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# LOCAL APPLICATION OF GROWTH HORMONE INDUCES INCREASED IMMUNOEXPRESSION OF IGF-I ON CRANIOFACIAL BONE DEFECTS REPAIR – A PILOT STUDY

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**ABSTRACT** The aim is to evaluate the local effect of different concentrations of growth hormone (GH) on the repair of craniofacial bone defects, through histological, histomorfometric, and insulin-like growth factor I (IGF-I) immunoexpression assessments. Critical defects (5 mm) were performed in 32 *Wistar* rats. The animals were divided into four groups: Group C (Control); Group S (Sponge-collagen); Group GH 0.08 mL; GH 0.104 mg; Group GH 0.1mL. Local applications were performed 3 times a week until the rats were euthanized at 60 days. The data were submitted to ANOVA and Tukey's test ($P < 0.05$). A healing process with predominance of collagen fibers and bone neoformation near the edges of the defect was observed in groups C and S. Islands of bone neoformation were observed at the center and edges of the defect in groups GH 0.08 and GH 0.1. In GH 0.1, the bone was more compact, and the defect was completely closed in some specimens. Bone neoformation was significantly higher in the GH-treated groups. All the specimens stained positive for IGF-I, and this immunoexpression was significantly higher in Group GH 0.1. In conclusion, locally applied GH significantly favored bone repair in rat calvaria, and a higher dose of GH increased the immunoexpression of IGF-I.

**KEYWORDS :** Growth hormone; insulin-like growth factor I; wound healing; bone eration.

## INTRODUCTION

In recent years, therapies that accelerate bone regeneration and stimulate osteoblastic activity have attracted the attention of researchers. Focus has been placed on therapies using stem cells, bisphosphonates and hormones, such as growth hormone (GH) and parathyroid hormone (PTH). GH therapy stands out, owing to its interference with bone physiology. [1,2,3,4]

Initially, GH was only indicated for children who presented growth retardation caused by a secretion deficiency. Currently, GH replacement therapy has been gaining prominence in adults. GH replacement in elderly patients who are deficient in this hormone has been found to improve the treatment of osteoporosis and promote an increase in muscle and bone mass, thus increasing the physical capacity of these patients. [5,6]

One of the first studies on the use of GH to treat bone defects was conducted by Wittbjer et al. in 1983. The authors studied the influence of local administration of human biosynthetic GH on bone formation in bone defects created in rabbits and grafted with autogenous bone. Since then, some studies have addressed this issue. [7] Studies have shown that the use of systemic GH favors the repair of bone defects [1,2] and improves the initial stability of implants [8], owing to direct or indirect stimulation of osteogenic cells [9,10] ; however, few studies to date have investigated the local or topical effect of GH on craniofacial bone repair. [4,11,12]

Therefore, the aim of this pilot study is to assess the influence of local application of different concentrations of GH on cranial bone repair, and evaluate the results of this application by performing histological and histomorfometric analyses, and by assessing the immunoexpression of insulin-like growth factor I (IGF-I).

## MATERIAL AND METHODS

The animal protocol was designed to minimize pain and discomfort to the animals. The experiments were carried out in the Vivarium at Positivo University, following approval by the Ethics Committee on the Use of Animals (ECUA 253). This study followed the guidelines of ARRIVE (Animal Research: Reporting in Vivo Experiment). The animals were kept in cages (n = 2/per cage) and throughout the experiment, the ambient light, temperature and humidity of each room were controlled by a digital panel in order to maintain a photoperiod of 12 h, a temperature range of 18 °C - 22 °C, and 65% humidity.

### Study design

Thirty-two male rats (Rattus norvegicus, albinus, *Wistar*), aged between 5 and 6 months and weighing between 340 and 450 g were used. The animals were randomly divided into 4 experimental groups: Group C (Control), clot and local application of saline solution; Group S (Sponge), collagen sponge associated to local application of saline solution; Group GH 0.08, collagen sponge associated to local application of 0.08 mL of GH (0.104 mg); Group GH 0.1, collagen sponge associated to local application of 0.1 mL of GH (0.130 mg). The local applications were performed by subcutaneous route.

### Surgical procedures

The animals were positioned individually inside a bell jar, then submitted to inhalation induction of anesthesia with oxygen and isoflurane (Cristália, Itapira, SP, Brazil), and

subsequently anesthetized by intramuscular injection in the posterior part of the thigh with 2.3 g of xylazine (0.52 mg/kg) (Vetbrands, Paulínia, SP, Brazil) and 1.16 g of ketamine (1.04 mg/kg) (Vetbrands, Paulínia, SP, Brazil). Anesthesia was maintained by vaporized isoflurane (Cristália, Itapira, SP, Brazil), using a face mask when necessary. The surgical procedure was performed by a single trained operator.

### Creation of critical calvarial defect

After anesthetic induction, hair removal and antisepsis with topical polyvinylpyrrolidone-iodine (PVP-I) (10% active iodine) were performed on the calvaria of the animals. A U-shaped incision was then made with a #15c scalpel blade for surgical access to the calvarial region, and a full-thickness flap was raised towards its posterior.

A critical-size transosseous defect (CTD) with a diameter of 5 mm[13] was created in the calvaria of each rat with a trephine (Neodent, Curitiba, PR, Brazil) coupled to an implant contra-angle (20:1; Kavo, Joinville, SC, Brazil) under copious irrigation with sterile saline solution. The defect reached the sagittal suture region. Removal of the bone block was carefully performed with spatulas to avoid rupture of the meninges.

The defect was filled only with a blood clot in the animals of Group C. In the animals of Group S, the defect was filled with a hydrolyzed collagen sponge (half the trephine size) (Hemospon, Technew, Rio de Janeiro, RJ, Brazil); 0.1 mL of saline was then applied with an insulin syringe and needle, and the defect was covered with a bovine biological membrane (8 x 8 mm) (GenDerm, Baumer, Mogi Mirim, SP, Brazil) (Figure 1).

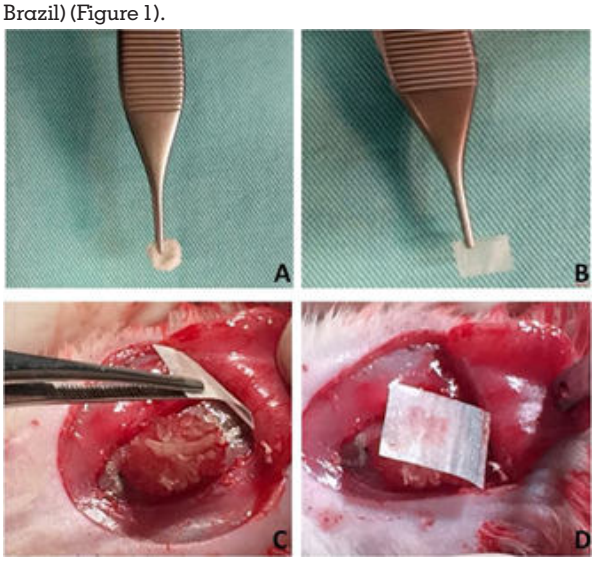


**Figure 1** – Group E: (A) Collagen sponge (Hemospon) 5 mm in diameter, cut with a trephine of the same diameter as that of the defect created in rat calvaria; (B) Genderm 8 x 8 mm membrane being positioned in the center of the defect; (C) collagen sponge being placed in the defect; (D) membrane over the sponge before tissue suturing.

In the animals of Group GH 0.08, the defect was filled with a hydrolyzed collagen sponge (with half the trephine size) (Hemospon, Technew, Rio de Janeiro, RJ, Brazil); 0.08 mL of GH was then applied with an insulin syringe and needle, and the defect was covered with a bovine biological membrane (8 x 8 mm) (GenDerm, Baumer, Mogi Mirim, SP, Brazil). In the animals of Group GH 0.1, the defect was filled with a hydrolyzed collagen sponge (with half the trephine size) (Hemospon, Technew, Rio de Janeiro, RJ, Brazil); 0.1 mL of GH was then applied with an insulin syringe and needle, and the defect was covered with a bovine biological membrane (8 x 8 mm) (GenDerm, Baumer, Mogi Mirim, SP, Brazil).

The soft tissues were repositioned and sutured using simple sutures. In the animals of Groups C and S, 3 weekly applications of 0.1 mL of saline were performed every other day, for 60 days. In the animals of Group GH 0.08, 3 weekly applications of 0.08 mL of GH (0.104 mg dose) were performed every other day, for 60 days. In the animals of Group GH 0.1, 3 weekly applications of 0.1 mL of GH (0.130 mg dose) were performed every other day, for 60 days.

As post-operative medication, each animal received an intramuscular injection of 24,000 units of penicillin G-benzathine in a single dose (Pentabiotico Veterinário Pequeno Porte, Fort Dodge Saúde Animal Ltda., Campinas, SP, Brazil), a single, 1 mg/kg subcutaneous dose of morphine, and 5 drops of paracetamol diluted in 100 mL of water ad libitum for 3 days.

### Euthanasia

The animals were euthanized 60 days after the surgical procedure, and then placed in a gas chamber ($CO_2$) and kept there for 10 min.

### Bone block removal

The bone blocks were removed and placed in vials with 10% formalin. The 4 livers and 8 kidneys of the animals of Groups C, GH 0.08 and GH 0.1 were removed and fixed in 10% formalin.

### Tissue processing

The bone blocks containing the surgical defect were decalcified in a 18% ethylenediaminetetraacetic acid (EDTA) solution. After decalcification, the specimens were cut in half, parallel to the sagittal suture.

Bone blocks and organs (livers and kidneys) underwent laboratory processing and were embedded in paraffin. Five-$\mu$m-thick longitudinal serial cuts were performed, starting from the center of the surgical defect. Five-$\mu$m-thick longitudinal serial cuts were also performed in the livers and kidneys. All the sections were stained with hematoxylin and eosin (HE) for analysis under light microscopy. For the histological and histomorphometric analyses, two cuts from each animal were selected to represent the center of the original surgical defect. The analyses were performed by a single, previously trained operator.

### Histological analysis

The images were analyzed using an optical microscope (Quimis, Diadema, SP, Brazil), and the following parameters of the surgically created bone defects were evaluated: bone defect closure, connective tissue characteristics, presence of osteoid matrix, and progression and type of repair. An assessment was also made to ascertain whether there were cellular or tissular changes in the livers and kidneys of the test animals, compared to the organs of the control animals that did not receive the locally applied GH.

### Histomorphometric analysis

The slides were serially photographed with a digital camera (EOS Rebel T5, Canon, Tokyo, Japan) coupled to a microscope (Olympus BX41, Melville, NY, USA) under 40X magnification. The individual images were then assembled using presentation software (Microsoft Powerpoint; Microsoft Corporation, Redmond, WA, USA) to form a single continuous image containing the two edges of the defect. Next, the images were submitted to the following histomorphometric measurements using ImageJ 1.6.0 software (Wayne Rasband [NIH], Bethesda, MA, USA):

1. Total area (TA): total area of the surgically created defect.

This measurement was performed from edge to edge of the original defect, including the variation in the skull thickness of each animal.

2. Area of osteoid matrix (AOM): areas of bone neoformation.

The TA was measured in $mm^2$, and was considered to be 100% of the area to be analyzed. AOM was also measured in $mm^2$, and was calculated as a percentage of TA, according to the following formula: AOM ($mm^2$) / TA ($mm^2$) x 100.

### Immunohistochemical analysis

The serial sections were submitted to dewaxing in xylol for 30 min at 40°C and rehydrated in decreasing alcohol concentration and distilled water solutions. Antigen retrieval was performed by proteolytic enzymatic digestion with a 5% pepsin solution (pepsin from porcine gastric mucosa; Sigma-Aldrich, Germany) for 60 min at 37°C in a humid chamber. Subsequently, the sections were washed in phosphate buffer (PBS, 0.05 M, pH 7.2) and submitted to blocking of endogenous peroxidase with 50% hydrogen peroxide. Background blocking was then performed with a 5% skim milk solution for 15 min at 36°C, followed by washing in PBS. The sections were then submitted to IGF-1 primary antibody incubation for 16 hours at 4°C in a moist chamber, using IGF-1 antibody, H-70, and rabbit polyclonal IgG at dilutions of 1:150, 1:200 and 1:100, respectively (Santa Cruz, Cambridge, MA, USA). After washing with PBS buffer, the sections were incubated with the avidin-biotin peroxidase complex (RTU, Vectasin, ABC Kit; Vector Laboratories Inc, Burlingame, CA, USA) for 30 min for each step, at room temperature. Afterwards, the reaction was developed with 0.06 mL of a substrate-chromogen diaminobenzidine solution (DAB-3,3 diaminobenzidine, Diagnostic BioSystems, Pleasanton, CA, USA) for 5 min. The slides were then washed with PBS, counterstained with Harris's haematoxylin, and mounted in a resin medium (Entellan, Merk Millipore, Darmstadt, Germany). A qualitative analysis of the distribution pattern of protein immunostaining was performed for reading and interpretation. The cuts underwent the same protocol for the negative control of the slides, except for the primary antibody, which was replaced by PBS.

For the qualitative immunohistochemical analysis, the images were analyzed using an optical microscope (Olympus BX41, Melville, NY, USA) with 400X magnification. IGF-I immunostaining was observed in the areas of extracellular matrix and osteoid matrix, and the presence of IGF-I-positive cells was also observed.

For the quantitative immunohistochemical analysis, each specimen was photographed three times along the length of the defect (two photos near the edges and one in the center) using a digital camera (EOS Rebel T5, Canon, Tokyo, Japan) coupled to an optical microscope (Olympus BX41, Melville, NY, USA) at 400X magnification. Image J software was used to perform the IGF-I immunoexpression count, and the area of the images was counted in square pixels.

### Statistical analysis

The research methodology, statistical applications and results were reviewed by an independent statistician. The area measurements for each animal (experimental unit) were represented as percentages. Mean, standard deviation and coefficient of variation were calculated. The Shapiro-Wilk test was used to assess the normality of the data from the histometric and immunohistochemical analyses. The Levene test was used to assess homoscedasticity. One-way ANOVA and Tukey's test were used to compare the study groups. All the tests were performed using a level of significance of 5%.

## RESULTS

### Histological analysis

*Group C (control)*

Histological analysis revealed that most of the defect was filled with a bundle of collagen fibers arranged in parallel in most specimens in Group C. Few areas of bone neoformation were observed near the edges of the defect (Figure 2a).

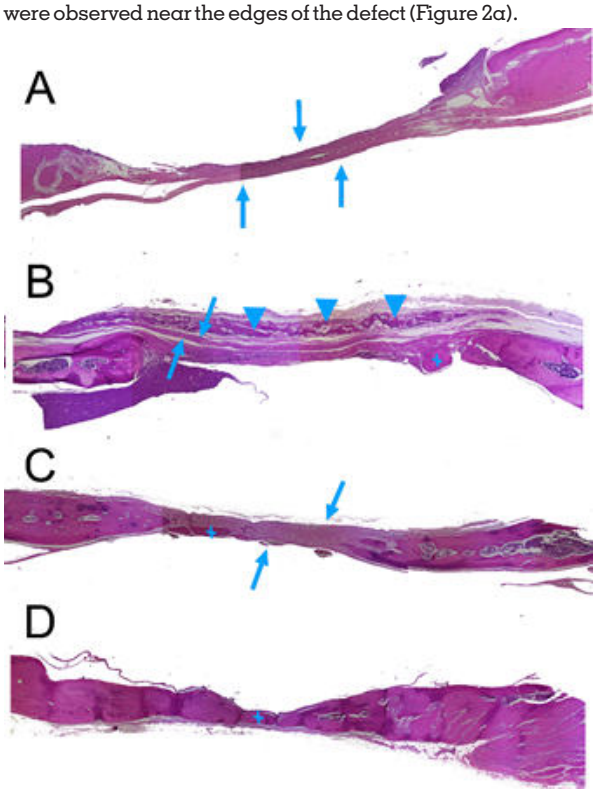


**Figure 2** –Histological image of the craniofacial bone defects created in the study animals. (A) Group C (60 days after surgery): bone neoformation near the edges of the defect; most of the inner part of the defect is filled with collagen fibers arranged in parallel (arrows) (HE; original magnification 40X). (B) Group S (60 days after surgery): presence of granulation tissue composed of collagen fibers (arrows), chronic inflammatory infiltrate, and remnants of collagen sponge and bovine cortical membrane (head arrows) and bone neoformation near the edges of the defect (asterisk) (HE; original magnification 40X). Group GH 0.08 (60 days after surgery): (c) islands of bone neoformation among collagen fibers (asterisk), bone neoformation near the edges of the defect, and thick collagen fibers (arrows) (HE; original magnification 40X). Group GH 0.1 (60 days after surgery): (d) bone neoformation in the entire length of the defect (HE; original magnification 40X).

*Group S (sponge)*

In most specimens, the defect was filled by granulation tissue composed of collagen fibers, chronic inflammatory infiltrate and remnants of bovine cortical membrane. Some areas of bone neoformation were observed near the edges of the defect (Figure 2b).

*Group GH 0.08*

In most specimens, there were thick collagen fibers arranged in parallel, islands of bone neoformation permeating the collagen fibers, and areas of bone neoformation near the edges of the defect. In none of the specimens was the defect completely closed (Figure 2c).

*Group GH 0.1*

In most of the defects of this group, the neoformed bone at the

edges of the defect was more compact than in the other groups, and the center of the defect had thick collagen fibers and islands of bone neoformation. The defect was completely closed in some specimens of this group (Figure 2d).

*Kidneys and Liver*

Histological images of the kidneys and liver of the animals of groups C, GH 0.08 and GH 0.1 are shown in Figure 3. Both organs had histological characteristics consistent with normality, both in the control group and in the groups treated with GH. In the liver, the centrilobular vein and porta hepatis were normal. The hepatocytes were hexagonal in appearance and were organized in a cordonal pattern; the sinusoidal capillaries appeared normal.

**Histomorphometric analysis**

Table 1 shows that the local application of GH in both concentrations resulted in a greater amount of bone repair. A significant increase in bone formation was observed in the groups where GH was used. Although no difference in bone neoformation was found between the GH 0.08 and GH 0.1 groups, there was a tendency towards larger bone areas when the higher GH dose was used.

**Immunohistochemical analysis**

*Qualitative analysis of IGF-I immunoexpression*

Presence of the IGF-I protein was observed in all the specimens of all the groups analyzed. There was less staining of IGF-I-positive cells in Groups C, S and GH 0.08 than in Group GH 0.1. Higher staining levels were observed in osteoblasts, fibroblasts and endothelial cells. Intense staining of the extracellular matrix within the defect was observed in all the specimens of all the groups (Figure 3).

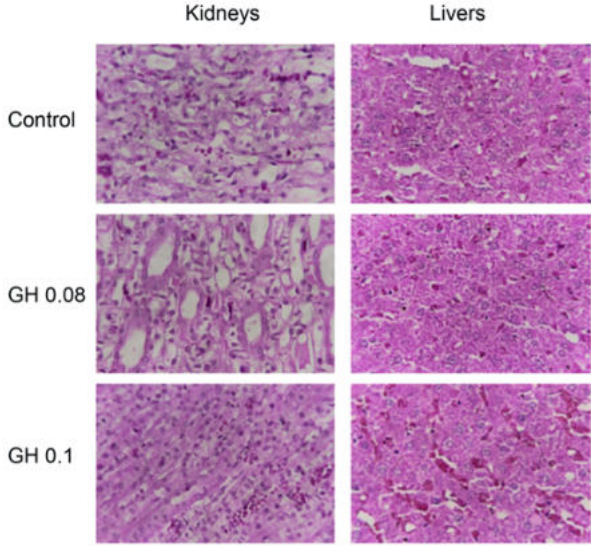


**Figure 3** – Histological images of the kidneys and livers of the animals of the different study groups (HE; original magnification 40X).

*Quantitative analysis of IGF-I immunoexpression*

Table 2 shows that there were no significant differences between groups C and S and between groups E and GH 0.08 in terms of IGF-I immunoexpression. In contrast, there were significant differences between Group C and Groups GH 0.08 and GH 0.1, and between Group S and Group GH 0.1. IGF-I immunoexpression increased with the application of a greater dose of hormone.

## DISCUSSION

In recent years, hormone therapy has been investigated in dentistry as a means of promoting bone repair and increasing osseointegration of dental implants. [8] GH stimulates the production of collagen and the synthesis and release of IGF-I, which, in turn, promotes an increase in the deposition of proteins by chondrocytes and osteoblasts, and in the number of mitoses, as well as the conversion of chondrocytes into osteoblasts.[14,15] Considering the positive effect of GH action on the bone healing process in critical defects, the aim of the present study can be justified by the clinical possibility of performing local GH applications. The results of this pilot study showed significant differences between the control groups (C and S) and the test groups (GH 0.08 and GH 0.1), regarding osteoneogenesis, thus confirming the action of GH in promoting bone repair.

Other studies using systemically, and locally applied GH also obtained positive osteoneogenesis results. When administered systemically for 10 consecutive days, a higher torsional strength of porcine tibiae and a faster ossification were observed in distractions performed on these bones.[16] An increase in bone regeneration in critical defects covered with expanded polytetrafluoroethylene (PTFE-e) membrane in rat calvaria was also observed after systemic application of 1.35 mg/kg of GH twice daily for 28 days.[10] Local use of GH led to accelerated condylar cartilage growth in rabbits [11], and GH associated to tricalcium phosphate cement for the treatment of critical defects in rabbit tibia led to enhanced bone repair.[12] Our results, despite being a pilot study, corroborate those findings.

However, the dose used in most studies was higher than those used in this study (0.104 mg and 0.130 mg), and the methods used were also different. [11, 12] Hence, it may be suggested that smaller doses can also be applied locally to stimulate cranial bone repair without causing systemic changes in distant organs. No hepatic or nephrological changes were observed in the animals of any of the groups, justifying the local application of GH to take advantage of the benefits of this hormone in the repair of bone lesions.

As for the results found in the different studies, some important aspects should be borne in mind. The results of animal experiments are influenced by factors, such as the species and type of animal used, animal age, defect stability, anatomic location of the defect, defect size, postoperative evaluation periods, and, lastly, the methods and criteria used for evaluation.[17]

The creation of critical defects in rat calvaria was chosen in this study because this method is commonly used in the related literature. [12,13] The critical defect simulates a difficult-to-heal defect and enables an assessment of how well the grafting material, whether associated to other stimulating factors like GH, aids in the repair process. In addition, this experimental model was chosen because it is easy to perform, involves a rapid tissue response, and is not costly.

Defect stability is an important factor when evaluating bone repair [17], and one way to achieve this goal is to use biological membranes. Bovine membrane was used in this research as a barrier for the hemostatic sponge graft. Its permeability allows the exchange of nutrients and prevents the invagination of non-osteogenic cells, ensuring favorable conditions for bone remodeling.[18]

Drugs in liquid form used in local applications need carriers to remain in the destination site. [12] The hemostatic sponge of porcine freeze-dried collagen was used as the GH carrier in the GH 0.08 and GH 0.1 groups. This sponge is helps to control bleeding, stabilizes the blood clot and protects the defect area.[19]

In addition, it can be assumed that the sponge contributes to clot stability by preventing the collapse of tissues, thereby maintaining the thickness of the defect. This may have favored

the migration of osteoblasts into the defect, possibly explaining the intense IGF-I staining observed in Group S, which was statistically like that observed in Group GH 0.08.

GH may have both anabolic and catabolic effects, depending directly on the dose administered. Furthermore, the optimal dose for bone gain promotion has yet to be established in the literature. High-dose GH therapy may lead to insulin resistance and functional impairment of the kidneys, owing to increased serum creatinine. [20] Since no renal alteration was observed, it could be stated that the dose used in this study was not high.

GH acts directly on the liver, which, stimulated by its action, releases IGF-I to exert its metabolic effect on the cells of bone, muscle, and other tissues. IGFs are synthesized by the liver and by most osteogenic cells. [21] A higher level of IGF-I immunostaining was observed in the groups where GH was applied locally to rat calvarial bone defects than in the control groups (C and S). IGF-I stimulates osteoneogenesis by inducing osteoblast proliferation and differentiation and inhibits the degradation of bone collagen by blocking collagenase activity. [22] Therefore, it is suggested that the GH-induced production of IGF-I (the actual agent responsible for the GH effects) promotes a more favorable environment for the bone repair process.

In our study, and within the limitations of our methodology, mainly involving few animals, GH proved to have a positive effect on bone repair Future research is warranted to assess the effect of GH on bone healing using different experimental designs, administration routes and doses, as well as different time periods such as one to four weeks postoperatively, aiming at further elucidating the mechanism of action of GH and its possible interactions with bone physiology.

## CONCLUSION

In conclusion, locally applied GH significantly favored bone repair in rat calvaria, and a higher dose of GH increased the immunoexpression of IGF-I.

### Acknowledgements

The authors declare there are no conflicts of interest in this study.

**Table 1** – Mean percentage area and Standard Deviation of osteoid matrix observed in the study groups.

| Group | % bone |
|---|---|
| Control | $7.04^{a} \pm 0.47$ |
| Sponge | $11.73^{a} \pm 0.33$ |
| GH 0.08 | $25.93^{b} \pm 0.18$ |
| GH 0.1 | $29.05^{b} \pm 1.35$ |

ANOVA and Tukey's HSD test ($p \leq 0.05$). Different letters indicate the existence of a statistically significant difference.

**Table 2** – Mean value and Standard Deviation of IGF-I immunoexpression (in pixels) observed in the study groups.

| Group | IGF-I immunoexpression |
|---|---|
| Control | $7.67^{a} \pm 2.12$ |
| Sponge | $9.32^{ab} \pm 0.54$ |
| GH 0.08 | $9.93^{bc} \pm 0.86$ |
| GH 0.1 | $11.37^{cd} \pm 0.52$ |

ANOVA and Tukey's HSD test (p 0.05). Different letters indicate the existence of a statistically significant difference.

## REFERENCES

1. Simpson AH, Mills L, Noble B. The role of growth factors and related agents in accelerating fracture healing. *J Bone Joint Surg Br*. 2006;88:701-705.
2. Tran GT, Pagkalos J, Tsiridis E, Narvani AA, Heliotis M, Mantalaris A, et al. Growth hormone: does it have a therapeutic role in fracture healing? *Expert Opin Investig Drugs* 2009;18:887-911.
3. Dioufa N, Schally AV, Ioulia C, Moustou E, Block NL, Owens GK, et al. Accelaration of wound healing by growth hormone-releasing hormone and its agonists. *Proc Natl Acad Sci USA* 2010;107:18611-18615.
4. Martin-Monge E, Tresguerres IF, Clemente C, Tresguerres JA. Local application of growth hormone to enhance osseointegration in osteoporotic bones: A morphometric and densitometric study. *Int J Oral Maxillofac Implants* 2017;32:751-758.
5. Kolbeck S, Bail H, Schmidmaier G, Alquiza M, Raun K, Kappelgard A, et al. Homologous growth hormone accelerates bone healing – a biomechanical and histological study. *Bone* 2003;33:628-637
6. Stenport VF, Olsson B, Morberg P, Tornell J, Johansson CB. Systemically administered human growth hormone improves initial implant stability: an experimental study in the rabbit. *Clin Implant Dent Relat Res* 2001;3:135-141.
7. Wittbjer J, Rohlin M, Thorngren KG. Bone formation in demineralized bone transplants treated with biosynthetic human growth hormone. *Scand J Plast Reconstr Surg* 1983;17:109-117.
8. Calvo-Guirado JL, Mate-Sanchez J, Delgado-Ruiz R, Ramirez-Fernández MP, Cutando-Soriano A, Peña M.. Effects of growth hormone on initial bone formation around dental implants: a dog study. *Clin Oral Implants Res* 2011;22:587-593
9. Andreassen TT, Jorgensen PH, Flyvbjerg A, Orskov H, Oxlund H. Growth hormone stimulates bone formation and strength of cortical bone in aged rats. *J Bone Miner Res* 1995;10:1057–1067
10. Cacciafesta V, Dalstra M, Bosch C, Melsen B, Andreassen TT. Growth hormone treatment promotes guided bone regeneration in rat calvarial defects. *Eur J Orthod* 2001;23:733-740.
11. Masood F, Mohammad R, Mohsen M, Fatemeh T, Sepide D, Shahlaa M. The effect of local injection of the human growth hormone on the mandibular condyle growth in rabbit. *Dent Res J (Isfahan)* 2014;11:436-441
12. Emilov-Velev K, Clemente-de-Arriba C, Alobera-García MÁ, Moreno-Sansalvador EM, Campo-Loarte J. Bone regeneration in experimental animals using calcium phosphate cement combined with platelet growth factors and human growth hormone. *Rev Esp Cir Ortop Traumatol* 2015;59:200-210.
13. Messora MR, Nagata MJ, Mariano RC, Dornelles RC, Bomfim SR, Fucini SE, et al. Bone healing in critical-size defects treated with platelet-rich plasma: a histologic and histometric study in rat calvaria. *J Periodontal Res* 2008;43:217-223.
14. Isaksson OG, Jannsson JO, Gause IA. Growth hormone stimulates longitudinal bone growth directly. *Science* 1982;216:1237-1239.
15. Feizbakhsh M, Razavi M, Minaian M, Teimoori F, Dadgar S, Maghsoodi S. The effect of local injection of the human growth hormone on the mandibular condyle growth in rabbit. *Dent Res J (Isfahan)*. 2014;11(4):436-41.
16. Raschke MJ, Bail H, Windhagen HJ, Kolbeck SF, Weiler A, Raun K, et al. Recombinant growth hormone accelerates bone regenerate consolidation in distraction osteogenesis. *Bone* 1999;24:81-88.
17. Bosch C, Melsen B, Vargervik K. Importance of the critical-size bone defect in testing bone-regeneration materials. *J Craniofac Surg* 1998;9:310-316.
18. Tal H, Pitaru S, Moses O, Kozlovsky A. Collagen gel and membrane in guided tissue regeneration in periodontal fenestration defects in dogs. *Journal of Clinical Periodontology*, 1996;23:1-6
19. Alpaslan C, Alpaslan GH, Oygur T. Tissue reaction to three subcutaneously implanted local hemostatic agents. *Br J Oral Maxillofac Surg*. 1997;35:129-32.
20. Cutfield WS, Wilton P, Bennmarker H, Albertsson-Wikland K, Chatelain P, Ranke MB, Price DA. Incidence of diabetes mellitus and impaired glucose tolerance in children and adolescentes receiving growth hormone treatment. *Lancet* 2000;355:610-613
21. Lobie PE, Breipohl W, Lincoln DT, García-Aragón J, Waters MJ. Localization of the growth hormone receptor/binding protein in skin. *J Endocrinol* 1990;126:467-471.
22. Stavropoulos A, Wikesjö UM. Growth and differentiation factors for periodontal regeneration: a review on factors with clinical testing. *J Periodontal Res* 2012;47:545-553.