



UNIVERSITY OF SEVILLA

DEPARTMENT OF STOMATOLOGY

DOCTORAL PROGRAM IN DENTISTRY

**LEPTIN AND ITS RECEPTOR IN
HUMAN DENTAL PULP AND
PERIAPICAL TISSUES**

DOCTORAL THESIS

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2014



**UNIVERSIDAD DE SEVILLA
DEPARTAMENTO DE ESTOMATOLOGÍA**

LEPTIN AND ITS RECEPTOR IN HUMAN DENTAL PULP AND PERIAPICAL TISSUES

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Sevilla, 24 de Junio de 2014

Fdo.: JUAN J. SEGURA EGEA

Fdo.: VÍCTOR SÁNCHEZ MARGALET

Acknowledgements

This Ph.D. thesis was carried out at the Department of Stomatology in the University of Seville. The underlying research work was in the framework of the project entitled “Leptin and its receptor in healthy and inflamed human dental tissues”, which was financed by a Grant from the University of Seville.

First of all, I am deeply grateful to my supervisors, Dr. Juan José Segura Egea and Dr. Víctor Sánchez Margalet, for giving me the opportunity to work with them and for their guidance, strong support and valuable advices during this time.

I am deeply owed to Dr. Fionnula Lundy for her supervision during my research stay in the Centre for Infection and Immunity, School of Medicine, Dentistry and Biomedical Sciences, Queen’s University Belfast.

I am also grateful to Dr. Aníbal Diogenes for his guidance during my research stay at the University of Texas Health Science Center at San Antonio.

I would also like to thank all my colleagues at the Department of Medical Biochemistry, Molecular Biology and Immunology. Particularly, I want to give a special mention to Dr. Antonio Pérez Pérez, Dr. Flora Sánchez Jiménez and M.Sc. Antonio Carmona Fernández for their invaluable help and for the good moments spent together.

I cannot forget to thank my close friends. I am really lucky having your friendship.

Finally, my deepest thanks go to my family for its unconditional love and support during my life.

Notice

The present research has been published, accepted or submitted for publication:

International Journals with peer review

- 1) **Martín-González J**, Sánchez-Jiménez F, Pérez-Pérez A, Carmona-Fernández A, Sánchez-Margalet V, Segura-Egea JJ. *Leptin expression in healthy and inflamed human dental pulp*. International Endodontic Journal 2013; 46:442-8.
- 2) **Martín-González J**, Pérez-Pérez A, Sánchez-Jiménez F, Carmona-Fernández A, Sánchez-Margalet V, Segura-Egea JJ. *Leptin receptor (LEPR) is up-regulated in inflamed human dental pulp*. Journal of Endodontic 2013 Dec; 39(12):1567-71.
- 3) **Martín-González J**, Carmona-Fernández A., Pérez-Pérez A, Sánchez-Jiménez F, Sánchez-Margalet V, Segura-Egea JJ. *Expression and Immunohistochemical Localization of Leptin Receptor in Human Periapical Granulomas*. International Endodontic Journal-accepted May 2014.
- 4) **Martín-González J**, Sánchez-Margalet V., Segura-Egea JJ. *Expression and immunohistochemical localization in human periapical granulomas*. Archives of Oral Biology-submitted April 2014.
- 5) **Martín-González J**, Pérez-Pérez A, Sánchez-Jiménez F, Carmona-Fernández A, Sánchez-Margalet V, Segura-Egea JJ. *Leptin Signalling in Normal Human Dental Pulp: Involvement of MAPK, PKB and STAT-3 Pathways*. International Endodontic Journal-submitted May 2014.

- 6) **Martín-González J**, Pérez-Pérez A, Sánchez-Jiménez F, Carmona-Fernández A, Sánchez-Margalet V, Segura-Egea JJ. *Leptin increases DSPP levels in human dental pulp: Immunohistochemical localization of LEPR.* Journal of Endodontic-submitted April 2014.
- 7) Pérez-Pérez A, Fernández-Riejos P, Sánchez-Jiménez F, Carmona-Fernández A, Santos-Alvarez J, **Martín-González J**, Segura-Egea JJ, Sánchez-Margalet V. *Leptin, a mediator of inflammation.* Journal of Endocrinology-submitted April 2014.

Abstracts published in journals or abstract books

Martín-González J, Pérez-Pérez A, Sánchez-Jiménez F, Segura-Egea JJ, Sánchez –Margalet V. *Leptin Promotes Dentin Sialophosphoprotein Expression in Human Dental Pulp.* 37th National Congress of the Spanish Biochemistry and Molecular Biology Society, 2014.

Martín-González J, Sánchez-Domínguez B, Crespo-Gallardo I, Martín-Jiménez M, Segura-Egea JJ. *Stimulatory effect of leptin in the dentin sialophosphoprotein (DSPP) production in human dental pulp.* 10th National and 7th International Congress of the Spanish Society of Conservative Dentistry, 2014.

Sánchez-Domínguez B, **Martín-González J**, Martín-Jiménez M, Crespo-Gallardo I, Segura-Egea JJ. *Leptin Signalling in Normal Human Dental Pulp.* 10th National and 7th International Congress of the Spanish Society of Conservative Dentistry, 2014

Martín-Jiménez M., **Martín-González J.**, Crespo-Gallardo I., Sánchez-Domínguez B., Segura-Egea JJ. *Activation of PKB Pathway signaling by leptin in human dental pulp*. 10th National and 7th International Congress of the Spanish Society of Conservative Dentistry, 2014.

Crespo-Gallardo I, **Martín-González J.**, Martín-Jiménez M, Sánchez-Domínguez B, Segura-Egea JJ. *Leptin activates STAT-3 signaling pathway in human dental pulp*. 34th National Congress of the Spanish Endodontic Society and 33th International Congress of the Iberoamerican Endodontic Society, 2013.

Segura-Egea JJ, **Martín-González J.**, Sánchez-Domínguez B, Castellanos-Cosano L, Martín-Jiménez M. *Up-regulation of leptin receptor (LEPR) in inflamed dental pulp*. 34th National Congress of the Spanish Endodontic Society and 33th International Congress of the Iberoamerican Endodontic Society, 2013.

López-Morales S., **Martín-González J.**, Castellanos-Cosano L., Martín-Jiménez M., Segura-Egea J.J. *Up-regulation of leptin receptor (LEPR) in inflamed dental pulp*. 33th National Congress of the Spanish Endodontic Society, 2012.

Martín-Jiménez M., **Martín-González J.**, Sánchez-Domínguez B., López-Morales S., Segura-Egea J.J. *RNA expression in the granulomatous periapical tissue. Determination by QRT-PCR*. 33th National Congress of the Spanish Endodontic Society, 2012.

Sánchez-Domínguez B., **Martín-González J.**, López-Morales S., Martín Jiméñez M., Segura-Egea J.J. *Presence of the leptin protein in inflammatory periapical tissue. Characterization by Western Blot*. 33th National Congress of the Spanish Endodontic Society, 2012.

Martín-González J, Sánchez-Domínguez B, Castellanos-Cosano L, Torres-Lagares D, Segura-Egea JJ. *Expression of leptin mRNA in normal human dental pulp*. XIX National Congress and VI International of the Spanish Society of Conservative Dentistry. 2012.

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Martín-Jiménez M, **Martín-González J**, Sánchez-Domínguez B, López-Frias FJ, Segura-Egea JJ. *Expression of mRNA leptin receptor (Ob-R) in normal human dental pulp: Implications in pulp revascularization*. XIX National Congress and VI International of the Spanish Society of Conservative Dentistry. 2012

Sánchez-Domínguez B, Castellanos-Cosano L, **Martín-González J**, Segura-Egea JJ. *Expression of leptin in normal human dental pulp*. XIX National Congress and VI International of the Spanish Society of Conservative Dentistry. 2012.

Conference awards

Award for best oral presentation: “Martín-González J, Sánchez-Domínguez B, Crespo-Gallardo I, Martín-Jiménez M, Segura-Egea JJ. *IV. Leptin Promotes Dentin Sialophosphoprotein Expression in Human Dental Pulp*. 10th National and 7th International Congress of the Spanish Society of Conservative Dentistry, 2014.

Award for best paper published in 2013: “Martín-González J, Pérez-Pérez A, Sánchez-Jiménez F, Carmona-Fernández A, Torres-Lagares D, Sánchez-Margalet V, Segura-Egea JJ. *Leptin receptor is up-regulated in inflamed human dental pulp*. J Endod. 2013 Dec; 39(12):1567-71”.10th National and 7th International Congress of the Spanish Society of Conservative Dentistry, 2014.

Table of contents

GENERAL INTRODUCTION	6
INTRODUCCIÓN GENERAL	16
OBJECTIVES	34
OBJETIVOS	36
CHAPTER 1: LEPTIN EXPRESSION IN HEALTHY AND INFLAMED HUMAN DENTAL PULP	38
CHAPTER 2: LEPTIN RECEPTOR IS UP-REGULATED IN INFLAMED HUMAN DENTAL PULP	58
CHAPTER 3: LEPTIN SIGNALLING IN HUMAN DENTAL PULP: INVOLVEMENT OF MAPK, PI3K AND JAK/STAT3 PATHWAYS	82
CHAPTER 4: LEPTIN PROMOTES DENTIN SIALOPHOSPHOPROTEIN EXPRESSION IN HUMAN DENTAL PULP	104
CHAPTER 5: LEPTIN EXPRESSION AND IMMUNOHISTOCHEMICAL LOCALIZATION IN HUMAN PERIAPICAL GRANULOMAS	127
CHAPTER 6: EXPRESSION AND IMMUNOHISTOCHEMICAL LOCALIZATION OF LEPTIN RECEPTOR IN HUMAN PERIAPICAL GRANULOMA	148
FINAL CONCLUSIONS	175
CONCLUSIONES FINALES	177
CURRICULUM VITAE	
JOURNAL PAPERS	180
BOOK CHAPTERS	183
CONFERENCES	183
RESEARCH STAYS	189
RESEARCH PROYECT DOCUMENTS	191

General introduction

General introduction

Nowdays, one of the most interesting aspects that medical and dental scientific community researches is the possible connection between oral inflammatory processes due to infection and systemic health (Seymour et al., 2009). Numerous epidemiological studies have found an association between general health and these oral inflammatory processes (Soskolne et al 2001; Janket et al 2003; Jimenez Pinzon et al 2004; Segura-Egea et al 2005; Caplan et al 2006; Ridao-Sate et al 2007). The evidence for this association has led to greater attention to the diagnosis and treatment of these oral diseases in different clinical situations, thereby improving the oral and systemic health of patients.

However, the pathophysiology of oral inflammatory processes due to infection is still not fully understood concerning basic, molecular and cellular aspects. Further research is needed to fully discern the underlying mechanisms by which local chronic infections can have an impact on systemic health, and in this endeavor pulpitis and apical periodontitis may serve as ideal disease models. The better knowledge would entail a better understanding of their potential impact systemically.

Pulpitis or dental pulp inflammation occurs when, either by caries or trauma, noxa and microorganisms invade dental pulp tissue triggering an immune and inflammatory response (Jontell et al. 1998). The bacteria that cause tooth caries are also the main cause of pulp infection and inflammation. Pulp injury is the result of a dynamic process in which, on the one hand, are the invading microorganisms and, on the other hand, is the immune and inflammatory host response (Bergenholtz et al. 2000). Inflammation of the dental pulp does not take place only when the bacteria from the caries have reached the pulp. Bacterial antigens and/or metabolic products may reach the pulp much earlier through the dentinal tubules and begin the inflammatory response. The inflammation may be acute or chronic because just like other tissues in the body, the pulp will react to irritants with innate and/or adaptive immune responses. (Warfvinge et al. 1985; Hahn et al., 2007). During the pulp immune response can also be formed pulp immune complexes and extracellular proteolytic enzymes that increase and worsen the pulp inflammation (Bergenholtz et al 1977). Definitely, the pulpitis is caused by

an immunopathological mechanism (Hahn et al 2007). In mild or moderate injuries, pulp inflammation can repair the damage caused (reversible pulpitis), maintaining pulp vitality. In these cases, pain appears with certain stimuli and never is spontaneous. Contrarily, if the aggressive factors are not eliminated and a pulp conservation and protection is not carried out, will end producing an irreversible pulpitis and, ultimately, pulp necrosis. Thus, irreversible pulpitis is the acute inflammatory response to persistence, growth and progression of the bacteria, or other aggressive agent in the dental pulp (Canalda et al 2001). In these cases, the patient has pain of increasing intensity, spontaneous, increasingly frequent painful episodes and pain that persists after removal of the stimulus. However, in some cases, irreversible pulpitis may be present without symptoms. So, two irreversible pulpitis clinical forms are distinguished: symptomatic and asymptomatic (Pumarola et al 2001).

The apical periodontitis is acute or chronic inflammation of the tissue around the tooth root, usually around the root apex, caused by bacterial infection of the dental pulp (Eriksen et al.1998). The apical periodontitis occurs in over 90% of cases, as a consequence of dental caries when it reaches the dental pulp causing pulpitis and / or necrosis of the pulp. The polymicrobial and / or antigenic content of the root canal goes through the apical foramen, or lateral canals, and invades the periapical or periradicular connective tissue triggering an inflammatory and immune response. Apical periodontitis can be acute and symptomatic or chronic and asymptomatic. Acute apical periodontitis causes minimal pain and bone resorption; sometimes they might be reversible. Chronic apical periodontitis is a consequence of necrotic pulp and therefore is irreversible. Periapical or periradicular observable radiologically osteolytic lesions are the result of bone destruction that occurs during periapical or periradicular chronic inflammatory process. The prevalence of apical periodontitis is very high in the general population. In Spain, 61% of patients and 4% of the teeth studied had chronic apical periodontitis (Jiménez-Pinzón et al., 2004). Four pathological forms are distinguished: periapical granuloma, chronic apical abscess, apical cyst and condensing osteitis (Pumarola et al 2001). The most common periapical lesions are periapical granulomas (Gbolahan *et al.* 2008). Periapical granuloma is a chronic inflammatory lesion at the apex of a non-vital tooth consisting of granulation tissue and scar. The inflammatory cell

infiltrate in these chronic periapical lesions consists of a mix of plasma cells, T- and B-lymphocytes, macrophages, polynuclear leucocytes (PMNs), dendritic cells (DCs), natural killer cells (NK cells), and mast cells, present in different proportions within the granulation tissue of periapical lesions (Márton & Kiss 1993, Stashenko *et al.* 1998, Liapatas *et al.* 2003, Márton & Kiss 2014). The inflammatory infiltrate constitutes approximately 50% of the cells present in periapical granulomas, with non-inflammatory connective tissue cells, including fibroblasts, vascular endothelium, proliferating epithelium, osteoblasts, and osteoclasts comprising the balance (Langeland *et al.* 1977, Yu *et al.* 1987).

The link between pulp/periapical inflammation and pulp/apical repair is not yet well established, however, inflammatory response appears as a prerequisite for tissue repair (Goldberg *et al.* 2004a, Goldberg *et al.* 2008b). When host cells recognize lipoteichoic acid (LTA) of Gram-positive bacteria and/or lipopolysaccharide (LPS) of Gram-negative bacteria, mediate local inflammatory process directly through production of antimicrobial peptides and cytokines, and indirectly through activation of migratory immune cells (Horst *et al.*, 2011). The release of local cytokines promotes expression of molecules implicated in mineralization (Linde *et al.*, 1993, Asgary *et al.*, 2014). At this respect, dental defensive and reparative responses could be influenced by leptin, a pro-inflammatory cytokine.

Leptin is an adipocyte-derived non-glycosylated hormone of 146 aminoacids encoded by the *Ob* gene (Zhang *et al.* 1994) with a tertiary structure resembling that of members of the long-chain helical cytokine family (Figure 1) (that includes IL-6, IL-11, IL-12, LIF, G-CSF, CNTF, and oncostatin M) (Flier, 1995). Primarily synthesized and released from adipose tissue (Maffei *et al.*, 1995a) to regulate weight control in a central manner and, at lower levels, by other tissues such as the stomach, skeletal muscle, placenta and bone marrow (Friedman *et al.*, 1998). Circulating leptin levels (normal range 1–15 ng/mL) directly reflect the amount of energy stored in the adipose tissue and are proportional to the body adipose mass both in mice and in humans; thus, obese individuals typically produce higher leptin than leaner individuals (Frederich *et al.* 1995, Iikuni *et al.*, 2008, Maffei *et al.*, 1995b, Zavalza-Gomez *et al.*, 2008.). During fasting period and after reduction of body fat mass, there is a decrease in leptin levels that leads to a

reduction in total energy expenditure to provide enough energy for the function of vital organs, that is, the brain, the heart, and the liver (Ahima et al., 1996a). However, there is now increasing evidence that leptin has systemic effects apart from those related to energy homeostasis, including regulation of neuroendocrine, reproductive, hematopoietic and immune functions (Ahima et al., 2000b). It has been shown that leptin regulates the immune response, both innate and adaptive responses, not only in normal but also in pathological conditions (Fernández-Riejos et al. 2010). Consistent with this role of leptin in the mechanisms of immune response and host defense, leptin levels are increased upon infectious and inflammatory stimuli such as LPS, turpentine, and cytokines (Matarese et al., 2000). Even though these effects of leptin decrease are aimed to improve the survival chances under starving conditions, the fall in leptin levels may lead to immune suppression (Lord et al., 1998), in addition to other neuroendocrine alterations affecting adrenal, thyroid, and sexual/reproductive function (Fantuzzi et al., 2000). At least, these alterations observed during fasting parallel the decrease in circulating leptin levels. In fact, both ob/ob mice (lacking leptin secretion) and db/db mice (lacking leptin receptor) are not only obese but they also show the immune/endocrine deficiencies observed during starvation (Fantuzzi et al., 2000), Howard et al., 1999).

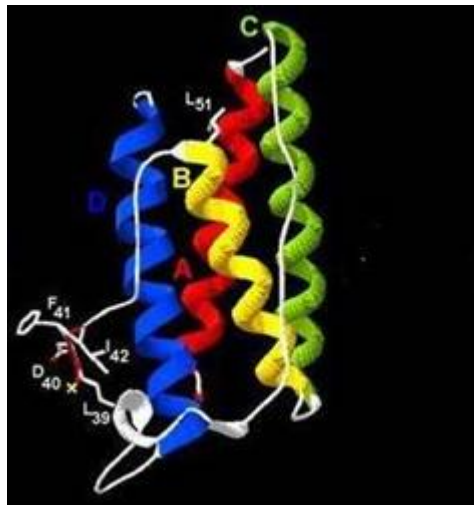


Figure 1. Three-dimensional structure of leptin. Leptin molecule has a set of four helices, similar to the classic long helical cytokines.

The pleiotropic nature of leptin is supported by the universal distribution of leptin receptor (LEPR) (Myers et al., 2004). LEPR also shows structural similarity to the class I cytokine receptor family (Myers et al., 2004, Tartaglia et al., 1995, Tartaglia 1997b) and similar to other receptors of this class. LEPR is expressed in six isoforms, as product of alternative RNA splicing. According to its structural differences, the receptor's isoforms are divided into three classes: long, short, and secretory isoforms (Figure 2). Among all LEPR isoforms, only full-length isoform (LEPRb) is able to fully transduce the activation signals into the cell since its cytoplasmic region contains several motifs required for signal transduction (Santos-Alvarez et al. 1999, 19). According to the multifunctional role of leptin, this fully-active isoform of LEPR is expressed not only in the hypothalamus, where it takes part in energy homeostasis, but also is present on peripheral tissues as well as on hematopoietic cells and on all types of immune cells involved in both innate and adaptive immunity (Cioffi et al., 1996, Sánchez-Margalet et al. 2003d, Loffreda et al., 1998). LEPR lacks intrinsic tyrosine kinase activity, but requires the activation of receptor-associated kinases of the Janus family (JAKs) (Tartaglia et al., 1995) which initiate downstream signalling, including members of the STAT (signal transducers and activators of transcription) family of transcription factors (Tartaglia 1997b). After ligand binding, JAKs autophosphorylate and tyrosine phosphorylates various STATs. Activated STATs then dimerize and translocate to the nucleus, where specific gene responses are elicited (Tartaglia 1997b, Gualillo et al., 2002). Different pathways in addition to STATs are known to be involved in LEPR signaling, including the mitogen-activated protein kinase (MAPK) family and the phosphatidylinositol 3-kinase (PI3K) signaling cascade (Figure 3) (Sánchez-Margalet et al., 2002c, Santos-Alvarez et al. 1999, Perez-Perez et al., 2008a, Perez-Perez et al., 2009b Perez-Perez et al., 2010c).

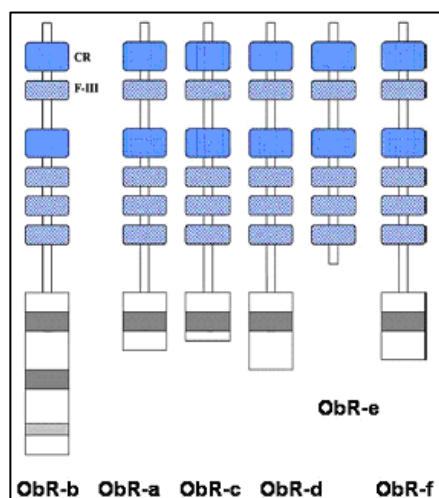


Figure 2: leptin receptor isoforms; (Hegyí et al 2004)

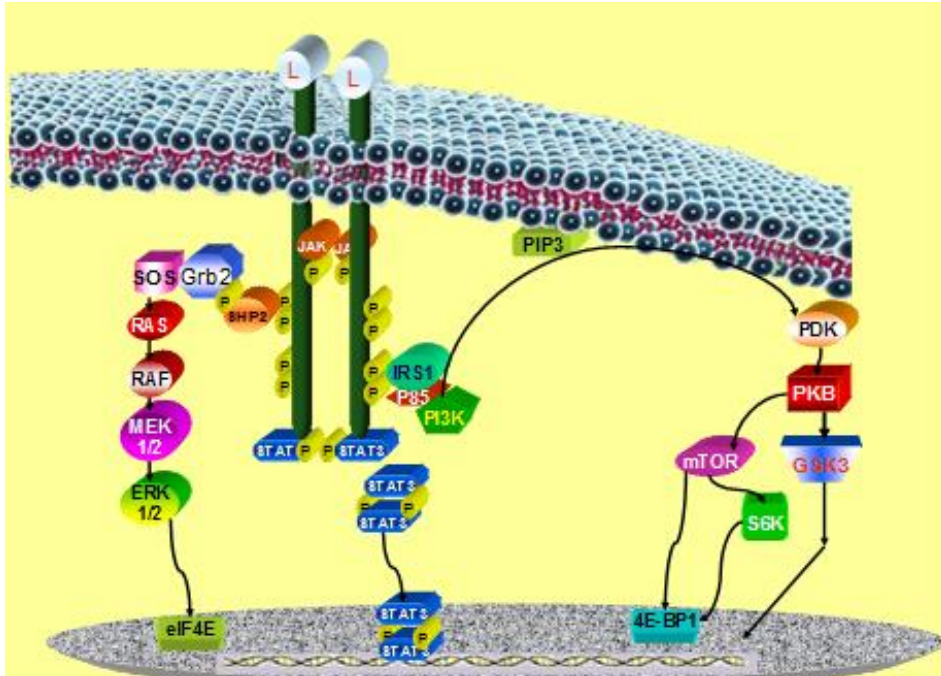


Figura 3. Leptin signaling pathways

Compelling evidence has implicated leptin in oral biology functions, thus, it has also been reported the presence of leptin and soluble LEPR in gingival tissue extract both in healthy and inflamed gingival tissues (Bozkurt et al., 2006, Dilsiza et al., 2010), in gingival crevicular fluid (Karthikeyan et al., 2007a, Karthikeyan et al., 2007b) as well as in human chronic periapical lesions (Kangarlou et al., 2010) and in saliva (Groschl et al., 2001, Randeva et al., 2003). Elevated serum leptin concentration has been associated with increased chronic periodontitis (Gundala et al., 2012). Moreover, the first evidence has emerged that leptin has effects on dental pulp stem cells, acting as an important modulator of pulpal mesenchymal stem cell differentiation (Um et al., 2011) being expressed in ameloblasts, odontoblasts, dental papilla cells and stratum intermedium cells in rat and human tooth germs at the late bell stage (Ide et al., 2011). Intriguingly, it has been reported that leptin is synthesized and secreted in vitro by pulp fibroblasts derived from extracted healthy molar teeth (El Karim et al., 2009). However, other dental cells might also be a source of leptin and LEPR. Thus, which dental cell type may

expresses leptin and LEPR, remain to be elucidated. Therefore, leptin could act by autocrine as well as paracrine pathways and therefore it may play a role in pulpal/periapical inflammatory and immune responses similar to that of the white adipose tissues. In this sense, although, adipocytes are not a normal cellular component in dental pulp, human dental pulp stem cells (DPSCs) are capable of differentiating into oil red-O-positive lipid-containing adipocytes (Gronthos et al., 2002), expressing in vitro the adipogenic master genes PPAR γ 2 (peroxisome proliferator-activated receptor gamma two) and lipoprotein lipase (LPL), two adipocyte-specific transcripts (Koyama et al., 2009). So, pulpal leptin could be secreted by DPSCs suffering adipogenic differentiation.

The dental pulp is a highly innervated tissue with good healing potential after injury and inflammation and angiogenesis is crucial for tooth development and a prerequisite for successful repair after injury and inflammation. Angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF-2), and transforming growth factor (TGF- β) have been identified in human dental pulp and dentin matrix (Grando et al., 2007, Guven et al., 2007, Roberts-Clark et al., 2000). In fact, these factors are up-regulated in dental pulp from carious teeth. Therefore, leptin might play an important role mediating angiogenesis by regulating angiogenic growth factor expression by pulpal, similar to that of the others inflamed tissues and cancer (Vansaun, 2013).

Although bacteria are the main etiologic agent in apical periodontitis or pulpitis, its presence is enough to initiate the disease, whereas the initiation and progression of these diseases depends on the quality of the host immune response. The infiltration of inflamed tissues by natural immune cells such as neutrophils, eosinophils, and macrophages is an important feature of inflammation. Macrophage infiltration of the adipose tissue is present in obesity, where it could expand adipocytes or neighboring pre-adipocytes (in turn responsible for the production of chemotactic signals leading to macrophage recruitment) (Bourlier et al., 2009, Bilan et al., 2009). Similarly to what occurs in obesity inflamed pulp or periapical are characterized by the infiltration of inflammatory cells such as lymphocytes, macrophages, dendritic cells and neutrophils, and consequently, pulpitis, is formed (Hahn et al 2000a). Moreover, it is well known that chemokines regulate the

trafficking of lymphocytes, and CC-chemokine ligand 20 (CCL20) shows to play a crucial role in the recruitment of memory T cells (Liao et al., 1999) and immature dendritic cells into inflammatory lesions (Dieu et al., 1998). Leptin is associated with an increased expression of CCL20 (Farquharson et al., 2012) and it has been demonstrated that CCL20 expression is induced by stimulation with caries-related bacteria invading deeply into the dentinal tubules as well as by proinflammatory cytokines in the inflamed pulpal lesions (Takahashi et al., 2008). Thus, leptin could regulate the production of chemotactic signals and the trafficking of lymphocytes during pulpal inflammatory response. When the number of macrophages increases during the innate response of the dentin/pulp complex to caries (Kamal et al., 1997), leptin can regulate monocyte function as assessed by *in vitro* experiments measuring free radical production via PKC-dependent pathways (Maingrette et al., 2003, Sanchez-Pozo et al., 2003). Moreover, as previously demonstrated in monocytes in immune system, leptin could stimulate the production of pro-inflammatory cytokines such as TNF- α and IL-6, and enhances CC-chemokine ligand expression in human dental pulp macrophage, through phosphorylation-activation of the JAK-STAT pathway (Kiguchi et al., 2009, Zhou et al., 2011).

The link between an adipokine, such as leptin, which first functional role is the control of appetite and hunger (Flier, 1995, Zhang et al., 1994), and the dental pulp/periapical defensive and reparative responses, could provide a new evidence of the relationship between obesity, inflammation and oral infections. Several possible pathways involving the host response and bacterial challenge have been proposed to explain the association between obesity and inflammation (D'Aiuto et al., 2012). Obesity could alter the host immune responses to oral infections increasing individuals' susceptibility. Thus, pro-inflammatory molecules and processes implicated in the pathogenesis of oral infections, such as pulpitis and apical periodontitis, including cytokines (e.g., Interleukin-IL-6), chemokines, and T-cell function, could be influenced by obesity (Falagas et al., 2006). An association between diet content in fatty acids and dental pulp cell population has been suggested (Vermelin et al., 1995). Rats fed with essential fatty acids deficiency (EFAD) diet, showed an increased dental pulp cell

For all the foregoing, it seems plausible to hypothesize that leptin and its receptor are expressed in the pulp and periapical human dental tissues, playing a role in local modulation of inflammatory and immune responses. Because no study has investigated the presence of leptin or its receptor in pulp and periapical human dental tissues, this thesis will focus specifically on the current situation in order to provide a scientific basis for discerning the underlying mechanisms in the pathophysiology of oral inflammatory processes.

Introducción general

Introducción general

Uno de los aspectos más interesantes a los que en la actualidad se enfrenta la comunidad científica médica y odontológica es la posible conexión entre los procesos inflamatorios orales de origen infeccioso y el estado de salud sistémico (Seymour et al. 2009). Son numerosos los estudios epidemiológicos que han encontrado asociación entre el estado de salud general y estos procesos inflamatorios orales (Soskolne et al. 2001; Janket et al. 2003; Jiménez Pinzón et al. 2004; Segura-Egea et al. 2005; Caplan et al. 2006; Ridao-Sacie et al. 2007)). La evidencia de esta asociación ha conllevado a una mayor atención al diagnóstico y tratamiento de estas enfermedades orales en diversas situaciones clínicas, con la consiguiente mejora de la salud oral y sistémica de los pacientes.

Sin embargo, no se conoce en profundidad la fisiopatología de los procesos inflamatorios orales de origen infeccioso en cuanto a aspectos básicos, moleculares y celulares. Se necesitan más investigaciones para discernir plenamente los mecanismos subyacentes por los cuales las infecciones crónicas locales pueden tener un impacto en la salud sistémica, y en este aspecto, la pulpitis y la periodontitis apical pueden servir como modelos ideales de enfermedades inflamatoria orales. Su mejor conocimiento conllevaría un mejor conocimiento de su posible repercusión a nivel sistémico.

La pulpitis o inflamación pulpar se produce cuando, bien sea por caries o por traumatismos, las noxas y los microorganismos alcanzan el tejido pulpar desencadenando una respuesta inmune e inflamatoria (Jontell et al. 1998). Las bacterias causantes de la caries son también la causa principal de la infección y la inflamación pulpar, siendo la lesión que sufrirá el tejido pulpar el resultado de un proceso dinámico en el que, de un lado, están los microorganismos invasores y, de otro, la respuesta inmune e inflamatoria del huésped (Bergenholtz et al 2000). Para que se produzca la respuesta inflamatoria pulpar no es necesario que las bacterias alcancen físicamente la pulpa. Por el contrario, existen evidencias experimentales que demuestran que antígenos bacterianos y/o subproductos metabólicos pueden difundir a través de los túbulos dentinarios y provocar respuestas inmunes en la pulpa dental (Warfvinge et al 1985). Durante la respuesta inmune pulpar pueden

formarse también complejos inmunes y liberarse, por fagocitosis, enzimas proteolíticos extracelulares que agravan y empeoran la inflamación pulpar (Bergenholtz et al 1977). En definitiva, la pulpitis se produce por un mecanismo inmunopatológico (Hahn et al 2007). Frente a agresiones leves o moderadas, la inflamación de la pulpa puede reparar el daño causado (pulpitis reversible), manteniéndose la vitalidad pulpar. En estos casos, el dolor aparece con ciertos estímulos y nunca es espontáneo. Por el contrario, si no se eliminan los factores agresivos y no se aplica una terapia de conservación y protección pulpar, terminará produciéndose una pulpitis irreversible y, en definitiva, la necrosis pulpar. La pulpitis irreversible es, pues, la respuesta inflamatoria aguda de la pulpa frente a la persistencia, crecimiento y progresión de las bacterias, u otro agente agresivo, en la cavidad pulpar (Canalda et al 2001). Cuando el proceso inflamatorio pulpar es irreversible, el paciente presenta dolor de intensidad creciente, espontáneo, episodios dolorosos cada vez más frecuentes y dolor que persiste después de eliminar el estímulo. Suele haber, antecedentes de lesiones cariosas profundas, exposición pulpar o restauraciones filtradas (Montgomery et al 1986). Sin embargo, en algunos casos la pulpitis irreversible puede cursar sin sintomatología, por lo que se distinguen dos formas clínicas de pulpitis irreversible: la sintomática y la asintomática (Pumarola et al. 2001)

La periodontitis apical es la inflamación aguda o crónica del tejido que rodea a la raíz dentaria, generalmente entorno al ápice radicular, producida por la infección bacteriana de la pulpa dental (endodonto) (Eriksen et al.1998). La periodontitis apical se produce, en más del 90% de los casos, como secuela de la caries dental, una vez que ésta alcanza la pulpa dentaria provocando pulpitis y/o necrosis pulpar. El contenido polimicrobiano y/o antigénico del conducto radicular sale por el foramen apical, o por conductos laterales, e invade el tejido conectivo periapical o periradicular desencadenando una respuesta inflamatoria e inmune. La periodontitis apical puede ser aguda y sintomática o crónica y asintomática. La periodontitis apical aguda cursa con dolor y mínima reabsorción ósea, pudiendo, en ocasiones, ser reversible. La periodontitis apical crónica es una consecuencia de la necrosis pulpar y es, por ello, irreversible. Puede presentarse con cuatro cuadros anatomopatológicos: granuloma apical, absceso apical crónico, quiste apical y osteitis condensante (Pumarola et al 2001). Las lesiones osteolíticas periapicales o periradiculares, observables radiológicamente, son

consecuencia de la destrucción ósea que conlleva el proceso inflamatorio crónico periapical o periradicular. La prevalencia de la periodontitis apical es muy alta en la población general, llegando en España al 61% de los individuos y al 4% de los dientes estudiados (Jiménez-Pinzón et al. 2004). Las lesiones periapicales más comunes son los granulomas periapicales (Gbolahan et al. 2008). El granuloma periapical es una lesión inflamatoria crónica en el ápice de un diente no vital que consiste de tejido de granulación. El infiltrado de células inflamatorias en estas lesiones periapicales crónicas consta de una mezcla de células plasmáticas, linfocitos T y B, macrófagos, leucocitos polinucleares (PMN), células dendríticas (DCs), las células asesinas naturales (células NK), y mastocitos, presentes dentro del tejido de granulación de las lesiones periapicales en diferentes proporciones (Márton y beso 1993, Stashenko et al. 1998, Liapatas et al. 2003, Márton y beso 2014). El infiltrado inflamatorio constituye aproximadamente el 50% de las células presentes en los granulomas periapicales, con células de tejido conectivo no inflamatorias, incluyendo fibroblastos, endotelio vascular, proliferación de epitelio, osteoblastos y osteoclastos (Langeland et al. 1977, Yu et al. 1987).

La conexión entre la inflamación pulpar y periapical y sus respectivas respuestas reparativas aún no están bien establecida, sin embargo, la respuesta inflamatoria aparece como un requisito previo para la reparación tisular (Goldberg et al. 2004a, Goldberg et al. 2008b). Cuando las células huésped reconocen al ácido lipoteicoico (LTA) de bacterias Gram-negativas y / o al lipopolisacárido (LPS) de las bacterias Gram-positivas, median el proceso inflamatorio local directamente a través de la producción de péptidos antimicrobianos y citoquinas, e indirectamente a través de la activación de las células inmunes migratorias (Horst et al., 2011). La liberación de citoquinas locales promueve la expresión de moléculas implicadas en la mineralización (Linde et al., 1993, Asgary et al., 2014). A este respecto, las respuestas defensivas y reparadoras dentales podrían ser influenciados por la leptina, una citoquina pro-inflamatoria.

La leptina es una hormona no glicosilada derivada de los adipocitos de 146 aminoácidos codificadas por el gen *ob* (Zhang et al. 1994) con una estructura terciaria semejante a la de los miembros de la familia de citoquinas de larga helicoidal (Figura 1) (IL- 6, IL-11, IL-12, LIF, G-CSF,

CNTF, y oncostatina M) (Flier, 1995). Principalmente es sintetizada y liberada por el tejido adiposo (Maffei et al., 1995a) para regular el control de peso de una manera central aunque también es sintetizada por otros tejidos tales como el estómago, músculo esquelético, placenta y la médula ósea (Friedman et al., 1998). Los niveles circulantes de leptina (rango normal 1-15 ng / ml) reflejan directamente la cantidad de energía almacenada en el tejido adiposo y son proporcionales a la masa adiposa del cuerpo, tanto en ratones como en seres humanos; por lo tanto, las personas obesas suelen producir mayor leptina que los individuos más delgados (Frederich et al. 1995, Iikuni et al., 2008, Maffei et al., 1995b, Zavalza-Gómez et al., 2008,). Durante el período de ayuno y después de la reducción de la masa de grasa corporal, hay una disminución en los niveles de leptina que conduce a una reducción en el gasto total de energía para proporcionar la energía suficiente para la función de los órganos vitales, es decir, el cerebro, el corazón, y el hígado (Ahima et al., 1996a). Sin embargo, ahora hay una evidencia creciente de que la leptina tiene efectos sistémicos aparte de las relacionadas con la homeostasis de la energía, incluyendo la regulación neuroendocrina, reproductiva, hematopoyética y funciones inmunes (Ahima et al., 2000b). Se ha demostrado que la leptina regula la respuesta inmune, tanto las respuestas innatas y adaptativas, no sólo en condiciones normales, sino también en condiciones patológicas (Fernández-Riejos et al. 2010). De acuerdo con este papel de la leptina en los mecanismos de respuesta inmune y defensa del huésped, los niveles de leptina se incrementan ante estímulos infecciosos e inflamatorios tales como LPS, trementina, y citoquinas (Matarese et al., 2000). A pesar de que estos efectos de disminución de leptina están dirigidas a mejorar las posibilidades de supervivencia en condiciones de hambre, la caída de los niveles de leptina puede conducir a la supresión inmune (Lord et al., 1998), además de otras alteraciones neuroendocrinas que afectan a las glándulas suprarrenales, tiroides y sexual / función reproductiva (Fantuzzi et al., 2000). Estas alteraciones fueron observadas durante el período de ayuno en paralelo a la disminución de los niveles circulantes de leptina. De hecho, tanto los ratones ob / ob (que carecen de secreción de leptina) y ratones db / db (que carecen de receptor de leptina) no sólo son obesos sino que también muestran las deficiencias inmunes / endocrinos observados durante el periodo de ayuno (Fantuzzi et al., 2000), Howard et . al, 1999)

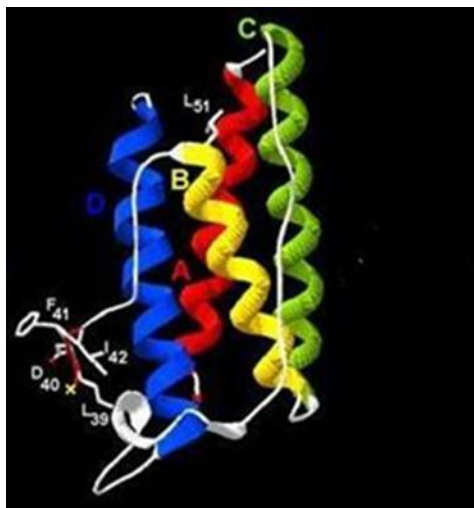


Figura 1. Estructura tridimensional de la leptina. La molécula de leptina tiene un conjunto de cuatro hélices, similar a las citoquinas clásicas de larga cadena helicoidal.

La naturaleza pleiotrópica de la leptina se debe a la distribución universal del receptor de leptina (LEPR) (Myers et al., 2004). LEPR también muestra similitud estructural con la familia de citoquinas de clase I (Myers et al., 2004, Tartaglia et al., 1995, Tartaglia 1997b) y es similar a otros receptores de esta clase. LEPR se expresa en seis isoformas. De acuerdo a sus diferencias estructurales, las isoformas del receptor se dividen en tres clases: las isoformas largas, cortas y de secreción (Figura 2). Entre todas las isoformas de LEPR, sólo la isoforma de longitud completa (LEPRb) es capaz de transducir totalmente las señales de activación en la célula desde su región citoplásmica conteniendo varios fragmentos necesarios para la transducción de señales (Santos-Álvarez et al. 1999, 19). De acuerdo con el carácter multifuncional de la leptina, esta isoforma completamente activa de LEPR no sólo se expresa en el hipotálamo, donde participa en la homeostasis de la energía, sino también está presente en los tejidos periféricos, así como en las células hematopoyéticas y en todo tipo de células inmunes implicadas en la inmunidad innata y adaptativa (Cioffi et al., 1996, Sánchez-Margalet et al. 2003d, Loffreda et al., 1998). LEPR carece de actividad intrínseca tirosina-quinasa, pero requiere la activación de las quinasas asociadas al receptor de la familia Janus (JAK) (Tartaglia et al., 1995) que inician la señalización, y activan miembros de la familia de factores de transcripción (STAT)

(Tartaglia 1997b). La vía STAT activada se dimerizan y se translocan al núcleo, donde se regula la expresión de genes específicos (Tartaglia 1997b, Gualillo et al., 2002). Además de STAT, la leptina activa otras vías de señalización; entre ellas se encuentran la familia de proteína quinasa mitogena activada (MAPK), la cascada de señalización fosfatidilinositol 3-quinasa (PI3K) (Figura 3) (Sánchez-Margalet et al., 2002c, Santos-Alvarez et al. 1999, Pérez-Pérez et al., 2008a, Pérez-Pérez et al., 2009b Pérez-Pérez et al., 2010c).

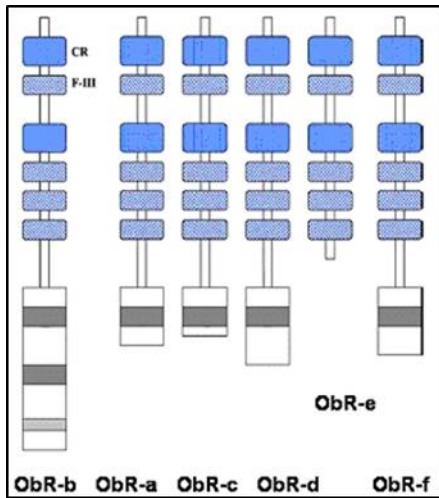


Figure 2: leptin receptor isoforms; (Hegyí et al 2004)

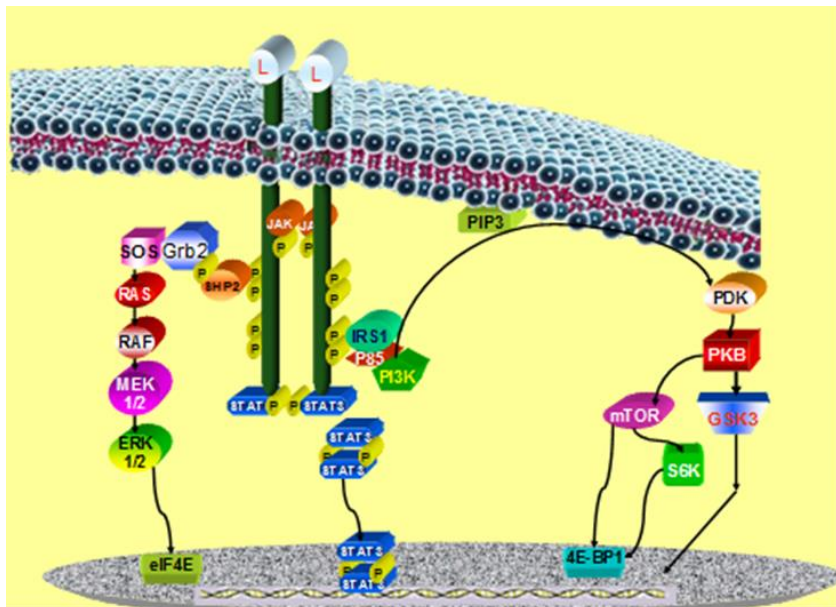


Figura 3. Leptin signaling pathways

La evidencia científica ha implicado a la leptina en las funciones de biología oral. Se ha demostrado la presencia de leptina y LEPR soluble en tejido gingival, tanto sanos e inflamadas (Bozkurt et al., 2006, Dilsiza et al., 2010), en el fluido crevicular gingival (Karthikeyan et al., 2007a, Karthikeyan et al., 2007b), así como en lesiones periapicales crónicas humanas (Kangarlou et al., 2010) y en la saliva (Gröschl et al., 2001, Randeva y col, 2003). Se ha observado una asociación entre una elevada concentración de leptina en sangre con mayor prevalencia de periodontitis apical crónica (Gundala et al., 2012). Por otra parte, ha surgido la primera evidencia de que la leptina tiene efectos sobre las células madre de la pulpa dental, actuando como un modulador importante de la diferenciación de las células madre mesenquimales pulpares (Um et al., 2011). Esta hormona se expresa en ameloblastos, odontoblastos, células de la papila dental y células del estrato intermedio en ratas y en gérmenes dentarios humanos en la fase de corona (Ide et al., 2011). Además, se ha demostrado de que la leptina es sintetizada y secretada por fibroblastos *in vitro* extraídos de los dientes molares extraídos sanos (El Karim y col., 2009). Sin embargo, otras células dentales también pueden ser una fuente de leptina y LEPR y qué tipo de célula dentaria puede expresar leptina y LEPR, aún no se ha dilucidado. Por lo tanto, la leptina podría actuar tanto por vía autocrina como paracrina y por lo tanto puede desempeñar un papel en la respuestas inflamatorias e inmunes pulpares y periapicales similares a la de los tejidos adiposos blancos. En este sentido, aunque los adipocitos no son un componente celular normal en la pulpa dental, las células madre de la pulpa dental humana (DPSCs) son capaces de diferenciarse en adipocitos (Gronthos et al., 2002), expresando *in vitro* genes adipogénicos PPAR γ 2 (peroxisoma proliferador activado del receptor gamma dos) y lipoproteína lipasa (LPL), dos marcadores específicos de adipocitos (Koyama et al., 2009). Por lo tanto, la leptina pulpar podría ser secretada por DPSCs que han experimentado diferenciación adipogénica.

La pulpa dental es un tejido muy innervado con buen potencial de curación después de la agresión e inflamación siendo la angiogénesis crucial para el desarrollo de los dientes y un requisito previo para la reparación exitosa después de la lesión y la inflamación. Los factores de crecimiento angiogénicos tales como el factor de crecimiento endotelial vascular (VEGF), factor de crecimiento de fibroblastos básico (bFGF-2), y el factor de crecimiento transformante (TGF- β) se han identificado en la pulpa dental

humana (Grando et al., 2007, Guven et al., 2007, Roberts-Clark et al., 2000). De hecho, estos factores son sobre-reguladas en la pulpa dental de los dientes cariados. Por lo tanto, la leptina podría desempeñar un papel importante sobre la angiogénesis mediante la regulación de la expresión del factor de crecimiento angiogénico pulpar, similar a la de los otros tejidos inflamados y al cáncer (Vansaun, 2013).

Aunque las bacterias son el principal agente etiológico de la periodontitis apical o la pulpitis, su presencia es suficiente para iniciar la enfermedad, mientras que la iniciación y progresión de estas enfermedades depende de la calidad de la respuesta inmune del huésped. La infiltración de los tejidos inflamados por las células inmunes naturales tales como neutrófilos, eosinófilos y macrófagos es una característica importante de la inflamación. De manera similar a lo que ocurre en la obesidad, la inflamación pulpar o periapical se caracterizan por la infiltración de células inflamatorias tales como linfocitos, macrófagos, células dendríticas y neutrófilos (Hahn et al 2000a). Por otra parte, es bien sabido que las citoquinas regulan el tráfico de linfocitos, y de citoquinas ligando CC 20 (CCL20) que muestra un papel clave en el reclutamiento de células T (Liao et al., 1999) y las células dendríticas inmaduras en lesiones inflamatorias (Dieu et al., 1998). La leptina se asocia con un aumento de la expresión de CCL20 (Farquharson et al., 2012) y se ha demostrado que la expresión de CCL20 es está influenciada por la estimulación de bacterias relacionadas con la caries dentinaria profunda, así como por citoquinas proinflamatorias en la pulpitis (Takahashi et al., 2008). Por lo tanto, la leptina podría regular la producción de señales quimiotácticas y el tráfico de linfocitos durante la respuesta inflamatoria pulpar. Durante la respuesta inmune innata del complejo dentino-pulpar a la caries, se produce un aumento de macrófagos (Kamal et al., 1997); la leptina puede regular la función de los monocitos según la evaluación de los experimentos in vitro que miden la producción de radicales libres a través de las vías PKC-dependiente (Maingrette et al., 2003, Sánchez-Pozo et al., 2003). Por otra parte, como se ha demostrado anteriormente en los monocitos en el sistema inmunológico, la leptina podría estimular la producción de citoquinas pro-inflamatorias tales como TNF- α e IL-6, y aumentar la expresión de citoquinas CC en los macrófagos de la pulpa dental humana, a través de la fosforilación-activación de la vía JAK-STAT Kiguchi et al., 2009, Zhou et al., 2011).

La conexión entre un adipoquina, como la leptina, cuyo principal papel funcional es el control del apetito y el hambre (Flier, 1995, Zhang et al., 1994), y las respuestas defensivas y reparativas pulpar y periapicales, podría proporcionar una nueva evidencia de la relación entre la obesidad, la inflamación y las infecciones orales. Se han propuesto varias posibles vías que implican la respuesta del huésped y la exposición bacteriana para explicar la asociación entre la obesidad y la inflamación (D'Aiuto et al., 2012). La obesidad puede alterar las respuestas inmunes del huésped a las infecciones orales aumentando la susceptibilidad de los individuos. Por lo tanto, las moléculas pro-inflamatorias y procesos implicados en la patogénesis de las infecciones orales, tales como pulpitis y la periodontitis apical, incluyendo citoquinas (por ejemplo, interleucina-IL-6), quimioquinas, y la función de las células T, podrían ser influenciadas por la obesidad (Falagas et al., 2006). Se ha sugerido una asociación entre el contenido de la dieta en ácidos grasos y la población de células de la pulpa dental (Vermelin et al., 1995). Las ratas alimentadas con déficit de ácidos grasos esenciales (EFAD) en la dieta, mostraron un aumento de células en la pulpa dental

Por todo lo anterior, parece plausible hipotetizar de que la leptina y su receptor se expresan en la pulpa y en los tejidos periapicales humanos, jugando un papel en la modulación local de la respuesta inflamatoria e inmune. Debido a que ningún estudio ha investigado la presencia de leptina o su receptor en la pulpa y en los tejidos dentales humanos periapicales, esta tesis se centrará específicamente en la situación actual con el fin de proporcionar una base científica para profundizar en los mecanismos que subyacen en la fisiopatología de los procesos inflamatorios orales.

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Objectives

Objectives

The main objective of this Ph. D thesis was to study the expression and the possible role of leptin and its receptor in human dental pulp and periapical tissues analyzing especially if there are differences in expression between healthy and inflamed tissues assessing their involvement in pathophysiology of inflammatory pulpo-periapical conditions (pulpitis and apical periodontitis). In order to achieve this main objective, the research carried out was divided into the following specific goals

1. To determine leptin expression in human dental pulp and to compare the leptin expression level in healthy and in experimentally induced inflamed dental pulps pulp (Chapter I).
2. To determine leptin receptor expression (LEPR) in human dental pulp and to compare the LEPR expression level in healthy and in experimentally induced inflamed dental pulps (Chapter II).
3. To characterize the signaling pathways stimulated by leptin in human dental pulp (Chapter III).
4. To identify the cell type expressing LEPR in human dental pulp (Chapter IV)
5. To characterize the possible functional effect of leptin on pulp cells (Chapter IV).
6. To determine the expression and immunolocalization of leptin in periapical inflammatory tissues (Chapters V and VI).

Objetivos

Objetivos

El objetivo general de esta tesis doctoral es estudiar la posible expresión y papel de la Leptina y de su receptor en la pulpa dental humana así como en los tejidos periapicales, analizando, especialmente si existen diferencias de expresión entre tejidos sanos e inflamados valorando su implicación en la fisiopatología de los procesos inflamatorios pulpo-periapicales (pulpitis y periodontitis apical). Para el cumplimiento de este objetivo general se plantean los siguientes objetivos concretos:

- 1.- Determinar la expresión de leptina en pulpa dental humana sana e inflamada, estudiando si existe asociación entre el grado de inflamación del tejido pulpar dental y el nivel de expresión de leptina (Capítulo I).
- 2.- Determinar la expresión de receptor de leptina en pulpa dental humana sana e inflamada, estudiando si existe asociación entre el grado de inflamación del tejido pulpar dental y el nivel de expresión de receptor de leptina (Capítulo II).
- 3.- Caracterizar las rutas de señalización estimuladas por la leptina (Capítulo III).
- 4.- Identificar las células que expresan receptor de leptina en la pulpa dental humana (Capítulo IV)
- 5.- Caracterizar el posible efecto funcional de la leptina sobre células pulpares (Capítulo IV).
- 6.- Determinar la expresión y la localización tisular de leptina y su receptor en tejido periapical humanos inflamados (Capítulos V y VI).

Chapter 1: **Leptin expression in healthy and inflamed human dental pulp.**

This chapter has been published as:

Jénifer Martín-González, Flora Sánchez-Jiménez, Antonio Pérez-Pérez, Antonio Carmona-Fernández, Víctor Sánchez-Margalet, Juan José Segura-Egea (2013): Leptin expression in healthy and inflamed human dental pulp. *International Endodontic Journal*; 46:442-448.

ABSTRACT

Aim. Leptin is an adipocyte-derived hormone that has been shown to regulate the immune response. This study aims to investigate the expression of leptin in healthy and inflamed human dental pulp.

Methodology. Twenty one pulp samples were obtained from freshly caries- and restoration-free extracted human third molars. In 7 third molars (inflamed pulp group) inflammation was induced prior to extraction. Pulp samples were processed and leptin expression was determined by quantitative real time-PCR (qRT-PCR), and the amount of leptin by immunoblot.

Results. All healthy and inflamed dental pulp samples expressed leptin. Western blot analysis revealed the presence of a protein with at an apparent molecular weight of ~16 kDa in human dental pulp, which corresponds to the estimated molecular weight of leptin. The expression of leptin mRNA in dental pulp was confirmed by qRT-PCR analysis and the size of the amplified fragments (296 bp for leptin and 194 bp for cyclophilin) were confirmed by agarose gel electrophoresis. The expression of leptin in the inflamed pulp group was significantly greater than in healthy teeth. The relative amount of leptin in inflamed pulps was almost twice than in healthy pulps ($p < 0.05$).

Conclusions. For the first time, the presence of leptin in human dental pulp tissues has been demonstrated. The upregulation of leptin expression in inflamed pulp samples suggests that leptin can play a role in pulpal inflammatory and immune responses.

1. INTRODUCTION

The adipocyte-derived hormone leptin has been shown to regulate the immune response, both innate and adaptive responses, not only in normal but also in pathological conditions (Fernández-Riejos *et al.* 2010). Leptin is a 16 kDa-glycosylated protein product of the *Ob* gene (Zhang *et al.* 1994). Leptin is mainly synthesized and secreted from adipose tissue (Ahima & Flier 2000) and was originally described as an adipocyte-derived hormone to regulate weight control in a central manner, via its cognate receptor in the hypothalamus (Flier 1995). However, leptin has been classified as a cytokine because its primary amino acid sequence shows structural similarities to the long chain helical cytokine family (Zhang *et al.* 1994, Sánchez-Margalet *et al.* 2003). Moreover, the leptin receptor (Ob-R) is expressed not only in the central nervous system, but also in peripheral tissues, such as haematopoietic and immune systems (Sánchez-Margalet *et al.* 2003). Therefore, a role for leptin in haematopoiesis and the immune system has been proposed (Cioffi *et al.* 1996, Sánchez-Margalet *et al.* 2003). It has been suggested that leptin orchestrates the immune host response by enhancing cytokine production and phagocytosis by macrophages (Fantuzzi & Faggioni 2000, Sánchez-Margalet *et al.* 2003, Fernández-Riejos *et al.* 2010).

The presence of leptin has been reported both in healthy and inflamed gingival tissues (Johnson & Serio 2001), in gingival crevicular fluid (Bozkurt *et al.* 2006, Karthikeyan & Pradeep 2007, Karthikeyan & Pradeep 2007, Dilsiza *et al.* 2010), and in human chronic periapical lesions (Haghighi *et al.* 2010). Elevated serum leptin concentration has been associated with increased chronic periodontitis (Gundala *et al.*

2012). Recently, it has been provided the first evidence that leptin has effects on dental pulp stem cells, acting as an important modulator of pulpal mesenchymal stem cells differentiation (Um *et al.* 2011), being expressed in ameloblasts, odontoblasts, dental papilla cells and stratum intermedium cells in rat and human tooth germs at the late bell stage (Ide *et al.* 2011).

It can be hypothesized that leptin is expressed by human dental pulp and that plays a role in the modulation of pulpal inflammatory and immune responses. However, even though it has been previously shown that leptin is synthesized and secreted *in vitro* by pulp fibroblasts derived from extracted healthy molar teeth (El Karim *et al.* 2009), to date no study has investigated its presence in human dental pulp. The aim of this study was to investigate the expression of leptin in healthy and inflamed human dental pulp.

2. MATERIAL AND METHODS

The study was carried out with the understanding and written consent of each subject and according to the principles of the Declaration of Helsinki. The protocol was approved by the Ethical Board of the University of Sevilla, Spain.

2.1. - Dental pulp samples

Human dental pulp was obtained from 21 freshly extracted third molars from nineteen healthy, nonsmoking, human donors (22–35 years old) who gave their written informed consent to donate their pulp tissue. All

teeth used in this study were caries- and restoration-free and without signs of periodontal disease. Fourteen third molars were simply extracted and processed (healthy pulp group). However, in 7 third molars (inflamed pulp group) inflammation was induced prior to extraction using the method described previously by Caviedes-Bucheli *et al.* (2005). Briefly, the inflammatory process was generated by mechanical exposure of the pulp chamber using a no.1 round carbide bur in a highspeed handpiece without irrigation. After a period of 10 min, the teeth were extracted and processed.

The extracted teeth were washed with 5.25% sodium hypochlorite after extraction to eliminate remains of periodontal ligament that could contaminate the pulp sample, and they were kept at -80°C until use. The teeth were then sectioned by using a Zekrya bur (Dentsply Maillefer, Tulsa, OK) in a high-speed hand-piece irrigated with saline solution. The pulp tissue was obtained by using a sterile endodontic excavator, washed 2–3 times in sterile PBS to remove excess blood, and placed on an Eppendorf tube. Each sample was divided into two parts, one for the Western blotting analysis and another one for the RNA extraction and quantitative real time PCR (qRT-PCR) assay.

2.2. - Antibodies and reagents.

The monoclonal mouse anti-tubulin (1:1000) was provided from Santa Cruz Biotechnology and the polyclonal rabbit anti-human leptin Y20 (1:1000) antibodies from Sigma (Sigma Diagnostics, St Louis, MO, USA). Horseradish peroxidase-linked anti-mouse/anti-rabbit (1:10000) immunoglobulins were purchased from Amersham Pharmacia.

2.3. - Western blotting analysis

The pulp tissue samples were incubated in 75 µl of lysis buffer containing 20 mM Tris, pH 8, 1% Nonidet P-40, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mM sodium orthovanadate, at 4 °C for 30 min on an orbital shaker and then centrifuged at 13,000 rpm for 15 min. Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific) using bovine serum albumin as standard. We added SDS-stop buffer containing 100 mM of DTT to the pulp tissue samples followed by boiling for 5 min. The samples were then resolved by 15% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Martín-Romero *et al.* 2000). The membranes were blocked with buffered saline–0.05% Tween 20 (PBST) containing 3% albumin 1 h at 23 °C. The blots were then incubated with primary antibody for 1 h, washed in PBST and further incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/anti-rabbit immunoglobulins (Santa Cruz). Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal from Pierce) (Sánchez-Margalet *et al.* 2003a, Sánchez-Margalet *et al.* 2003b). The bands obtained in the blots were scanned and analyzed by the PCBAS2.0 program. The relative optical density of the different bands was normalized by the corresponding intensity of the tubulin immunoblot in each individual experiment.

2.4. - RNA extraction and quantitative real time PCR (qRT-PCR) assay and agarose gel electrophoresis.

Abundance of leptin mRNA was determined by quantitative real time PCR reaction (qRT-PCR). Total RNA was extracted from human dental pulp tissue samples using TRISURE reagent (Chomczynski 1993). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 µg of total RNA was reverse transcribed at 55°C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche). Quantitative real time PCR was performed using the following primers based on the sequences of the National Center for Biotechnology Information GenBank database (Table 1). Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a Chromo 4 DNA Engine (Bio-Rad). A typical reaction contained 10µM of forward and reverse primer, 3µL of cDNA, and the final reaction volume was 25µL. The reaction was initiated by preheating at 50°C for 2 min, followed by heating at 95°C for 10 min. Subsequently, 41 amplification cycles were carried out as follows: denaturation 15 s at 95°C and 1min annealing and extension at 58°C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $1/\Delta Ct$ method. For the treated samples, evaluation of $1/\Delta Ct$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin).

The retro-transcribed DNA samples were then resolved by 1% agarose gel. After the run of the electrophoresis, gels were visualized directly upon illumination with UV light (Voytas 2001).

Table 1. Sequence of PCR primers used in this study.

Gene	Primer sequences
Leptin	
Forward:	5'-GAACCCTGTGCGGATTCT-3'
Reverse:	5'CCAGGTCGTTGGATATTTGG-3'
Cyclophilin	
Forward:	5'-CTTCCCCGATACTTCA-3'
Reverse:	5'-TCTTGGTGCTACCTC-3'

2.5. -Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as means \pm standard deviation (SD) in arbitrary units (AU). Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the Graph Pad Prism computer program (GraphPad Software). Statistically significant differences between healthy and inflamed dental pulp samples were tested using the Mann–Whitney rank sum test. Significance levels were set at $p < 0.05$.

3. RESULTS

All healthy human dental pulp samples expressed leptin at both protein and mRNA levels. Western blot revealed the expression in dental pulp of an immune-reactive protein that migrated with recombinant leptin protein at an apparent molecular weight of ~16 kDa, which corresponds to the estimated molecular weight of leptin (Fig. 1).

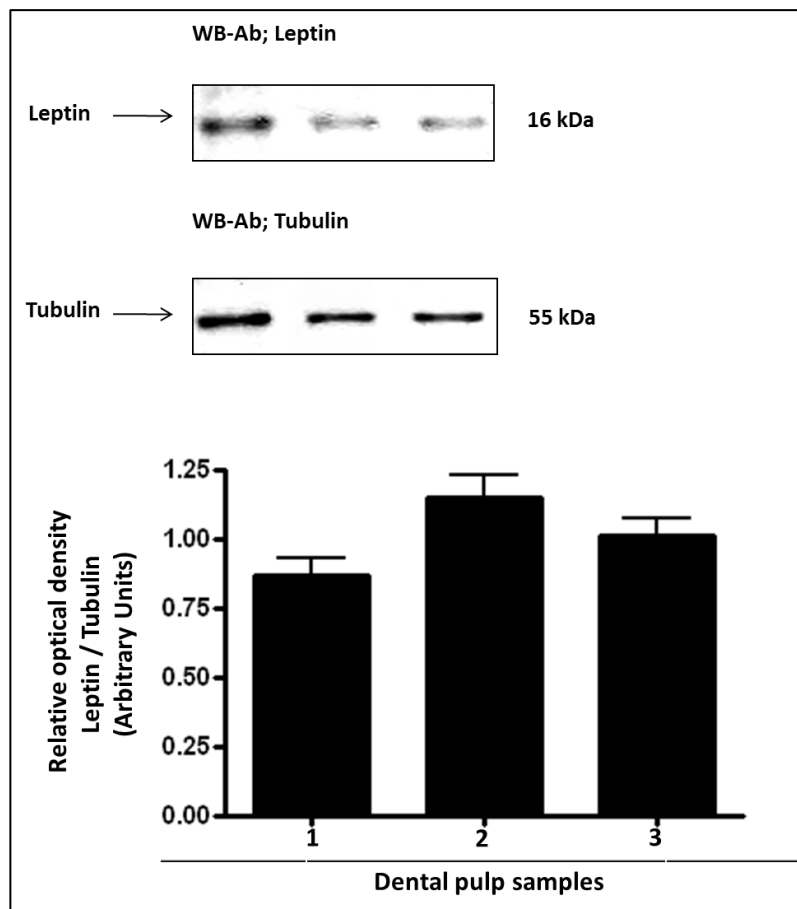


Figure 1. Expression of leptin in healthy human dental pulp. To calculate relative amounts of leptin expression, the band intensity of each sample was normalized with tubulin. Densitograms are expressed as means \pm standard deviations in arbitrary units,

calculated as normalized band intensity. Results shown in the immunoblot are from a representative experiment with three dental pulp samples repeated three times.

To confirm the leptin expression in dental pulp, quantitative real time PCR (qRT-PCR) assay was carried out to identify the leptin mRNA (Fig. 2, upper panel). The size of the amplified fragments (296 bp for leptin and 194 bp for cyclophilin) was confirmed by agarose gel electrophoresis (Fig. 2, lower panel).

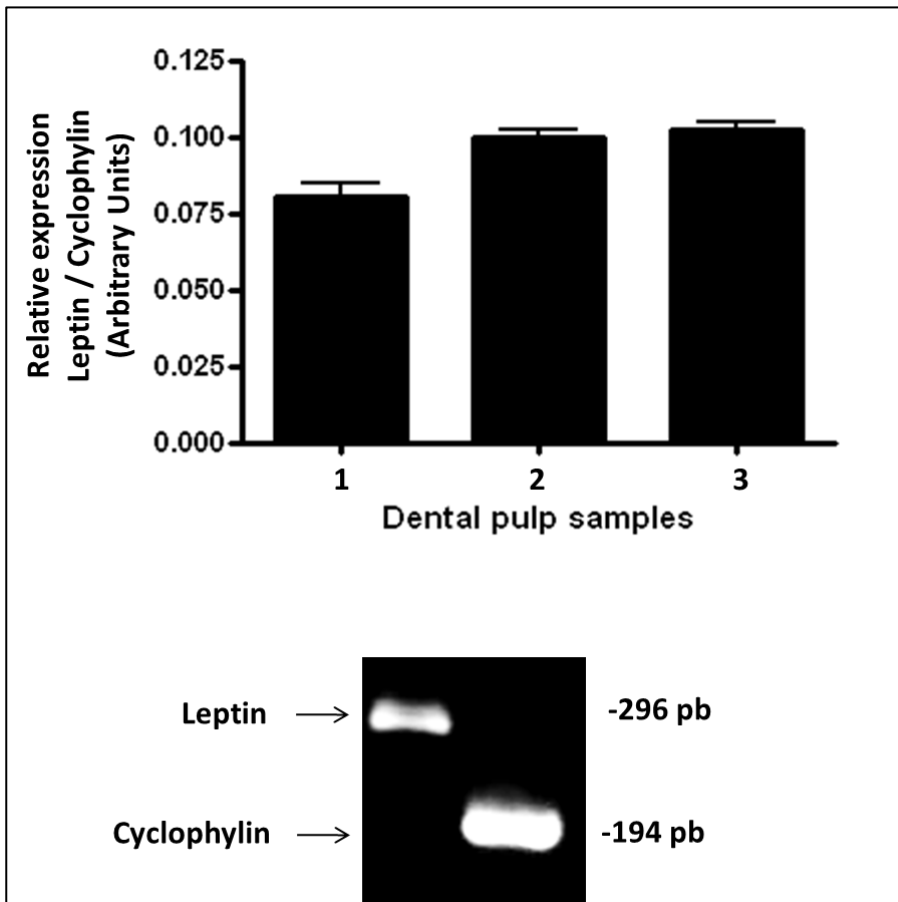


Figure 2. Leptin mRNA expression in healthy human dental pulp. Total RNA was extracted from each healthy human dental pulp sample. Leptin mRNA was

quantified using quantitative real time PCR (qRT-PCR) assay, and visualized by agarose gel electrophoresis. Cyclophilin was used as internal standard. Results from a representative experiment with three dental pulp samples are shown. Data represent fold change from the mean values of cyclophilin and are expressed as means \pm SD.

All inflamed dental pulp samples expressed the protein leptin. The expression of leptin in the dental pulp of teeth with inflamed pulps was significantly greater than in healthy teeth ($p < 0.05$) (Fig. 3). The relative amount of leptin in inflamed pulps was almost twice than in healthy pulps.

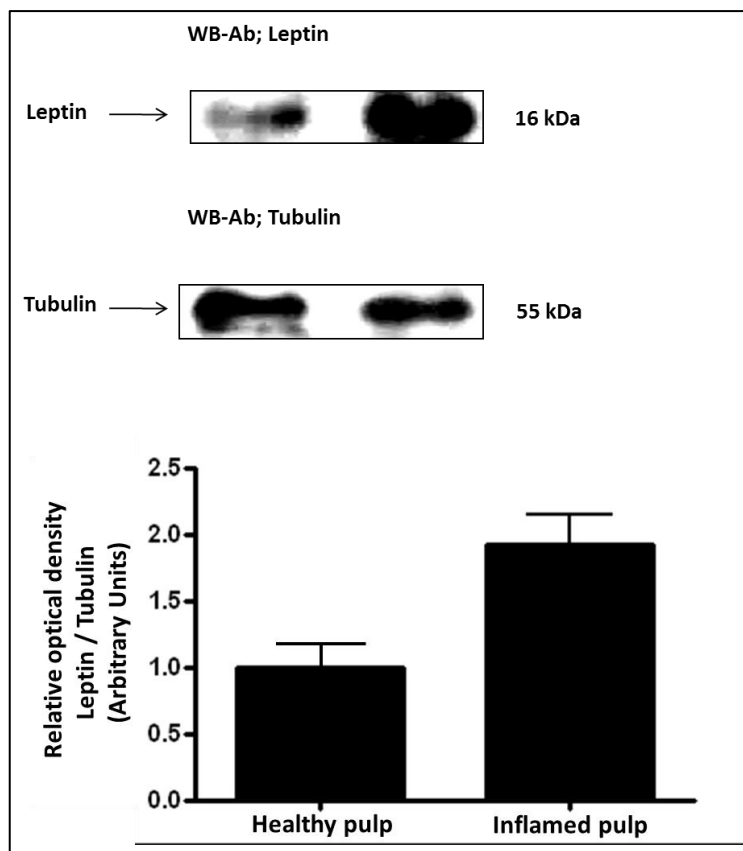


Figure 3. Expression of leptin in healthy and inflamed human dental pulp samples. To calculate relative amounts of leptin expression, the band intensity of

each sample was normalized with tubulin. Densitograms are expressed as means \pm standard deviations in arbitrary units. The data indicate a significant increase of leptin in the inflamed dental pulp (n = 7) compared with healthy dental pulp (n = 14) ($p < 0.05$). Results shown in the immunoblot are from a representative experiment with two dental pulp samples (one healthy and one inflamed) repeated three times.

4. DISCUSSION AND CONCLUSION

The present study is the first to demonstrate the expression of leptin in human healthy and inflamed dental pulp tissues. A recently study has also demonstrated the expression of leptin in rat dental pulp by Western blot analysis (Ide *et al.* 2011). As well, this study is the first to demonstrate that leptin is upregulated in the inflamed dental pulp.

Leptin is one of the most important hormones secreted by adipose tissue and its implication in energetic homeostasis at central level has been largely described. Leptin is also secreted by other type of tissues, such as placenta (trophoblast), ovaries, skeletal muscle, stomach, mammary epithelial cells, bone marrow, pituitary and liver (Margetic *et al.* 2002). Although adipocytes are not a normal cellular component in dental pulp, human dental pulp stem cells (DPSCs) have been found to be capable of differentiating into oil red O-positive lipid-containing adipocytes (Gronthos *et al.* 2002), expressing in vitro the early adipogenic master gene PPAR γ 2 (peroxisome proliferator-activated receptor gamma two) and the late marker lipoprotein lipase (LPL), two adipocyte-specific transcripts (Koyama *et al.* 2009). So, pulpal leptin could be secreted by DPSCs suffering adipogenic differentiation. Nevertheless, another dental pulp cells can also be the source of leptin found in human dental pulp tissue. Thus, it has been demonstrated that human pulp fibroblasts in culture are able to synthesize and secrete leptin (El Karim *et al.*

2009), and it has been found that leptin is expressed in odontoblasts in rat and human tooth germs (Ide *et al.* 2011). Therefore, these cell types could be also the origin of pulpal leptin found in the present study.

One of the physiological roles of leptin is the connection between nutritional status and immune competence (Sánchez-Margalet *et al.* 2003, Fernández-Riejos *et al.* 2010). Leptin modulates the immune system at the development, proliferation, antiapoptotic, maturation, and activation levels. In fact, leptin receptors have been found in neutrophils, monocytes, and lymphocytes, and the leptin receptor belongs to the family of class I cytokine receptors. Moreover, leptin activates similar signaling pathways to those engaged by other members of the family (Sánchez-Margalet *et al.* 2003b, Fernández-Riejos *et al.* 2010). Leptin signaling deficiency impairs humoral and cellular immune responses (Bennet *et al.* 1996).

The overall leptin action in the immune system is a pro-inflammatory effect during adaptive immune response, activating pro-inflammatory cells, promoting T-helper 1 responses, and mediating the production of the other pro-inflammatory cytokines, such as tumor necrosis factor- α , IL-2, or IL-6 (Fernández-Riejos *et al.* 2010). In processes involving innate immunity, leptin circulating levels increase acutely upon infectious and inflammatory stimuli such as LPS, turpentine, and cytokines (Sarraf *et al.* 1997, Bernotiene *et al.* 2006).

The expression of leptin in human dental pulp showed in the present study suggests that this protein could modulate pulpal immune and inflammatory responses. Moreover, the present investigation has demonstrated the upregulation of leptin in inflamed dental pulp. The significantly higher relative amount of leptin in inflamed dental pulps found in the present study,

support the concept that leptin plays a role in dental pulp inflammatory processes. Pulp inflammation is characterized by the infiltration of inflammatory cells such as lymphocytes, macrophages, dendritic cells and neutrophils, and consequently pulpitis is formed (Hahn *et al.* 2000). It is well known that chemokines regulate the trafficking of lymphocytes, and CC chemokine ligand 20 (CCL20) has been shown to play a crucial role in the recruitment of memory T cells (Liao *et al.* 1999) and immature dendritic cells into inflammatory lesions (Dieu *et al.* 1998). Taking into account that leptin is associated to an increased expression of CCL20 (Farquarson *et al.* 2011), the high relative amount of leptin in inflamed pulp suggests that leptin could regulate the trafficking of lymphocytes during pulpal inflammatory response. It has been demonstrated that CCL20 expression is induced by stimulation with caries-related bacteria invading deeply into the dentinal tubules as well as by proinflammatory cytokines in the inflamed pulpal lesions (Takahashi *et al.* 2008).

When the number of macrophages increases during the innate response of the dentin/pulp complex to caries (Kamal *et al.* 1997), leptin can regulate monocyte function as assessed by *in vitro* experiments measuring free radical production (Sánchez-Pozo *et al.* 2003, Maingrette & Renier 2003). Thus, leptin has been found to stimulate the oxidative burst in control monocytes (Sánchez-Pozo *et al.* 2003) and macrophages (Maingrette & Renier 2003), and binding of leptin at the macrophage cell surface increases lipoprotein lipase expression through oxidative stress- and PKC-dependent pathways (Maingrette & Renier 2003).

Leptin dose-dependently stimulates the production of pro-inflammatory cytokines by monocytes, such as TNF- α and IL-6 and enhances CC-chemokine ligand expression in cultured murine macrophage, through

phosphorylation of Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) pathway (Kiguchi *et al.* 2009). Taking into account that JAK2-STAT3 signaling pathway has been identified in human dental pulp (Zhou *et al.* 2011), leptin could use the same signaling pathway in human dental pulp.

As a result of the nature of the present study, it was not possible to identify which type of human pulp cell expresses leptin. Further investigations must be carried out to elucidate which pulp cell type express leptin, as well as if leptin-receptors are expressed by human dental pulp cells. Nevertheless, the upregulation of leptin expression in inflamed pulp samples showed in this study suggests that leptin plays a role in pulpal inflammatory and immune responses.

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Chapter 2: Leptin Receptor is Up-Regulated in Inflamed Human Dental Pulp.

This chapter has been published as:

Jénifer Martín-González, Antonio Pérez-Pérez, Flora Sánchez-Jiménez, Antonio Carmona-Fernández, Víctor Sánchez-Margalet, Juan José Segura-Egea (2013). Leptin Receptor is Up-Regulated in Inflamed Human Dental Pulp. *Journal of Endodontics*; 39:1567-1571.

ABSTRACT

Introduction. After leptin receptor (LEPR) identification in haematopoietic, immune system and other tissues, a role for leptin regulating inflammation and immune response has been accepted. This study aims to describe the possible expression of LEPR in healthy human dental pulp, and to compare it with LEPR expression in inflamed human dental pulp.

Methodology. Twenty-one pulp samples were obtained from freshly caries- and restoration-free extracted human third molars. In seven third molars (inflamed pulp group), inflammation was experimentally induced prior to extraction. Pulp samples were processed, and LEPR expression was determined by quantitative realtime PCR (qRT-PCR) and the amount of LEPR protein was analysed by immunoblot.

Results. All healthy and inflamed dental pulp samples expressed LEPR. Western blot analysis of human dental pulp revealed the presence of a protein with an apparent molecular weight of approximately 120 kDa, which corresponds to the estimated molecular weight of LEPR. The expression of LEPR mRNA was confirmed by qRT-PCR analysis and the size of the amplified fragment (338 bp for LEPR and 194 bp for cyclophilin) was assessed by agarose gel electrophoresis. The relative amount of LEPR in inflamed pulps was approximately 50% higher than in healthy pulps ($p < 0.05$).

Conclusions. The presence of LEPR in human dental pulp tissues has been demonstrated for the first time. The up-regulation of LEPR expression in inflamed pulp samples suggests that leptin can play a role in inflammatory and local immune responses in human dental pulp.

1. INTRODUCTION

Leptin is a 16 kDa protein hormone of 146 amino acids encoded by the *Ob* gene (1) primarily synthesized and released from adipose tissue (2) and, at lower levels, by other tissues such as the stomach, skeletal muscle, placenta and bone marrow (3). Initially, leptin was described as an adipocyte-derived signaling molecule playing a key role in metabolism and homeostasis, regulating the body weight through the control of energy intake and energy expenditure at hypothalamic level (4). According to its primary amino acid sequence, that shows structural similarities to the long chain helical cytokine family, such as IL-2, IL-12 and GH, leptin has been classified as a pro-inflammatory cytokine (1, 5). Consequently, a role for leptin regulating immunity, inflammation and haematopoiesis has been accepted (6-9).

Leptin affects both innate and adaptive immunity exerting an effect on T-cells, monocytes, neutrophils, and endothelial cells. In innate immunity, leptin modulates the activity and function of neutrophils and mononuclear cells (8,10). Moreover, leptin up-regulates both phagocytosis and the production of proinflammatory cytokines of the acute-phase response (11,12). In adaptive immunity, leptin affects the generation, maturation and survival of thymic T cells and the switch towards a pro-inflammatory Th1 immune response (13, 14). Consistent with this role of leptin in the

mechanisms of immune response and host defense, leptin levels are increased upon infectious and inflammatory stimuli such as LPS, turpentine, and cytokines (15).

Leptin receptor (LEPR) shows sequence homology to members of the class I cytokine receptor (gp130) superfamily (16), which includes the IL-6-type cytokine receptors, leucocyte inhibitory factor and granulocyte colony-stimulating factor (17). LEPR is expressed in six isoforms, as product of alternative RNA splicing. According to its structural differences, the receptor's isoforms are divided into three classes: long, short, and secretory isoforms. Among all LEPR isoforms, only full-length isoform (LEPRb) is able to fully transduce the activation signals into the cell since its cytoplasmic region contains several motifs required for signal transduction (18,19). According to the multifunctional role of leptin, this fully-active isoform of LEPR is expressed not only in the hypothalamus, where it takes part in energy homeostasis, but also is present on peripheral tissues as well as on hematopoietic cells and on all types of immune cells involved in both innate and adaptive immunity (6,8,11,13,20,21).

LEPR has been shown in mice monocytes and lymphocytes (11, 13). The presence of both the short and long isoforms of the LEPR has been confirmed

in human peripheral blood T lymphocytes (both CD4 and CD8) by Western blot and flow cytometry analysis (8,21).

In relation to oral tissues, leptin has been identified both in healthy and inflamed gingival tissues (22,23), in crevicular fluid (24-26) and in cultured human pulp fibroblasts derived from extracted healthy molar teeth where leptin production seems to be regulated by neuropeptides (27). Moreover, LEPR immunoreactivity has been found in the gingival epithelium (23) and LEPR gene has been detected in experimental rats periapical lesions (28), but no study has described yet the expression of LEPR mRNA and protein in these tissues.

Recently, it has been described the expression of leptin in human healthy dental pulp (29). Moreover, experimentally induced dental pulp inflammation (after mechanical pulp injury with short exposure in mouth before tooth extraction) produces the up-regulation of leptin expression, suggesting a possible role of this cytokine-like hormone mediating a tissue response to inflammation in dental pulp (29). Since there is no evidence of the expression of LEPR in normal human dental pulp so far, the aim of this study was to analyze the possible LEPR expression in human dental pulp tissue samples,

and in addition, to compare the LEPR expression level in healthy and in experimentally induced inflamed dental pulps.

2. MATERIAL AND METHODS

The study was carried out with the understanding and written consent of each subject and according to the principles of the World Medical Association Declaration of Helsinki. The protocol was approved by the Ethical Board of the University.

2.1.-Human dental pulp samples

Human dental pulps from nineteen healthy, non-smoking, human donors (22–32 years old), who gave their written informed consent, were obtained from 21 freshly extracted third molars, as previously described (30). All teeth used in this study were caries- and restoration-free and without signs of periodontal disease.

Fourteen third molars were simply extracted and processed (healthy pulp group). However, in seven-third molars (inflamed pulp group), inflammation was induced prior to extraction using the method described previously by Caviedes-Bucheli *et al.* (31). Briefly, the inflammatory process was generated by mechanical exposure of the pulp chamber using a no.1 round

carbide bur in a high-speed hand piece without irrigation. After a period of 10 min, the teeth were extracted and processed.

The extracted teeth were washed with 5.25% sodium hypochlorite after extraction to eliminate remains of periodontal ligament that could contaminate the pulp sample, and they were kept at -80°C until use. The teeth were then sectioned by using a Zekrya bur (Dentsply Maillefer, Tulsa, OK, USA) in a high-speed hand-piece irrigated with saline solution. The pulp tissue was obtained by using a sterile endodontic excavator, washed 2–3 times in sterile PBS to remove excess blood, and placed on an Eppendorf tube. Each sample was divided into two parts, one for the Western blotting analysis and another one for the RNA extraction and quantitative real time PCR (qRT-PCR) assay.

2.2. - Antibodies and reagents

The monoclonal mouse anti- β -tubulin (1:1000) and the polyclonal rabbit antibodies against the long isoform of leptin receptor (C-terminal), were provided from Santa Cruz Biotechnology (CA, USA). Horseradish peroxidase-linked anti-mouse/anti-rabbit (1: 10 000) immunoglobulins were purchased from Amersham Pharmacia (Amersham Pharmacia Biotech, Barcelona, Spain).

2.3.- Western blotting analysis

The pulp tissue samples were incubated in 75 μ l of lysis buffer containing 20 mM Tris, pH 8, 1% Nonidet P-40, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mM sodium orthovanadate, at 4 °C for 30 min on an orbital shaker and then centrifuged at 13,000 rpm for 15 min. The supernatants were transferred to new tubes. Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as standard. We added SDS-stop buffer containing 100 mM of DTT to the pulp tissue samples followed by boiling for 5 min. 50 μ g protein were loaded in each lane. The samples were then resolved by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (21). The membranes were blocked with buffered saline–0.05% Tween 20 (PBST) containing 3% bovine serum albumin during 1 h at 23 °C. The blots were then incubated with primary antibody for 1 h, washed in PBST, and further incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/antirabbit immunoglobulins. Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal from Pierce). The bands obtained in the blots were scanned and analyzed by

the PCBAS 2.0 program. The relative optical density of the different bands was normalized by the corresponding intensity of the tubulin immunoblot in each individual experiment.

2.4. -RNA extraction and quantitative real time PCR (qRT-PCR) assay and agarose gel electrophoresis.

Abundance of LEPR mRNA was determined by quantitative real time PCR reaction (qRT-PCR). Total RNA was extracted from human dental pulp tissue samples using TRISURE reagent (32). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 µg of total RNA was reverse transcribed at 55°C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche, Mannheim, Germany). Quantitative real time PCR was performed using the primers based on the sequences of the National Center for Biotechnology Information GenBank database (Table 1). Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a MiniOpticon (Bio- Rad, Hercules, CA, USA). A typical reaction contained 10 µM of forward and reverse primer, 3 µL of cDNA, and the final reaction volume was 25 µL. The reaction was initiated by preheating at 50°C for 2 min, followed by heating at 95°C for 10 min. Subsequently, 41 amplification cycles were carried out as follows: denaturation 15 s at 95°C

and 1 min annealing and extension at 58°C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin), and relative to the untreated control. The retro-transcribed DNA samples were then resolved by 1% agarose gel. 20 μ l samples were loaded in each lane. After running the electrophoresis, gels were visualized directly upon illumination with UV light.

Table 1. Sequence of PCR primers used in this study.

Gene	Primer sequences
Leptin receptor	
Forward:	5'-ATAGTTCAGTCACCAAGTGC-3'
Reverse:	5'-GTCCTGGAGAACTCTGATGTC-3'
Cyclophilin	
Forward:	5'-CTTCCCCGATACTTCA-3'
Reverse:	5'-TCTTGGTGCTACCTC-3'

2.5. - Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as mean \pm standard deviation (SD) in arbitrary units (AU). Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the GraphPad Prism computer program (GraphPad Software, San Diego, CA, USA). Statistically significant differences between healthy and inflamed dental pulp samples were tested using the Mann–Whitney rank sum test. Significance levels were set at $p < 0.05$.

3. RESULTS

All specimens obtained from human dental pulp samples expressed LEPR at both protein and mRNA levels.

To study the presence of the LEPR in pulp tissue samples, Western blot was carried out using antibodies that specifically recognize the long isoform of leptin receptor (C-terminal). The amount of LEPR in every sample was standardized by anti- β -tubulin immunoblot. LEPR was detected as a band

with molecular mass of about 120 kDa, which corresponds to the estimated molecular weight of LEPR (Fig. 1).

Relative mRNA levels of LEPR were determined by quantitative real time PCR (qRT-PCR). The mRNA level was normalized to internal control cyclophilin. The synthesis of LEPR mRNA was identified in all specimens (Fig. 2), both in healthy pulps (Fig. 2.A) and in inflamed pulps (Fig. 2.B). Forty cycles were run for RT-PCR. The CT (cross-over threshold) value for cyclophilin was 21 cycles and 26-27 cycles for LEPR. The size of the amplified fragments (338 bp for LEPR and 194 bp for cyclophilin) was confirmed by agarose gel electrophoresis (Fig. 2.C). Data indicate a significant increase of LEPR in the inflamed dental pulps compared with healthy dental pulps (Fig. 2.D). The relative amount of LEPR mRNA in inflamed pulps increased almost 50% compared to that observed in healthy pulps ($p < 0.05$)

All inflamed dental pulp samples expressed the LEPR protein (Fig. 3). The expression of LEPR in the inflamed pulps was significantly greater than that observed in healthy teeth. The relative amount of LEPR in inflamed dental pulps increased nearly 50% compared to that detected in healthy pulps ($p < 0.05$).

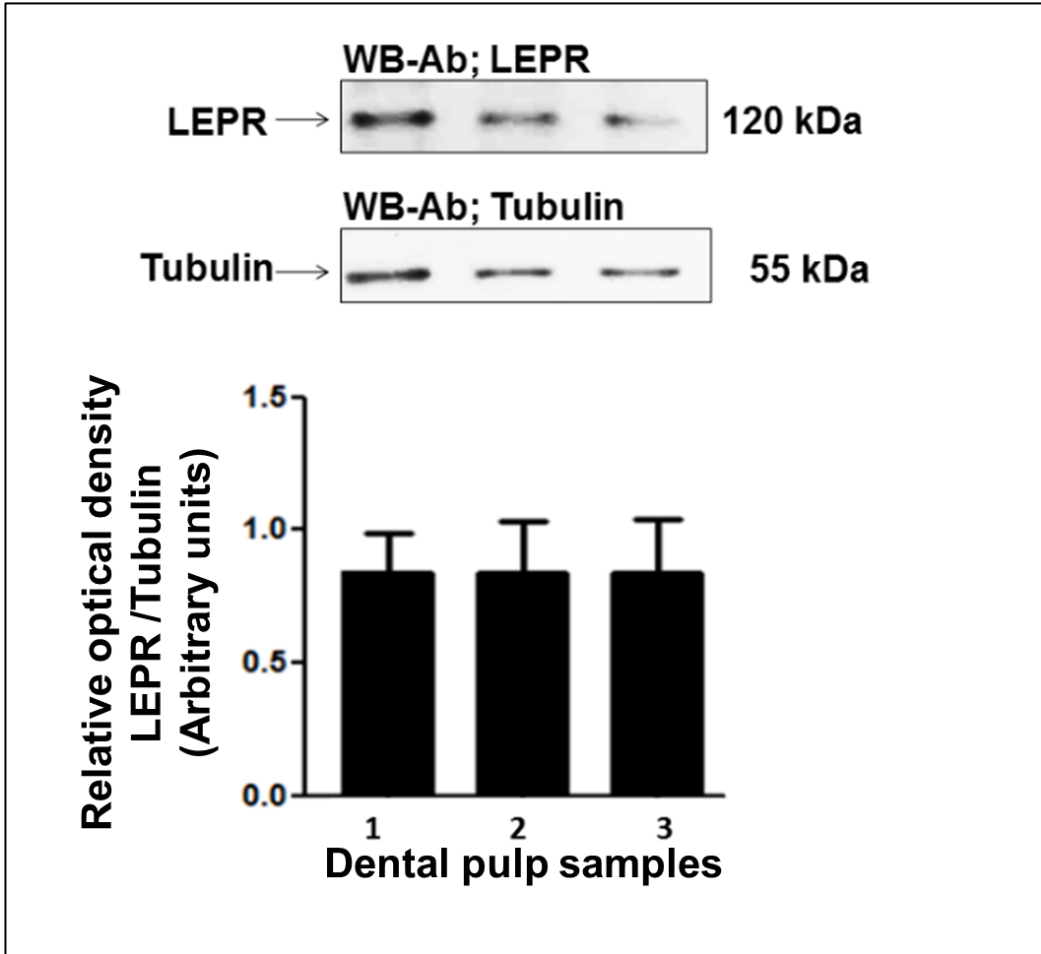


Figure 1. Relative amounts of LEPR in three samples of human dental pulp. Results shown are from a representative experiment with three dental pulp samples repeated three times. Densitograms are expressed as means \pm standard deviation in arbitrary optical density units, calculated as normalized band intensity, i.e. the band intensity of each sample was normalized with tubulin.

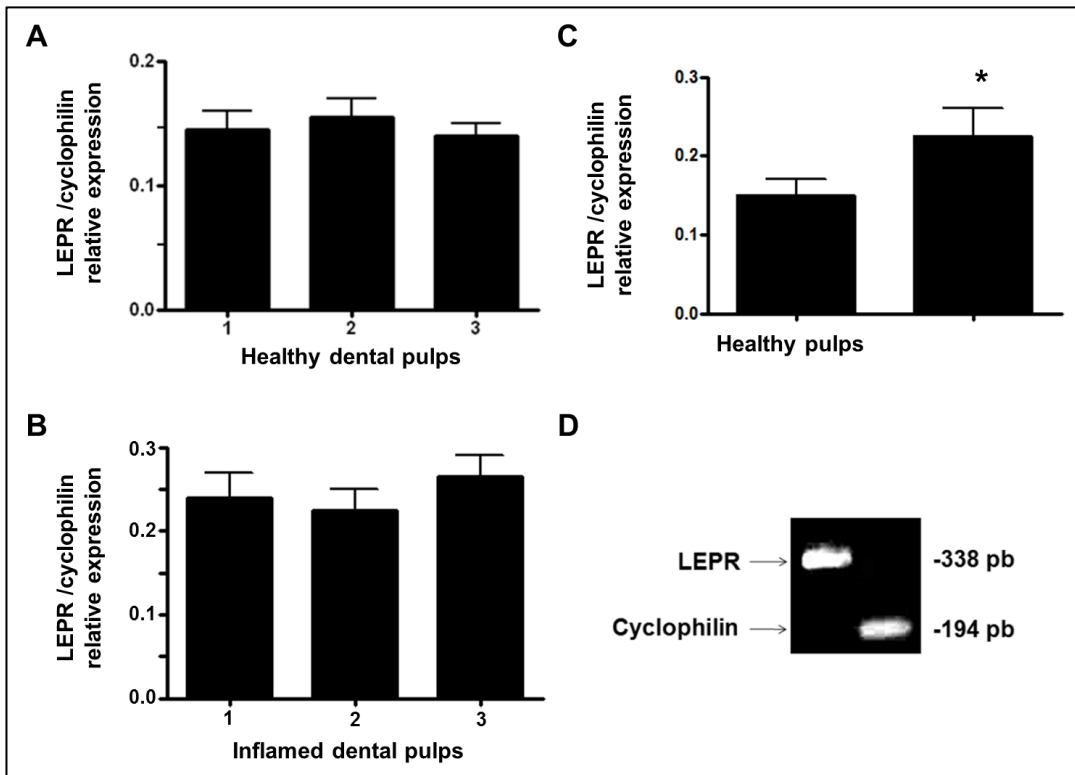


Figure 2. LEPR mRNA expression in normal and inflamed human dental pulp.

Total RNA was extracted from each human dental pulp sample. LEPR mRNA was quantified using quantitative real time PCR (qRT-PCR) assay, and visualized by agarose gel electrophoresis. Cyclophilin was used as internal standard. Results from a representative experiment with three dental pulp samples are shown. Data represent fold change from the mean values of cyclophilin and are expressed as means \pm standard deviation. A) Healthy pulps. B) Inflamed pulps. C) Comparison between healthy and inflamed pulps. The data indicate a significant increase of LEPR mRNA in inflamed dental pulp (n = 7) compared with healthy dental pulp (n = 14) (*p < 0.05). D) Size of the amplified fragments confirmed by agarose gel electrophoresis.

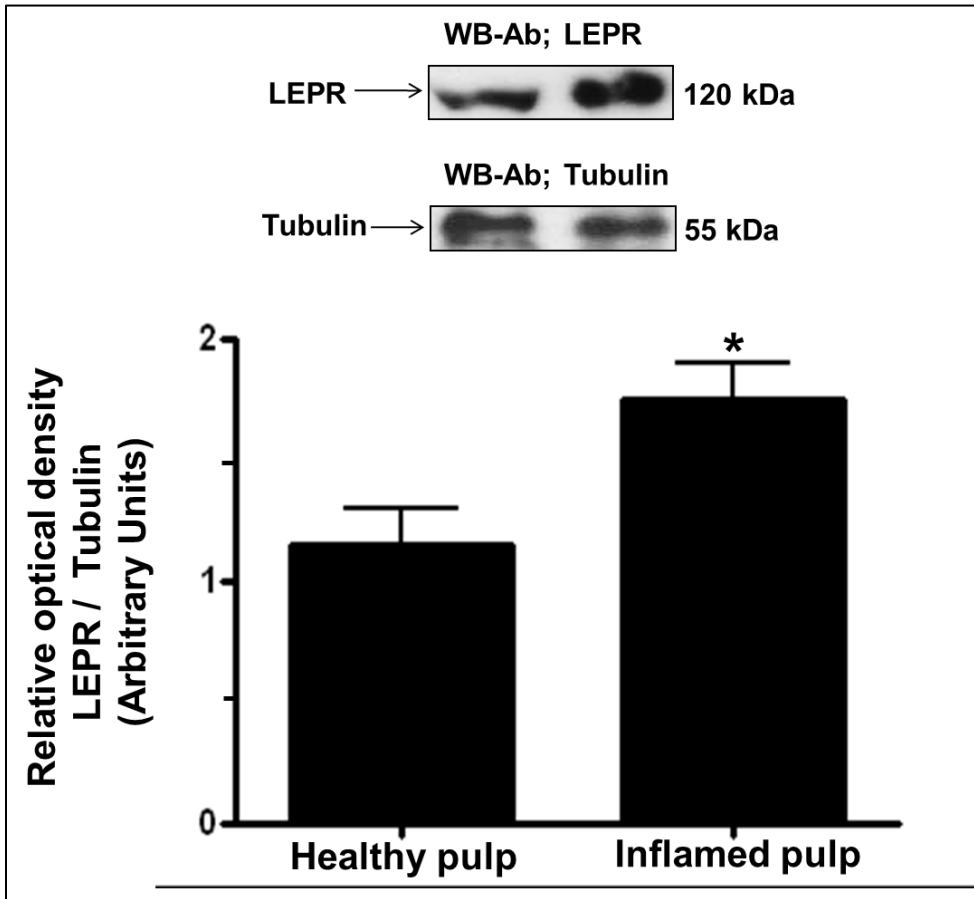


Figure 3. Expression of LEPR in healthy and inflamed human dental pulp samples. To calculate relative amounts of LEPR expression, the band intensity of each sample was normalized with tubulin. Densitograms are expressed as means \pm standard deviations in arbitrary units. The data indicate a significant increase of LEPR in inflamed dental pulp ($n = 7$) compared with healthy dental pulp ($n = 14$) (* $p < 0.05$). Results shown in the immunoblot are from a representative experiment with two dental pulp samples (one healthy and one inflamed) repeated three times.

4. DISCUSSION AND CONCLUSION

For the first time, the expression of LEPR in human healthy and inflamed dental pulp has been demonstrated. Moreover, this is the first study demonstrating that LEPR is up-regulated in the inflamed dental pulp. LEPR has also been detected previously in experimental rats periapical lesions (28), but mRNA and protein expression studies are still pending.

Recently, it has been provided evidence that leptin is expressed in ameloblasts, odontoblasts, dental papilla cells and stratum intermedium cells in tooth germs of mandibular third molars at the late bell stage (33), in rat dental pulp (33), and, more recently, in human dental pulp (29). Therefore, the result of the present investigation, together with those previous findings, suggests that leptin and LEPR expressing cells could be implicated in the physiology of the human dental pulp.

The expression of LEPR in human dental pulp can be explained because this tissue contained several cells types that can express LEPR, such as monocytes (11,13), NK cells (34) and CD4 T and CD8 T cells (35). Taking into account that it has been reported that leptin acts as an important modulator of dental pulp stem cells (DPSCs) differentiation, promoting the cemento / odontoblastic differentiation and suppressing the adipogenic

differentiation in DPSCs (36), the results of the present investigation suggest that DPSCs also express LEPR. Given that DPSCs could produce leptin (27, 29), leptin may play a role in defensive and reparative responses of dental pulp against deep carious lesions, likely involving a negative feedback. However, the possible messengers involved in this feedback mechanism are unknown, representing an interesting area of future research.

Both leptin and LEPR share structural and functional similarities with the IL-6 family of cytokines (5,16), and leptin production is increased during infection and inflammation (7,37). Indeed, leptin has been linked with a stress-related reaction (38), and it has been suggested that leptin could be the link connecting the obesity and the proinflammatory phenotype that occurs in obese subjects (39, 40).

It has been demonstrated that leptin is synthesized and secreted in cultured human pulp fibroblasts derived from extracted healthy molar teeth (27). Leptin production in these cultures seems to be regulated by neuropeptides: SP and NPY decreased leptin levels in the first 24 h., increasing them at 48 h.; on the contrary, CGRP increased leptin levels at 48 hours. On the other hand, the inflammation model used in this study, i.e. high-speed drilling and mechanical pulp exposure, are effective stimulus to release neuropeptides in

dental pulp (41). Moreover, the expression of CGRP, SP and NPY is significantly higher in the inflamed human pulp compared with healthy pulp (42). Taking into account that leptin has proangiogenic effects, including induction of neovascularization and formation of capillary-like structures (27, 43, 44), and that NPY operates with leptin in the regulation of food intake and energy expenditure (45), El-Karim *et al.* (27) have suggested that NPY can indeed regulate leptin production by pulp fibroblasts. Thus, the expression of LEPR in vascular endothelial cells in human dental pulp must be investigated in order to determine if leptin has pro-angiogenic effect in dental pulp.

The results obtained in this study must be interpreted prudently, because the inflammation model used, high-speed drilling and mechanical pulp exposure, is not totally comparable to infectious acute pulpitis in carious teeth. Although mechanical pulp exposure was successful in inducing inflammation, as stated in previous studies (31, 41), it has been demonstrated a greater expression of neuropeptides in acute irreversible pulpitis than in pulps having induced inflammation, which could be explained by the evolution time of the inflammatory process (42). In the mechanically induced pulpitis, pulps are obtained 10 min after the stimulus was applied; on the

contrary, inflammation in acute irreversible pulpitis had at least 24-h duration (42).

In conclusion, the expression of LEPR in human dental pulp showed in the present study points to a possible role for leptin and LEPR in the physiology as well as the pathophysiology of the dental pulp, probably acting as a modulator of pulp immune and inflammatory responses against caries or/and as a link between these processes and cytokine genotype/phenotype.

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Chapter 3: Leptin Signalling in Human Dental Pulp: Involvement of MAPK, PI3K and JAK/STAT3 Pathways

This chapter has been submitted for publication as:

Jenifer Martín-González, Antonio Pérez-Pérez, Flora Sánchez-Jiménez, Antonio Carmona-Fernández, Víctor Sánchez-Margalet, Juan José Segura-Egea (2014). Leptin Signalling in Human Dental Pulp: Involvement of MAPK, PI3K and JAK/STAT3 Pathways. *International Endodontic Journal*. Submitted.

ABSTRACT

Introduction. Leptin is the peripheral signal produced by the adipocyte to regulate energy metabolism. It has been demonstrated that leptin receptor (LEPR) is expressed by human dental pulp cells, being up-regulated in experimental pulpitis. This study aims to describe the possible signal transduction pathways of leptin in human dental pulp.

Methodology. Fifteen dental pulp samples were obtained from freshly caries- and restoration- free extracted human third molars. Pulp samples were processed, and leptin signalling was determined analyzing JAK-STAT, PI3K and MAPK phosphorylation by immunoblot.

Results. Leptin stimulated JAK-STAT pathway by promoting STAT3 tyrosine phosphorylation. PI3K pathway was also triggered by leptin stimulation as assessed by the study of PKB phosphorylation. Leptin also stimulated tyrosine/threonine phosphorylation of MAPK by studying phosphorylation of MAPK 1/3. These signalling pathways were confirmed in all human dental pulps. Western blot analysis of leptin-stimulated human dental pulp samples revealed the presence of proteins with an apparent molecular weight of approximately 42-44, 60 and 93 kDa, which corresponds, respectively to the estimated molecular weight of tyrosine phosphorylated forms of MAPK, PKB and STAT3.

Conclusions. The present study demonstrates for the first time that leptin stimulates human dental pulp cells through distinct signalling pathways: MAPK, PI3K and JAK/STAT3. These results provide evidence supporting the role of leptin in pulp physiology.

1. INTRODUCTION

Leptin is a 16-kDa-glycosylated protein product of the Ob gene (Zhang *et al.* 1994) synthesized mainly in adipose cells (Ahima *et al.* 2000) to regulate weight control in a central manner (Flier 1995). However, leptin can also be expressed in other tissues, such as placenta, stomach and skeletal muscle (Masuzaki *et al.* 1997, Bado *et al.* 1998). Recently, it has been described the expression of leptin in human dental pulp (Martín-González *et al.* 2013a), being its production regulated by neuropeptides (El Karim *et al.* 2009).

There is increasing evidence that leptin has systemic effects apart from those related to energy homeostasis, including regulation of neuroendocrine, reproductive, hematopoietic and immune functions (Ahima *et al.* 2000). Additionally, leptin has been classified as a cytokine because its primary amino acid sequence shows structural similarities to the long-chain helical cytokine family (Zhang *et al.* 1994, Sánchez-Margalet *et al.* 2003). Leptin has been shown to regulate both innate and adaptive immune responses, not only in normal but also in pathological conditions (Fernández-Riejo *et al.* 2009). On the other hand, leptin modulate pulp mesenchymal stem cell differentiation (Um *et al.* 2010).

Leptin exerts its biological activity through a specific membrane receptor, the leptin receptor (LEPR) which is expressed in 6 isoforms as a product of alternative RNA splicing. According to its structural differences, the receptor's isoforms are divided into 3 classes: long, short, and secretory isoforms. Among all LEPR isoforms, only full length isoform (LEPRb) is able to fully transduce the activation signals into the cell because its cytoplasmic region contains several motifs required for signal transduction (Tartaglia 1997). According to the multifunctional role of leptin, this fully active isoform of LEPR is expressed not only in the hypothalamus but also in

peripheral tissues as well as in hematopoietic cells and in all types of immune cells involved in both innate and adaptive immunity (Sánchez-Margalet *et al.* 2003, Lord *et al.* 1998, Bennett *et al.* 1996, Martín-Romero *et al.* 2000a). Currently, there is evidence that this full length isoform of LEPR is also expressed in human dental pulp (Martín-González *et al.* 2013b) and in human periapical granuloma (Martín-González *et al.* 2014). The up-regulation of leptin and LEPRb expression in inflamed pulp samples suggests that leptin can play a role in inflammatory and immune responses in human dental pulp (Martín-González *et al.* 2013a, Martín-González *et al.* 2013b). Moreover, it has been demonstrated the expression of LEPRb by macrophages in human periapical granulomas (Martín-González *et al.* 2014).

The binding of leptin to LEPRb results in auto-phosphorylation of LEPRb and activation of several intracellular signalling cascades, including JAK/STAT3 (Janus kinase/signal transducer and activator of transcription), PI3K (phosphatidylinositol-3-kinase) and MAPK (mitogen-activated protein kinase) signalling pathways (Saxena *et al.* 2007).

The intracellular signalling pathways activated by leptin have been studied in many different systems (Fruhbeck 2006). However, little is known about the molecular mechanisms underlying the effects of leptin on dental pulp cells. The aim of the present study was to investigate the signal transduction pathways activated by leptin in normal human dental pulp.

2. MATERIALS AND METHODS

The study was carried out with the understanding and written consent of each subject and according to the principles of the World Medical Association

Declaration of Helsinki. The protocol was approved by the Ethical Board of the University of Sevilla.

2.1. - Reagents

Human recombinant leptin was provided from R&D Systems (Minneapolis, MN, USA). Polyclonal rabbit anti-phospho-PKB (Ser472/473, 1:1500) and polyclonal rabbit anti-phospho-STAT3 (Tyr705, 1:1500) were purchased from Cell signalling Technology. Polyclonal rabbit anti-phospho-mitogen-activated protein kinases 1 and 3 (MAPK1/3; Thr202/Tyr204, 1:3000) were from Sigma-Aldrich. The monoclonal mouse anti- β -tubulin (1:5000) was provided from Sigma-Aldrich. Horseradish peroxidase-linked anti-mouse/anti-rabbit (1:10000) immunoglobulins were purchased from Amersham Pharmacia (Amersham Pharmacia Biotech, Barcelona, Spain).

2.2. - Human Dental Pulp Samples Preparation

Human dental pulps from 13, non-smoking, human donors (22–32 years old), who gave their written informed consent, were obtained from 15 freshly extracted third molars. All teeth used in this study were caries- and restoration-free and without signs of periodontal disease.

The extracted teeth were washed with 5.25% sodium hypochlorite after extraction to eliminate remains of periodontal ligament that could contaminate the pulp sample. The teeth were then sectioned by using a Zekrya bur (Dentsply Maillefer, Tulsa, OK, USA) in a high-speed hand-piece irrigated with saline solution. The pulp tissue was obtained by using a sterile endodontic excavator, washed 2–3 times in sterile ice-cold phosphate-buffered saline (PBS) to remove excess blood. Each dental pulp was cut into different segments and thoroughly rinsed with sterile ice-cold PBS.

Human dental pulps were randomly distributed in tubes containing 1 ml of a DMEN-F12 medium (2 replicates per treatment). Dental pulps were maintained in a shaking water bath at 37°C during 5 min to equilibrate temperature. Dental pulps were incubated at 37°C in 5% CO₂ (Magarinos *et al.* 2007) for 10 min in the same medium supplemented or not with 0.1, 1 and 10 nM human recombinant leptin (R&D Systems, Minneapolis, MN). After all this, dental pulps were removed and resuspended in 75 µl of lysis buffer (1 x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 10 mg/ml phenylmethanesulfonyl fluoride (PMSF)) during 30 min at 4°C on an orbital shaker and later centrifuged at 10000 g for 20 min. Supernatants were analyzed by Western blot.

2.3. - Western Blotting Analysis

Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA), using bovine serum albumin as standard. SDS-stop buffer containing 100 mM of DTT was added to the pulp tissue samples, followed by 5 min boiling. In each lane 50 µg protein were loaded. The samples were then resolved by 12% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Hybond, Amersham Pharmacia). The membranes were blocked with buffered saline – 0.05% Tween 20 (PBST) containing 3% bovine serum albumin during 1 h at 23°C. Membranes were then immunoblotted overnight with polyclonal rabbit anti-phospho-STAT3 (Tyr705, 1:1500, Cell signalling Technology), with polyclonal rabbit anti-phospho-mitogen-activated protein kinases 1 and 3 (MAPK1/3; Thr202/Tyr204, 1:3000, Sigma-Aldrich) and polyclonal rabbit anti-phospho-PKB (Ser472/473, 1:1500, Cell signalling Technology). Loading controls were performed by immunoblotting the same membranes with monoclonal anti-β-tubulin (1:5000, Sigma-Aldrich). Then, the blots

were washed in PBST and further incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/antirabbit immunoglobulins (1:10000, Santa Cruz). Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal; Pierce Biotechnology, Rockford, IL, USA). Quantification of protein bands was determined by densitometry using the PCBAS 2.0 program. The relative optical density of the different bands was normalized by the corresponding intensity of the tubulin immunoblot in each individual experiment.

2.4. - Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as means \pm SD in arbitrary units (AU). Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the GraphPad Prism computer program (GraphPad Software, San Diego, CA. USA). Statistically significant differences between stimulated or not dental pulp samples were tested using the Mann–Whitney rank sum test and multivariate ANOVA. Significance levels were set at $p < 0.05$.

3. RESULTS

Leptin activation of JAK-STAT3 signalling pathway in human dental pulp

To study the possible activation of STAT3 by leptin, human dental pulp was stimulated with human leptin and solubilized lysed samples were analyzed by Western blot using antibodies that specifically recognize the tyrosine phosphorylated form of STAT3 (P-STAT3) (Fig. 1, upper panel). Tyrosine phosphorylation of STAT3 was observed in response to human leptin

stimulation. The relative amount of P-STAT3 in stimulated pulps was significantly higher than in unstimulated pulps ($p < 0.05$). Activation of STAT3 was observed at 0.1, 1 and 10 nM leptin, reaching a maximum at 0.1 nM leptin.

Western blot analysis revealed the presence in the pulp samples of a protein with apparent molecular weight of 93 kDa, corresponding to the estimated molecular weight of P-STAT3 (Fig. 1, lower panel). The amount of P-STAT3 in every sample was controlled with anti- β -tubulin immunoblot.

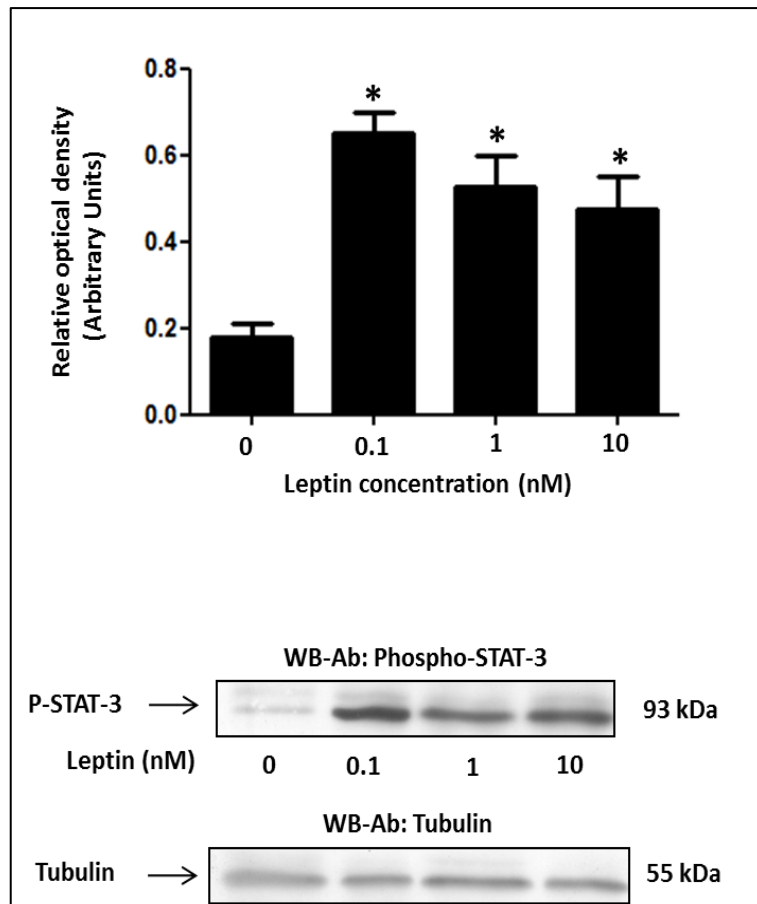


Figure 1. Leptin activation of JAK/STAT3 signalling pathway in human dental pulp. Human dental pulp were incubated in the absence or presence of 0.1, 1 and 10 nM leptin for

10 min in DMEM 0% FCS. For the activation of STAT3, cell lysates were analyzed by immunoblotting using the specific antibody against the phosphorylated form of STAT3 (P-STAT3). The amount of protein in every sample was checked by immunoblot using anti- β -tubulin (1:5000) antibody. Data shown are representative of three independent experiments. Densitograms with standard error are shown. * $p < 0.05$ versus control (0 nM leptin).

Leptin activation of PI3K pathway in human dental pulp

To analyse the effect of PI3K pathway in human dental pulp in response to human leptin, the activation of the central kinase of this pathway, i.e. PKB, was measured by immunoblot using antibodies that specifically recognize the phosphorylated form of PKB (P-PKB) (Fig. 2, upper panel). Leptin stimulated PKB phosphorylation. The relative amount of PKB in stimulated pulps was significantly higher compared to control pulps ($p < 0.05$). Activation of PKB was observed at 0.1, 1 and 10 nM leptin, reaching a maximum at 0.1 nM leptin.

Western blot analysis revealed the presence in the pulp samples of a protein with apparent molecular weight of 60 kDa, which corresponds to the estimated molecular weight of P-PKB (Fig. 2, lower panel). Anti- β -tubulin antibodies were used for the control of the immunoblot.

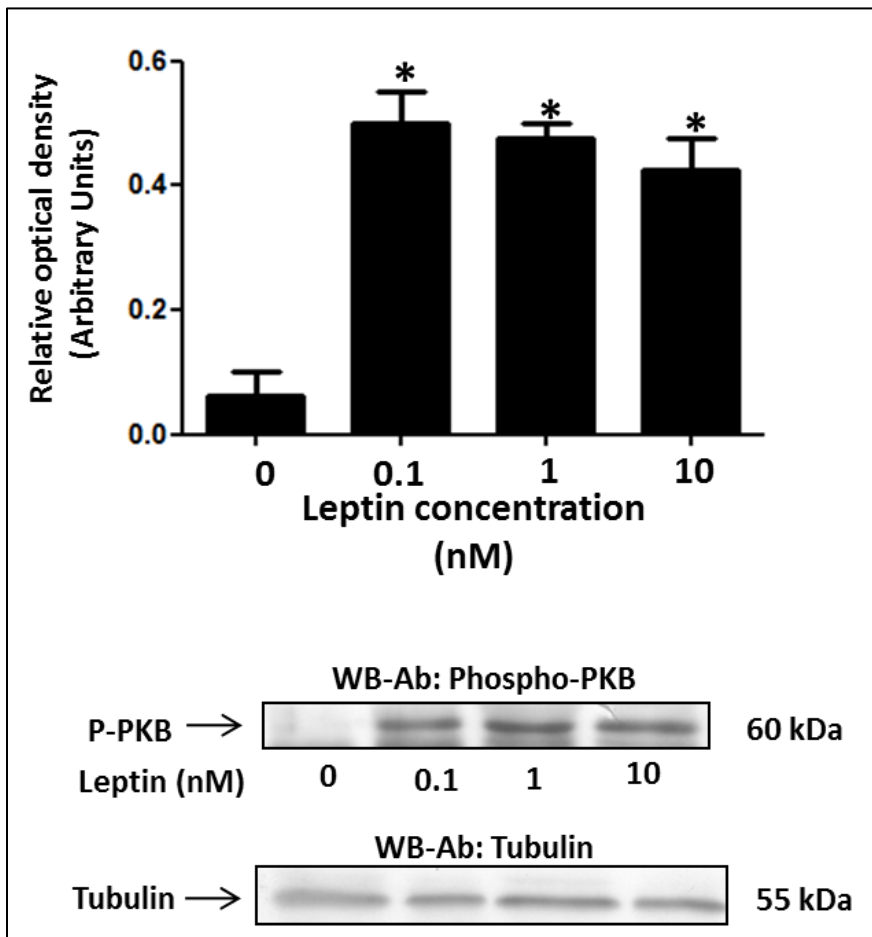


Figure 2. Leptin activation of PI3K signalling pathway in human dental pulp. Human dental pulp were incubated in the absence or presence of 0.1, 1 and 10 nM leptin for 10 min in DMEM 0% FCS. For the activation of PKB, cell lysates were analyzed by immunoblotting using the specific antibody against the phosphorylated form of PKB (P-PKB). The amount of protein in every sample was checked by immunoblot using anti- β -tubulin (1:5000) antibody. Data shown are representative of three independent experiments. Densitograms with standard error are shown. * $p < 0.05$ versus control (0 nM leptin).

Leptin activation of MAPK pathway in human dental pulp

To investigate the effect of leptin on MAPK pathways, the phosphorylation level of MAPK was studied. Antibodies that specifically recognize the phosphorylated forms of the MAPK (P-p42 MAPK, P-p44 MAPK) were used. Leptin 0.1, 1 and 10 nM stimulated tyrosine/threonine phosphorylation of MAPK as assessed by specific immunoblot with the anti-doubly phosphorylated MAPK antibody (Fig. 3, upper panel). The relative amount of MAPK in stimulated pulps was almost four-times higher than in unstimulated pulps ($p < 0.05$). Maximal phosphorylation was observed at 0.1 nM leptin.

Western blot analysis revealed the presence in the pulp samples of two proteins with apparent molecular weights of 42 kDa and 44 kDa, which corresponds, respectively, to the estimated molecular weights of P-p42 MAPK and P-p44 MAPK (Fig. 3, lower panel). Anti- β -tubulin antibodies were used for the control of the immunoblot.

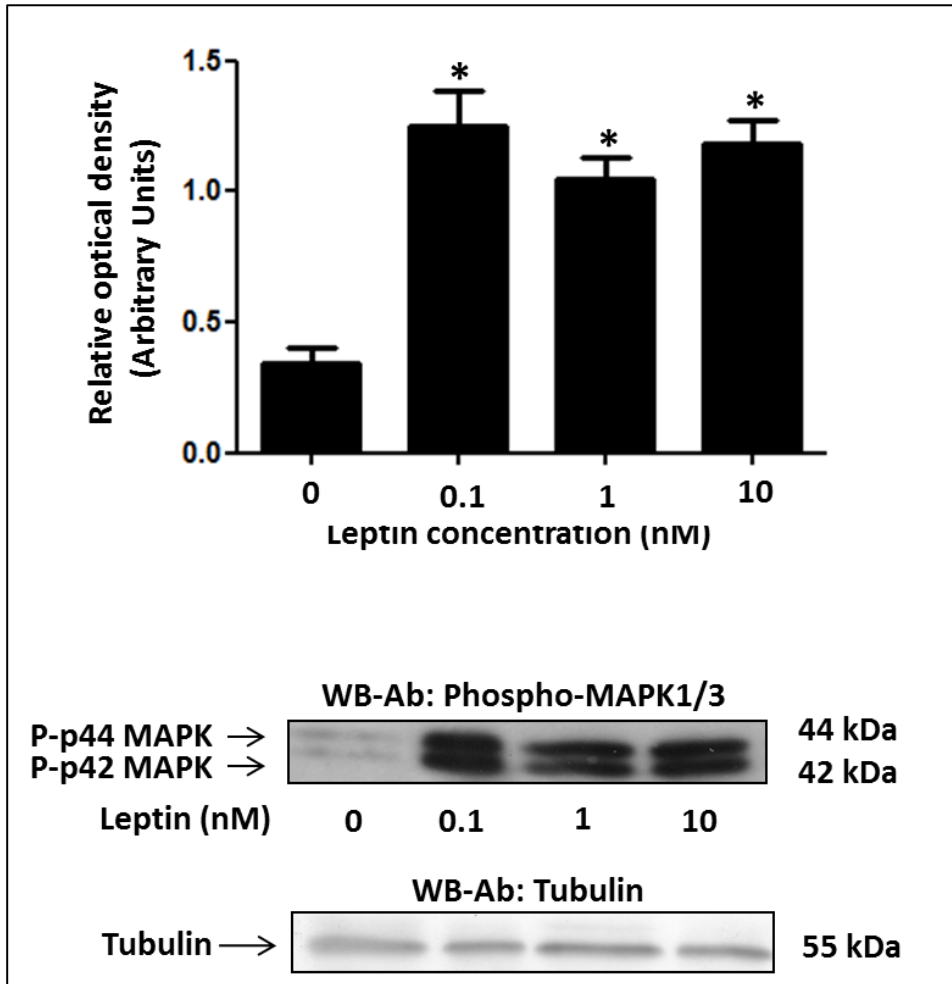


Figure 3. Leptin activation of MAPK signalling pathway in human dental pulp. Human dental pulp were incubated in the absence or presence of 0.1, 1 and 10 nM leptin for 10 min in DMEM 0% FCS. For the activation of MAPK, cell lysates were analyzed by immunoblotting using the specific antibody against the phosphorylated form of MAPK (P-p44-MAPK and P-p42-MAPK). The amount of protein in every sample was checked by immunoblot using anti- β -tubulin (1:5000) antibody. Data shown are representative of three independent experiments. Densitograms with standard error are shown. * $p < 0.05$ versus control (0 nM leptin).

4. DISCUSSION AND CONCLUSION

The present study demonstrates for the time that leptin stimulates human dental pulp cells through distinct signalling pathways, including JAK-STAT, PI3K and MAPK pathways.

Previously published results have demonstrated that leptin is synthesized and secreted in cultured human dental pulp fibroblasts derived from extracted healthy molar teeth (Vaisse *et al.* 1996), as well as in human healthy and inflamed dental pulp (Martín-González *et al.* 2013a). Moreover, it has been found that dental pulp leptin levels increases during experimentally induced pulpitis (Martín-González *et al.* 2013a). In addition, the presence of LEPR in human dental pulp and its up-regulation in inflamed dental pulp, indicates that leptin may have a role in the physiology and in pathophysiology of the dental pulp, probably acting as a modulator of pulp immune and inflammatory responses (Martín-González *et al.* 2013b). On the other hand, it has been provided evidence that leptin promotes odontoblastic differentiation and suppress adipogenic differentiation in dental pulp stem cells (DPSCs) (Um *et al.* 2010). Taking into account that DPSCs could express LEPR and produce leptin (Martín-González *et al.* 2013a, El Karim *et al.* 2007, Martín-González *et al.* 2013b), leptin could play a role in defensive and reparative responses of dental pulp against deep dentin carious lesions, likely involving negative feedback. However, the molecular mechanisms underlying the effect of leptin on dental pulp cells are not completely understood and the possible messengers involved in this feedback mechanism are unknown, representing an interesting area of future research.

To further understand the signal transduction of leptin in human dental pulp, it is important to assess the major signalling pathways known to be activated by leptin receptor in other systems (Sánchez-Margalet *et al.* 2003, Vaisse *et al.* 1996, Martin-Romero *et al.* 2001b, Wang *et al.* 1997, Kellerer *et al.* 1997, Szanto *et al.* 2000, Zhao *et al.* 2000, Liu *et al.* 2014), these are JAK-STAT, PI3K and MAPK pathways (Fig. 4).

Mitogen-activated protein kinase (MAPK) is a well-known signalling pathway activated by leptin receptor (Pérez-Pérez *et al.* 2008a). MAPKs are an essential component in many physiologic processes, such as cell growth, proliferation, differentiation, and apoptosis (Neary 1997). In dental pulp, MAPK is activated during odontoblast stimulation in tertiary dentinogenesis and is one of the known kinases that is required for late-stage odontoblastic differentiation and angiogenic potential of DPSCs (Qin *et al.* 2014, Kim *et al.* 2014). Recently it has been demonstrated that MTA, Biodentine, Bioaggregate and calcium silicate cements are able to stimulate odontoblastic differentiation and mineralization nodule formation by activating the MAPK pathway (Huang *et al.* 2014, Chou *et al.* 2014, Jung *et al.* 2014). MAPK pathway plays an important role in regulating the angiogenic behavior of dental pulp cells cultured on MTA (Huang *et al.* 2014). The result that leptin activates strongly MAPK pathway in human dental pulp cells, are in accordance with previous findings showing that leptin promotes odontoblastic differentiation in dental pulp stem cells (DPSCs) (Um *et al.* 2010) and further support the role of leptin in dentinogenesis. Studies on leptin stimulation of dentin sialo-phosphoprotein (DSPP) in cultured human dental pulp cells must be carried out to advance in this research field.

In relation with the PI3K pathway, it has been implicated in the regulation of many cellular processes, including resistance to apoptosis, cell motility, differentiation, and proliferation (Pérez-Pérez *et al.* 2009b, Pérez-Pérez *et al.* 2010c, Vanhaesebroeck *et al.* 1997). Previous studies have shown that leptin activates PI3K in myotubes, β -cells, hepatocytes and PBMC (Vaisse *et al.* 1996, Martin-Romero *et al.* 2001, Wang *et al.* 1997, Kellerer *et al.* 1997, Szanto *et al.* 2000, Zhao *et al.* 2000, Liu *et al.* 2014). In dental pulp, toll-like receptor 2 (TLR2) signaling through the PI3K pathway is necessary for lipoteichoic acid (LTA) -induced VEGF expression in pulp cells (Soden *et al.* 2009). Interaction between lipopolysaccharide (LPS) and toll-like receptor 4 (TLR4) also signaling through the PI3K pathway in DPSCs (He *et al.* 2014).

On the other hand, the JAK-STAT signalling pathway plays critical, non-redundant roles in mediating cellular transcriptional responses to cytokines, and in cell activation, survival and proliferation (Liu *et al.* 2014, Pérez-Pérez *et al.* 2009b, Pérez-Pérez *et al.* 2010c, Silvennoinen *et al.* 1997). It has been showed that leukemia inhibitory factor (LIF) inhibits the odontoblastic differentiation of DPSCs via the JAK-STAT3 signaling pathway (Zhou *et al.* 2011).

In this context, the demonstration of leptin activation pathways in human dental pulp strongly support the concept that MAPK, PI3K and JAK/STAT3 pathways are involved in the stimulation of dental pulp cells proliferation and differentiation by leptin. Thus, these results further support the possible role of leptin as an important modulator of pulp immune and inflammatory responses as well as its effect on odontoblastic differentiation and proliferation of DPSCs. It can be hypothesized that depending upon the

predominant pathway activated, leptin exerts either growth or proliferation effect on dental pulp cells.

In conclusion, major LEPR signalling pathways, MAPK, PI3K and JAK/STAT3, are triggered by leptin in human dental pulp cells. These results provide evidence supporting the possible role of leptin in the pulp physiology. Further studies are necessary to elucidate the physiological events in which are involved each one of the leptin intracellular signalling pathways.

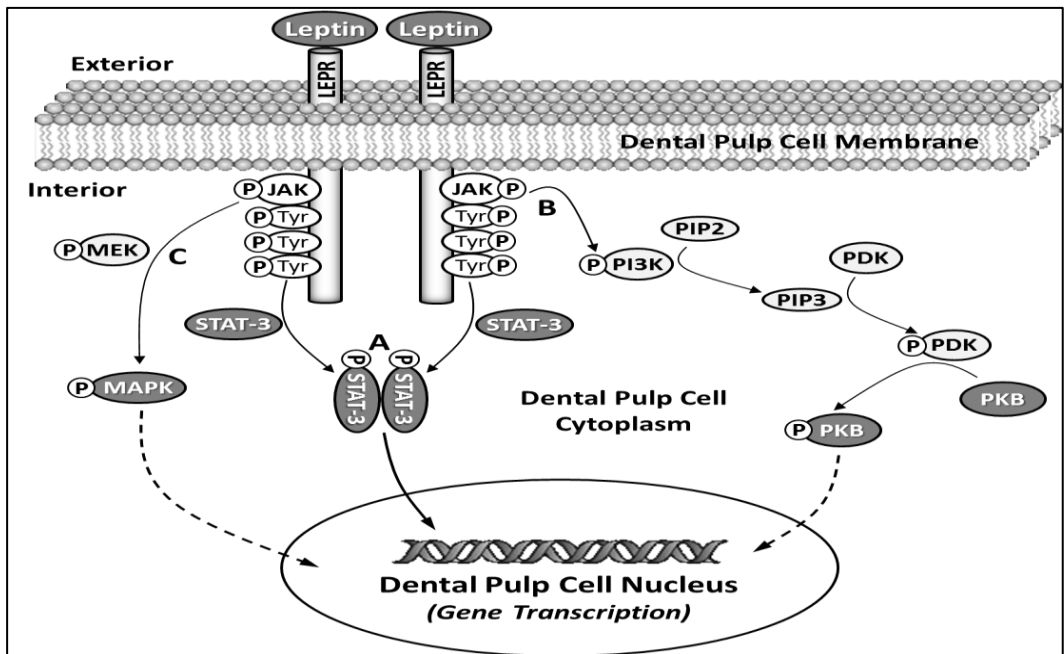


Figure 4. Proposed leptin signalling pathways in human dental pulp cells.

A) *Leptin receptor signalling via JAK-STAT3 pathway:* leptin receptor LEPR is associated with receptor-associated kinases of the Janus family (JAKs); upon leptin stimulation, JAK activity is increased and signal transducer and

activator of transcription - 3 (STAT3) is tyrosine phosphorylated, dimerized and translocated to the nucleus to activate gene expression.

B) *Leptin receptor signalling via PI3K pathway*: leptin stimulation promotes the tyrosine phosphorylation of the intracellular domain of the leptin receptor, providing a molecular mechanism for phosphatidylinositol 3-kinase (PI3K) activation, following phosphatidylinositol-bisphosphate (PIP₂) phosphorylation to PIP₃, which phosphorylates phosphoinositol-dependent kinase (PDK) and, finally, protein-kinase B (PKB).

C) *Leptin receptor signalling via MAPK pathway*: JAK activation phosphorylates MAP kinase kinase (MEK), which in turn phosphorylates mitogen-activated protein kinase (MAPK), promoting a phosphorylation cascade and, finally, inducing the expression of specific target genes in the nucleus. Solid lines: direct actions. Dashed lines: indirect actions.

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Chapter 4

Leptin Promotes Dentin Sialophosphoprotein Expression in Human Dental Pulp

This chapter has been submitted for publication as:

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Leptin Promotes Dentin Sialophosphoprotein Expression in Human Dental Pulp.
Journal of Endodontics. Submitted

ABSTRACT

Introduction. Leptin, an inflammation-related adipokine, and its receptor (LEPR) are expressed in human dental pulp. Dentin sialophosphoprotein (DSPP) is involved in dentinogenesis and in dental pulp reparative response. The cell type expressing LEPR in dental human pulp and the resultant effect of the binding of leptin to LEPR remain unknown. This study describes the immunohistochemical localization of LEPR and the effect of leptin on DSPP expression in human dental pulp.

Material and Methods. Twenty-five dental pulp specimens were obtained from freshly caries-free and restoration-free extracted human third molars. LEPR localization was examined by immunohistochemistry using anti-human LEPR monoclonal antibody. The effect of leptin on DSPP expression was determined by immunoblot and quantitative real-time PCR (qRT-PCR).

Results. Immunoreactivity for LEPR concentrated in odontoblast layer and predentin zone, but was not evident in the central zone of the dental pulp. Leptin dose-dependently stimulated DSPP expression. Western blot analysis revealed the presence of a protein with an apparent molecular weight of ~ 100 kDa, the estimated molecular weight of DSPP. The expression of DSPP mRNA was confirmed by qRT-PCR and the size of the amplified fragments (298 bp) was confirmed by agarose gel electrophoresis.

Conclusions. The expression of LEPR by odontoblasts has been demonstrated. Leptin enhances DSPP levels and DSPP mRNA expression in human dental pulp cells. These findings suggest that leptin plays a functional role in dental pulp physiology.

1. INTRODUCTION

Leptin, a non-glycosylated hormone of 146 aminoacids released by adipocytes, regulates energy intake and expenditure, appetite and hunger, metabolism, and behavior (1). Recent evidence shows that leptin is an inflammation-related adipokine, acting as pro-inflammatory (1, 2). Consequently, a role for leptin regulating immunity, inflammation, and hematopoiesis has been accepted (3-5). In addition, it has been shown that leptin regulates both innate and adaptive immune responses, not only in normal but also in pathological conditions (6). Leptin levels increase during acute infection, inflammation and sepsis, favored particularly by LPS and cytokines such as TNF- α , IL-6, and IL-1b (7).

Lately, compelling evidences implicate leptin in dental pulp biology. Leptin acts as modulator of pulp mesenchymal stem cell differentiation (8); leptin is synthesized and secreted in vitro by pulp fibroblasts derived from extracted healthy molar teeth (9); and, finally, it has been reported the up-regulation of leptin and its receptor (LEPR) expression in inflamed human dental pulp (10, 11).

On the other hand, dentin sialophosphoprotein (DSPP), secreted by odontoblasts, is the most abundant non-collagenous protein (NCP) in the predentin (12). DSPP plays a main role in the biomineralization process of predentin (13, 14), being expressed by odontoblast-like cells underlying reparative dentin (15, 16). Although there are no specific odontoblastic markers, DSPP has been used as indicator of odontoblastic differentiation (17, 18).

There are evidences supporting the concept that dental pulp inflammatory response to caries alters DSPP expression. Thus, DSPP gene expression is down-regulated by lipoteichoic acid (LTA) (19) and tumor necrosis factor- α (TNF- α), an inflammatory cytokine, increases the expression of DSPP in dental pulp cells (20).

The cell type expressing LEPR in dental pulp and the resultant effect of leptin binding to LEPR remain unknown. The aim of the present study was to describe the immunohistochemical localization of LEPR and the effect of leptin on DSPP expression in human dental pulp.

2. MATERIALS AND METHODS

The study was carried out with the understanding and written consent of each subject and according to the principles of the World Medical Association Declaration of Helsinki. The protocol was approved by the Ethical Committee.

2.1.-Reagents

Human recombinant leptin was provided from R&D Systems (Minneapolis, MN, USA). Monoclonal mouse anti-DSPP (LFMb-21, 1:1500) and the monoclonal rabbit antibodies against the long isoform of LEPR (C-terminal) were purchased from Santa Cruz Biotechnology (CA, USA). The monoclonal mouse anti- β -tubulin (1:5000) was provided from Sigma-Aldrich. Horseradish peroxidase-linked anti-mouse/anti-rabbit (1:10000) immunoglobulins were purchased from Amersham Pharmacia (Amersham Pharmacia Biotech, Barcelona, Spain).

2.2. - Human dental pulp

Human dental pulp was obtained from 25 freshly extracted third molars from twenty-two healthy, nonsmoking, human donors (20- to 54-year old) who gave their written informed consent to donate their pulp tissue. All teeth were caries-and restoration-free and without signs of periodontal disease. The extracted teeth were washed with 5.25% sodium hypochlorite after extraction to eliminate remains of periodontal ligament that could contaminate the pulp sample. The teeth were then sectioned by using a Zekrya bur (Dentsply Maillefer, Tulsa, OK, USA) in a high-speed hand-piece irrigated with saline solution. The pulp tissue was obtained by using a sterile endodontic excavator, washed 2–3 times in sterile ice-cold phosphate-buffered saline (PBS) to remove excess blood. Fifteen samples were cut into two parts, one for the Western blotting analysis and another one for RNA extraction and quantitative real-time PCR (qRT-PCR) assay, and ten samples were analyzed by immunohistochemistry.

2.3. - Dental Pulp Samples

Human dental pulps were randomly distributed in tubes containing 1 ml of DMEN-F12 medium (2 replicates per treatment). Dental pulps were maintained in a shaking water bath at 37°C during 5 min to equilibrate temperature. Dental pulps were incubated at 37°C in 5% CO₂ (21) for 12 hours in the same medium supplemented or not with 1 and 10 nM human recombinant leptin (R&D Systems, Minneapolis, MN). Then, dental pulps were removed and resuspended in 75 µl of lysis buffer (1 x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 10 mg/ml phenylmethanesulfonyl fluoride (PMSF)) during 30 min at 4°C on

an orbital shaker and later centrifuged at 10000 g for 20 min. Supernatants were analyzed by Western blot.

2.4. - Western Blotting Analysis

Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA), using bovine serum albumin as standard. SDS-stop buffer containing 100 mM of DTT was added to the pulp tissue samples, followed by 5 min boiling. In each lane 50 µg protein were loaded. Then, samples were resolved by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Hybond, Amersham Pharmacia). The membranes were blocked with buffered saline – 0.05% Tween 20 (PBST) containing 3% bovine serum albumin during 1 h at 23°C. After this, membranes were immunoblotted overnight with monoclonal mouse anti-sialophosphoprotein (LFMb-21, 1:1500, Santa Cruz Biotechnology). Loading controls were performed by immunoblotting the same membranes with monoclonal anti-β-tubulin (1:5000, Sigma-Aldrich). Then, the blots were washed in PBST and further incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/antirabbit immunoglobulins (1:10000, Amersham Pharmacia). Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal; Pierce Biotechnology, Rockford, IL, USA). Quantification of protein bands was determined by densitometry using the PCBAS 2.0 program. The relative optical density of the different bands was normalized by the corresponding intensity of the tubulin immunoblot in each individual experiment.

2.5.-RNA extraction and quantitative real-time PCR (qRT-PCR) assay and agarose gel electrophoresis.

Abundance of dentin sialophosphoprotein mRNA was determined by quantitative real-time PCR reaction (qRT-PCR). Total RNA was extracted from human dental pulp tissue samples using TRISURE reagent (21). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 µg of total RNA was reverse transcribed at 55°C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche, Mannheim, Germany). Quantitative real time PCR was performed using the primers based on the sequences of the National Center for Biotechnology Information GenBank database (Table 1). Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a MiniOpticon (Bio- Rad, Hercules, CA, USA). A typical reaction contained 10 µmol/L of forward and reverse primer, 3 µL of cDNA, and the final reaction volume was 25 µL. The reaction was initiated by preheating at 50°C for 2 min, followed by heating at 95°C for 10 min. Subsequently, 40 amplification cycles were carried out as follows: denaturation 15 s at 95°C and 1 min annealing and extension at 59°C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. For the treated samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin) and relative to the untreated control. The retro-transcribed DNA samples were then resolved by 1% agarose gel. After the run of the electrophoresis, gels were visualized directly upon illumination with UV light (22).

Table 1. Sequence of PCR primers used in this study.

Gene	Primer sequences
Dentin sialophosphoprotein	
Forward:	5' AGAAGGACCTGGCCAAAAAT-3'
Reverse:	5'-TCTCCTCGGCTACTGCTGTT-3'
Cyclophilin	
Forward:	5'-CTTCCCCGATACTTCA-3'
Reverse:	5'-TCTTGGTGCTACCTC-3'

2.6. - Immunohistochemistry

The dental pulp samples were fixed in 10% formalin for at least 24 hours and then processed in paraffin and processed routinely. A series of 4- μ m sections from each tissue sample were cut. The first section of each serie was stained with haematoxylin and eosin (HE) to study the histology; the second section was used for immunohistochemical staining for expression of LEPR.

Pilot studies were undertaken to establish the optimum dilution of the antibody as well as confirming concentration and application times for the chemicals used during the immunohistochemistry (IHC) procedure. Sections were picked up onto a slide, deparaffinized in xylene, rehydrated by sequential immersion in a graded series of alcohols and transferred into water for 10 minutes and then heat treated in sodium citrate buffer (pH 6.0), for 40 min at 96 °C, to unmask antigens. The sections were cooled and washed in phosphate-buffered saline (PBS, pH 7.2). Specimens were incubated in blocking agent (5% albumin bovine serum in PBS) for 1 hour. Endogenous

peroxidase activity was quenched by incubating the sections in a solution of 3% H₂O₂ in 10% methanol and in PBS for 15 min. Sections were incubated with rabbit antihuman LEPR monoclonal primary antibodies at 1: 100 dilution overnight at 4°C. The sections were then washed in PBS (3X, 5 minutes each) and incubated with secondary antibodies. The manufacturer's instructions were followed for the sequential incubation and durations for the exposure to the secondary antibodies. After washing with PBS, the sections were incubated with diaminobenzidine substrate kit (Dako, Carpinteria, CA, USA) for 5 minutes that resulted in a brown-coloured precipitate at the antigen-antibody binding sites and the reaction was stopped in distilled water. After removing the slides from water all the sections were dehydrated and one drop of aqueous mounting medium (Dako Faramount) was applied, and the sections were coverslipped.

Finally, the immunohistochemical specimens were examined using a Leica Laborlux S Microscope (Leica Microsystem GmbH Wetzlar, Germany) with a Nikon DSL2 photo digital system (Nikon Corp, Tokyo, Japan). Each sample was analyzed with a double-blind system by two different operators under magnifications up to 100x. A cell was considered as positive when it demonstrated distinct brown surface staining. Representative sites in each sample were photographed at 1, 4, 10, 40, 60 and 100x magnification and captured with a software system (CS3, version 10.0.1; Adobe photoshop, San Jose, CA, USA).

2.6. - Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as means \pm SD in arbitrary units

(AU). Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the GraphPad Prism computer program (GraphPad Software, San Diego, CA. USA). Significance levels were set at $p < 0.05$.

3. RESULTS

Microscopic analyses of all human dental pulp samples included in the study ($n = 10$) showed that dental pulp specimens were reactive to anti-LEPR antibody. The presence and localization of LEPR in human dental pulp are illustrated in Figure 1, 2 3 and 4. Immunoreactivity for LEPR concentrated in odontoblast layer and predentin zone (Figs. 1 2, 3 and 4), but was not evident in the central zone of the dental pulp.

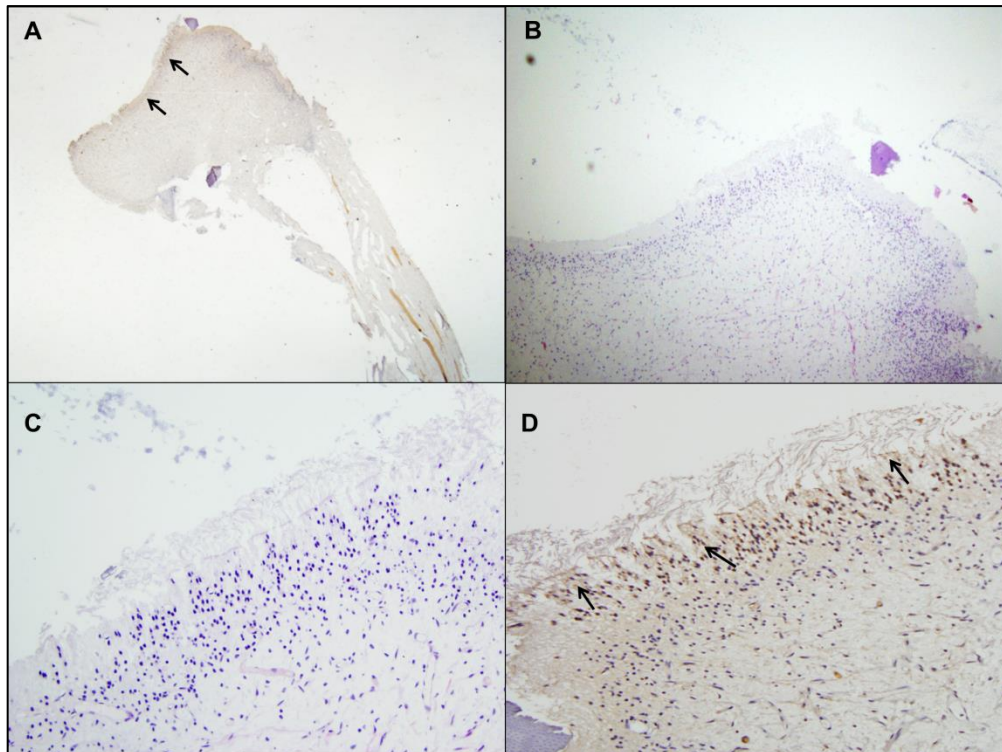


Figure 1. Immunohistochemical localization and expression of LEPR in human dental pulp. Representative sections from one of the specimens of human dental pulp (Haematoxylin-Eosin, magnification x4 (B) and x10 (C)). In situ LEPR expression by immunohistochemical (arrows); magnification x1 (A) and x10 (D).

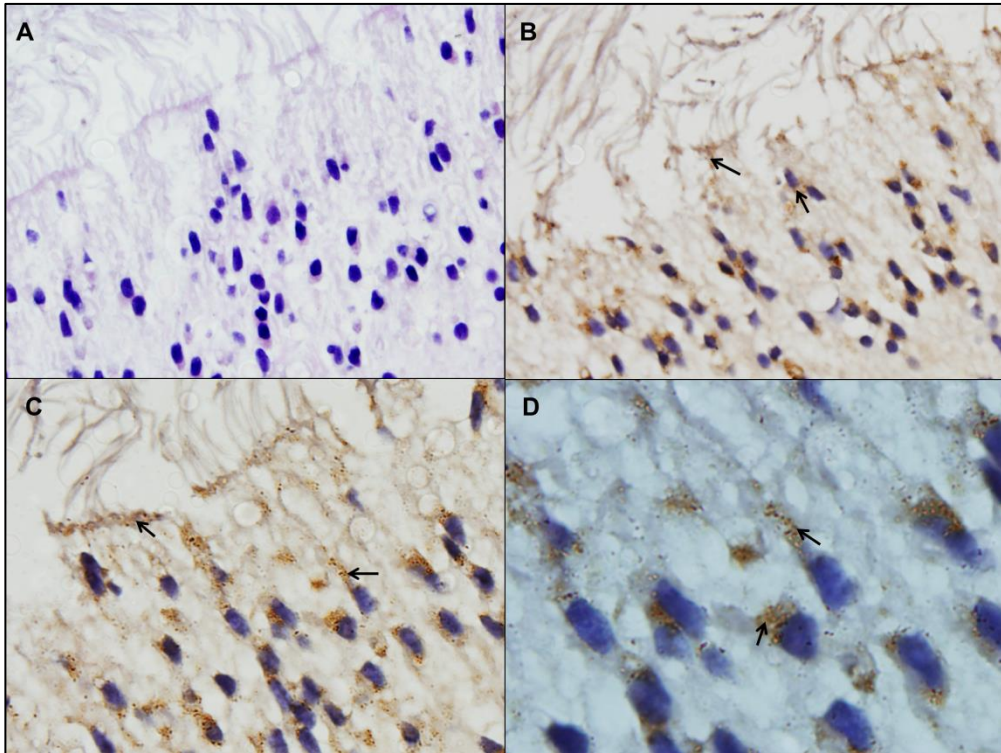


Figure 2. Immunohistochemical localization and expression of LEPR in human dental pulp. Representative sections from one of the specimens of human dental pulp (Haematoxylin-Eosin, magnification x40 (A)). In situ LEPR expression by immunohistochemical (arrows); magnification x40 (B), x60 (C) and x100 (D).

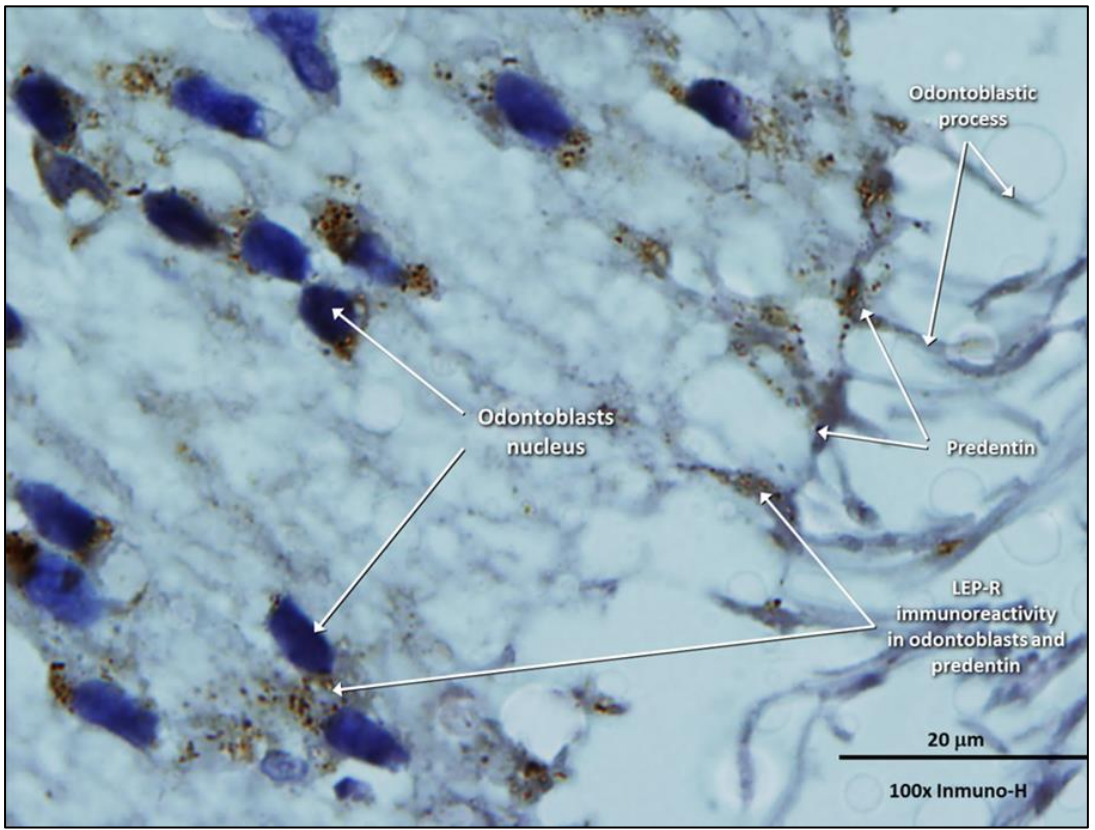


Figure 3. Immunohistochemical localization and expression of LEP-R in human dental pulp. In situ LEP-R expression by immunohistochemical. Magnification x100.

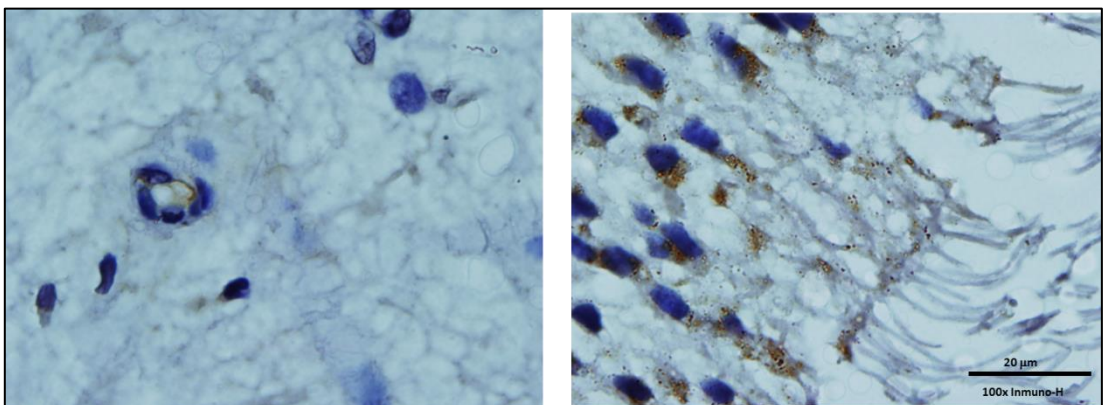


Figure 4. Immunohistochemical localization and expression of LEP-R in human dental pulp. Absence of immunoreactivity for LEP-R in the central pulp. Positive immunoreactivity for LEP-R in the odontoblast layer and predentin.

In order to check whether leptin may stimulate DSPP expression in human dental pulp, dose-response experiments stimulating the pulp samples with human leptin were performed, analyzing the amount of leptin protein and ARNm levels.

The amount of DSPP proteins in every sample was controlled by anti-tubulin immunoblot. As shown in Figure 4, the effect of leptin on DSPP protein synthesis was dose dependent. Although 1 nM leptin was sufficient to increase the expression of DSPP, maximal expression of DSPP was observed at 10 nM leptin, which increased ten-fold the basal protein synthesis rate ($p < 0.05$).

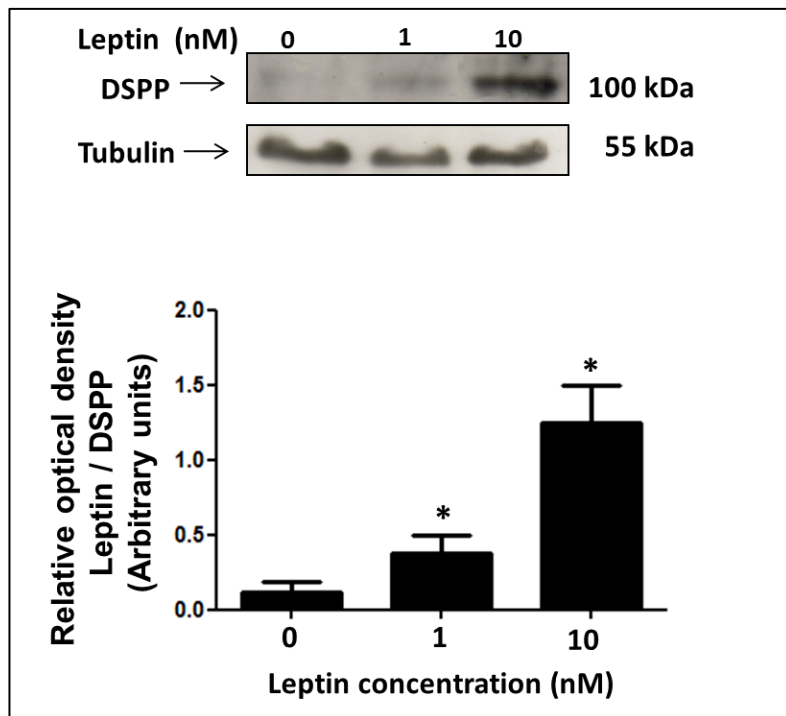


Figure 4. Stimulatory effect of leptin on DSPP expression in human dental pulp samples. Human dental pulp were incubated in the absence or

presence of 1 and 10 nM leptin for 12 hours in DMEM 0% FCS. Samples lysates were analyzed by immunoblotting using the specific antibody against the DSPP. The amount of protein in every sample was checked by immunoblot using anti- β -tubulin (1:5000) antibody. Data shown are representative of three independent experiments. Densitograms with standard error are shown. * $p < 0.05$ versus control (0 nM leptin).

To further validate the effect of leptin on DSPP expression in a dose dependent manner in human dental pulp tissue, real-time PCR assay was performed to examine the mRNA levels of DSPP in these tissue samples. As it can be seen in figure 5 (upper panel), leptin enhanced mRNA DSPP levels in a dose-dependent manner. Maximal effect was achieved at 10 nM leptin, dose at which the expression level of DSPP increased almost 10-fold ($p < 0.05$). The size of the amplified fragments (298 bp for DSPP) was confirmed by agarose gel electrophoresis (Fig. 5, lower panel).

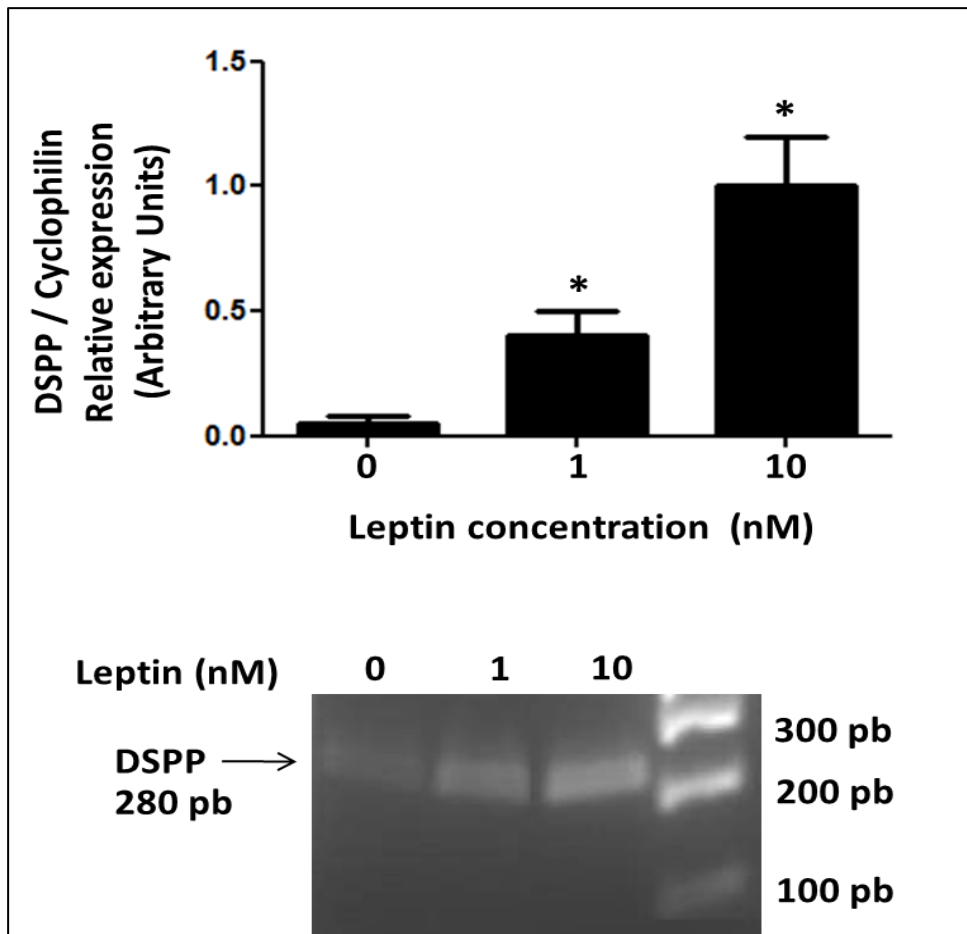


Figure 4. Stimulatory effect of leptin on DSPP mRNA expression in human dental pulp samples. Human dental pulp were incubated for 12 hours in the presence of different concentrations of leptin (1 and 10 nM), washed and lysed. Total RNA was extracted from each sample. DSPP mRNA was quantified using quantitative real time PCR (qRT-PCR) assay and visualized by agarose gel electrophoresis. Cyclophilin was used as internal standard. Results from a representative experiment performed in triplicate are shown. Data represent fold change from the mean values of cyclophilin and are expressed as means \pm SD. * $p < 0.05$ versus control (0 nM leptin).

4. DISCUSSION AND CONCLUSION

The results of the present study provide, for the first time, evidence indicating that odontoblasts express LEPR. Moreover, these results demonstrated that leptin has a functional role in dental pulp physiology, enhancing the expression of DSPP in dental pulp cells. Taking into account that leptin is expressed in ameloblasts, odontoblasts, dental papilla cells and stratum intermedium cells in tooth germs of mandibular third molars at the late bell stage (23), in rat dental pulp (23), and in normal and inflamed human dental pulp (10, 11), the results of the present investigation, together with those previous findings, further support the role of leptin in dentin biomineralization and/or in dental pulp reparative and immune responses.

The expression of LEPR has been demonstrated in experimental rats periapical lesions (24) and in normal and inflamed human dental pulp (11). Moreover, it has been shown the up-regulation of LEPR in experimentally inflamed human dental pulps (11). Although human dental pulp contained several cells types expressing LEPR, such as monocytes (25), NK cells (26) and CD4 T and CD8 T cells (27), the results of the present study demonstrated for the first time that odontoblast also express LEPR. The presence of LEPR immunoreactivity not only in the odontoblast layer but also in the predentin zone, suggests that leptin could be implicated in dentin biomineralization process. Moreover, the stimulation of DSPP mRNA and protein expression in human dental pulp by leptin, showed in this investigation, suggests that leptin-LEPR interaction in odontoblasts, directly triggers a signaling pathway transduction cascade that induces DSPP mRNA transduction and DSPP synthesis. This result is in accordance with previous findings showing that leptin promotes the cemento / odontoblastic

differentiation and suppress the adipogenic differentiation of dental pulp stem cells (DPSCs) (8). Furthermore, DPSCs could produce leptin (9, 10) and, through a negative feedback loop, enhance self-odontoblastic differentiation and suppress adipogenic one, playing a role in both defensive and reparative responses of dental pulp against deep carious lesions (11). To investigate which are the messengers involved in this feedback, represents an interesting area of future research.

Dentin formation, dentinogenesis, involves the secretion by odontoblasts of an unmineralized type I collagen-rich extracellular matrix termed predentin (28). The deposition of hydroxyapatite crystals transforms the predentin to the mineralized phase, the dentin. In this process, non-collagenous proteins (NCPs), also secreted by odontoblasts, are believed to actively promote and control the mineralization of collagen fibers and crystal growth within predentin (29-31). Dentin sialophosphoprotein (DSPP), a member of the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) family of NCPs (12), is the most abundant NCPs, playing a main role in the biomineralization process of predentin (13, 14). Mutations in the DSPP gene are associated with dentinogenesis imperfecta (DGI), an autosomal dominant disorder causing dentin hypomineralization and significant tooth decay (27, 31, 32).

The link between pulp inflammation and pulp repair is not yet well established, however, inflammatory response appears as a prerequisite for tissue repair (30, 33). When dentin is being destroyed by caries, odontoblasts recognizes lipoteichoic acid (LTA) of Gram-positive bacteria, and lipopolysaccharide (LPS) of Gram-negative bacteria, mediating pulp inflammatory process directly through production of antimicrobial peptides

and cytokines, and indirectly through activation of migratory immune cells (34). The release of cytokines within the pulp tissue activates reparative dentin formation promoting expression of molecules implicated in mineralization (28, 35). At this respect, it has been shown that TNF- α increases the expression of DSPP in dental pulp cells (20).

On the contrary, odontoblasts challenged with LTA down-regulate the major dentin matrix components type I collagen and DSPP (DSPP) (19). A similar DSPP down-regulation has been observed in an odontoblast-like cell line after stimulation with LP (36). This repression of odontoblasts specialized function of dentin matrix synthesis and mineralization stimulated by bacterial byproducts, LTA and LPS, resembles the progression of caries bacteria in actively deep caries lesions (33).

The link between an adipokine, such as leptin, which first functional role is the control of appetite and hunger (1, 2), and the expression of DSPP, a dentin protein implicated in dental pulp defensive and reparative responses, could also be interpreted as a new evidence of the relationship between obesity, inflammation and oral infections. At this respect, it has been shown that comorbidities of obesity and periodontitis are associated with significant local up-regulation of several micro RNA species that share inflammatory and metabolic mRNA targets (37). There is also clinical evidence suggesting that obesity is a risk factor for periodontitis (38, 39). Several possible pathways involving the host response and bacterial challenge have been proposed to explain the association between obesity and inflammation (40). Obesity could alter the host immune responses to oral infections increasing individuals' susceptibility (41).

Thus, pro-inflammatory molecules and processes implicated in the pathogenesis of chronic oral infections, such as periodontal disease and chronic apical periodontitis, including cytokines (e.g., Interleukin-IL-6), chemokines, and T-cell function, could be influenced by obesity (Falagas and Kompoti, 2006). An association between diet content in fatty acids and dental pulp cell population has been suggested (42). Rats fed with essential fatty acids deficiency (EFAD) diet, showed an increased dental pulp cell density, but not modified the number or shape of the odontoblasts (33, 42).

In conclusion, the results of the present study demonstrate that odontoblasts express LEPR and that leptin enhances the expression of DSPP in dental pulp cells. These findings further support the functional role of leptin in dentin mineralization process and/or in dental pulp reparative and immune responses.

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Chapter 5: Leptin expression and immunohistochemical localization in human periapical granulomas

This chapter has been submitted for publication:

Jenifer Martín-González, Víctor Sánchez-Margalet, Juan J. Segura-Egea (2014). Leptin expression and immunohistochemical localization in human periapical granulomas. *Archives of Oral Biology*. Submitted.

ABSTRACT

Objective. Leptin, initially described as an adipocyte-derived hormone to regulate weight control, is expressed in normal and inflamed human dental pulp, being up-regulated during pulp experimental inflammation. This study aims to investigate the expression of leptin in human periapical granuloma.

Design. Periapical inflammatory lesions were obtained from extracted human teeth and teeth which underwent periapical surgery. After their histopathological categorization as periapical granulomas (n = 20), they were examined by immunohistochemistry using human leptin polyclonal antibodies. Leptin mRNA expression was also determined by quantitative real-time PCR (qRT-PCR) and the amount of leptin protein was analyzed by immunoblot.

Results. Leptin was expressed in all periapical granuloma samples. Inflammatory cells were reactive to leptin antibodies. Western blot analysis revealed the presence in the samples of a protein with apparent molecular weight of approximately 16 kDa, corresponding to the estimated molecular weights of leptin. The expression of leptin mRNA was confirmed by qRT-PCR analysis and the size of the amplified fragment (296 bp for leptin and 194 bp for cyclophilin) was assessed by agarose gel electrophoresis.

Conclusion. Periapical granuloma expresses leptin. This finding suggests that leptin can play a role in inflammatory and immune periapical responses.

1. INTRODUCTION

Chronic periapical lesions occur as a result of the immunological response to continuous antigenic stimulation from root canals and are one of the most significant causes of tooth loss in adults and the effects on the systemic health of patient have been investigated^{1,2}. The most common periapical lesions are periapical granulomas³. Periapical granuloma is a chronic inflammatory lesion at the apex of a non-vital tooth consisting of granulation tissue and scar. The inflammatory cell infiltrate in these chronic periapical lesions consists of a mix of T- and B-lymphocytes, polymorphonuclear neutrophils (PMNs), macrophages, dendritic cells (DCs), plasma cells, NK cells, eosinophils, and mast cells, present in different proportions within the granulation tissue of periapical lesions⁴⁻⁷. The inflammatory infiltrate constitutes approximately 50% of the cells present in periapical granulomas, with non-inflammatory connective tissue cells, including fibroblasts, vascular endothelium, proliferating epithelium, osteoblasts, and osteoclasts comprising the balance^{8,9}. During periapical inflammation, host cells in the periapical tissues release many inflammatory mediators, proinflammatory cytokines, and growth factors through innate and adaptive immune responses¹⁰.

Leptin, an adipocyte-derived hormone of 16 kDa encoded by the *Ob gene*¹¹, regulates weight control¹² but also it has been accepted a role for it regulating immunity, inflammation and hematopoiesis¹³⁻¹⁵. In fact, it has been classified as a pro-inflammatory cytokine because its primary aminoacid sequence shows structural similarities to the long chain helical cytokine family, such as IL-2, IL-12 and growth hormone^{11,16}. Therefore, leptin affects both innate and adaptive immunity exerting an effect on T-cells, monocytes, neutrophils,

and endothelial cells¹⁷. Consistent with this role of leptin in the mechanisms of immune response and host defense, leptin levels are increased upon infectious and inflammatory stimuli such as LPS, turpentine, and cytokines¹⁸. Accordingly, leptin receptor (LEPR) shows sequence homology to members of the class I cytokine receptor (gp130) superfamily¹⁹ and is expressed not only in the central nervous system, but also in hematopoietic and immune systems¹⁶, in mice monocytes and lymphocytes^{17,20} and in human peripheral blood T lymphocytes (both CD4 and CD8)^{16,21}.

In relation with oral tissues, it has been proposed that leptin can be implicated in inflammatory and local immune responses in human dental pulp²²⁻²⁴. Moreover, its presence has been reported in healthy and inflamed human dental pulp²³, gingival tissues^{25,26} and in gingival crevicular fluid²⁷⁻²⁸. On the other hand, LEPR immunoreactivity has been found in the gingival epithelium²⁶, LEPR gene has been detected in experimental rats periapical lesions²⁹, and both LEPR protein and LEPR mRNA have been described in healthy and inflamed human dental pulp²⁴.

Leptin has been quantified in supernatant fluids of explants cultures of chronic periapical lesions³⁰. However, the expression and immunolocalization of leptin in periapical inflammatory tissues has not been studied. The aim of this study was to analyze and characterize the expression of leptin in human periapical granulomas.

2. METHODS

The study was carried out with the understanding and written consent of each subject and according to the principles of the World Medical Association

Declaration of Helsinki. The protocol was approved by the Ethical Board of the University.

2.1. Human chronic periapical lesions samples

Twenty human chronic periapical lesions from twenty healthy, nonsmoking, human donors (45–72 years old), who gave their written informed consent, were obtained from 14 freshly extracted teeth and 6 teeth which undergone periapical surgery treatment. Every teeth used in this study was caries-free and restoration-free. Inflammatory tissues surrounding periapical area were dissected. Each sample was then divided into three parts, one for the Western blotting analysis, other for RNA extraction and quantitative real-time PCR (qRT-PCR) assay, and another one for pathologic evaluation and immunohistochemistry.

2.2. Antibodies and reagents

The monoclonal mouse anti- β -tubulin (1:1000) and the polyclonal rabbit anti-human leptin Y20 (1:1000) antibodies were provided from were provided from Santa Cruz Biotechnology (CA, USA). Horseradish peroxidase-linked anti-mouse/anti-rabbit (1:10.000) immunoglobulins were purchased from Amersham Pharmacia (Amersham Pharmacia Biotech, Barcelona, Spain).

2.3. Western blotting analysis

The granuloma samples were incubated in 75 μ l of lysis buffer containing 20 mM Tris, pH 8, 1% Nonidet P-40, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mM sodium orthovanadate, at 4 °C for 30 min on an orbital shaker and then centrifuged at 12,000 g for 15 min. The supernatants were transferred to new tubes. Total protein levels were

determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as standard. We added SDS-stop buffer containing 100 mM of DTT to the periapical tissue samples followed by boiling for 5 min. 50 µg protein were loaded in each lane. The samples were then resolved by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes¹². The membranes were blocked with buffered saline–0.05% Tween 20 (PBST) containing 3% bovine serum albumin during 1 h at 23 °C. The blots were then incubated with primary antibody for 1 h, washed in PBST, and further incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/antirabbit immunoglobulins. Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal from Pierce)⁶. The bands obtained in the blots were scanned and analyzed by the PCBAS 2.0 program. The relative optical density of the different bands was normalized by the corresponding intensity of the tubulin immunoblot in each individual experiment.

2.4. RNA extraction, quantitative real-time PCR (qRT-PCR) assay and agarose gel electrophoresis.

Abundance of leptin mRNA was determined by quantitative real-time PCR reaction (qRT-PCR). Total RNA was extracted from periapical tissue samples using TRISURE reagent³¹. Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 µg of total RNA was reverse transcribed at 55°C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche, Mannheim, Germany). Quantitative real time PCR was performed using the primers based on the sequences of the National Center for Biotechnology Information GenBank database (Table 1). Quantitative RT-PCR Master Mix Reagent kit

was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a MiniOpticon (Bio- Rad, Hercules, CA, USA). A typical reaction contained 10 μ M of forward and reverse primer, 3 μ L of cDNA, and the final reaction volume was 25 μ L. The reaction was initiated by preheating at 50°C for 2 min, followed by heating at 95°C for 10 min. Subsequently, 40 amplification cycles were carried out as follows: denaturation 15 s at 95°C and 1 min annealing and extension at 58°C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. For the processed samples, evaluation of $2^{-\Delta\Delta CT}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin). After running the electrophoresis, gels were visualized directly upon illumination with UV light.

Table 1. Sequence of PCR primers used in this study.

Gene	Primer sequences
Leptin	
Forward:	5'-GAACCCTGTGCGGATTCT-3'
Reverse:	5'CCAGGTCGTTGGATATTTGG-3'
Cyclophilin	
Forward:	5'-CTTCCCCGATACTTCA-3'
Reverse:	5'-TCTTGGTGCTACCTC-3'

2.5. Immunohistochemistry

The excised periapical lesions were fixed in 10% formalin for at least 24 hours and then embedded in paraffin and processed routinely. A series of 5- μ m sections from each tissue sample were cut. The first section of each serie

was stained with haematoxylin-eosin (H&E) to study the histology and to confirm the diagnosis of periapical granuloma. The second section was used for immunohistochemical staining for expression of leptin.

Pilot studies were undertaken to establish the optimum dilution of the antibody as well as confirming concentration and application times for the chemicals used during the immunohistochemistry (IHC) procedure. Second sections was picked up onto a slide, deparaffined in xylene, rehydrated by sequential immersion in a graded series of alcohols and transferred into water for 10 min. Then, specimens were heat treated in sodium citrate buffer (pH 6.0), for 40 min at 96 °C, to unmask antigens. The sections were cooled and washed in phosphate-buffered saline (PBS, pH 7.2). Specimens were incubated in blocking agent (5% albumin bovine serum in PBS) for 1 hour. Endogenous peroxidase activity was quenched by incubating the sections in a solution of 3% H₂O₂ in 10% methanol and in PBS for 15 min. The sections were incubated with rabbit antihuman leptin polyclonal primary antibody at 1:100 dilutions overnight at 4°C. The sections were then washed in PBS (3X, 5 minutes each) and incubated with the secondary antibody. The manufacturer's instructions were followed for the sequential incubation and durations for the exposure to the secondary antibody. After washing with PBS, the sections were incubated with diaminobenzidine substrate kit (Dako, Carpinteria, CA, USA) for 5 minutes that resulted in a brown-coloured precipitate at the antigen-antibody binding sites and the reaction was stopped in distilled water. After removing the slides from water, all the sections were dehydrated and one drop of aqueous mounting medium (Dako Faramount) was applied, and the sections were coverslipped. Human placenta was used as a positive control for leptin expression³².

Finally, the immunohistochemical specimens were examined using a Leica Laborlux S Microscope (Leica Microsystem GmbH Wetzlar, Germany) with a Nikon DSL2 photo digital system (Nikon Corp, Tokyo, Japan). Each sample was analyzed with a double-blind system by two different operators under magnifications up to x100. A cell was considered as positive when it demonstrated distinct brown surface staining. Six representative sites in each sample were photographed at 1.25, 4, 10, 20, 60 and 100x magnification and captured with a software system (CS3, version 10.0.1; Adobe Photoshop, San Jose, CA, USA).

2.6. Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as mean \pm standard deviation (SD) in arbitrary units (AU). Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the GraphPad Prism computer program (GraphPad Software, San Diego, CA, USA).

3. RESULTS

3.1 Histological analysis

To define the pathological features of the periapical lesions, paraffin sections of 20 samples were evaluated histologically using hematoxylin and eosin staining. All periapical lesions examined by light microscopy exhibited a large number of infiltrated inflammatory cells, showing the characteristic of the chronic granulomatous inflammatory process: a dense accumulation of

polymorphonuclear (PMN) leukocytes, lined by granulomatous tissue containing lymphocytes, macrophages and plasmatic cells (Fig. 1A). No epithelial cells were observed in these samples and the specimens were classified as periapical granulomas.

3.2. Leptin immunolocalization

Immunohistochemical detection of leptin was assessed. The positive control (human placenta) staining pattern resembled that of leptin immunoreactivity (Fig. 1,B). Inflammatory tissue in all periapical granuloma samples was reactive to leptin antibodies. Immunoreactivity was detected independently of the inflammatory status of the periapical granuloma (Fig. 1,C,D). Endothelial cells were also reactive to leptin antibodies (Fig. 1,E).

3.3. Western blot analysis of leptin expression

All human periapical granuloma samples expressed leptin at both protein and mRNA levels. Western blot analysis revealed the presence in the samples of a protein with apparent molecular weights of 16 kDa, which corresponds to the estimated molecular weight of leptin (Fig. 2).

3.4. Quantitative real-time PCR assay of leptin mRNA

To further validate the expression of leptin in human periapical granulomas, a real-time PCR assay was performed to examine the messenger RNA (mRNA) levels of leptin in the inflammatory tissues (leptin/cyclophilin ratio: 0.91 ± 0.18) (Fig. 3, upper panel) The size of the amplified fragments (296 bp for leptin and 194 bp for cyclophilin) was confirmed by agarose gel electrophoresis (Fig. 3, lower panel).

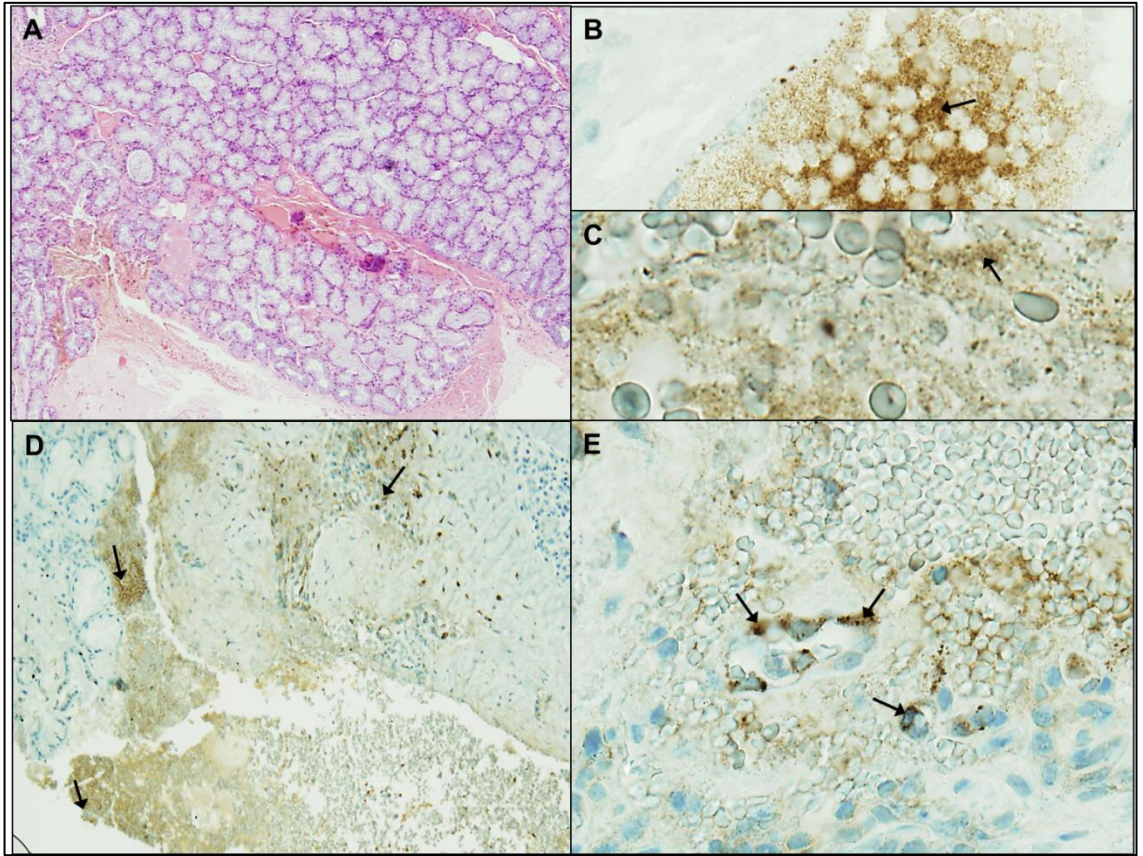


Figure 1. Histological and immunohistochemical study of periapical granulomas. Representative sections from a periapical granuloma showing early stage with predominantly foamy macrophages. A: Periapical granuloma H&E X60. B: Leptin expression by immunohistochemical, positive control in human placenta, X60. C, D, E: Leptin expression by immunohistochemical in periapical granuloma (arrows), X60, X20, X40.

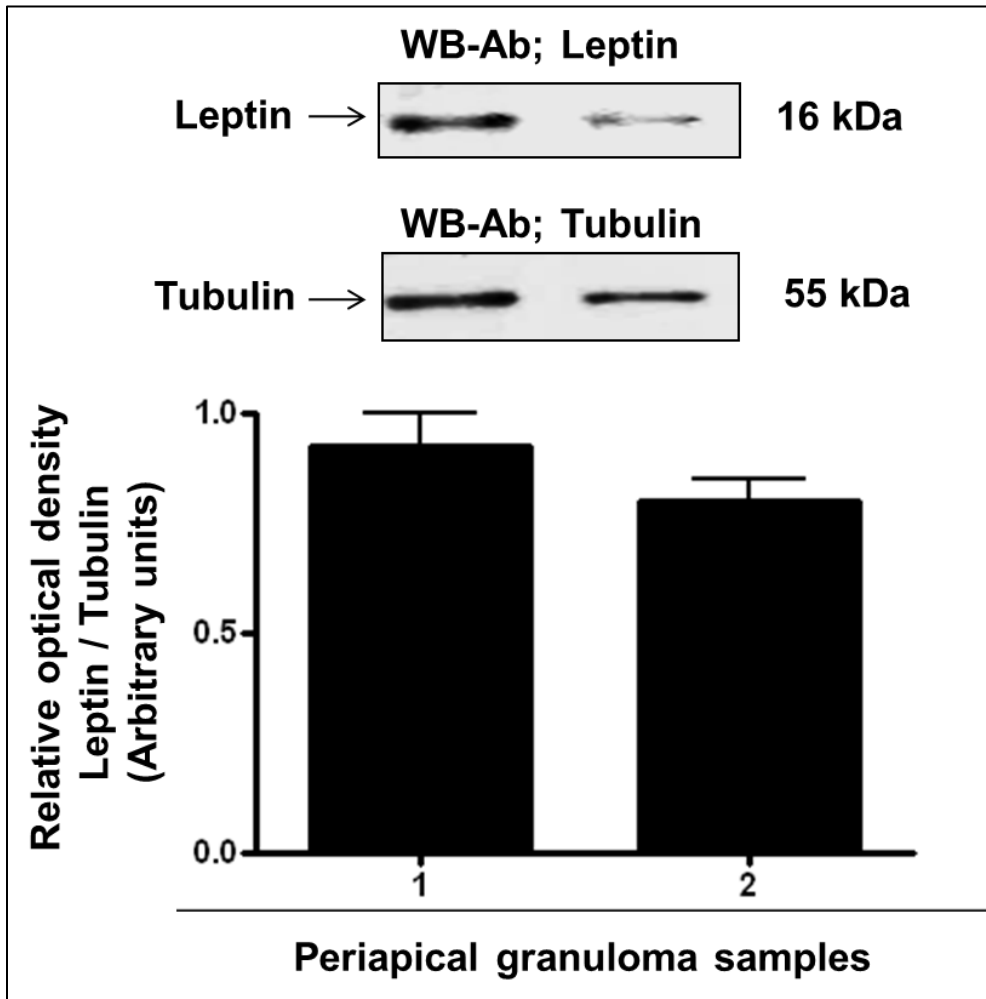


Figure 2. Relative amounts of leptin in two samples of human periapical granuloma. Results presented are from the specimens showing the minimum (1) and maximum (2) leptin expression levels (representative experiments repeated three times). Densitograms are expressed as means \pm SD in arbitrary optical density units, calculated as normalized band intensity, i.e. the band intensity of each sample was normalized with tubulin.

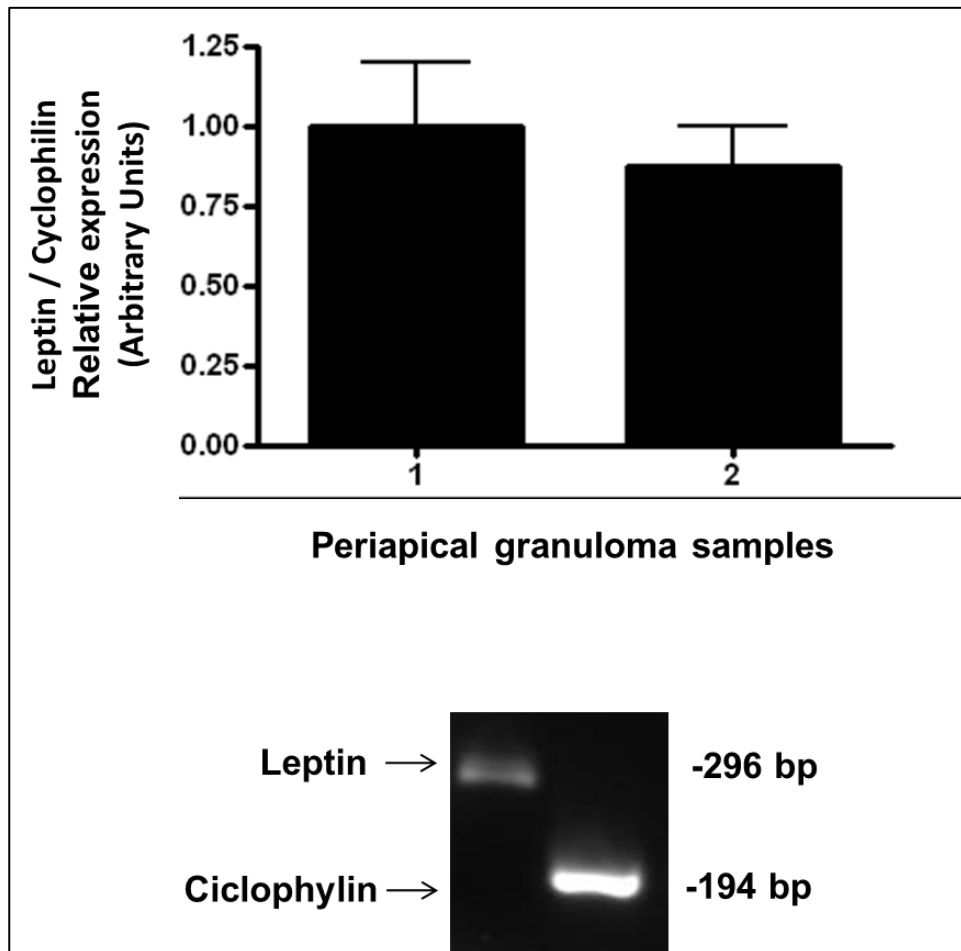


Figure 3. Leptin mRNA expression in human periapical granulomas. Total RNA was extracted from each human periapical granuloma sample. Leptin mRNA was quantified using quantitative real time PCR (qRT-PCR) assay. Cyclophilin was used as internal standard. Results presented are from the specimens showing the minimum (1) and maximum (2) leptin mRNA expression levels (representative experiments performed in triplicate). Data represent fold change from the mean values of cyclophilin and are expressed as means \pm SD (upper panel). The size of the amplified fragments was confirmed by agarose gel electrophoresis (lower panel).

4. DISCUSSION AND CONCLUSION

This study is the first to demonstrate, both immunohistochemically and at the level of mRNA and protein, the expression of leptin in human periapical granuloma samples.

Leptin is an important hormone secreted by adipose tissue¹¹. Leptin implication in the control of metabolism and energy homeostasis at central level has been largely described¹². One of these functions is the connection between nutritional status and immune competence¹⁶. The adipocyte-derived hormone leptin has been shown to regulate the immune response, innate and adaptive response, both in normal and pathological conditions^{16,17,18}. Leptin share helical structure, functions and receptor subunit makeup, with the IL-6 family of cytokines^{3,9}. IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), and cardiotrophin-like cytokine (CLC) are included in the IL-6 family of cytokines, being all pleiotropic and exhibiting overlapping biological functions³³. Leptin production is increased during infection and inflammation^{5,34}, up-regulating both phagocytosis and the production of pro-inflammatory cytokines of the acute-phase response^{7,34,35}.

The results of the present study show that inflammatory tissue in all periapical granuloma samples is reactive to leptin antibodies. Moreover, the level of leptin mRNA found in periapical granulomas (leptin/cyclophilin ratio: 0.91 ± 0.18) was higher compared to that observed in human pulp samples (leptin/cyclophilin ratio: 0.085 ± 0.015)²³. Previously, ELISA has been used to demonstrate the expression of leptin in supernatant fluids of explants cultures of human chronic periapical lesions³⁰, finding a greater leptin concentration in small periapical lesions (< 5mm) than large lesions.

Earlier studies have also shown that human dental pulp express leptin, being increased its expression in the presence of pulp inflammation²³. Taking into account the detection of LEPR in normal and inflamed human dental pulp²⁴ and in experimentally induced rats periapical lesions³⁰, together with the immunohistochemical demonstration of leptin expression in human periapical granulomas, showed in the present study, support the role of leptin in periapical inflammatory process. Moreover, previous and present results suggest that leptin expression correlated with the grade of periodontal^{26,27,28}, pulp^{23,24} and periapical tissue inflammation³⁰.

In human periapical granulomas is characteristic an organization of profuse collagen fibers in diverse directions appearing as irregular dense connective tissue with vascular elements. Inflammatory elements of the connective tissue, such as histiocytes/macrophages, lymphocytes, neutrophils, eosinophils, and multinucleated giant cells are present in granulomatous inflammation^{3,4}. In the present study, the expression of leptin in the inflammatory tissue of periapical granulomas has been demonstrated immunohistochemically. Leptin detected in this tissue can has two origins: circulating leptin or leptin bound to its receptor in immune and endothelial cells. Thus, LEPR has been detected in human CD4⁺ T lymphocytes³⁶, CD8⁺ T lymphocytes³⁷, B lymphocytes³⁸, human monocyte/macrophages³⁹, and human neutrophils⁴⁰. Accordingly, increased expression of LEPR has been observed in macrophages/foam cells and the endothelial lining of the intima of neorevascularized regions of human atherosclerotic aorta⁴¹. Since leptin induces endothelial cell proliferation and angiogenesis⁴¹, the presence of leptin in human periapical granulomas suggest a role of leptin in periapical healing.

It has been shown that leptin regulates bone formation^{42,43} and positively promotes ossification through multiple ways, including bone mineralization, remodeling, resorption and osteoblast differentiation⁴⁴. Because of this, several studies have assessed the possible implication of leptin in periodontal disease. The presence of leptin has been reported in healthy and inflamed gingival tissues^{13,14} and in gingival crevicular fluid¹⁵⁻¹⁷. A decrease in serum leptin levels following non-surgical periodontal treatment has been reported^{45,46}. Moreover, LEPR gene has been detected experimentally in periapical lesions in rats²⁰, and LEPR immunoreactivity has been detected gingival epithelium¹⁴.

In conclusion, the expression of leptin in human periapical granulomas demonstrated in the present study, points to a possible role of leptin in periapical immune, inflammatory and reparative responses.

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Chapter 6: Expression and Immunohistochemical Localization of Leptin Receptor in Human Periapical Granuloma

This chapter has been accepted for publication as:

Jenifer Martín-González, Antonio Carmona-Fernández, Antonio Pérez-Pérez, Flora Sánchez-Jiménez, Víctor Sánchez-Margalet, Juan J. Segura-Egea (2014). Expression and Immunohistochemical Localization of Leptin Receptor in Human Periapical Granuloma. *International Endodontic Journal*. Accepted.

ABSTRACT

Introduction. Leptin, initially described as an adipocyte-derived hormone to regulate weight control, as well as its receptor (LEPR), are expressed in human dental pulp. LEPR are up-regulated during pulp inflammation. This study aims to investigate the expression and immunohistochemical localization of LEPR in human periapical granulomas.

Methodology. Periapical inflammatory lesions were obtained from extracted human teeth and teeth which underwent periapical surgery. After their histopathological categorization as periapical granulomas (n = 20), they were examined by immunohistochemistry using human LEPR monoclonal antibodies. LEPR mRNA expression was also determined by quantitative real-time PCR (qRT-PCR) and the amount of LEPR protein was analyzed by immunoblot.

Results. All granuloma samples expressed LEPR. Amongst inflammatory cells, only macrophages showed expression of LEPR. Western blot analysis revealed the presence in the samples of a protein with apparent molecular weight of ~ 120 kDa, corresponding to the estimated molecular weight of LEPR. The qRT-PCR analysis demonstrated the expression of LEPR mRNA, corresponding the size of the amplified fragment (338 bp), assessed by agarose gel electrophoresis, to that of LEPR mRNA.

Conclusions. Human periapical granulomas express LEPR. In periapical granulomas only macrophages showed expression of LEPR. This finding suggests that leptin can play a role in inflammatory and immune periapical responses.

1. INTRODUCTION

Leptin is a 16 kDa non-glycosylated polypeptide hormone of 146 amino acids encoded by the *Ob* gene (Zhang *et al.* 1994). Leptin was initially described as an adipocyte-derived hormone to regulate weight control (Flier 1995), but it has also been classified as a pro-inflammatory cytokine (Zhang *et al.* 1994, Sánchez-Margalet *et al.* 2003). A role for leptin regulating immunity, inflammation and hematopoiesis has been accepted (Cioffi *et al.* 1996, Fantuzzi & Faggioni 2000, Sánchez-Margalet *et al.* 2003). Consistent with this role of leptin in the mechanisms of immune response and host defense, leptin levels are increased upon infectious and inflammatory stimuli such as LPS, turpentine, and cytokines (Matarese 2000).

Accordingly, leptin receptor (LEPR) shows sequence homology to members of the class I cytokine receptor superfamily (Tartaglia *et al.* 1995), such as the IL-6-type cytokine receptors, leucocyte inhibitory factor and granulocyte colony-stimulating factor (Baumann *et al.* 1996). LEPR is expressed not only in the central nervous system, but also in hematopoietic and immune systems (Sánchez-Margalet *et al.* 2003), in mice monocytes and lymphocytes (Loffreda *et al.* 1998 , Lord *et al.* 1998) and in human peripheral blood T lymphocytes (both CD4 and CD8) (Sánchez-Margalet *et al.* 2003, Martín-Romero *et al.* 2000).

In relation with oral tissues, LEPR immunoreactivity has been found in the gingival epithelium (Yetkin *et al.* 2012), LEPR gene has been detected in experimental rats periapical lesions (Martínez *et al.* 2007), and both LEPR protein and LEPR mRNA have been described in healthy and inflamed human dental pulp (Martín-González *et al.* 2013a). The up-regulation of LEPR (Martín-González *et al.* 2013a) and leptin (Martín-González *et al.* 2013b) expression in inflamed human pulp samples suggests that leptin can play a role in inflammatory and local immune responses in human dental pulp.

Chronic periapical lesions results from a localized inflammatory reaction to the bacteria and by-products of the root canal system. The most common periapical lesions are periapical granulomas (Gbolahan *et al.* 2008). Periapical granuloma is a chronic inflammatory lesion at the apex of a non-vital tooth consisting of granulation tissue and scar. The inflammatory cell infiltrate in these chronic periapical lesions consists of a mix of plasma cells, T- and B-lymphocytes, macrophages, polynuclear leucocytes (PMNs), dendritic cells (DCs), natural killer cells (NK cells), and mast cells, present in different proportions within the granulation tissue of periapical lesions (Márton & Kiss 1993, Stashenko *et al.* 1998, Liapatas *et al.* 2003, Márton & Kiss 2014). The inflammatory infiltrate constitutes approximately 50% of the

cells present in periapical granulomas, with non-inflammatory connective tissue cells, including fibroblasts, vascular endothelium, proliferating epithelium, osteoblasts, and osteoclasts comprising the balance (Langeland et al. 1977, Yu et al. 1987).

The presence of leptin has been demonstrated in chronic periapical lesions (Haghighi *et al.* 2013) suggesting that leptin could play a role in the inflammatory and immune responses in periapical tissues. However, the expression of LEPR by inflammatory cells present in periapical granulomas has not been studied.

The aim of this study was to investigate the expression of LEPR in human periapical granulomas. Immunohistochemistry was used to determine whether LEPR is expressed on the surface of infiltrating cells.

2. MATERIALS AND METHODS

The study was carried out with the understanding and written consent of each subject and according to the principles of the World Medical Association Declaration of Helsinki. The protocol was approved by the Ethical Board of the University.

2.1. - Human chronic periapical lesions samples

Twenty human chronic periapical lesions from twenty healthy, nonsmoking, human donors (45–72 years old), who gave their written informed consent, were obtained from 14 freshly extracted teeth and 6 teeth which undergone periapical surgery treatment. Inflammatory tissues surrounding periapical area were dissected. Each sample was then divided into three parts, one for the Western blotting analysis, other for RNA extraction and quantitative real-time PCR (qRT-PCR) assay, and another one for pathologic evaluation and immunohistochemistry.

2.2. - Antibodies and reagents

The monoclonal mouse anti- β -tubulin (1:1000) and the polyclonal rabbit antibodies against the long isoform of LEPR (C-terminal), were provided from Santa Cruz Biotechnology (CA, USA). Horseradish peroxidase-linked anti-mouse/anti-rabbit (1:10.000) immunoglobulins were purchased from Amersham Pharmacia (Amersham Pharmacia Biotech, Barcelona, Spain).

2.3.- Western blotting analysis

The granuloma samples were incubated in 75 μ l of lysis buffer containing 20 mM Tris, pH 8, 1% Nonidet P-40, 137 mM NaCl, 1 mM MgCl₂, 1 mM

CaCl₂, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mM sodium orthovanadate, at 4 °C for 30 min on an orbital shaker and then centrifuged at 12,000 g for 15 min. The supernatants were transferred to new tubes. Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as standard. We added SDS-stop buffer containing 100 mM of DTT to the periapical tissue samples followed by boiling for 5 min. 50 µg protein were loaded in each lane. The samples were then resolved by 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Martin-Romero *et al.* 2000). The membranes were blocked with buffered saline–0.05% Tween 20 (PBST) containing 3% bovine serum albumin during 1 h at 23 °C. The blots were then incubated with primary antibody for 1 h, washed in PBST, and further incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/antirabbit immunoglobulins. Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal from Pierce) (Sánchez-Margalet *et al.* 2003). The bands obtained in the blots were scanned and analyzed by the PCBAS 2.0 program. The relative optical density of the different bands was normalized by the corresponding intensity of the tubulin immunoblot in each individual experiment.

2.5. - RNA extraction and quantitative real-time PCR (qRT-PCR) assay and agarose gel electrophoresis.

Abundance of LEPR mRNA was determined by quantitative real-time PCR reaction (qRT-PCR). Total RNA was extracted from periapical tissue samples using TRISURE reagent (Chomczynski 1993). Concentration and purity of the isolated RNA were estimated spectrophotometrically at 260 and 280 nm. For cDNA synthesis, 5 µg of total RNA was reverse transcribed at 55°C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche, Mannheim, Germany). Quantitative real time PCR was performed using the primers based on the sequences of the National Center for Biotechnology Information GenBank database (Table 1). Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green), and PCRs were performed on a MiniOpticon (Bio- Rad, Hercules, CA, USA). A typical reaction contained 10 µM of forward and reverse primer, 3 µL of cDNA, and the final reaction volume was 25 µL. The reaction was initiated by preheating at 50°C for 2 min, followed by heating at 95°C for 10 min. Subsequently, 40 amplification cycles were carried out as follows: denaturation 15 s at 95°C and 1 min annealing and extension at 58°C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. For the processed samples, evaluation of $2^{-\Delta\Delta CT}$

indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin). The retro-transcribed DNA samples were then resolved by 1% agarose gel; 20 µl samples were loaded in each lane. After running the electrophoresis, gels were visualized directly upon illumination with UV light.

Table 1. Sequence of PCR primers used in this study.

Gene	Primer sequences
Leptin receptor	
Forward:	5'-ATAGTTCAGTCACCAAGTGC-3'
Reverse:	5'-GTCCTGGAGAACTCTGATGTC-3'
Cyclophilin	
Forward:	5'-CTTCCCCGATACTTCA-3'
Reverse:	5'-TCTTGGTGCTACCTC-3'

2.6. - Immunohistochemistry

The excised periapical lesions were fixed in 10% formalin for at least 24 hours and then embedded in paraffin and processed routinely. A series of 5-µm sections from each tissue sample were cut. One section of each serie was stained with haematoxylin-eosin (H&E) to study the histology and to confirm

the diagnosis of periapical granuloma. A second section was used for immunohistochemical staining for expression of LEPR.

Pilot studies were undertaken to establish the optimum dilution of the antibody as well as confirming concentration and application times for the chemicals used during the immunohistochemistry (IHC) procedure. Second and third sections were picked up onto a slide, deparaffined in xylene, rehydrated by sequential immersion in a graded series of alcohols and transferred into water for 10 min. Then, specimens were heat treated in sodium citrate buffer (pH 6.0), for 40 min at 96 °C, to unmask antigens. The sections were cooled and washed in phosphate-buffered saline (PBS, pH 7.2). Specimens were incubated in blocking agent (5% albumin bovine serum in PBS) for 1 hour. Endogenous peroxidase activity was quenched by incubating the sections in a solution of 3% H₂O₂ in 10% methanol and in PBS for 15 min. Second sections were incubated with rabbit antihuman LEPR monoclonal primary antibodies, respectively, at 1: 100 dilutions overnight at 4°C. The sections were then washed in PBS (3X, 5 minutes each) and incubated with their respective secondary antibodies. The manufacturer's instructions were followed for the sequential incubation and durations for the exposure to the secondary antibodies. After washing with PBS, the sections were incubated with diaminobenzidine substrate kit (Dako, Carpinteria, CA,

USA) for 5 minutes that resulted in a brown-coloured precipitate at the antigen–antibody binding sites and the reaction was stopped in distilled water. After removing the slides from water, all the sections were dehydrated and one drop of aqueous mounting medium (Dako Faramount) was applied, and the sections were cover-slipped.

Finally, the immunohistochemical specimens were examined using a Leica Laborlux S Microscope (Leica Microsystem GmbH Wetzlar, Germany) with a Nikon DSL2 photo digital system (Nikon Corp, Tokyo, Japan). Each sample was analyzed with a double-blind system by two different operators under magnifications up to x100. A cell was considered as positive when it demonstrated distinct brown surface staining. Six representative sites in each sample were photographed at 1.25, 4, 10, 20, 60 and 100x magnification and captured with a software system (CS3, version 10.0.1; Adobe Photoshop, San Jose, CA, USA).

2.7. - Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as mean \pm standard deviation (SD) in arbitrary units (AU). Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using

the GraphPad Prism computer program (GraphPad Software, San Diego, CA, USA).

3. RESULTS

To define the pathological features of the periapical lesions, paraffin sections of 20 samples were evaluated histologically using hematoxylin and eosin staining. Periapical lesions examined by light microscopy exhibited a large number of infiltrated inflammatory cells, showing zones with the characteristic of the chronic granulomatous inflammatory process: many small capillaries, fibroblasts, numerous connective tissue fibers, and inflammatory infiltrate constituted by a dense accumulation of round cells (plasma cells and small lymphocytes), macrophages, foam cells and occasional polymorphonuclear (PMN) leukocytes (Fig. 1, A and B). No epithelial cells were observed in these samples and the specimens were classified as periapical granulomas. Amongst the inflammatory cells in the periapical granulomas, only macrophages were reactive to LEPR antibodies. Plasma cells, lymphocytes, PMNs, fibroblasts, or endothelial cells did not show expression of LEPR (Fig. 1, C and D).

All human periapical granuloma samples expressed LEPR at both protein and mRNA levels. Western blot analysis revealed the presence in the samples of

a protein with apparent molecular weight of 120 kDa, which corresponds to the estimated molecular weight of LEPR (Fig. 2).

LEPR expression levels (representative experiments repeated three times). Densitograms are expressed as means \pm SD in arbitrary optical density units, calculated as normalized band intensity, i.e. the band intensity of each sample was normalized with tubulin.

To further validate the expression of leptin and LEPR in human periapical granulomas, a real-time PCR assay was performed to examine the messenger RNA (mRNA) levels of LEPR (LEPR/cyclophilin ratio: 1.11 \pm 0.16) (Fig. 3, upper panel). The size of the amplified fragments (338 bp for LEPR and 194 bp for cyclophilin) was confirmed by agarose gel electrophoresis (Fig.3, lower panel).

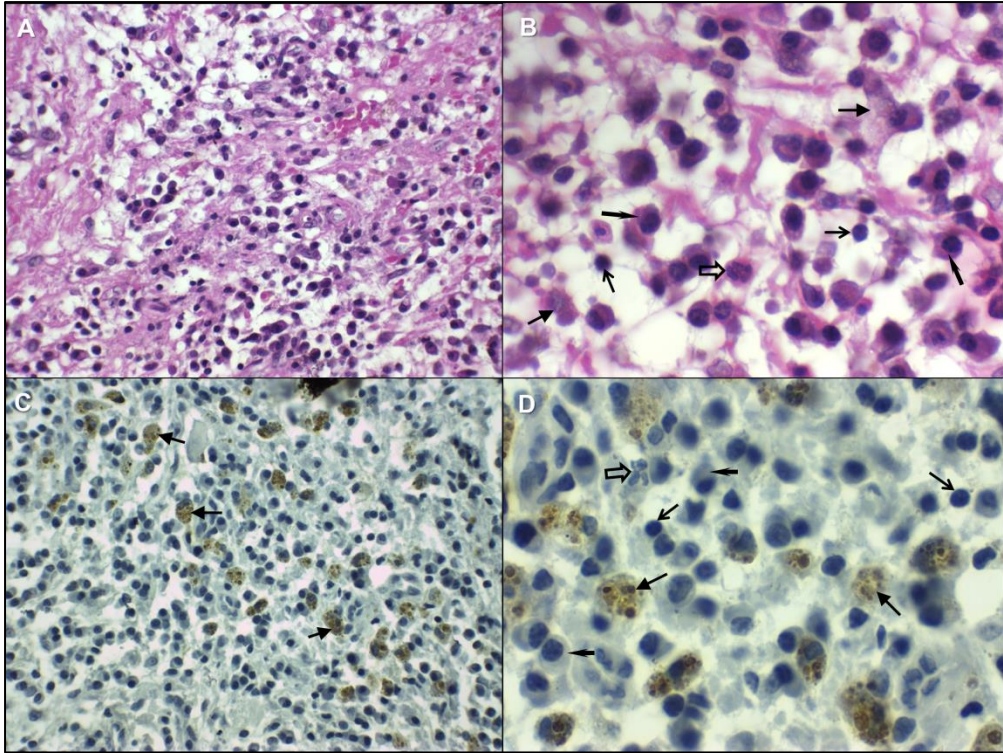


Figure 1 *In situ* expression of Leptin receptor in human periapical granuloma. Photomicrographs of haematoxylin-eosin (A, B) and immuno stained (C,D) sections from periapical granuloma. A: H&E, 40X. B: H&E, 100X. C: In situ LEPR expression by immunohistochemical, 40X. D: In situ LEPR expression by immunohistochemical, 100X. Note: plasma cells (→), lymphocytes (→), polynuclear cells (⇨) and macrophages (→).

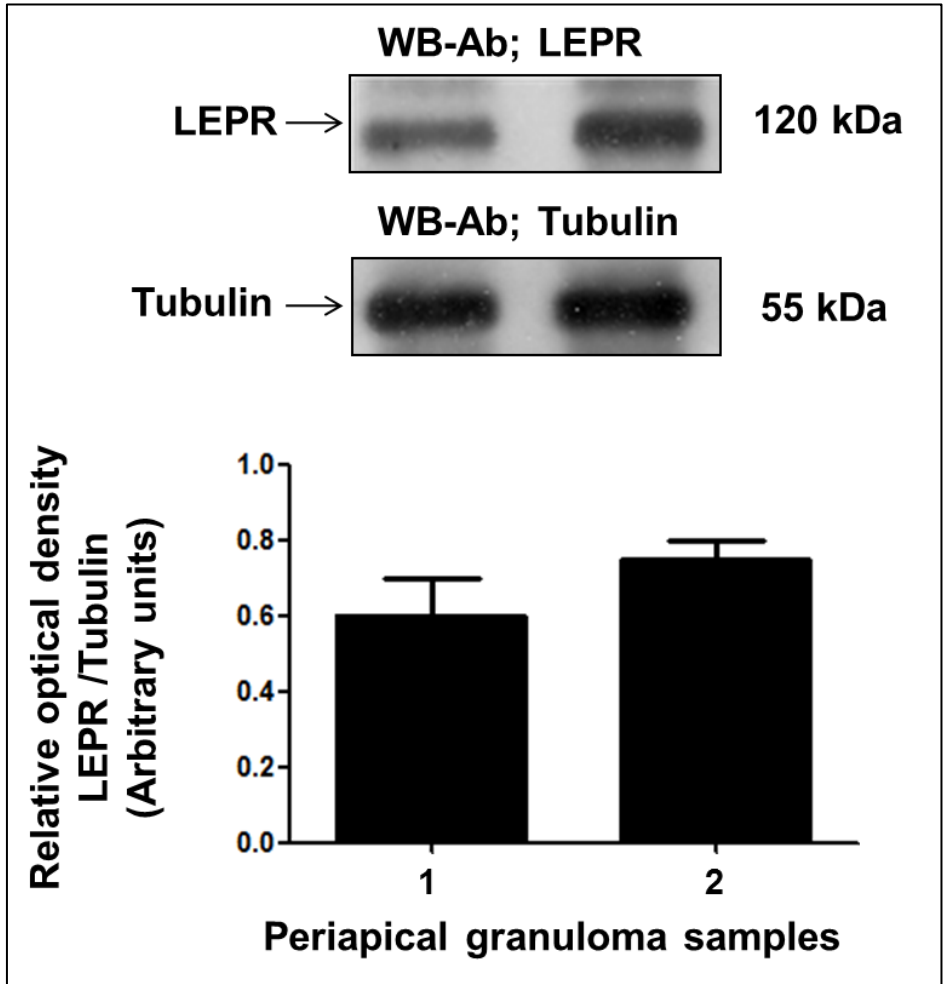


Figure 2. Relative amounts of LEPR in two samples of human periapical granuloma. Results presented are from the specimens showing the minimum (1) and maximum (2).

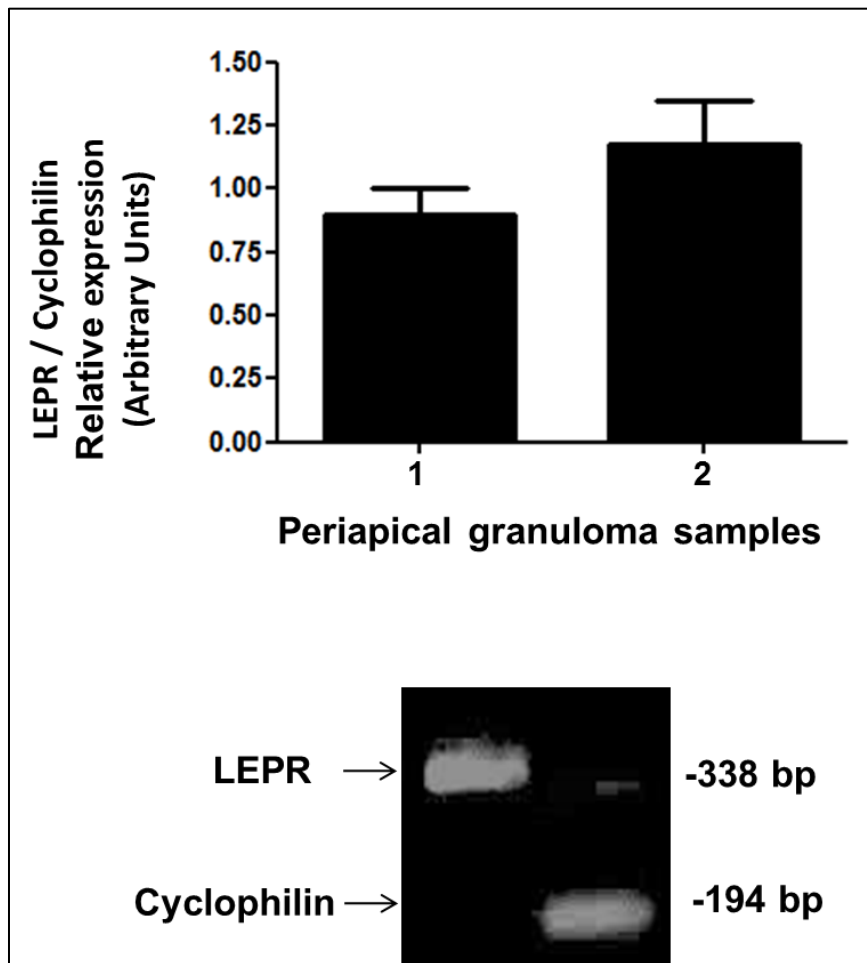


Figure 3. LEPR mRNA expression in human periapical granulomas. Total RNA was extracted from each human periapical granuloma sample. LEPR mRNA was quantified using quantitative real time PCR (qRT-PCR) assay. Cyclophilin was used as internal standard. Results presented are from the specimens showing the minimum (1) and maximum (2) LEPR mRNA expression levels (representative experiments performed in triplicate). Data represent fold change from the mean values of cyclophilin and are expressed as means \pm SD (upper panel). The size of the amplified fragments was confirmed by agarose gel electrophoresis (lower panel).

4. DISCUSSION AND CONCLUSION

This study is the first to demonstrate, both immunohistochemically and at the level of mRNA and protein, the expression of LEPR in human periapical granulomas. The expression of leptin in supernatant fluids of explants cultures of human chronic periapical lesions have been previously demonstrated using the ELISA method (Haghighi *et al.* 2010). LEPR had also been detected previously in experimental rats periapical lesions (Martíne *et al.* 2007), but mRNA and protein expression studies and the immunohistochemical demonstration of LEPR expression in human periapical inflammatory tissue had not been previously reported. The result of the present investigation, together with those previous findings, suggests that leptin and LEPR expressing cells could be implicated in the pathophysiology of the human periapical lesions.

The presence of leptin has been reported in healthy and inflamed gingival tissues (Johnson & Serio 2001, Yetkin *et al.* 2012), in gingival crevicular fluid (Bozkurt *et al.* 2006, Karthikeyan *et al.* 2007a, Karthikeyan *et al.* 2007b), and in supernatant fluid from cultures of human pulp fibroblasts derived from extracted healthy molar teeth, being its production regulated by neuropeptides (El Karim *et al.* 2009). Recently, it has been described the expression of leptin in human healthy and experimentally induced inflamed

dental pulp (Martín-González *et al.* 2013b). Likewise, the expression of LEPR mRNA in healthy (LEPR/cyclophilin ratio: 0.15 +/- 0.02) and experimentally inflamed human dental pulp (LEPR/cyclophilin ratio: 0.23 +/- 0.04) ($p < 0.05$) has been previously quantified (Martín-González *et al.* 2013a). The LEPR mRNA level found in the pulp is low compared to that found in periapical granuloma in the present work (LEPR/cyclophilin ratio: 1.11 +/- 0.16). Taking together these results, it can be suggested that LEPR expression correlated with the grade of tissue inflammation. The model of experimental pulp inflammation used in the previous study (Martín-González *et al.* 2013a), i.e. high-speed drilling and mechanical pulp exposure followed by pulp extraction after 10 min, could explain the lower level of LEPR mRNA found, compared to its level in periapical granuloma. However, further studies are needed to show the actual expression of this receptor in acute infectious pulpitis in carious teeth.

The primary aminoacid sequence of leptin shows structural similarities to the long chain helical cytokine family, such as IL-2, IL-12 and growth hormone (Zhang *et al.* 1994, Madej *et al.* 1995). Leptin and LEPR share structural and functional similarities with the IL-6 family of cytokines (Madej *et al.* 1995, Tartaglia *et al.* 1995) and leptin production is increased during infection and inflammation (Fantuzzi & Faggioni 2000, Sánchez-Margalet *et al.* 2002) up-

regulating both phagocytosis and the production of pro-inflammatory cytokines by murine macrophages in the acute-phase response (Loffreda *et al.* 1998, Kiguchi *et al.* 2009). Leptin affects both innate and adaptive immunity exerting an effect on T-cells, monocytes, neutrophils, and endothelial cells (Sánchez-Margalet *et al.* 2003).

In the human periapical granuloma is characteristic an organization of profuse collagen fibers in diverse directions appearing as irregular dense connective tissue with vascular elements. All inflammatory elements of the connective tissue were commonly observed, with a great variety of cell shapes and different cell nuclear organization (Rodríguez *et al.* 2011). In the present study, all inflammatory cell types characteristic of human periapical granuloma were evident. However, immunohistochemical examination of periapical inflammatory samples only demonstrated the expression of LEPR in macrophages. These findings are in accordance with the previous detection of LEPR mRNA expression in human monocyte/macrophages (Zarkesh-Esfahani *et al.* 2001). Taking into account that leptin is a potent macrophage chemoattractant (Gruen *et al.* 2007), the result of the present investigation suggest that leptin plays a role in macrophage chemotaxis during periapical chronic inflammatory response. Lymphocytes, plasma cells and PMNs present in granuloma samples did not express LEPR. On the contrary, LEPR

expression has been described during lymphocyte activation in CD8+ T lymphocytes (Martín-Romero *et al.* 2000) and B lymphocytes (Papathanassoglou *et al.* 2006).

On the other hand, it has been showed that leptin regulates bone formation (Cirmanová *et al.* 2008, Turner *et al.* 2013). The overall effect of leptin on bone might be bimodal. In a model of osteoblast, leptin positively promotes ossification through multiple ways including bone mineralization, remodeling, resorption and osteoblast differentiation (Zhang *et al.* 2013). Therefore, several studies have assessed the correlation between leptin and periodontal disease. A decrease in serum leptin levels following non-surgical periodontal treatment has been reported (Shimada *et al.* 2010, Altay *et al.* 2013). Moreover, LEPR immunoreactivity has been found in the gingival epithelium (Yetkin *et al.* 2012) and LEPR gene has been detected experimentally in periapical lesions in rats (Martínez *et al.* 2007), but no study has yet described the expression of LEPR mRNA and protein in these tissues.

Apical periodontitis is an acute or chronic inflammatory lesion around the apex of a tooth caused by bacterial infection of the dental pulp. The susceptibility to develop pulp and periapical inflammatory processes has

been shown to depend upon genetic factors, being influenced by the cytokine genotype/phenotype. Thus, symptomatic dental abscesses predominate in individuals displaying high-producer IL6 genotype (de Sá et al. 2007). Increased IL-1 β production contributes to increased susceptibility to apical periodontitis (Morsani *et al.* 2011), and interleukin (IL)-1 gene polymorphism is associated to root resorption (Bastos Lages *et al.* 2009). In conclusion, the expression of LEPR in human periapical granulomas shown in the present study points to a possible role for leptin in the physiology as well as the pathophysiology of the periapical tissues, probably acting as a modulator of periapical immune and inflammatory responses or/and as a link between these processes and cytokine genotype/phenotype.

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Final conclusions

Based on the research carried out, the following conclusions were drawn:

1. The present study is the first to demonstrate the expression of leptin and its receptor (LEPR) in human healthy and inflamed dental pulp tissues.
2. The present investigation has demonstrated the upregulation of leptin and its receptor in inflamed dental pulp. The significantly higher relative amount of both of them in inflamed dental pulps found in the present study, support the concept that leptin plays a role in dental pulp inflammatory processes.
3. The main leptin receptor signalling pathways, MAPK, PI3K and JAK/STAT3, are triggered by leptin in human dental pulp cells. These results provide further support the possible role of leptin in the pulp physiology.
4. Odontoblasts express leptin receptor. Leptin receptor is localized in the odontoblast layer and in the predentin.
5. Leptin stimulates dentin sialophosphoprotein (DSPP) expression. Leptin plays a role in mineralization of dentine.
6. This study is the first to demonstrate, both immunohistochemically and at the level of mRNA and protein, the expression of leptin and its receptor in human periapical granulomas. These results support the role of leptin in periapical inflammatory process.
7. Endothelial cells were reactive to leptin antibodies in human periapical granulomas. Amongst the inflammatory cells in the periapical granulomas, only macrophages were reactive to LEPR antibodies. Plasma cells, lymphocytes, PMNs, fibroblasts, or endothelial cells did not show expression of LEPR. These findings further support the role of leptin in periapical inflammatory and immune responses.

Conclusiones finales

De la investigación desarrollada se extraen las siguientes conclusiones:

1. El presente estudio es el primero en demostrar la expresión de la leptina y su receptor (LEPR) en el tejido pulpar dental humano sano e inflamado.
2. La presente investigación ha demostrado la sobre-regulación de la leptina y su receptor en la pulpa dental inflamada. Estos hallazgos, apoyan la idea de que la leptina participa en los procesos inflamatorios pulpares.
3. Las principales vías de señalización del receptor de leptina, MAPK, PI3K y JAK/STAT3, son activadas por la leptina en las células de la pulpa dental humana. Estos resultados proporcionan apoyo del posible papel de la leptina en la fisiología de la pulpa.
4. Los odontoblastos expresan receptor de leptina. La expresión del receptor de leptina se localiza en la capa de odontoblastos y en la pre dentina.
5. La leptina estimula la expresión de sialofosfoproteína (DSPP). Por tanto, la leptina juega un papel en la mineralización de la dentina.
6. Este estudio es el primero en demostrar, tanto por inmunohistoquímica como por niveles de ARNm y de proteína, la expresión de la leptina y su receptor en los granulomas periapicales humanos. Estos resultados apoyan la hipótesis de que la leptina participa en el proceso inflamatorio periapical.
7. Las células endoteliales mostraron inmunoreactividad a los anticuerpos de leptina en granulomas periapicales humanos. Entre las células inflamatorias presentes en los granulomas periapicales, sólo los macrófagos fueron reactivos a anticuerpos de LEPR. Las células plasmáticas, linfocitos, PMN, fibroblastos, o células endoteliales no mostraron expresión de LEPR. Estos hallazgos apoyan aún más el posible papel de la leptina en las respuestas inflamatorias e inmunes periapicales.

Curriculum Vitae

JOURNAL PAPERS

1. **Martín-González J**, Carmona-Fernández A., Pérez-Pérez A, Sánchez-Jiménez F, Sánchez-Margalet V, Segura-Egea JJ. *Expression and Immunohistochemical Localization of Leptin Receptor in Human Periapical Granulomas*. In press, International Endodontic Journal.
2. **Martín-González J**, Sánchez-Margalet V., Segura-Egea JJ. *Expression and immunohistochemical localization in human periapical granulomas*. Archives of Oral Biology. Under review.
3. **Martín-González J**, Pérez-Pérez A, Sánchez-Jiménez F et al. *Leptin Signalling in Normal Human Dental Pulp: Involvement of MAPK, PKB and STAT-3 Pathways*. Int Endod J. Under review.
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6. Alonso-Ezpeleta O, Martín PJ, López-López J, Castellanos-Cosano L, **Martín-González J**, Segura-Egea JJ. *Pregabalin in the treatment of inferior alveolar nerve paraesthesia following overfilling of endodontic sealer*. J Clin Exp Dent. 2014 Apr 1; 6(2):e197-202.
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11. Rodríguez-Ortega L, Velasco-Ortega E, Castellanos-Cosano L, **Martín-González J**, López-Frías FJ, Segura-Egea JJ. *Endodontic-implant pathology: Concept, types, diagnosis, treatment and prevention*. *Av Periodon Implantol*. 2013; 25, 2: 83-90.
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13. **Martín-González J**, Echevarría-Pérez M, Sánchez-Domínguez B, Tarilonte-Delgado ML, Castellanos-Cosano L, López-Frías FJ, Segura-Egea JJ. *Influence of root canal instrumentation and obturation techniques on intra-operative pain during endodontic therapy*. *Med Oral Patol Oral Cir Bucal*. 2012 Sep 1; 17(5):e912-8.
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BOOK CHAPTERS

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2. **Martín-González J**, Mateo-Aguilar E, López-Martín I, Martín-González LJ, Montes Galdeano FL, Alférez-Maldonado AD. *Oral health in the elderly. 1st International Congress of Research on Health and Aging, 2014.*

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4. **Martín-González J**, Pérez-Pérez A, Sánchez-Jiménez F, Segura-Egea JJ, Sánchez -Margalet V. *Leptin Promotes Dentin Sialophosphoprotein*

Expression in Human Dental Pulp. 37th National Congress of the Spanish Biochemistry and Molecular Biology Society, 2014.

5. Martín-González J, Sánchez-Domínguez B, Crespo-Gallardo I, Martín-Jiménez M, Segura-Egea JJ. *Stimulatory effect of leptin in the dentin sialophosphoprotein (DSPP) production in human dental pulp*. 10th National and 7th International Congress of the Spanish Society of Conservative Dentistry, 2014.

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23. Sánchez-Domínguez B, Castellanos-Cosano L, **Martin-González J**, Segura-Egea JJ. *Expression of leptin in normal human dental pulp.* XIX National Congress and VI International of the Spanish Society of Conservative Dentistry. 2012.

24- **Martin-González J**, Castellanos-Cosano L, Raya-Pérez E, Sánchez-Domínguez B, Segura-Egea JJ. *Retrograde peri-implant and cross infection implant-tooth endodontic.* 9th National-4th International Congress of Spanish Society of Gerodontology, 2011.

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33. Calvo-Monroy C, López-Frias FJ, Sánchez-Domínguez B, **Martín-González J,** Castellano-Cosano L. *Influence of root canal instrumentation*

and other root canal filling techniques on discomfort felt by the patient during root canal treatment. 31st National Congress of the Spanish Endodontic Society, 2010.

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36. Castellano-Cosano L, Sánchez-Domínguez B, **Martín-González J,** Jiménez-Moreno E, Segura Egea JJ. *Periapical and endodontic status of a sample of hypertensive patients: influence of smoking.* 31st National Congress of the Spanish Endodontic Society, 2010.

37. Castellano-Cosano L, Mendoza-Mendoza A, Sánchez-Domínguez B, **Martín-González J,** Segura Egea JJ. *Root fracture of a maxillary central incisor with an immature apex.* 31st National Congress of the Spanish Endodontic Society, 2010.

38. Segura Egea JJ, Sánchez-Domínguez B Castellano-Cosano L, **Martín-González J,** López-Frías FJ. *Effect of the methacrylate-based endodontic sealer Epiphany on rat peritoneal macrophages viability.* 31st National Congress of the Spanish Endodontic Society, 2010.

CONFERENCE AWARDS

1. Award for best oral presentation: “Martín-González J, Sánchez-Domínguez B, Crespo-Gallardo I, Martín-Jiménez M, Segura-Egea JJ. *IV. Leptin Promotes Dentin Sialophosphoprotein Expression in Human Dental Pulp.* 10th National and 7th International Congress of the Spanish Society of Conservative Dentistry, 2014.

2. Award for best paper published in 2013: “Martín-González J, Pérez-Pérez A, Sánchez-Jiménez F, Carmona-Fernández A, Torres-Lagares D,

Sánchez-Margalet V, Segura-Egea JJ. *Leptin receptor is up-regulated in inflamed human dental pulp*. J Endod. 2013 Dec; 39(12):1567-71".10th National and 7th International Congress of the Spanish Society of Conservative Dentistry, 2014.

RESEARCH STAYS

1) CENTRE: **Centre for Infection and Immunity, School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast.**

PERIOD OF STUDY: **September 21st, 2013-December 19th, 2013.**

RESEARCH PROYECT: Effect of Leptin on proliferation and diferentation of human pulp stem cells from dental pulp.

DIRECTOR OF RESEARCH: Dr. Fionnula Lundy.

2) CENTRE: **UT Health Science Center. Department of Endodontics, Dental School (Neuropharmacology Research Group). San Antonio, Texas.**

PERIOD OF STUDY: **September 19th, 2012-December 19th, 2012.**

RESEARCH PROYECT: Extraction of growth factors from the dentin.

DIRECTOR OF RESEARCH: Dr. Aníbal Diogenes.

Research project documents



A quien pueda interesar:

El Comité Ético de Experimentación de la Universidad de Sevilla, habiendo examinado el Proyecto "**IMPLICACIONES SISTÉMICAS DE LA PATOLOGÍA Y TERAPÉUTICA ENDODÓNCICAS: ESTUDIO DE SU ASOCIACIÓN CON LA DIABETES MELLITUS Y EL SÍNDROME METABÓLICO**" presentado por D. Juan José Segura Egca, emite el siguiente informe.

El proyecto cumple los requisitos exigidos para experimentación en sujetos humanos y en animales, y se ajusta a las normativas vigentes en España y en la Unión Europea.

Sevilla, a 01 de febrero de 2010.

EL PRESIDENTE DEL COMITE,

Fdo.: P.D. Prof. Dr. Fernando Rodríguez Fernández.

DECLARACIÓN DE CONSENTIMIENTO INFORMADO

D./Dña, de años de edad y con DNI nº, manifiesta que ha sido informado/a sobre el Proyecto de Investigación titulado **IMPLICACIONES SISTÉMICAS DE LA PATOLOGÍA Y TERAPÉUTICA ENDODÓNICAS: ESTUDIO DE SU ASOCIACIÓN CON LA DIABETES MELLITUS Y EL SÍNDROME METABÓLICO**, cuyo investigador principal es el Dr. Juan J. Segura Egea.

He leído la hoja de información que se me ha entregado.

He podido hacer preguntas sobre el estudio.

He recibido suficiente información sobre el estudio.

He hablado con: _____

Comprendo que mi participación es voluntaria.

Comprendo que puedo retirarme del estudio:

1. Cuando quiera.
2. Sin tener que dar explicaciones.
3. Sin que esto repercuta en mis cuidados médicos.

Sevilla, a de de 2011.

Fdo. D/Dña



Servicio Andaluz de Salud
CONSEJERÍA DE SALUD

C.I.F.: Q9150013B

NOMBRE: José Luís Gutiérrez Pérez

CARGO: Director Gerente del Servicio Andaluz de Salud

MANIFIESTA:

Que en relación con el Proyecto de Investigación titulado "**IMPLICACIONES SISTÉMICAS DE LA PATOLOGÍA Y TERAPÉUTICA ENDODÓNCICAS: ESTUDIO DE SU ASOCIACIÓN CON LA DIABETES MELLITUS Y EL SÍNDROME METABÓLICO**" cuyo investigador principal es el Dr. Juan J. Segura Egea, del Dpto. de Estomatología de la Universidad de Sevilla, este Centro Directivo considera de interés las investigaciones planteadas en el mismo, que están incluidas en nuestro *PLAN INTEGRAL DE DIABETES*. Muy especialmente, consideramos de interés el estudio propuesto sobre la interrelación entre las infecciones orales de origen endodóncico y la evolución de los enfermos diabéticos. Los resultados de este proyecto de investigación podrían ayudar a un mejor control de los enfermos diabéticos y aportarían evidencias sobre la probable relación entre el estado de salud oral, la diabetes mellitus y el síndrome metabólico.

Sevilla, 2 de febrero de 2010

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