

RESEARCH ARTICLE

The Enalapril Use in Arterial Hypertension Stimulates The Reparative Processes in Fractures of The Proximal Femur

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Received date: Oct 21, 2021; Revised date: Dec 31, 2021; Accepted date: Jan 12, 2022

Abstract

BACKGROUND: In patients with a fracture of the proximal femur and concomitant arterial hypertension, there is a disturbance of the reparative processes of bone tissue. This research aimed to study the regulation of the reparative processes of fractures of the proximal femur with intramedullary osteosynthesis during the correction of concomitant hypertension, which was examined based on some markers using the rat model.

METHODS: The study involved healthy Wistar rats and spontaneously hypertensive rats (SHR). The subjects were then grouped into healthy rats without exposure (1.1) SHR without exposure (2.1), healthy rats with modeled fractures of the proximal femur (1.2), SHR with modeled fractures of the proximal femur (2.2), SHR underwent hypertension correction with enalapril in subgroups without fracture (2.3) and SHR underwent hypertension correction with enalapril in subgroups with fracture (2.4). The levels of interleukin (IL)-6, tumor necrosis factor alpha (TNF- α), IL-10, amino-terminal propeptide procollagen type III (PIIINP), glucose, uric acid, creatinine, urea, cholesterol, and

albumin were determined in the blood serum of the animals. Femur preparations were examined after the removal of intramedullary fixation.

RESULTS: Serum IL-6 level of animal in group 2.4 (2.297 ± 0.361 pg/mL) were reduced compared to the corresponding indicators of rats in group 2.3 (4.054 ± 0.491 pg/mL, $p < 0.05$). Serum glucose and urea levels of animal in group 2.4 (3.951 ± 0.156 mmol/L, 6.552 ± 0.426 mmol/L, respectively) were significantly reduced in comparison with the group 2.3 (6.384 ± 0.890 mmol/L, 10.369 ± 0.888 mmol/L, respectively). The histological results indicated a positive effect of the drug enalapril on the healing of fractures of the proximal femur in animals with hypertension.

CONCLUSION: Correction of arterial hypertension with enalapril in fractures of the proximal femur improves the reparative processes of bone tissue.

KEYWORDS: injury healing, remodeling, concomitant diseases, angiotensin-converting enzyme inhibitors, cytokines, growth factor, collagen, biochemical parameters

Indones Biomed J. 2022; 14(1): 36-44

Introduction

Fractures of the proximal femur are a serious medical problem, especially with an aging population.(1) Hip fractures are associated with a high overall mortality rate (up to 30%) after the incident.(2) Often, such injuries occur

in patients with a history of concomitant diseases, for example, arterial hypertension. Hypertension is one of the main risk factors for diseases causing mortality worldwide of approximately 7-18%.(3) Angiotensin-converting enzyme (ACE) inhibitors, to which enalapril belongs, together with angiotensin receptor blockers (ARBs), are drugs of the first choice for hypertension. Recent studies

demonstrated that the bone tissue renin-angiotensin system (RAS) is directly involved in bone metabolism.(4) It has been suggested that the risk of hip fractures is reduced with the use of ACE inhibitors and ARBs.(5) A study demonstrated a positive effect on the healing of fractures of enalapril, as an ACE inhibitor, in contrast to losartan, as an ARB, which did not show a positive effect.(6) The use of enalapril leads to increased blood flow and improved microcirculation in the fracture zone, which provides an appropriate microenvironment for osteoblast activity to produce bone matrix. Therefore, enalapril was chosen in our work. Correction of hypertension with ACE inhibitors has a positive effect on fracture healing.(7) However, the mechanisms underlying fracture healing in the setting of concomitant hypertension are not fully understood.

Cytokines and cellular signals in fracture healing continue to be studied. It is relevant to study the effect of enalapril on reparative processes in the remodeling phase. In this phase of fracture healing, the strength characteristics and structure of the bone are restored in accordance with the lines of mechanical stress. This happens by resorption of the primitive bone structure and the formation of lamellar bone, which contributes to a strong connection at the fracture site. (8) The regulation of disturbances in reparative processes at this stage will make it possible to influence the restoration of damage to the initial configuration (normal structure) of the bone. Studies of molecular regulators of remodeling processes are important in the search for strategies and methods of treating fractures associated with hypertension to optimize bone recovery.

Bone healing is a complex regenerative process characterized by three overlapping phases: the inflammatory phase, the recovery phase, and the remodeling phase.(9) The healing process is driven by a complex network of inflammatory, angiogenic, osteoanabolic, and osteocatabolic mediators.(10) Interleukin (IL)-6 plays an important role in fracture healing. IL-6 is known to act as a modulator of osteoclast differentiation and is involved in collagen accumulation.(11,12) IL-6 signals are transmitted using two mechanisms. This is the mechanism of classical signaling through the membrane-bound receptor (mIL-6R), as well as the signaling mechanism through its soluble form (sIL-6R). (13) Tumor necrosis factor alpha (TNF- α), like IL-6, also has both pro-inflammatory and immunoregulatory properties. (14) TNF- α , as well as IL-6, have a close relationship with fracture healing.(15) The cells involved in healing express growth factors and cytokines. To control the ability of cells to inhibit or initiate reparative processes, cytokines and growth factors allow the formation of positive and negative

feedback loops.(16) IL-10 is a potent anti-inflammatory cytokine and may affect bone formation.(17) It is known that low physiological concentrations of IL-10 (0.01-1.0 ng/mL) promote osteogenesis by activating the p38/MAPK signaling pathway. At the same time, higher doses of IL-10 (10-100 ng/mL) suppress osteogenesis by inhibiting the p38/MAPK signaling pathway by activating NF- κ B.(18) Amino-terminal propeptide procollagen type III (PIIINP) is used as a biomarker for increased collagen III synthesis.(19) PIIINP level may reflect degradation of soft callus during bone fracture healing.(20)

The study of the biochemical composition of blood provides high accuracy in assessing the state of the body. Therefore, it is used to monitor the healing process of fractures to correct treatment tactics. Purpose of this work is to study the regulation of the reparative processes of fractures of the proximal femur with intramedullary osteosynthesis during the correction of concomitant arterial hypertension on IL-6, TNF- α , IL-10, PIIINP, and other biochemical parameters.

Methods

The study was approved by the Committee on Ethical Animal Care and Use of the Kharkiv Medical Academy of Postgraduate Education, Ukraine (Protocol No. 5 dated November 12, 2019). The conditions for keeping laboratory animals included a natural light regime, an optimal temperature (20-22°C), a standard diet, and free access to water.(21) The experiments were carried out in accordance with the principles of the “European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes” (Strasbourg, 1986).

Study Design and Animal Model

This study involved two groups of animals: healthy Wistar rats (n=12) and spontaneously hypertensive rats (SHR) (n=24) weighing 250±30 g at the age of 8–9 months. The subjects were then grouped based on Figure 1.

The healthy rats were randomly assigned to two groups, with each group consist of 6 rats. Group 1.1 was represented by intact rats, while group 1.2 included animals with a modeled fracture.

SHR were also randomly distributed into four groups, with each group consist of 6 rats. Group 2.1 was represented by SHR animals, group 2.2 included SHR animals with a modeled fracture, group 2.3 comprised SHR animals, undergoing hypertension correction, and group 2.4 was

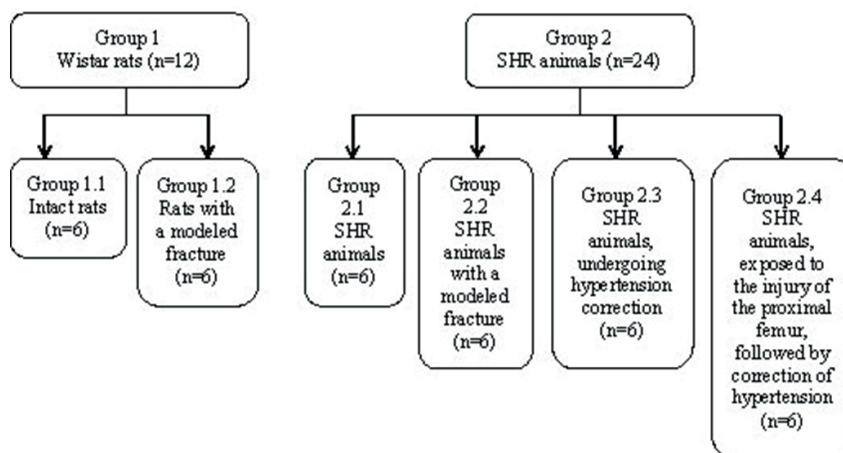


Figure 1. Subjects grouping scheme.

represented by SHR animals, exposed to the injury of the proximal femur, followed by correction of hypertension.

In animal groups 1.2, 2.2, and 2.4, surgery was performed under anesthesia with 10 mg/kg BW of zoletil (tiletamine hydrochloride and zolazepam hydrochloride, Virbac, Carros, France) intramuscularly. Without incision of soft tissues, using crampoms, a mechanical effect was made on the proximal femur perpendicular to the axis of the bone until a fracture appeared in it. Closed mini-invasive intramedullary osteosynthesis was performed to fix and stabilize the fracture. Then the stability of fixation was checked for the absence of rotational mobility of bone fragments, as well as for the absence of restrictions on mobility in the knee and hip joints.

In groups 2.3 and 2.4, the animals received enalapril from the day after the operation until the day of euthanasia. The drug was administered once a day at a dose of 1 mg/kg. Animals of groups 2.1 and 2.2 received an aqueous solution 14 days after the procedure, the animals were euthanized by inhalation of chloroform in a confined space.

Bioactive Molecules and Biochemical Parameters

Blood for the research was taken by open cardiac puncture. The levels of IL-6, TNF- α , IL-10 were determined using a solid-phase type of enzyme immunoassay kits (Vector-Best, Novosibirsk, Russia). The PIIINP level was determined using the Enzyme-Linked Immunosorbent Assay kit (Elabscience, Houston, TX, USA). Serum glucose (glucose oxidase method), uric acid (uricase method with peroxidase), creatinine (kinetic method with alkaline picrate (Jaffe)), urea (kinetic method, urease with glutamate dehydrogenase), cholesterol (enzymatic method), and albumin (bromocresol green method) levels were determined using diagnostic kits for clinical biochemistry SpineLab (Kharkiv, Ukraine). All

studies were carried out according to the manufacturer's protocols.

Histological Examinations

For histological examination, sections of the femur were removed from animals. The sections of the femurs of experimental rats were fixed in 10% neutral formalin, decalcified in 7% nitric acid, dehydrated in alcohols of increasing concentration (50°, 70°, and twice 96°), embedded in paraffin.(22) Paraffin sections prepared on a sled microtome were stained with hematoxylin and eosin, as well as according to Van Gieson, and analyzed in the field of view of a Primo Star microscope (Zeiss, Jena, Germany). Micrographs of preparations were performed using a Microocular digital camera.

Statistical Analysis

Statistical processing of the results was carried out using the Statistica 6.0 (StatSoft Inc., Tusla, OK, USA) analysis package using the non-parametric Mann–Whitney U test for independent samples. The results were presented as mean \pm SE, where SE was the standard error of the arithmetic mean. Differences were considered statistically significant at $p < 0.05$. Histograms were plotted by GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

Results

Bioactive Molecules and Biochemical Parameters

Modeling a fracture and correction of hypertension with enalapril led to a change in the concentration of bioactive molecules and biochemical parameters in the blood serum of experimental animals (Table 1, Figure 2).

Table 1. Concentrations of bioactive molecules in the blood serum of experimental animals.
Presented in mean±SE, n=6.

Group of Animals	Parameters			
	IL-6 (pg/mL)	IL-10 (pg/mL)	TNF- α (pg/mL)	PIINP (pg/mL)
1.1	2.761±0.497	217.607±24.743	1.835±0.131	20.126±2.048
1.2	2.415±0.174	167.769±18.817	2.168±0.247	34.167±0.719**
2.1	2.472±0.453	244.123±40.895	1.899±0.128	37.341±2.098
2.2	3.314±0.352	207.485±28.167	2.253±0.165	35.219±2.055
2.3	4.054±0.491	250.567±12.810	1.835±0.087	32.048±0.879
2.4	2.297±0.361*	215.631±24.920	2.215±0.251	32.862±1.097

Statistically significant differences were taken into account compared with the groups without surgery: * $p < 0.05$, ** $p < 0.01$; the selections were compared within one line.

Histological Analysis

On histological preparations, the site of the diaphyseal fracture was identified, which was performed with excess bone callus, the microscopic structure of which was different in three groups of animals after surgery (groups 1.2, 2.2, 2.4).

In animal group 1.2, the callus consisted of newly formed bone tissue of a small-looped structure with reticular-fibrous tissue in the intertrabecular spaces (Figure 3, 1A). The trabeculae were of sufficient width with a large number of osteocytes evenly distributed in the bone tissue (Figure 3, 2A). Osteoblasts were located on the surface of the bone trabeculae. Outside, the callus was covered with connective tissue formed by densely packed bundles of collagen fibers (Figure 3, 3A).

Signs of edema were revealed in histopreparations of callus in animals of the SHR group, erythrocytes in blood vessels, perivascular hemorrhages, and thickening of the walls of blood vessels were often observed, which indicates a violation of the rheological properties of blood and tissue blood filling. The newly formed bone tissue was located mainly in “islets” among the fields of reticular-fibrous tissue (Figure 3, 1B). Narrow bone beams were connected to each other only in some areas, forming a sparse trabecular network. The distribution of osteocytes in the bone tissue was uneven; osteoblasts were observed in small numbers (Figure 3, 2B). Focal lymph-histiocytic infiltration, hemorrhages were observed in the sites. In the connective tissue covering the callus, there were sites of leukocyte infiltration, vibration and fragmentation of collagen fiber bundles, their chaotic arrangement, probably due to edema (Figure 3, 3B).

The microscopic structure of callus in animals of the SHR group, which underwent hypertension correction, had features similar to both those of groups 1.1 and 2.2. However,

the bone trabeculae in all areas formed a trabecular network, while their width was smaller than in group 1.1 (Figure 3, 1C). Osteoblasts and osteocytes were observed in sufficient numbers (Figure 3, 2C). The phenomena of edema in bone and connective tissues were less pronounced, erythrocytes in the vessels were not detected, and perivascular hemorrhages were isolated (Figure 3, 3C).

Discussion

In our work, no differences were observed in the levels of IL-6 in healthy rats that underwent an experimental fracture of the femur compared with the same indicator in healthy rats in the group without surgery. Which, apparently, is associated with the pleiotropy of IL-6 and with a wide spectrum of biological activity of the studied cytokine in immune regulation, hematopoiesis, and inflammation, as well as in metabolic, proliferative, and regenerative processes.(23,24) The results of our work show that in rats with arterial hypertension, IL-6 levels increased after fracture (group 2.2) compared to similar cytokine concentrations in SHR rats without surgery (group 2.1), and also significantly decreased in the serum of SHR animals with modeled fractures while taking enalapril (group 2.4) compared to levels IL-6 in SHR rats of group 2.3.

Apparently, a decrease in IL-6 levels during hypertension correction is associated with a decrease in the inflammatory process. It should also be borne in mind that, inflammation can be one of the factors associated with a decrease in bone mass.(25) A decrease in inflammation appears to lead to a decrease in resorption processes. The results of the histological study carried out in our work confirm the improvement of healing in the group of animals, which was modeled fracture of the proximal femur and

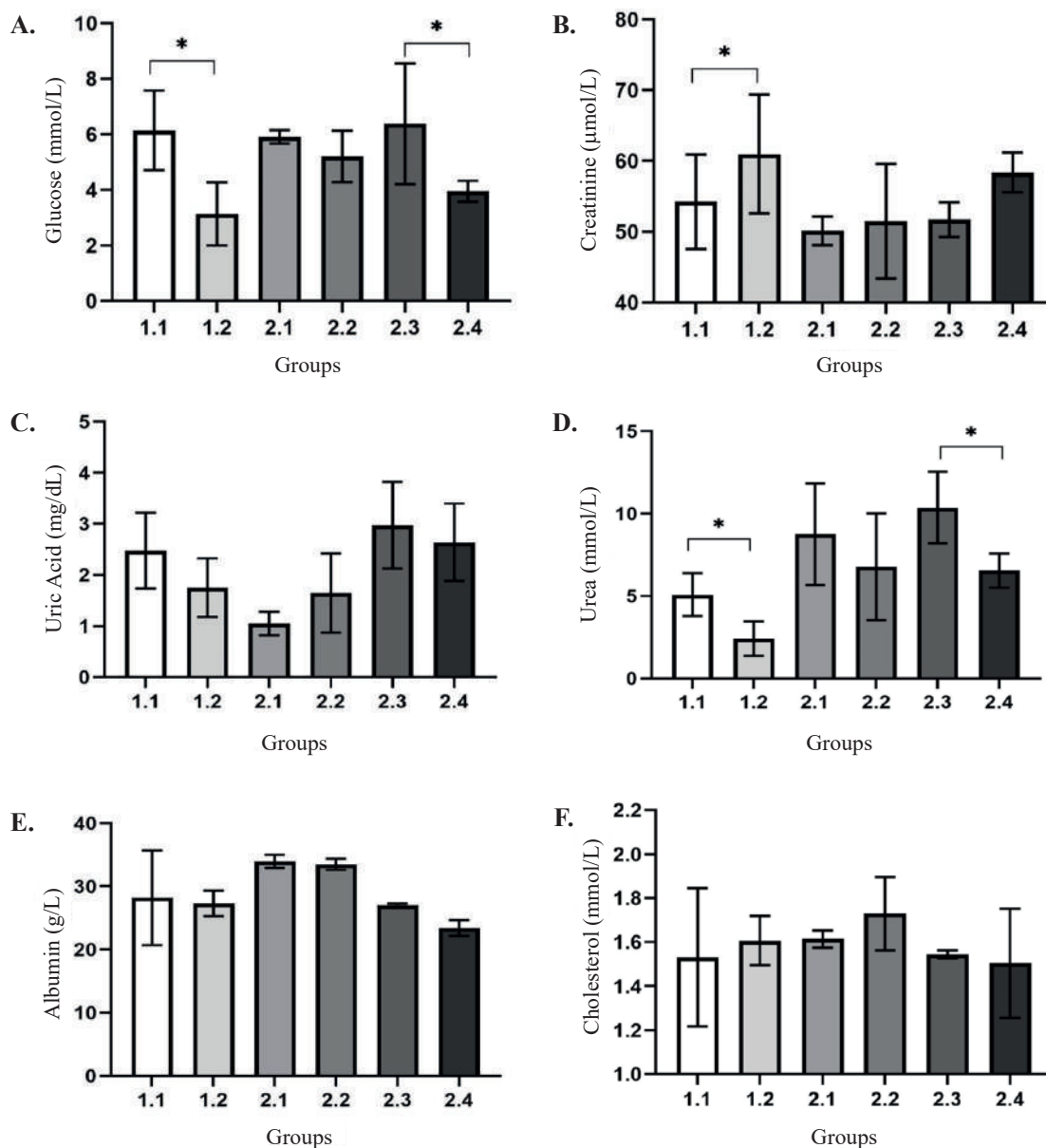


Figure 2. Levels of the studied biochemical parameters in the blood serum of animals. A: glucose; B: creatinine; C: uric acid; D: urea; E: albumin; F: cholesterol. The error bars represent the standard error of the arithmetic mean for each indicator, * $p < 0.05$ (n=6).

corrected hypertension with enalapril (group 2.4). Thus, a decrease in IL-6 production may be one of the mechanisms by which the correction of arterial hypertension stimulates the fracture healing process.

In our work, there was a tendency to an increase in the levels of TNF- α in the groups of animals that underwent an experimental fracture of the proximal femur, compared with the indices of the studied cytokine in the groups without surgery. Apparently, by this time, the healing of the fracture passes into the phase of late repair. As TNF- α levels are known to decrease during the early repair phase and increase during the late repair phase.(26) The literature also

contains data similar to those obtained by us. The authors reported that there were no statistically significant changes in serum TNF- α levels in older patients in the early stages of femoral fracture healing (1, 3, and 7 days after the fracture. (27) The literature also describes studies, the results of which differ from ours. Given that bone mineral density in SHR rats is reduced, studies of osteoporotic fractures in rats were analyzed. Thus, serum TNF- α levels in rats with these fractures were significantly higher at the 1st and 2nd weeks of healing.(28)

In our work, was shown a decrease in the serum level of IL-10 in all groups of animals, which had a simulated

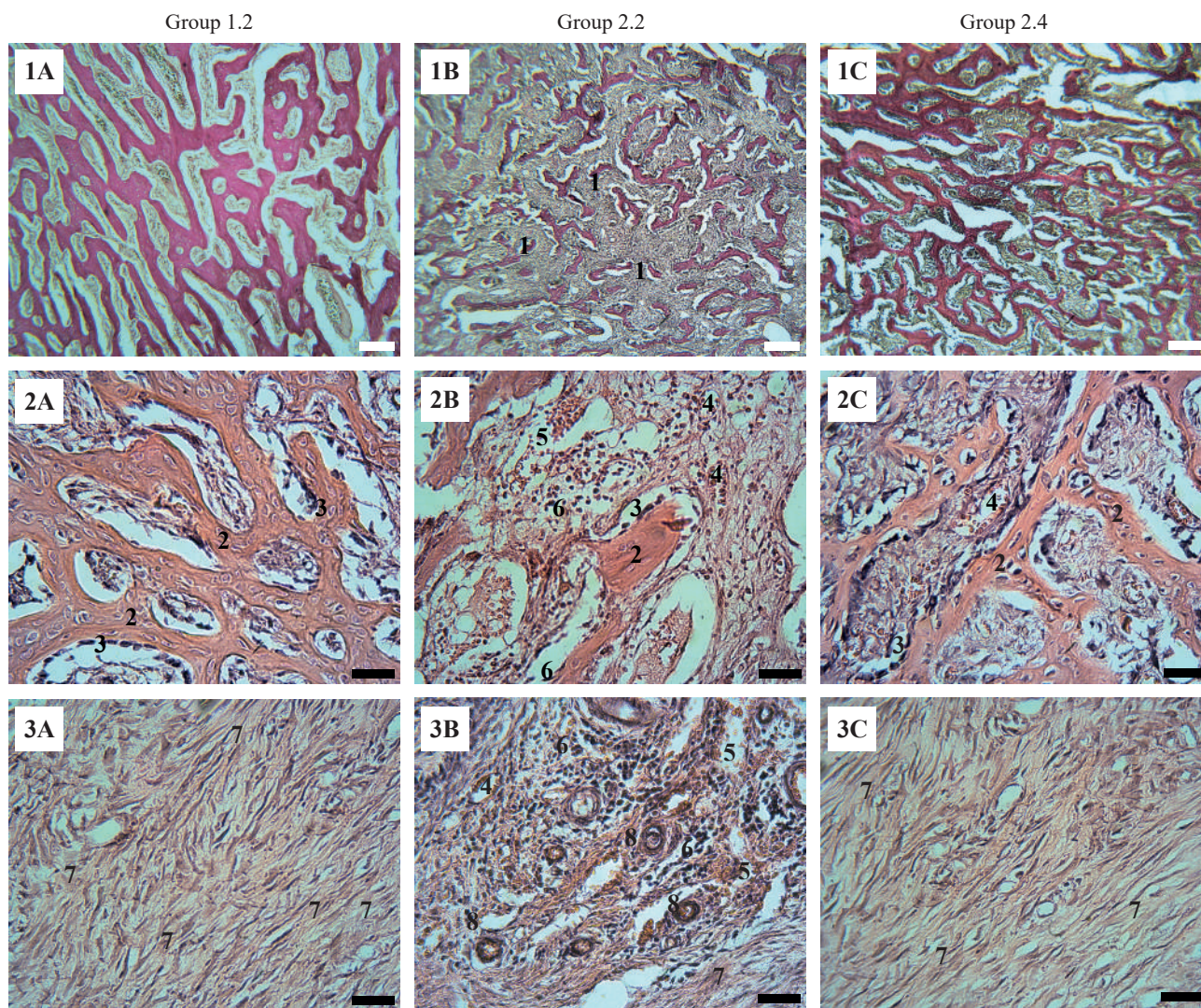


Figure 3. Site of bone callus in rats 2 weeks after surgery. 1A, 1B, 1C: trabecular meshwork, Van Gieson (white bar=100 μ m); 2A, 2B, 2C: bone trabeculae with osteocytes and osteoblasts, reticular-fibrous tissue, hematoxylin-eosin (black bar 25 μ m); 3A, 3B, 3C: connective tissue sheath, hematoxylin-eosin (black bar: 25 μ m). Annotation notes: 1: isolated trabeculae that do not form a network, 2: osteocytes, 3: osteoblasts, 4: dilated vessels, 5: hemorrhages, 6: foci of leukocyte infiltration, 7: dense connective tissue, 8: vessels with thickened walls and erythrocytes.

femoral fracture. The lowest concentrations of IL-10 were obtained in the serum of healthy Wistar rats after surgery. Given that a decrease in IL-10 concentration promotes osteogenesis, low levels of this cytokine in groups of animals with a fracture can be considered as a compensatory response to trauma. According to the literature, significantly lower levels of IL-10 in serum were found in rats at the 2nd week of fracture healing.(28) This is consistent with our results.

An increase in PIIINP level was shown in the group of healthy Wistar rats with surgery in comparison with similar concentrations of rats in group 1.1, which is possibly associated with an earlier onset of the remodeling

phase in this group of animals. At the same time, there were no differences in PIIINP levels in the groups of rats SHR 2.1 and 2.2, as well as in groups 2.3 and 2.4. Considering that SHR rats have low bone density, presumably due to dysregulation of bone remodeling together with impaired bone architecture prone to fractures (29), it can be assumed that changes in PIIINP levels in SHR rats of the experimental groups will occur in the more distant period of fracture healing. Our results are confirmed by the literature data. In one study, PIIINP levels peaked at 2 and 12 weeks after plate osteosynthesis of closed malleolus fractures and distal tibial fractures.(30) In another study, the peak of the rise in PIIINP concentrations occurred 42 days after bone injury in athletes

and remained significantly elevated up to 84 days.(31) For tibial shaft fractures with a fibular fracture with the delayed union, PIIINP levels were also significantly higher after ten weeks. At the same time, signs of an insufficient response of osteoblasts appeared only twenty weeks after the injury. Matrix collagen I and III production in the first ten weeks of healing was adequate.(32) Our results contradict the findings of a study that showed significantly higher serum levels of PIIINP in the early stages of the fracture healing process in the group of animals with impaired bone healing compared with animals in which there was a normal fracture healing process.(33)

Thus, the studied profile of intercellular mediators and the revealed relationships reflect changes in regulatory pathways that lead to disruption of reparative processes during fracture healing in SHR animals. Correction of arterial hypertension in fractures of the proximal femur in SHR rats leads to improved bone remodeling. Further studies will make it possible to assess the role of bioactive molecules in regulatory mechanisms in fracture healing both in arterial hypertension and in other chronic pathologies, which in the future will make it possible to optimize the treatment of patients with fractures of the proximal femur with concomitant hypertension.

The results of our study demonstrate a decrease in glucose levels 2 weeks after surgery in animals of groups 1.2 and 2.4 (Figure 2A). One of the reasons for the decrease in this indicator after the damage is the increased uptake of glucose by proliferating cells.(34)

In our work, urea levels were reduced in animals of all groups that underwent hip surgery (Figure 2D). The concentration of urea in the blood depends on the rate of protein metabolism, liver and kidney function. Since the liver is the main site of urea production, when organ function declines in response to injury, urea levels also drop. It is worth noting that urea levels were significantly higher in SHR rats compared to healthy Wistar rats. There is evidence in the literature that there is an increase in urea and creatinine levels with increasing blood pressure.(35) Hypertension, in turn, is associated with the risk of mortality after hip fractures.(36)

In our work, an increase in creatinine concentrations in animals of group 1.2 after surgery was shown, as well as a tendency to an increase in creatinine levels was observed in animals of group 2.4, who received enalapril after surgery (Figure 2B). It should be noted that ACE inhibitors are not nephrotoxic.(37) According to the literature, a high level of creatinine is one of the markers of the risk of early mortality in elderly patients with fractures of the proximal femur.(38)

There were no differences in creatinine levels in SHR rats compared to healthy Wistar rats.

In our study, the serum uric acid level was decreased in Wistar animals after traumatic exposure and increased in SHR animals that underwent surgery (Figure 2C). Considering that an increased level of uric acid in serum may be associated with hypertension (39), administration of enalapril in SHR rats after the operation contributed to a decrease in uric acid in the blood serum of animals of this group. The literature also shows an association of uric acid with an increased risk of hip fractures.(40)

In our study, in rats of groups with an experimental defect of the femur, lipid metabolism disorders were not revealed (Figure 2F). According to the literature, high total cholesterol levels are associated with a low risk of fractures.(40) The results obtained in our work demonstrate no difference in albumin levels in Wistar and SHR rats (Figure 2E). According to the literature, low albumin levels are another potent independent risk factor for death after surgery for geriatric hip fractures.(41) Hypoalbuminemia is also an independent predictor of many cardiovascular diseases.(42) However, there was no association of serum albumin with fracture risk in relation to blood pressure.(43)

As a result of the histological study, it can be noted that in the groups of SHR animals for which a fracture of the proximal femur was simulated, there was a violation of the process of reparative bone regeneration and a slowdown in the consolidation of bone fragments. The use of enalapril has a positive effect on the healing of fractures of the proximal femur in animals with arterial hypertension, which is reflected in the acceleration of repair processes at the stage of remodeling.

Conclusion

Correction of disturbances in the reparative processes of fractures of the proximal femur with the use of therapy correcting hypertension occurs, apparently, due to modulation with cytokines. The results of histological and biochemical studies have shown that the use of enalapril in arterial hypertension stimulates the reparative processes of bone tissue.

Acknowledgements

This study was funded by the Ministry of Health of Ukraine from the state budget.

Authors Contribution

VB and SR were involved in planning and supervised the work, MV and LB performed the measurements, TM and MK processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. MV and MK conducted a statistical analysis. VB, TM and LB aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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