (21) and has good bioavailability and a longer half-life in oral administration. The activity of ketorolac depends primarily on its analgesic effect, not its anti-inflammatory and anti-febrile activity. It seems that ketorolac has no negative effects on bone healing and can have an antioxidant role. Thus, ketorolac can effectively inhibit free radicals derived from 2, 2 azopes (2-amidinopropane) dihydrochloride in various non-cellular laboratory systems (22). It has also been reported that a number of NSAIDs, including ketorolac, do not promote oxidative status (23). However, the anti-inflammatory activity of NSAIDs may also be due in part to their ability to purify ROS and nitrogen-activated species, and inhibit neutrophil oxidative/oxidative bursting. They are created by various activated compounds (22).

Opioids are one of the most important and basic drugs used for pain management in the preoperative and postoperative periods of all surgeries. Orthopedic patients are no exception to this rule, and opioids are used to provide analgesia in about 80% of fracture patients (24). One of the opioids commonly used to relieve postoperative pain in laboratory animals is buprenorphine (25). Buprenorphine is a weak partial agonist at mu-opioid receptors, and its unique analgesic and medicinal properties distinguish it from methadone and other opioid analgesics. In addition to the early effects of buprenorphine on mu-opioid receptors, it also appears to act as a kappa receptor antagonist (possibly contributing to spinal analgesic and antiphysphoric effects), while delta receptor agonists and relative agonists act on opioid-receptor-like 1 (ORL-1) receptors (26). Opioid analgesics that can activate phospholipase D2 through nicotinamide adenine dinucleotide (NADH) / nicotinamide adenine dinucleotide phosphate (NADPH) oxidation cause the production of superoxide radicals, which increase the concentration of ROS which ultimately leads to oxidative stress. Nevertheless, opium that does not activate phospholipase D2 does not cause ROS production and oxidative stress (27). Some opioid analgesics, such as buprenorphine and morphine, do not induce phospholipase D2 activity, and thus, do not lead to oxidative stress, while some other opioids, such as methadone and fentanyl, produce ROS and oxidative stress through the induction of phospholipase D2 activity (27).

One of the drug delivery systems is the implant system. The main purpose of presenting these drug forms implant system is the local release of the drug in order to increase the presence and absorption of the drug at the desired site, which can be designed to be a controlled-release system. These systems first release an initial concentration of the drug, and after providing the therapeutic concentration of the drug in the blood, a certain dose of the drug is released periodically. A wide range of natural and synthetic polymers are abundantly used to design controlled drug delivery systems (28). Hydrogels are composed of crosslinked polymer networks that contain large amounts of hydrophilic groups and domains. The physicochemical properties of hydrogels are similar to the extracellular matrix both in composition and mechanically. In fact, hydrogels can be used to achieve two purposes: 1- as cell-supporting substances during tissue regeneration, and 2- for delivery of controlled drug systems (29, 30). Chitosan-based hydrogels are excellent for use in controlled drug delivery systems. Moreover, due to the lack of immune and inflammatory reactions to the use of chitosan in the living organism, it is the most preferred substance in its slow-releasing subcutaneous drug delivery system.

The present study was an investigation of the antioxidant changes and albumin levels following administering a controlled-releasing drug delivery system of chitosan hydrogel loaded with buprenorphine and ketorolac in an experimental defect in the proximal epiphysis of the tibia in the rat model. In addition, the relationship between the measured parameters and the role of different drugs on oxidative stress conditions and changes in albumin in bone defects were investigated.

Methods

This study was reviewed and approved by the research ethics committee of Shahrekord University, Iran, with the ethical code IR.SKU.REC.1399.018.

Ketorolac and buprenorphine were obtained from Daropakhsh (Raha Pharmaceutical Co., Iran) and Diosynth (Apeldoom, The Netherlands), respectively. Low molecular chitosan and glycerol 2-sodium disodium hydrate (βGP) were purchased from Merck (Darmstadt, Hesse, Germany). Other reagents and solvents were purchased from Merck (Darmstadt, Hesse, Germany).

Synthesis of Chitosan Hydrogel Loaded with Ketorolac and Buprenorphine: As per our previous research (31), to prepare chitosan hydrogel loaded with ketorolac and buprenorphine, 480 mg of chitosan was dissolved in 12 ml of 0.1 M acetic acid solution and stirred for 3 hours to obtain a homogeneous solution. Subsequently, 120 mg of ketorolac and 2 mg of buprenorphine dissolved in 5 ml of water were added to chitosan. In a separate container, 3.2 g of βGP was dissolved in 2 ml of deionized water, and then, both containers of chitosan and βGP were placed in an ice bath at 4 °C. When the containers reached a temperature of 4 °C, βGP solution was added dropwise to the chitosan solution for 10 minutes. The final solution was kept at 4 °C and used in subsequent steps (calculated values for hydrogel preparation for 19 rats) (32). To prepare chitosan hydrogel loaded with buprenorphine or ketorolac alone or to prepare chitosan hydrogel without buprenorphine or ketorolac, only 120 mg of ketorolac or 2 mg of buprenorphine or none were added, respectively. However, the amounts of all chitosan hydrogel solutions were the same as 5 ml. Although more characterization of the hydrogels are mentioned in our previous study (31). Moreover, further analysis of chitosan hydrogel, buprenorphine or ketorolac alone hydrogel, and ketorolac-buprenorphine hydrogel was done through methods such as Fourier transform infrared spectroscopy (FTIR) and the results were compared.

Animals and Experimental Surgery: The laboratory animals selected for the present study were female Wistar rats (weighing 200-250 g) kept in a standard environment. One hundred rats were randomly divided into 5 equal groups: (A) the control group in which only experimental surgical intervention was performed and did not receive any pharmacological intervention, (B) the chitosan hydrogel receiving group, (C) the group receiving chitosan hydrogel loaded with buprenorphine, (D) the group receiving chitosan hydrogel loaded with ketorolac, and (E) the group receiving chitosan hydrogel loaded with ketorolac and buprenorphine. To create an experimental bone defect in the proximal epiphysis of the tibia, the animals were placed supine, and surgery was performed under routine aseptic conditions. The animals were first anesthetized by intraperitoneal injection of a combination of ketamine (70 mg/kg) and xylazine (10 mg/kg) (33). The skin of the animals was clipped and disinfected at the surgical site, and then, to maintain anesthesia, the rats were subjected to inhalation anesthesia with isoflurane gas using a facial mask (34). A 1 cm long longitudinal incision was then made along the skin of the prepared area covering the tibial head (35). After incising the skin, the fascia and the local muscles below it were also incised to show the lateral surface of the head of the tibia (35). Thus, following the exposure of the right proximal epiphysis of the right tibia of each rat, using a low-velocity microdrill (150 rpm), an experimental defect with a diameter of 2 mm and a depth of 2 mm was applied (35). Finally, in all groups, the surgical site, including muscles, fascia, and skin, were sutured with 0-4 monocryl thread and 0-3 nylon, respectively, in a simple continuous pattern. In order to prevent possible infections after surgery, an intramuscular injection of ampicillin was administered every 12 hours for 5 days after surgery.

Assessment of Antioxidant Factors and Albumin: Cardiac blood samples were taken on days 0, 3, 7, and 21 after surgery following general anesthesia. Blood samples were collected in cold test tubes without anticoagulant (36), and the blood samples were centrifuged for 5 minutes at 1200 x g at room temperature to separate the blood sample into the upper and lower layers. Finally, blood serum was obtained to measure various parameters (36). Serum albumin was measured using the Bromocresol green (BCG) method in the wavelength range of 628 nm (37, 38). The basis for measuring serum SOD was the method of inhibiting the pyrogallol autoxidation reaction (31). In this reaction, by adding a sample containing SOD with an unknown concentration, the amount of inhibition of the pyrogallol oxidation reaction is measured at a specific time and is determined in comparison with controlling the amount of SOD concentration in the sample. The light absorption of the samples at 420 nm was performed using a spectrophotometer (39). To determine the rate of lipid peroxidation, serum MDA content was measured using the thiobarbituric acid method (40). GPX enzyme activity was measured through NADPH oxidation using a reaction system consisting of glutathione, GPX, and cumene hydroperoxidase. In this reaction, the used NADPH is considered a marker to determine the amount of GPX, and at the 340 nm wavelength, determines the activity of GPX. Finally, the light absorption of the samples was performed using a spectrophotometer at a wavelength of 340 nm (41). CAT activity was measured using the peroxidative function of this enzyme (41). Finally, potassium hydroxide terminated the reaction and the resulting formaldehyde in combination with chromogen (dye) was spectroscopically measured using a spectrophotometer at 550 nm (41).

Statistical Analysis: The mean ± SD of the assessed parameters were compared using ANOVA and Tukey's post hoc test in SPSS software (version 23; IBM Corp, Armonk, NY, USA) at the significance level of $P < 0.05$.

This study received no funding.

Results

Preparation and Characterization of Chitosan Hydrogel Loaded with Ketorolac and Buprenorphine: In our previous study (31), we have detailed every step in the preparation and characterization of the ketorolac and buprenorphine-loaded chitosan hydrogel. Here, we clarify some final results of hydrogel characterization for further research.

Fourier Transform Infrared Spectroscopy: The FTIR spectra of chitosan hydrogel, ketorolac, buprenorphine, and ketorolac-buprenorphine hydrogel are displayed in figure 1. FTIR was used to confirm the final formulation of ketorolac-buprenorphine hydrogel. The chitosan hydrogel composite sample is shown in figure 1A. The index peak of each component, including chitosan (31) and -GP, are shown in this graph. In figure 1A, as shown in figures 1B, 1C, and 1D, the distinctive NHCO, PO4, and P-O-C absorption bands are visible in the FTIR spectra of chitosan hydrogel at 1576, 1058, and 965 cm^{-1}, respectively.

There are several similar functional groups between ketorolac and buprenorphine, as aromatic rings and amino groups are seen in figures 1B and 1C. The stretching bands of aromatic rings of ketorolac hydrogel can be seen in figure 1B as stretching vibration of sp^2 and sp^3 C-H at 2931 and 2919 cm^{-1} and in buprenorphine hydrogel (Figure 1C) at 2926 and 2961 cm^{-1}, respectively. Furthermore, C=C aromatic ring stretching can be seen at 1618 and 1401 cm^{-1} for ketorolac hydrogel and 1617 and 1400 cm^{-1} for buprenorphine hydrogel, respectively. The ketone group is the only functional group in ketorolac that is absent in buprenorphine.

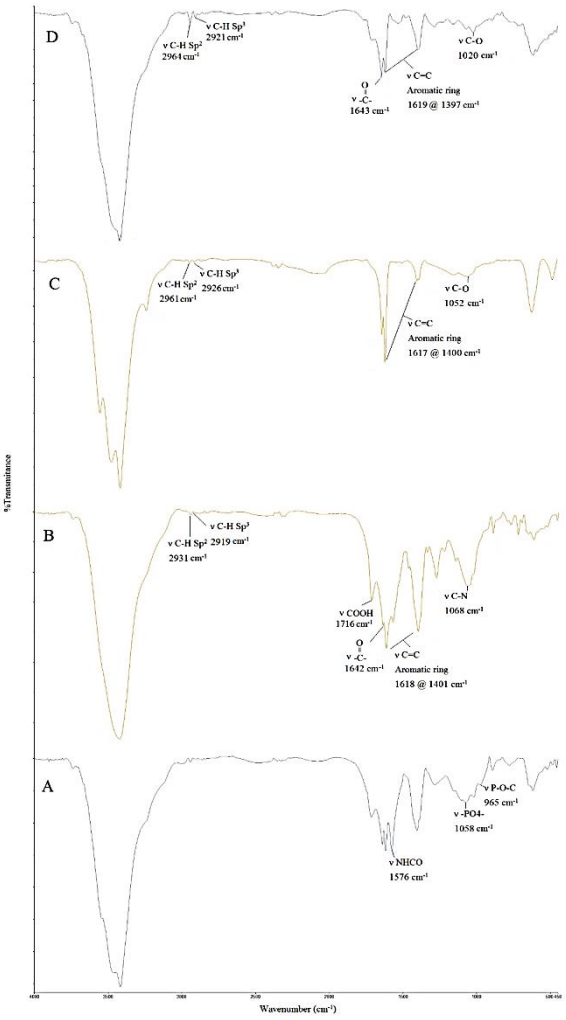

Figure 1. Fourier transform infrared spectroscopy of chitosan (a), ketorolac hydrogel (B), buprenorphine hydrogel (C), ketorolac hydrogel, and buprenorphine hydrogel (D)

As figure 1B shows, the stretching band of C=O has been shifted to a lower frequency of 1642 cm^{-1} by conjugating the carbonyl group with two aryl groups. Moreover, the peaks at 1716 and 1068 cm^{-1} were attributed to C=O (of the acidic group) and C-N (type III) stretching, respectively.

On the other hand, the ether group is the only differentiating group compared to ketorolac in the buprenorphine molecule. The most important band related to the tensile strength of C-O is observed at 1000 to 1300 cm^{-1}, which can be seen at 1052 cm^{-1} in figure 1C.

The FTIR data for the final hydrogel, ketorolac-buprenorphine hydrogel, showed that all the indicator peaks for chitosan hydrogel, ketorolac hydrogel, and buprenorphine hydrogel were simultaneously present (Figure 1D). Therefore, the combination of ketorolac and buprenorphine in the crosslinked hydrogel of chitosan was confirmed in this study.

Superoxide Dismutase: On the third day of the study, in the groups receiving chitosan, buprenorphine, and ketorolac-buprenorphine, compared to the group receiving ketorolac, a significant increase was observed in the mean of SOD, as well as a significant decrease in mean SOD in the control group. In fact, on the third day of the study, a significant increase was observed in mean SOD in the control group compared to the other four groups. On the seventh day of the study, a significant decrease was observed in mean SOD in the groups receiving chitosan, ketorolac, and buprenorphine compared to the control group and the groups receiving ketorolac-buprenorphine. However, on day 21 of the study, no significant difference was observed between the studied groups in terms of mean SOD (Figure 2).

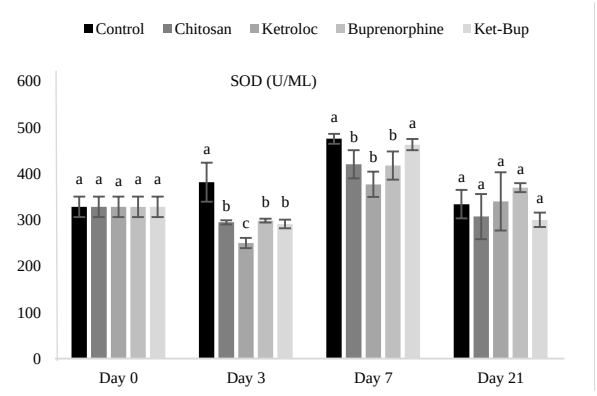

Figure 2. Measurement of the mean ± SD of SOD in different groups and times; Control (the group in which only empirical bone deficiency developed and did not receive any pharmacological intervention); Chitosan (chitosan hydrogel receiving group); Ketorolac (the group receiving ketorolac-loaded chitosan hydrogels); Buprenorphine (the group receiving buprenorphine-loaded chitosan hydrogels); Ket-Bup (the group receiving buprenorphine+ketorolac-loaded chitosan hydrogels); SOD (superoxide dismutase enzyme)

Catalase: On the third and seventh days of the study, in the groups receiving chitosan, ketorolac, buprenorphine, and ketorolac-buprenorphine, a significant decrease was observed in mean CAT compared to the control group. However, on day 21 of the study, no significant difference was observed in mean CAT in the different groups (Figure 3).

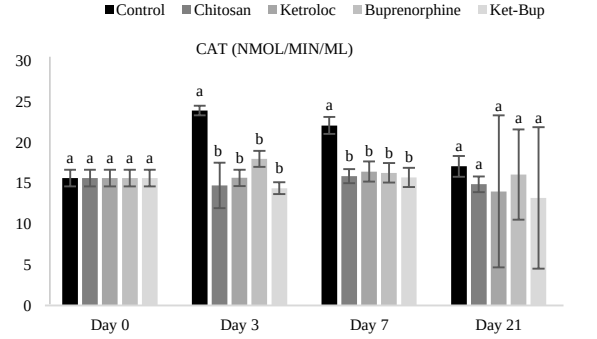

Figure 3. Measurement of the mean ± SD of catalase in different groups and times; Control (the group in which only empirical bone deficiency developed and did not receive any pharmacological intervention); Chitosan (chitosan hydrogel receiving group); Ketorolac (the group receiving ketorolac-loaded chitosan hydrogels); Buprenorphine (the group receiving buprenorphine-loaded chitosan hydrogels); Ket-Bup (the group receiving buprenorphine+ketorolac-loaded chitosan hydrogels); CAT (catalase)

Glutathione Peroxidase: On the third and seventh days of the study, in the four treatment groups receiving chitosan, ketorolac, buprenorphine, and ketorolac-buprenorphine, a significant decrease was observed in mean GPX compared to the control group.

However, on day 21 of the study, no significant difference was observed in mean GPX in the study groups (Figure 4).

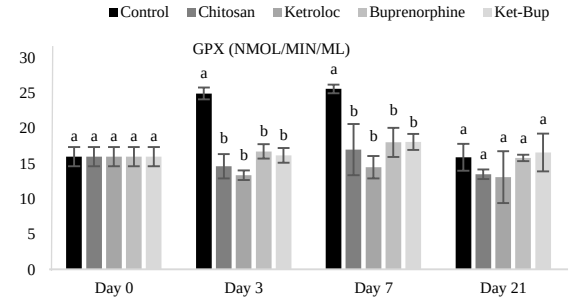

Figure 4. Mean ± SD of GPX in different groups and at different times; Control (the group in which only empirical bone deficiency developed and did not receive any pharmacological intervention); Chitosan (chitosan hydrogel receiving group); Ketorolac (the group receiving ketorolac-loaded chitosan hydrogels); Buprenorphine (the group receiving buprenorphine-loaded chitosan hydrogels); Ket-Bup (the group receiving buprenorphine+ketorolac-loaded chitosan hydrogels); GPX (glutathione peroxidase)

Malondialdehyde: On the third and seventh days of the study, in the 4 treatment groups receiving chitosan, ketorolac, buprenorphine, and ketorolac-buprenorphine, a significant decrease was observed in mean MDA compared to the control group. However, on day 21 of the study, no significant difference was observed in the mean MDA in the study groups (Figure 5).

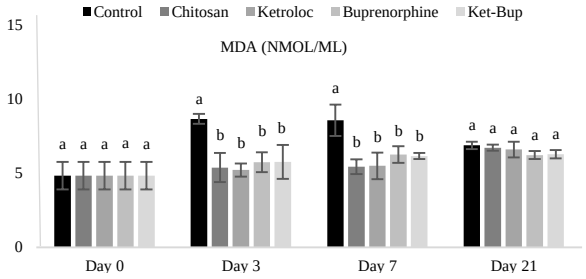

Figure 5. Comparison of the mean ± SD of MDA in different groups and at different times; Control (the group in which only empirical bone deficiency developed and did not receive any pharmacological intervention); Chitosan (chitosan hydrogel receiving group); Ketorolac (the group receiving ketorolac-loaded chitosan hydrogels); Buprenorphine (the group receiving buprenorphine-loaded chitosan hydrogels); Ket-Bup (the group receiving buprenorphine+ketorolac-loaded chitosan hydrogels); MDA (malondialdehyde)

Albumin: On the third day of the study, in the groups receiving chitosan, ketorolac, buprenorphine, and ketorolac-buprenorphine, a significant decrease was observed in mean albumin compared to the control group. However, on days 7 and 21 of the study, no significant difference was observed in the different groups studied (Figure 6).

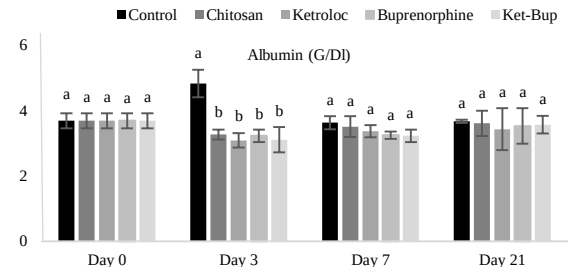

Figure 6. Comparison of the mean ± SD of albumin parameters at different times and in different groups; Control (the group in which only empirical bone deficiency developed and did not receive any pharmacological intervention); Chitosan (chitosan hydrogel receiving group); Ketorolac (the group receiving ketorolac-loaded chitosan hydrogels); Buprenorphine (the group receiving buprenorphine-loaded chitosan hydrogels); Ket-Bup (the group receiving buprenorphine+ketorolac-loaded chitosan hydrogels)

Discussion

Controlled drug delivery based on a hydrogel network has been designed and has shown great promise for treating many diseases, such as bone injury. Various polymers with biocompatible properties have been designed to release drugs at a specific pH value with biocompatible properties, and several of these carriers are currently being investigated. The application of chitosan as a hydrogel for the fabrication of drug molecules could represent a new approach to controlled drug treatment in bone injury. In this study, we created a novel formulation of buprenorphine and ketorolac-loaded controlled-release hydrogel for use in an experimental bone defect in the tibia. This complex network was created using the gradual medication delivery mechanism. This method of crosslinking chitosan hydrogels has been employed in numerous studies, and similar results in terms of the morphological structure have been obtained.

The FTIR method was used to validate the structure of buprenorphine-ketorolac hydrogel. As mentioned, some indicated peaks of ketorolac and buprenorphine as ketone and ether groups were found in figures 1B and 1C, respectively. As the results show, all indicated peaks were found for the final production as buprenorphine-ketorolac hydrogel. Moreover, other compounds, such as chitosan and βGP, had effects on the obtained FTIR in figure 1D, showing certain peaks.

In the present study, changes in biochemical markers related to the amount of ROS and the amount of oxidative stress, including SOD, CAT, GPX, and MDA, as well as albumin parameters following the use of a controlled releasing delivery drug system loaded with chitosan hydrogel, ketorolac, and buprenorphine were evaluated in rats with experimental bone defects in the tibia. The study of fracture patterns in the rat model is very important because it provides insight into the bone metabolism of animals and humans and potentially shows the positive and negative effects of various drugs on bone repair. In addition, these models reveal ways to study the physiological process of bone healing (42). It is also possible to study the antioxidant properties of some drugs and their effects on the amount of ROS, and consequently, the amount of oxidative stress in rat models. The proximal epiphysis of the tibia has a medial surface that is considered very suitable for inducing bone defects because it has a wide, broad, low-convex surface with no muscle insertion (43). Oxidative stress in cells occurs due to an imbalance between oxidative systems and antioxidants. When the production of free radicals exceeds the body's antioxidant defense capacity to detoxify them, a condition called oxidative stress occurs, so that the increase in oxidants and non-depletion of antioxidants shifts the oxidative / antioxidant balance to oxidative condition (9). Most of the body's energy is produced by the controlled enzymatic reaction of oxygen with hydrogen in oxidative phosphorylation in the oxidative metabolism that occurs in the mitochondria (44). Thus, the increase in oxidative stress is due to an imbalance between oxidation products and antioxidant defense. It has also been suggested that there is a link between oxidative stress and pain perception (45). Cyclooxygenase and lipoxygenase pathways, following any trauma or fracture, can produce ROS (peroxide and superoxide ions) whose peroxides intensify cyclooxygenase and lipoxygenase activity (46). Prostaglandins also participate in the oxidation of NADP (H) in neutrophils (47). SOD is the main defense agent

against oxygen radicals, which prevents endothelial and mitochondrial dysfunction by inactivating nitric oxide and inhibiting peroxynitrite formation. It also scavenges oxygen radicals produced in the respiratory chain (48).

When bone fractures occur, large amounts of free radicals are produced. Some of these produced radicals react with oxygen to form the metabolites of oxygen radicals (49). Increased osteoclastic activity and increased activity of ROS are involved in many bone injuries, but does increased ROS production destroy the antioxidant defense system and ultimately lead to hyperoxidative stress and impaired bone healing? While considering this question, it should be noted that GPX, CAT, and SOD are endogenous antioxidants that are increased by free radicals in the event of severe trauma (12). Osteoclasts degrade calcified tissue through complex advanced stages, but in particular, the controlled production of free radicals by the natural activity of osteoclasts accelerates the destruction of calcified tissues and helps bone remodeling (50). However, osteoclasts contain the enzyme NADPH oxidase, which can regulate the production of ROS by cytokines (51). The increase in osteoclastic activity observed in bone lesions may be responsible for the increased production of ROS (superoxide oxide), which is manifested by increased serum MDA levels. In addition, the enzyme SOD converts the superoxide anion ($O_{2\cdot}$) to hydrogen peroxide (H_2O_2), which is converted to H_2O and O_2 by GPX or CAT (52). In accordance with the above-mentioned mechanism, the results of our study in the control group compared to other treatment groups showed that on days 3 and 7 after the bone defect, the mean of 4 antioxidant parameters was significantly higher.

It seems that the highest production of oxygen free radicals is during the inflammatory phase of bone healing. Inflammatory cells include polymorphonuclear leukocytes, macrophages, and mast cells, the primary cells penetrating the fracture site (53, 54). During this phase (inflammatory phase), osteoclasts also become active and begin to remove necrotic bone (53). Inflammatory cells (55-57) and osteoclasts (58) have been shown to produce reactive oxygen mediators. In fact, in the early stages of fracture healing, inflammatory cells, and subsequently, osteoclasts play an important role in causing oxidative stress. Therefore, increasing the activity of the antioxidant defense system at this stage is not enough to overcome oxidative stress in order to heal the fracture. Finally, it can be stated that prescribing a drug with antioxidant properties may be beneficial to the fracture healing process by overcoming the negative effects of the produced oxygen free radicals.

In fact, SOD is the first detoxifying enzyme and also the most powerful antioxidant in the cell. SOD has been suggested to be an important endogenous antioxidant enzyme that acts as part of the first line of the defense system against ROS (59). This enzyme converts the superoxide anion ($O_{2\cdot}$) to hydrogen peroxide (H_2O_2), which is converted to H_2O and O_2 by GPX or CAT (52). A relationship has been observed between SOD deficiency and a number of harmful processes in animals and humans (60). Another mechanism that may contribute to the effectiveness of SOD is the regulation of neutrophil apoptosis. To relieve inflammation, activated neutrophils must be properly removed through apoptosis. SOD may be effective as an inhibitory mediator of neutrophil inflammation (61). Since SOD shows increased activity following severe trauma and free radical production (12), in our study on day 3, in the chitosan, buprenorphine, and ketorolac-buprenorphine groups, a significant increase was observed in the mean of this parameter compared to the group receiving ketorolac and a significant decrease was recorded in the mean of this parameter compared to the control group. However, this significant decrease in mean SOD was more noticeable in the group receiving ketorolac compared to the control group. Therefore, in all treatment groups, a significant decrease in mean SOD was observed compared to the control group, which is probably due to the antioxidant properties of chitosan (62). On the seventh day of our study, in the groups receiving chitosan, ketorolac, and buprenorphine compared to the control and ketorolac-buprenorphine groups, a significant decrease was observed in mean SOD, which could be due to a significant reduction in oxidative stress and antioxidant impacts of ketorolac, chitosan, and buprenorphine.

Moreover, the activity of SOD depends on oxygen concentration. In addition to mitochondria, phagocytic cells such as neutrophils and macrophages are also involved in producing ROS (63). It has also been reported that an increase in the concentration of this enzyme is associated with an increase in oxygen production by neutrophils (64). Therefore, a significant decrease in this enzyme in the group that received chitosan hydrogel loaded with ketorolac may indicate a decrease in the amount of ROS, and consequently, a decrease in SOD activity which is probably due to the anti-inflammatory effect of ketorolac.

Treatment with SOD mimics reduces the production of proinflammatory cytokines and inhibits the expression of adhesion molecules on endothelial cells, and prevents neutrophil infiltration (65). In fact, SOD mimics have been identified as inhibitors of cytokine production, including TNF-αIL-1βand IL-6 (66).

It has been stated that one of the effects of ROS is lipid peroxidation, the end product of which is MDA (67). MDA, in addition to being an indicator of lipid peroxidation, is also referred to as an index of osteoclastic activity. In one study, oxidative status during bone healing in a rat model was assessed by measuring MDA in bone samples as an indicator of oxidative stress (57). It was seen that MDA levels increased significantly on days 7 and 14 after the experimental bone fracture. Finally, the authors stated that oxidative stress is a strong factor in disrupting the bone healing process (57). The results of our study also showed that the MDA level in the control group on the third and seventh days after the bone defect was significantly higher than the mean of this parameter in other groups. In fact, mean MDA in the treatment groups decreased significantly on the 3rd and 7th days of the study. It can be concluded that destructive peroxidation was higher in the control group than in the other treatment groups.

The main physiological role of GPX is maintaining the proper level of hydrogen peroxide in the cell, thus reducing the potential for free radical damage. This enzyme plays an important role in inhibiting the process of lipid peroxidation, and thus, protects cells from oxidative stress (68). GPX is responsible for scavenging reactive oxygen radicals through glutathione along with glutathione reductase. Due to the ability of GPX to catalyze H_2O_2 and lipid peroxides, this enzyme plays a unique role in protecting tissues against ROS (69). In addition, as mentioned earlier, the enzyme SOD converts the superoxide anion ($O_{2\cdot}$) to hydrogen peroxide (H_2O_2), which is converted to H_2O and O_2 by GPX or CAT (52). According to the above, following a significant increase in mean SOD in

the control group on days 3 and 7, a significant increase was also observed in mean CAT and GPX in the control group at these times, indicating oxidative stress. In the 4 treatment groups, a significant decrease was observed in mean CAT and GPX on days 3 and 7, which indicates the control of oxidative stress conditions. Therefore, a significant decrease in mean SOD, CAT, and GPX indicates a decrease in the amount of reactive oxygen free radicals in the body.

Albumin levels are considered an acute phase reactive protein against severe disease or physiological stress (70, 71). Inflammation has been reported to mainly enhance vascular permeability through chemicals produced by neutrophils (15). Thus, increasing the albumin concentration in the inflamed area due to its antioxidant impacts is considered a positive result in the affected area (16). The indirect antioxidant activity of albumin is the ability to transport bilirubin, which has a high affinity for binding to Lys240 molecules (17). Albumin-bound bilirubin has also been shown to act as an inhibitor of lipid peroxidation (18). According to the above, on the third day of our study, in the control group, compared to the other four treatment groups, a significant increase was observed in mean albumin. On days 7 and 21, no significant difference was observed in the mean of this parameter in different groups. It seems that a compensatory increase in serum albumin synthesis is a beneficial response to inflammatory conditions that require an immune response, cell proliferation, healing, and tissue growth (72). Albumin acts in interstitial spaces as a free radical scavenger and antioxidant. Albumin in cells also rapidly provides amino acids as building elements of cell proliferation and tissue matrix deposition (72).

CAT is a highly efficient enzyme for degrading H_2O_2, especially in high amounts of peroxide, which is mainly found in the cytoplasm (red blood cells) and peroxisomes (most cells, especially the liver). H_2O_2 is produced by free radicals that, when accumulated, are toxic to body tissues or cells. CAT, which is abundant in peroxisomes, breaks down H_2O_2 into water and molecular oxygen, thereby reducing free radical damage. CAT is a highly efficient enzyme that can decompose millions of hydrogen peroxide molecules in one second (59). Mitochondria are both the main source and an important target of ROS. Mitochondrial CAT can protect these organs from exogenous or endogenous hydrogen peroxide damage. However, by changing the redox oxidation state in the mitochondria, cells may increase their sensitivity to TNF-α induced apoptosis. The antioxidant effect of CAT, when expressed in the mitochondrial chamber, depends on the oxidant and the ROS production site (73). On days 3 and 7 of the present study, in the groups receiving chitosan, ketorolac, buprenorphine, and ketorolac-buprenorphine, a significant decrease was observed in the mean of this parameter compared to the control group. However, on day 21 of the study, no significant difference was observed in the mean of this parameter in different groups. A significant decrease in mean CAT in the 4 treatment groups compared to the control group indicates the control of oxidative stress conditions in the treatment groups.

Evidence has shown that chitosan can prevent lipid oxidation in biological systems by scavenging free radicals and inhibiting the formation of ROS, mainly due to the presence of hydroxyl and amine groups in the chitosan (74-76). Chitosan has high antioxidant activity and antilipidemic effects on metabolic syndrome in rats (62, 77). The high antioxidant effects of chitosan and its protective effect have also been reported in rats with chronic renal failure (78). Several mechanisms have been reported for the antioxidant properties of chitosan, including the ability to scavenge free radicals and the ability to chelate and reduce the activity of free radicals (74, 75, 79). For these reasons, in the present study, a controlled-release drug delivery system was synthesized on the chitosan scaffold. Clinical and laboratory studies show that NSAIDs act as antioxidants (80). Any trauma that leads to the activation of cyclooxygenase and lipoxygenase pathways can lead to the production of ROS (peroxide and superoxide ions); moreover, peroxides themselves intensify cyclooxygenase and lipoxygenase activity (46). Therefore, NSAIDs can play an antioxidant role with their functional mechanism. The antioxidant effect of ketorolac has already been explained by the inhibition of free radicals (22, 81, 82). It has also been stated that ketorolac has no oxidative-promoting activity (23). It has also been suggested that the anti-inflammatory activity of NSAIDs may be due in part to their ability to scavenge ROS and active nitrogen species, and inhibit neutrophil respiratory/oxidative bursting (22). As mentioned earlier, the highest production of oxygen free radicals is during the inflammatory phase of bone healing, so the administration of NSAIDs, due to their anti-inflammatory effect, can also provide antioxidant results. In accordance with the abovementioned facts, in the treatment group receiving chitosan hydrogel loaded with ketorolac, the better control of oxidative stress conditions was possibly due to the antioxidant and anti-inflammatory properties of chitosan and ketorolac. Therefore, it is possible to control the oxidative stress conditions by correctly controlling the inflammatory phase of bone healing and benefiting from the anti-inflammatory properties of ketorolac. The challenge in synthesizing these slow-release systems is NSAIDs overdose, which can induce oxidative stress due to damage to the gastrointestinal tract (by inhibiting cyclooxygenase and suppressing the protective effects of prostaglandins on the GI mucosa) and hepatotoxicity (83, 84). However, the treatment group receiving chitosan loaded with ketorolac in the present study did not show an increase in antioxidants, which indicates the induction of oxidative stress.

There are several studies that show an association between opium and oxidative stress on nerve cells. Repeated use of opium due to increased dopamine secretion causes permanent damage to the mechanism of dopamine, the occurrence of autoxidation, and the production of 3 and 4-dihydroxyphenylacetic acid and hydrogen peroxide (85). These products subsequently react with the metal ions iron and calcium to form hydroxyl free radicals during the Fenton process, which is probably the most reactive free radical in the cellular environment and potentially leads to oxidative stress (86, 87). Narcotic analgesics that can activate phospholipase D_2 by NADH / NADPH oxidation cause the production of superoxide radicals, which converts superoxide anion ($O_2^{\cdot}$) to hydrogen peroxide (H_2O_2). Subsequently, H_2O_2 converts to H_2O and O_2 by CAT or GPX (52). Phospholipase D_2 hydrolyzes phosphatidylcholine to produce choline and phosphatidic acid and also plays a key role in the membrane transport of cellular receptors and signal transduction (27). Various studies have shown that phosphatidic acid derived from phospholipase D_2 regulates NADH / NADPH oxidase activity, thus leading to the production of ROS (88-91). Nevertheless, opiums that

do not activate phospholipase D_2, such as buprenorphine, do not cause the production of ROS and oxidative stress (27). In the present study, in the group receiving chitosan hydrogel loaded with buprenorphine, the oxidative stress conditions were well controlled, which was probably due to the antioxidant properties of chitosan and buprenorphine.

The limitation of the present clinical study was the use of the minimum number of laboratory animals (rats) according to the rules of ethics in research.

Conclusion

The results of this study showed that in the control group, in which only bone defect occurred and no therapeutic intervention was performed, serum levels of SOD and albumin on day 3, and GPX, CAT, and MDA on days 3 and 7 significantly increased compared to the other 4 treatment groups. The results of this study indicated the occurrence of oxidative stress conditions in the control group and the control of oxidative stress conditions in the treatment groups. The best control was observed in the chitosan hydrogel group loaded with ketorolac. In addition, the level of SOD enzyme significantly decreased on day 3 in the group receiving chitosan hydrogel loaded with ketorolac compared to the 3 other treatment groups and the control group, indicating excellent control of oxidative stress conditions in this treatment group. Control of oxidative stress in all treatment groups compared with the control group is related to the presence of chitosan and its antioxidant effects, as well as to the drugs involved in chitosan, namely ketorolac, and buprenorphine. Finally, it can be concluded that controlled-release systems loaded with ketorolac and buprenorphine can control oxidative stress when experimental bone defect is induced in rats.

Conflict of Interest

The authors declare no conflict of interest in this study.

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None.

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