SERUM GHRELIN LEVELS DURING FRACTURE HEALING AND IMMUNOHISTOCHEMICAL INVESTIGATION OF GHRELIN IN BONE TISSUE

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ABSTRACT

Background*: Ghrelin is a hormone with efficacy on increasing osteoblastic cell proliferation, differentiation, growth hormone release, and preventing apoptosis. The present study aimed to investigate whether ghrelin has a role in bone fracture healing process and presence in the bone tissue.*

Patients and methods*: Blood samples of 10 patients, who underwent surgery for fracture repair, were obtained for 4 times in the manner that; before fracture stabilization (preoperative) and on the postoperative 1st, 10th, and 60th days. Additionally, blood samples were obtained from 10 healthy subjects (control group). Ghrelin levels in serum samples were measured by enzyme-linked immunosorbent assay method and presence of ghrelin was immunohistochemically investigated in bone tissue samples .*

Results*: Although no difference was found between the groups in terms of blood levels of ghrelin, it was observed that acylated ghrelin level was increased beginning from the postoperative 1st day as compared to the control group. Des-acylated ghrelin levels were also increased as compared to the controls, except for the postoperative 10th day. Ghrelin was not detected in bone tissues. Osseous union was observed in all patients of the fracture group.*

Conclusions*: we thought that increased acylated and des-acylated ghrelin levels were contributed to fracture healing process by means of enhancing cell proliferation, preventing apoptosis, presenting anti-inflammatory and antimicrobial effect and increasing growth hormone release.*

Key words*: Fracture healing, ghrelin, bone tissue.*

Received *Septemper 18, 2013;* ***Accepted*** *October 24, 2013*

Introduction

Fracture healing is a complex biological process and repair of fractured bone begins at the time of fracture[(1)]. It has been reported that various degrees of healing problems appear in 5%-10% of the fractures occurring in a year in the Unites States[(2)]. Although the reason of these problems is usually not known, age, nutritional and hormonal status, inadequate reduction, concomitant diseases, infections, medications and characteristic of the trauma have been reported as the factors that influence fracture healing[(3)].

During fracture formation the amount of osteoblasts and osteoclasts is not adequate for fracture healing. In this period, fracture healing is provided by precursor and supporting cells, capillaries, lymphatic and nervous systems, and locally-mediated mechanisms. Coupling factors, which are locally produced in fracture site or reach through blood circulation and are able to maintain bone balance at regional level, are needed[(4-6)].

Ghrelin, also known as orexigenic hormone, was discovered in 1999 by Japanese scientists[(7)]. It is a 28-aminoacid lipopeptide hormone, mainly released from gastric fundus. This hormone is also synthesized in many tissues including the hypothalamus, small intestine, kidneys and heart[(7-10)].

Ghrelin is found in two forms in body fluids and tissues. Human ghrelin includes an 8-carbon fatty acid named as octanyl group. Ghrelin with octanyl group is the acylated (active) ghrelin

(aGAH). Ghrelin without fatty acid is the des-acylated ghrelin (dGAH) and also known as inactive ghrelin[8,11]. Des-acylated ghrelin accounts for 80%-90% of overall circulating ghrelin[12].

Ghrelin have many functions including cellular proliferation and differentiation, stimulation of food intake and growth hormone (GH) secretion, pancreatic exocrine and endocrine function, glucose metabolism, sleep and behavior, immune regulation, and cardiovascular function[13,14,15].

Ghrelin is reported to stimulate proliferation and differentiation of osteoblasts in rats[16]. Bone densitometry measurements in female rats have revealed that in vivo bone mineralization was increased after the use of GH-releasing hormone (GHRP-6) or ipamoreline, its peptide analogue, for 12 weeks[11, 17, 18]. However, there is less data about the functions of ghreline in bone tissue. In this study, we aimed to profile blood levels of ghrelin during fracture healing, and to investigate the levels of ghrelin in human fractured-bone tissue.

Materiald and methods

Ten adult patients (>18 years old) who were diagnosed with lower extremity fracture (femur or tibia) and underwent surgery for fracture repair in the Department of Orthopedics and Traumatology, Firat University Faculty of Medicine were enrolled. The control group consisted of 10 adult (>18 years old) volunteers, who were completely healthy and non-fractured bone formation. Approval of the Ethics Committee of the Firat University Faculty of Medicine and written informed consents of the patients were obtained for the study.

Detailed medical, surgical, and trauma histories (reason for fracture and the method of stabilizing the fracture) of the patients were obtained. Patients with open or pathological fracture, osteogenesis imperfecta, rachitism, suspicious malignancy, diabetes, chronic renal failure, osteomyelitis, history of gastric or intestinal surgery, hepatic or hematological diseases, and any endocrine disorder such as thyroid dysfunction were excluded.

Thirty-two different bone tissue samples were obtained to immunohistochemically investigate the presence of ghrelin in the femur heads removed from the patients, who had developed femur neck fracture and undergone hip prosthesis. Moreover, gastric tissue was used as positive control during immunohistochemical examination since ghrelin is known to be synthesized in the gastric tissue. Gastric tissues (n=3) were obtained from the archive of the Pathology Department of the Firat University.

A venous blood sample of 5 mL was obtained for four times (before stabilization of fracture, and on the postoperative 1st, 10th, and 60th days) in the fracture group and for once (between 08.00 a.m. and 10.00 a.m.) in the control group. Since the peptides are easily degraded by proteases in the cell, 20 μL-30 μL aprotinin, which is a protease inhibitor, was added for each mL of blood to measure the serum levels of ghrelin accurately. Furthermore, 1/10 volume of 1 N HCl was added to the serum samples obtained after centrifuge. By this way, the samples were allowed to remain stable at -20 ºC up to one year.

In serum samples, ghrelin levels were measured according to the instruction of the manufacturer using Human acylated ghrelin enzyme-linked immunosorbent assay (ELISA) kit (SPI-Bio Bertin Pharma, Bretonneux, France; Cat.A05106) and human des-acylated ghrelin ELISA kit (SPI Bio Bertin Pharma, Bretonneux, France; Cat.A05119). Total amount of ghrelin was obtained by mathematical addition of the amounts of aGAH and dGAH.

In order to determine ghrelin expression in bone tissues of femur heads and in gastric tissues of the controls by immunohistochemical method, 5 µm sections were obtained from formalin-fixed paraffin-embedded tissues and mounted on poly-L-lysine coated glass in the laboratory of the Pathology Department of Firat University. All sections were deparaffinized by incubating at 56 °C for 15 minutes. After deparaffinization performed by passages through 5 xylenes for 20 minutes, slides were rehydrated by again passing through gradually decreasing alcohol concentrations (96%, 90%, 80%, 70%) for 20 minutes. Slides were washed with distilled water for 5 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H2O2) for 10 minutes. They were prepared in accordance with avidin-biotin-peroxidase complex method. Tissue sections were kept in microwave oven in citrate buffer (pH=6) at 800 W for 5+5 minutes and at 640 W for 5 minutes. Following microwave oven application, they were cooled at room temperature for 20 minutes and then washed with 0.01 M phosphate buffered saline (PBS) (pH=7.4). Margin of the sections were dried and contoured using a glass pen. They were incubated with ultra V Block, a blocking agent, for 10 minutes to prevent nonspecific antibody binding.

Thereafter, the sections were incubated with rabbit anti-ghrelin primary antibody (human, diluted 1/400) (Phoenix Inc., CA, USA) at 38 °C for 30 minutes. They were then washed with PBS and incubated with biotinylated goat antiserum (Lab Vision Corporation, Fremont, CA, USA) at 38 °C for 10 minutes. After washed with PBS again, each section was incubated with streptavidin-biotin-peroxidase complex for 10 minutes. The sections were washed twice with PBS, for each 5 minutes. As chromogen, aminoethyl carbazole was dripped and left for incubation for 10 minutes until a color appears. All sections were washed with tap water and kept in Mayer's hematoxylin solution for 1-2 minutes to provide counterstaining. After washing with tap water for 5 minutes, their margins were wiped without damaging the tissues. The sections were mounted with Ultramount aqueous permanent mounting medium (Dako Denmark A/S, Glostrup, Denmark) and examined under a light microscope. Immunohistochemical assessment (positivity/negativity) of ghrelin in the sections was performed using semi-quantitative method.

Lane and Sandhu19 radiological scoring system was used to evaluate new bone formation in fracture line; 0 indicates no healing, 1 indicates callus formation, 2 indicates beginning of osseous union, 3 indicates beginning of disappearance of fracture line, and 4 indicates complete osseous union.

The statistical package for the social sciences (SPSS Inc., Chicago, IL, USA) version 17.0.1 was used for statistical analyses. In addition to descriptive statistical methods (mean, standard deviation), intergroup comparisons of normally distributed quantitative parameters were performed by independent samples t-test. Mann-Whitney U test was used for intergroup comparisons of non-homogeneous and non-normally distributed parameters. Paired samples t-test was used for intragroup comparison of the normally distributed parameters, whereas Wilcoxon signed-rank test was used for intragroup comparison of the non-normally distributed parameters. Results were evaluated within 95% confidence interval and a p value <0.05 was considered statistically significant.

Results

In the fracture group, the mean age was 43.4±12.8 years and 80% of the patients were male. In the control group, the mean age was 38.0±7.0 years and 70% of the patients were male. No significant differences were found between the groups in terms of age and gender (p>0.05, for each).

There were no significant differences between these two groups in terms of preoperative and postoperative 1st, 10th, and 60th day aGAH and dGAH values (Table 1).

	Fracture Group (n=10)	**Control Group (n=10)**	**p**
Preoperative			
aGAH	80.51±66.39	86.61±52.34	0.822
dGAH	233.28±151.81	119.70±93.05	0.062
Postoperative 1st day			
aGAH	64.01±38.73	86.61±52.34	0.288
dGAH	182.70±165.32	119.70±93.05	0.311
Postoperative 10th day			
aGAH	84.64±48.92	86.61±52.34	0.932
dGAH	119.15±104.79	119.70±93.05	0.990
Postoperative 60th day			
aGAH	115.94±77.77	86.61±52.34	0.337
dGAH	145.75±105.35	119.70±93.05	0.565

Table 1: Ghrelin levels of the fracture and control groups.
Blood samples were obtained from the control group for once.
Data are presented as mean±standard deviation.
Ghrelin levels are presented as pg/mL.
aGAH, acylated ghrelin; dGAH, des-acylated ghrelin.

In the fracture group, no significant differences were found in terms of both preoperative and postoperative 1st, 10th, and 60th day aGAH and dGAH values (p>0.05 for both). Total amount of ghrelin was 313.79±190.56 in the preoperative period, 246.73±179.01 on the postoperative 1st day, 203.79±145.63 on the postoperative 10th day, and 261.69±158.70 on the postoperative 60th day of the fracture group and 206.25±137.4 in the control group. No significant difference was determined between the groups (p=0.552, Fig. 1).

Immunohistochemical examination revealed ghrelin positivity in the enteroendocrine (P/D1) cells of the gastric tissue, which was used as positive control, whereas in the bone tissue, ghrelin positivity was not detected (Fig. 2).

Radiological examination which was per-

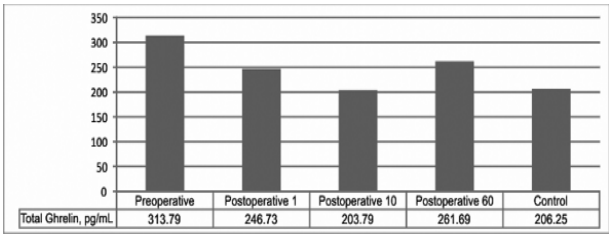

Figure 1: Total amount of ghrelin.

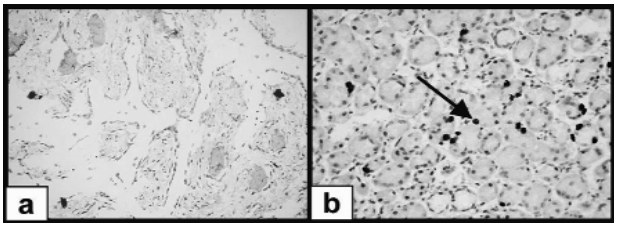

Figure 2: Ghrelin negativity in bone trabeculae and osteoblasts (a), Ghrelin positivity in the enteroendocrine cells of gastric tissue (arrow) (b) (immunoperoxidase X200).

formed in the postoperative 4th month for new bone formation demonstrated the beginning of osseous union in 9 and callus formation in 1 of 10 patients according to the system recommended by Lane and Sandhu[19]. Delayed union was considered in the patient with callus formation and dynamization was performed in the postoperative 5th month. Complete osseous union was observed on the postoperative 10th month radiographs. As a consequence, osseous union occurred in all patients.

Discussion

Despite numerous studies especially on cytokines and hormones concerning how fracture healing occurs and which factors play role, the factors that take place in fracture healing still remains unclear. In recent years, the basic concern that guides the studies to investigate whether ghrelin has a role in fracture healing is the demonstration of ghrelin synthesis in the odontoplast layer of tooth by Aydın et al[10]. Moreover, it has also been demonstrated that ghrelin stimulates intramembranous bone repair in rats[20], stimulates human osteoblastic TE85 cells by nitric oxide/cyclic guanosine monophosphate signal pathway[21], and inhibits apoptosis while stimulating proliferation and differentiation in osteoblastic MC3T3-E1 cells[22]. Although functional role of ghrelin in immune response has not been completely clarified yet, some studies demonstrated that it inhibits anorectic proinflammatory cytokines such as interleukin-1-beta, interleukin-6, and tumor necrosis factor-alpha[23]. Ghrelin is determined that it increases GH release dose-dependently, of which the efficacy on fracture healing has been demonstrated, both in vivo and in vitro[24, 25]. In a meta-analysis, Biver et al.[26] found no satisfactory evidence supporting the relation between ghrelin and bone mineral density. Under the light of this information, the present study, we aimed to determine whether ghrelin had a role in fracture healing process.

In this study ghrelin positivity was detected in the P/D1 cells which had endocrine functions in the gastric tissue taken as positive control for immunohistochemical examination; however, ghrelin positivity could not be detected in the bone tissue. Bone and tooth tissues are histologically similar. Immunohistochemical detection of ghrelin in the odontoblast layer of tooth by Aydın et al.[10] theoretically suggested the ghrelin synthesis in the osteoblast layer of bone. Absence of ghrelin, which is responsible for GH release, in any layer of bone tissue has indicated that bone and tooth tissues differ in terms of ghrelin metabolisms. However, recent studies have reported that ghrelin has four main forms containing 8-carbon fatty acid, containing 10-carbon fatty acid, containing 10-carbon unsaturated fatty acid, and dGAH[27] Considering that tissues and circulation contain approximately 80% dGAH and 20% aGAH, the probable cause of not detecting ghrelin in bone tissue may be low distribution percentage of aGAH in the tissue. Thus, it is important for further studies to investigate both dGAH and the other forms of ghrelin in bone tissue and to completely clarify this subject. Despite extensive literature review, there is no study investigating ghrelin in human bone tissue yet. Therefore, we are unable to discuss our results with those from the previous investigations.

The role of ghrelin in bone development, differentiation, and proliferation arises probably from the amount of circulating ghrelin. Therefore, in the present study, we investigated the change in the amounts of aGAH and dGAH in the course of fracture healing by analyzing blood samples obtained in preoperative period and on the postoperative days. In the present study, despite the absence of significant difference between the groups in terms of ghrelin values, preoperative aGAH value was lower in the fracture group as compared to that in the control group. In the fracture group, while the aGAH value detected on the postoperative 1st day was lower than the preoperative value, the values detected on the postoperative 10th and 60th days

were higher as compared to the preoperative value. The slight reduction detected in the preoperative period could be attributed to the fracture-related conditions such as inflammation and stress. Since ghrelin has anti-inflammatory effect and is a natural antioxidant of the body, we think that it is used to remove fracture-related free radicals and thus it decreases. Lower level of aGAH on the postoperative 1st day as compared to the basal level, in other words, probability of further decrease due to surgery-related heavy burden, stress, and anesthesia corroborates this opinion. The level of aGAH was increased on the postoperative 10th and 60th days due to disappearance of surgical-related stress. Moreover, an increase approximately by 20%-30% was observed in the aGAH levels as compared to the control group and the preoperative period. The potential cause of higher increase in the aGAH level compared to the basal level and that of controls may be the fact that defense system was enhanced due to increased ghrelin level, which has antimicrobial property and contributes to wound healing, by compensatory mechanism of the body. Levels of aGAH reported in the present study were slightly higher (80-86 pg/mL) than the accepted normal physiological range (33-66 pg/mL)[28, 29]. This might have caused due to the standard deviation of the measurements owing to kits from different companies. In fact, the results obtained by the kits of Lincon and Phoneix companies were 10 folds different from each other[30].

In the present study, although dGAH level did not differ between the groups, an increase by almost 100% was observed in the preoperative period as compared to the control group and this increment continued postoperatively, except the postoperative 10th day. However, when the values were compared with the preoperative period, dGAH value presented a gradual decrease. The mechanism valid for aGAH is also acceptable for dGAH. Nonetheless, when aGAH and dGAH values were compared in terms of periods, aGAH presented a time-dependent increase in fracture healing process, whereas dGAH presented time-dependent decrease in the same periods. This time-dependent change in aGAH may be considered to play a role in deposition or change of calcium, which is the main component of bone structure. Since one of the pathways of mechanism of aGAH action is the increase in calcium release by inositol trisphosphate, we are in the opinion that this free calcium contributes to fracture healing. In addition, since time-dependent increase in aGAH is responsible for GH release, it indirectly contributes to fracture healing by stimulating release of GH, which has a role in fracture healing. On the other hand, reason for the decrease in dGAH level during the fracture healing process as compared to the preoperative period suggests that it might have been used as anti-inflammatory and antioxidant. However, the reason for the increment in the dGAH level as compared to the controls might have been the compensatory increase of dGAH release in response to the decreased aGAH level due to the fracture-related and postoperative stresses.

In conclusion, the time-dependent increase in aGAH and dGAH levels contributed to the fracture healing process by increasing cell proliferation, preventing apoptosis, presenting anti-inflammatory and antimicrobial efficacy and above all by enhancing GH release, despite the limited number of study population.

References

1) Marsell R, Einhorn TA. *The biology of fracture healing. Injury.* 2011 Jun;42(6):551-5. DOI: 10.1016/j.injury.2011.03.031. Epub 2011 Apr 13. Cited in: PubMed; PMID 21489527.
2) Einhorn TA. *Enhancement of fracture-healing.* J Bone Joint Surg Am. 1995 Jun; 77(6): *940-56.* Cited in: PubMed; PMID 7782368.
3) Calori GM, Albisetti W, Agus A, Iori S, Tagliabue L. *Risk factors contributing to fracture non-unions.* Injury. 2007 May; 38 Suppl 2:S11-8. Cited in: PubMed; PMID 17920412.
4) Ozaki A, Tsunoda M, Kinoshita S, Saura R. *Role of fracture hematoma and periosteum during fracture healing in rats: interaction of fracture hematoma and the periosteum in the initial step of the healing process.* J Orthop Sci. 2000; 5(1): *64-70.* Cited in: PubMed; PMID 10664441.
5) Khan SN, Bostrom MP, Lane JM. *Bone growth factors.* Orthop Clin North Am. 2000 Jul; 31(3): *375-88.* Cited in: PubMed; PMID 10882464.
6) Yilmaz C, Erdemli E, Selek H, Kinik H, Arikan M, Erdemli B. *The contribution of vitamin C to healing of experimental fractures.* Arch Orthop Trauma Surg. 2001 Jul; 121(7): *426-8.* Cited in: PubMed; PMID 11510911.
7) Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. *Ghrelin is a growth-hormone-releasing acylated peptide from stomach.* Nature. 1999 Dec 9; 402(6762): *656-60.* Cited in: PubMed; PMID 10604470.
8) Aydin S, Ozkan Y, Caylak E, Aydin S. Ghrelin ve Biyokimyasal Fonksiyonları. *Ghrelin and its biochemical functions: Review* Türkiye Klinikleri J Med Sci. 2006 26(3): *272-83.* Turkish.
9) Aydin S, Aydin S, Ozkan Y, Kumru S. *Ghrelin is present in human colostrum, transitional and mature milk.*

Peptides. 2006 Apr; 27(4): *878-82*. Epub 2005 Sep 26. Cited in: PubMed; PMID 16191452.

10) Aydin S, Ozercan IH, Geckil H, Dagli F, Aydin S, Kumru S, Kilic N, Sahin I, Ozercan MR. *Ghrelin is present in teeth*. J Biochem Mol Biol. 2007 May 31; 40(3): *368-72*. Cited in: PubMed; PMID 17562288.
11) Kojima M, Kangawa K. *Ghrelin: structure and function*. Physiol Rev. 2005 Apr; 85(2): *495-522*. Cited in: PubMed; PMID 15788704.
12) Korbonits M, Goldstone AP, Gueorguiev M, Grossman AB. *Ghrelin a hormone with multiple functions*. Front Neuroendocrinol. 2004 Apr; 25(1): *27-68*. Cited in: PubMed; PMID 15183037.
13) Akamizu T, Kangawa K. *The physiological significance and potential clinical applications of ghrelin*. Eur J Intern Med. 2012 Apr; 23(3): *197-202*. DOI: 10.1016/j.ejim.2011.12.001. Epub 2011 Dec 30. Cited in: PubMed; PMID 22385874.
14) Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K. *Ghrelin strongly stimulates growth hormone release in humans*. J Clin Endocrinol Metab. 2000 Dec; 85(12): *4908-11*. Cited in: PubMed; PMID 11134161.
15) Hataya Y, Akamizu T, Takaya K, Kanamoto N, Ariyasu H, Saijo M, Moriyama K, Shimatsu A, Kojima M, Kangawa K, Nakao K. *A low dose of ghrelin stimulates growth hormone (GH) release synergistically with GH-releasing hormone in humans*. J Clin Endocrinol Metab. 2001 Sep; 86(9): *4552*. Cited in: PubMed; PMID 11549707.
16) Maccarinelli G, Sibilia V, Torsello A, Raimondo F, Pitto M, Giustina A, Netti C, Cocchi D. *Ghrelin regulates proliferation and differentiation of osteoblastic cells*. J Endocrinol. 2005 Jan; 184(1): *249-56*. Cited in: PubMed; PMID 15642801.
17) Ahnfelt-Rønne I, Nowak J, Olsen UB. Do growth hormone-releasing peptides act as ghrelin secretagogues? Endocrine. 2001 Feb;14(1):133-5. Cited in: PubMed; PMID 11322495.
18) Fukushima N, Hanada R, Teranishi H, Fukue Y, Tachibana T, Ishikawa H, Takeda S, Takeuchi Y, Fukumoto S, Kangawa K, Nagata K, Kojima M. *Ghrelin directly regulates bone formation*. J Bone Miner Res 2005 May; 20(5): *790-8*. Epub 2004 Dec 27. Cited in: PubMed; PMID 15824852.
19) Lane JM, Sandhu HS. *Current approaches to experimental bone grafting*. Orthop Clin North Am. 1987 Apr; 18(2): *213-25*. Cited in: PubMed; PMID 3550572.
20) Deng F, Ling J, Ma J, Liu C, Zhang W. *Stimulation of intramembranous bone repair in rats by ghrelin*. Exp Physiol. 2008 Jul; 93(7): *872-9*. DOI: 10.1113/expphysiol.2007.041962. Cited in: PubMed; PMID 18562476.
21) Wang DH, Hu YS, Du JJ, Hu YY, Zhong WD, Qin WJ. *Ghrelin stimulates proliferation of human osteoblastic TE85 cells via NO/cGMP signaling pathway. Endocrine*. 2009 Feb; 35(1): *112-7*. DOI: 10.1007/s12020-008-9117-3. Epub 2008 Oct 25. Cited in: PubMed; PMID 18953675.
22) Kim SW, Her SJ, Park SJ, Kim D, Park KS, Lee HK, Han BH, Kim MS, Shin CS, Kim SY. *Ghrelin stimulates proliferation and differentiation and inhibits apoptosis in osteoblastic MC3T3-E1 cells*. *Bone*. 2005 Sep; 37(3): *359-69*. Cited in: PubMed; PMID 15978880.
23) Arvat E, Maccario M, Di Vito L, Broglio F, Benso A, Gottero C, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E. *Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone*. J Clin Endocrinol Metab. 2001 Mar; 86(3): *1169-74*. Cited in: PubMed; PMID 11238504.
24) Date Y, Murakami N, Kojima M, Kuroiwa T, Matsukura S, Kangawa K, Nakazato M. *Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats*. Biochem Biophys Res Commun. 2000 Aug 28; 275(2): *477-80*. Cited in: PubMed; PMID 10964690.
25) Peino R, Baldelli R, Rodriguez-Garcia J, Rodriguez-Segade S, Kojima M, Kangawa K, Arvat E, Ghigo E, Dieguez C, Casanueva FF. *Ghrelin-induced growth hormone secretion in humans*. Eur J Endocrinol. 2000 Dec; 143(6): *R11-4*. Cited in: PubMed; PMID 11124868.
26) Biver E, Salliot C, Combescure C, Gossec L, Hardouin P, Legroux-Gerot I, Cortet B. *Influence of adipokines and ghrelin on bone mineral density and fracture risk: a systematic review and meta-analysis*. J Clin Endocrinol Metab. 2011 Sep; 96(9): *2703-13*. DOI: 10.1210/jc.2011-0047. Epub 2011 Jul 21. Cited in: PubMed; PMID 21778223.
27) Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. *Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by posttranslational processing*. J Biol Chem. 2003 Jan 3; 278(1): *64-70*. Epub 2002 Oct 31. Cited in: PubMed; PMID 12414809.
28) Nakahara K, Nakagawa M, Baba Y, Sato M, Toshinai K, Date Y, Nakazato M, Kojima M, Miyazato M, Kaiya H, Hosoda H, Kangawa K, Murakami N. *Maternal ghrelin plays an important role in rat fetal development during pregnancy*. Endocrinology. 2006 Mar; 147(3): *1333-42*. Epub 2005 Dec 8. Cited in: PubMed; PMID 16339208.
29) Popovic V, Svetel M, Djurovic M, Petrovic S, Doknic M, Pekic S, Miljic D, Milic N, Glodic J, Dieguez C, Casanueva FF, Kostic V. *Circulating and cerebrospinal fluid ghrelin and leptin: potential role in altered body weight in Huntington's disease*. Eur J Endocrinol. 2004 Oct; 151(4): *451-5*. Cited in: PubMed; PMID 15476444.
30) Gröschl M, Uhr M, Kraus T. *Evaluation of the comparability of commercial ghrelin assays*. Clin Chem. 2004 Feb; 50(2): *457-8*. Cited in: PubMed; PMID 14752024.

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