รายงานวิจัยฉบับสมบูรณ์

โครงการ

การศึกษาความผิดปกติของกระดูกอ่อนในภาวะเบาหวานแบบไม่พึ่งพาอินซูลิน (Non Insulin Dependent Diabetes Mellitus, NIDDM หรือ Type II DM, T2DM) โดยใช้ต้นแบบหนูเบาหวานสายพันธุ์ Goto-Kakizaki (GK)

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สัญญาเลขที่ TRG5680022

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โครงการ

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และ มหาวิทยาลัยมหิดล

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Abstract

Project Code : TRG5680022

Project Title : The study of cartilage and growth plate abnormalities in Non Insulin Dependent Diabetes Mellitus, NIDDM or Type II DM, T2DM using Goto-Kakizaki (GK) rat as a model of in vivo study

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Project Period : 2 years

Type 2 diabetes mellitus (T2DM) is a prevalent metabolic disorder caused by spontaneous target tissue-resistance to insulin and progessive degeneration of multiple organ systems. International Diabetes Federation (IDF) last year alerted the global population with number of 366 million diabetic's in 160 countries, and predicted an increase to over 552 million cases by the year 2030. The marginal bone loss that usually observed around dental implant has been well documented and expected. It has related with self-reaction to the forensic body of each patient as well as the osseointegrated interface. The investigation of bone quantity and quality of the implant site may help to define the implant-bone interface, which in turn affects primary stability of the immediate implant placement. Analysis of bone quality prior to surgery provides vital information during treatment planning for dental implant. Additionally, it helps in predicting postsurgical success in healthy and systemic diseases i.e., diabetic mellitus. We categorized bone type according to their density (D1–D4) in all regions of the jaws based on descriptive morphology and clinician tactile analog. The subjective hand-felt resistance during forty-two implant surgeries were recorded for comparing with the HU value obtained from CBCT and CT. The mean HU from CBCT shows proportional higher value to those from CT. Bone densities are graded among all
bone types. It is well acknowledged that clinical awareness of evaluating the amounts of bone surrounding the implant site by appropriate method is critical for a successful outcome.

Keywords : Bone formation; Osseointegration; Histomorphometry, Tactile anal

บทคัดย่อ

รหัสโครงการ: TRG5680022

ชื่อโครงการ: การศึกษาความผิดปกติของกระดูกอ่อนในภาวะเบาหวานแบบไม่พึ่งพาอินซูลิน (Non Insulin Dependent Diabetes Mellitus, NIDDM หรือ Type II DM, T2DM) โดยใช้ต้นแบบหนูเบาหวานสายพันธุ์ Goto-Kakizaki (GK)

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ระยะเวลาโครงการ: 2 ปี

โรคเบาหวานจัดเป็นหนึ่งในกลุ่มโรค Non communicable diseases (NCD) เป็นปัญหาสาธารณสุขอันดับต้นๆในประเทศทั่วโลกรวมทั้งประเทศไทยด้วย ทั้งนี้การเกิดโรคเบาหวานประเภทที่สองแบบไม่พึ่งพาอินซูลินเป็นประเภทที่พบบ่อยที่สุดในผู้ป่วยเบาหวาน โดยผู้ป่วยจะแสดงอาการเช่น ระดับน้ำตาลในเลือดสูงกว่าปกติ, หลั่งอินซูลินมากกว่าปกติ อย่างไรก็ตาม การศึกษาผลของภาวะเบาหวานในเนื้อเยื่อ กระดูกและฟันยังคงเป็นที่ถกเถียงกันและยังไม่สามารถอธิบายถึงความผิด ปกติของกระดูกขากรรไกรในผู้ที่เป็นโรคเบาหวานได้ นอกจากนี้ภาวะเบาหวานเป็นปัจจัยหลักที่ทำให้เกิดความล้มเหลวต่อการฝังรากฟันเทียมและการฟื้นฟูสภาพของกระดูกขากรรไกร ผู้วิจัยจึงมีความประสงค์ที่จะหาคุณสมบัติและความหนาแน่นของกระดูกขากรรไกรมนุษย์ในแต่ละตำแหน่งของขากรรไกรบนและล่าง เพื่อนำไปใช้ทำนายการฟื้นฟูของกระดูกหลังการผ่าตัดฝังรากฟันเทียมคุณภาพของกระดูกในแต่ละตำแหน่งของขากรรไกร และวิจัยหาสภาวะความรุนแรงของโรคปริทันต์ที่ส่งผลต่อการหายของแผลและการฟื้นฟูของกระดูกรอบบริเวณรากฟันเทียม จากผลการวิจัยพบว่ากระดูกขากรรไกรมนุษย์สามารถจัดประเภทได้ 4 ประเภทโดยใช้การประเมินทางรังสีเอกเรย์ นอกจากนี้การใช้ความรู้สึกด้านทานมือขณะเจาะกระดูกขากรรไกรมีความสัมพันธ์อย่างมีนัยสำคัญกับกระดูกขากรรไกรประเภทที่ 1และ 4 สภาวะความรุนแรงของโรคปริทันต์ มีความสัมพันธ์ต่อการหายของแผลและการฟื้นฟูของกระดูกรอบบริเวณรากฟันเทียม แต่ไม่มีความสัมพันธ์ต่อความหนาแน่นของกระดูกขากรรไกรมนุษย์ในแต่ละตำแหน่งของขากรรไกร ดังนั้นทันตแพทย์จะสามารถใช้แนวทางการจัดประเภทของกระดูกตามตำแหน่งของขากรรไกรนี้ เป็นข้อมูลประกอบการวางแผนการรักษาและยังสามารถคาดเดาอัตราความสำเร็จของรากฟันเทียม และการเชื่อมต่อของกระดูกและวัสดุรากเทียมที่ตำแหน่งต่างๆของกระดูกขากรรไกรมนุษย์ได้

คำหลัก : การสร้างกระดูก; การเชื่อมต่อของกระดูกและวัสดุ; ฮิสโตมอร์โฟเมทรี, ค่าความรู้สึกผ่านมือ

Introduction

The position of the mandibular and maxillary teeth is commonly divergent to the position of the basal bone. The alveolar bone and basal ridge are varied from each other as more evidence has been shown in the different resorbing patterns following tooth loss (1, 2). Using a radiographic scale to determine bone quality, the two highest density of alveolar and basal buccal cortical in maxilla were located in canine and premolar sites whereas incisor and premolar sites caught a similar range of density. The bony structure of human jawbone is irregular in shape and size due to a non-uniform modelling during embryogenesis and early life. The mandible shows a thicker cortical and denser trabecular bone compared with the maxilla while the trabecular in posterior parts in both arch are recognized to have lower density and thickness (3-5). The prospective clinical studies reviewed that total failure rate of implant stability is associated with bone quality surrounding the implant site (6, 7). These observations emphasize the promise of bone structural analysis in each dental implant surgery, especially in the thinner maxilla, wherein primary stability may be challenging to achieve. Despite studies in the past decades, integration of various tools used in bone type classification remained unclear.

Up to now, dental technology and surgical guidelines point to a need for knowledge on bone quality and mechanical behaviour of the bone (8-10). The stability of implant anchoring in bone crucially ensures implant success and therefore local bone quality and quantity are factors to be considered in routine assessment. The purpose of this study was to evaluate the alveolar bone type, elaborate the classification system as well as radiological structure of alveolar bone ridge in 4 dental quadrants.

Methodology

Study design: This study was a descriptive cross sectional study. The ethical approval was obtained from the Mahidol University Ethics Review Board, Mahidol University, Bangkok (MU-DT/PY-IRB 2013/033.0807)

Study site: Department of Dental Implant, Faculty of Dentistry, Mahidol University, Bangkok, Thailand.

Sample size: The study sample consisted of 30 dental implant sites. Subjects were selected based on following inclusion and exclusion criteria.

Inclusion Criteria

- Male or female
- Age more than 18 years
- Reasonable amounts of alveolar bone and no complex oral rehabilitation needs
- Willing and able to accept the protocol and to give a written informed consent for the surgical procedure
- Absence of soft or hard tissue inflammation
- Adequate oral hygiene, assessed by the plaque index, sulcus bleeding index, periodontal severity index (PSI)
- Good general health or controlled systemic disease

Exclusion Criteria

- Immediate implant placement
- Neurologic disease that contraindicates implant therapy
- Previous or current radiotherapy or chemotherapy

- Psychological or psychiatric conditions that could influence the treatment
- Blood dyscrasias and liver failure
- Poor metabolic control (Hb a 1c glycosylated hemoglobin > 13.0% or creatinine > 1.7 ml/dl)
- Smoking of >1 pack of cigarettes/day

Protocol of alveolar bone assessment

Step 1

1. After history taking and clinical examination, the participants were selected based on inclusion and exclusion criteria.

2. General information (age, sex, smoking history, concomitant systemic diseases, drug allergy), position of implant, posterior support, duration of edentulism, remaining teeth, periodontal disease, and length and width of edentulous space (mm) were assessed.

3. Radiographic images: periapical and panoramic radiographs and CBCT (Cone Beam Computed Tomography) were performed to evaluate if the bone sites had the minimal volume necessary to receive an implant (4.0 mm*11.5 mm; Intralock)

4. Classification of bone types was done by a radiologist and experienced surgeon/s according to the original classification system, based on radiography and surgeon's tactile perception during drilling. These classification methods was categorized bone types into four groups: I, II, III, & IV, according to the distribution of cortical and trabecular bone.

Step 2

1. Local anesthesia was injected and a flap was opened at the implant site. Then, the gingival thickness was measure by a probe.

2. A guild pin 2.2 mm was used to drilling at the implant site and bone was collected bone by autogenous bone harvester (Mega Gen Implant. Co., Ltd.) to obtain specimen from each site. Surgeon's tactile perception was noted during drilling and the bone was categorized into one of the four groups: I, II, III, & IV. Specimens were transferred into 2 ml Eppendorf tube and place on ice and then centrifuged and cleaned by normal saline, following which the specimen was placed in refrigerator at -80°C.

3. The titanium implant (Intralock Co., Ltd.) was then placed, and healing abutment was connected by a experienced surgeon.

4. The surgical site was sutured and medication were prescribed; Amoxicillin 500 mg (1*3) for 3 days, Paracetamol 500 mg (2*4) for 3 days, and Ibuprofen 400 mg (1*3) for 3 days.

Step 3

1. The wound healing was assessed and scored according to Mombelli index (Mpi).
2. Any pain or complication was noted.

Step 4

1. Periapical radiograph of the implant site was taken.

Data Analysis

All the analyses will be calculated using a 2-tailed, p-value < 0.05 as statistically significant cut off point. Descriptive analyses performed for type of alveolar bone. Spearman's correlation will be used to evaluate the relationships between bone classifications and Bone histomorphometric parameter. SSPS 17.0 for Windows (Chicago, IL, USA) was used for data analysis.

Results

Among 30 implant sites, 12 (40%) were from female and 18 (60%) from male. Regarding associated systemic diseases, 23 (76.7%) samples were from normal persons, 1 (3.3%) from a diabetic, 3 (10%) from a hypertensive, and 3 (10%) from hypertensive patients with hypothyroidism patients. All of the participants had no periodontal disease. Five out of 30 samples (16.7%) were collected from smoking patients. Regarding bone types according to CBCT, panoramic X-ray, and surgeon tactile sensation, the most common bone type was bone Type 3, 24 (80%), 21 (70%), and 14 (46.7%), respectively. Regarding gingival thickness, thick biotype was 27 (90%) and thin biotype was 3 (10%) as shown in Table 1.

Table 1. Bone type, CBCT, Radiological results and gingival biotype regarding site of implants

Information regarding site of implants (30 samples)		
		n (%)
CBCT result	Bone Type 2	5 (16.7)
	Bone Type 3	24 (80)
	Bone Type 4	1 (3.3)
Panoramic X-ray result	Bone Type 2	6 (20)
	Bone Type 3	21 (70)
	Bone Type 4	3 (10)
Type of bone	I	2 (6.7)
(Surgeon tactile sensation)	II	11 (36.7)
	III	14 (46.7)
	IV	3 (10)
Gimgival biotype	Thick	27 (90)
	Thin	3 (10)

We examined the total 30 implant-surgeries by following the Misch classification. We could categorize alveolar bone type into four density groups (D1–D4). In other words, all regions of the jaws were classified based on descriptive morphology and clinician tactile analog as shown in table 2. This data were used to compare with further investigation such as anatomical location and radiographic scale.

Table 2. Our modified classification of alveolar bone quality and quantity based on L&Z and Misch

Reference	Tool used in classification	Type of bone	Images
Lekholm & Zarb (1985)	Plain radiography, Morphology	Type 1: Homogeneous cortical bone	
		Type 2: Thick cortical bone with marrow cavity	
		Type 3: Thin cortical bone with dense trabecular bone of good strength	
		Type 4: Very thin cortical bone with low density trabecular bone of poor strength	
Misch (1990, 1993)	Clinician perception (Hand feel resistance)	D1 = Oak wood	
		D2 = Pine wood	
		D3 = Balsa wood	
		D4 = Styrofoam	

The tactile assessment of alveolar bone quality

The tactile assessments are the noninvasive method for evaluating bone quality. They are the most applicable, yet subjective for drilling resistance assessment. These methods are recorded during the surgery or after surgery (i.e, insertion torque, Periotest). The subjective hand-felt resistance during forty-two implant surgeries were recorded for comparing with the HU value obtained from CBCT and CT. The mean HU from CBCT shows proportional higher value to those from CT. Bone densities are graded among all bone types as shown in Figure 1.

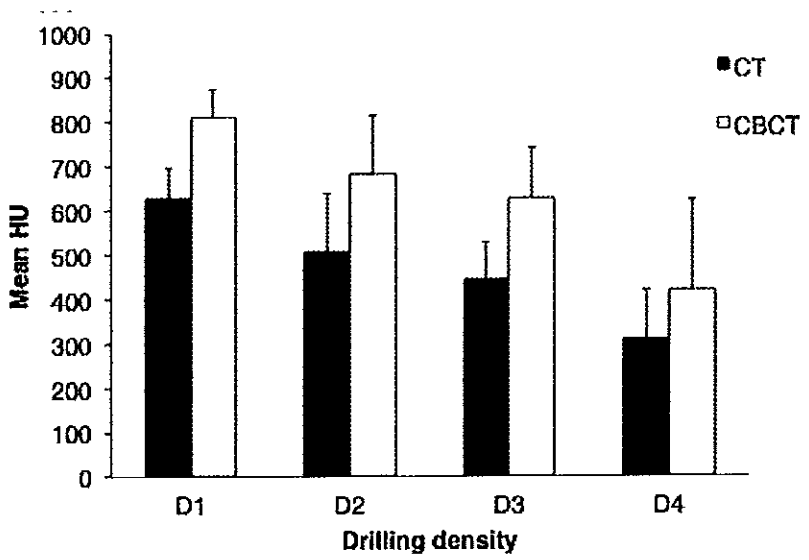

Figure 1: Correlation of drilling density with CT and CBCT analysis: Hounsfield unit (HU); D1 >1250; D2: 850-1250; D3: 350-850; D4:150-350; D5 <150

Histomorphometrical analysis

Bone histomorphometry has been inferred as a gold standard method for the evaluation of bone microarchitecture, as it allows two-dimensional analysis, inferred estimation of the spatial organisation of the trabecular net configuration measured from a set of histological sections (11). Histomorphometric parameters were obtained using the formulas proposed by Parfitt et.al 1983 to explain bone compositions (12, 13). The positive correlation between the Lekholm and Zarb classification and histomorphometric parameter was found significantly only when compared with BV/TV and Tb.Sp. It suggested that a single variable should not be solely used to define a type of bone. Another study was done in comparison among anatomical quadrants of the jawbone. The posterior maxilla was reported to be the lowest trabecular

volume with thinner thickness and lower trabecular number (Tb.N) when compared with other quadrants. Mean percentage of histomorphometric bone volume (BV/TV) from biopsies was used to correlate with clinical scoring based on the Misch classification, as shown in Figure 2.

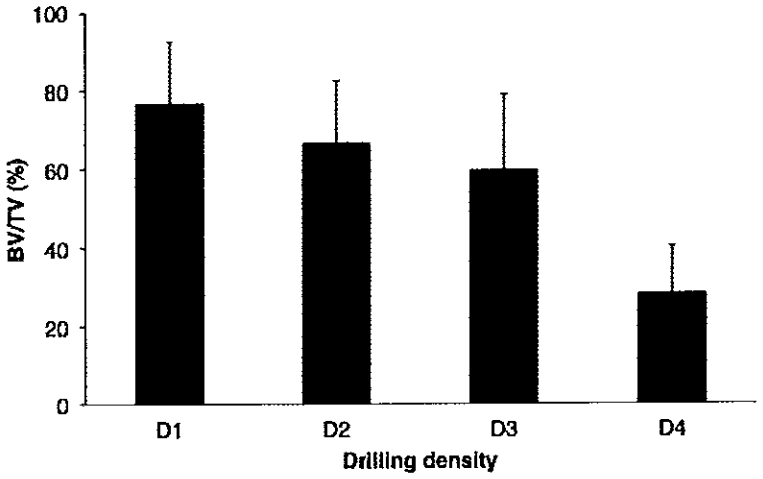

Figure 2: Histomorphometric parameter, mean percentage of bone volume (BV/TV) in different hand assessed scoring

Discussion

In our study, human jawbone could be classified into four density groups (D1–D4) in all regions of the jaws based on descriptive morphology and clinician tactile analog. The most common locations for this type of bone are the posterior region of the maxilla. It is rarely observed in mandible. The bone trabeculae may be up to 10 times weaker than the cortical bone of D1. In addition, the bone-implant contact after initial loading is often less than 25% as they are sparse bone, initial fixation of any implant design presents a surgical challenge. Implant failure after initial loading reported the highest score in this type. Moreover, it takes the longest

time to integrate with the implant after placement. Bone grafting or expansion is often required for improving initial implant fixation as well as incremental loading of the implants over time was suggested to improve stability in this type.

In accordance to the bone classification proposed by Lekholm and Zarb, Type I indicated a homogeneous cortical bone with small marrow cavity while Type III or Type IV are more heterogeneous, which is mostly composed of the trabeculae portion with a thin layer cortical bone. Thus, the correlations found between BS/BV, Tb.Th, Tb.Sp and the Lekholm and Zarb classification may be not be harmonized. Pereira and colleagues studied a histomorphometrical analysis correlated with the Lekholm and Zarb classification in bone biopsy from 32 implant sites. At least two fields in average were analyzed in each bone specimen. Total bone length, bone area and total tissue (soft and hard) area were measured. The results demonstrated that Type I or II were associated with lower BS/BV, higher Tb.Th, and lower Tb.Sp while Type III and IV were associated with lower Tb.Th, higher Tb.Sp, and BS/BV (14). Nevertheless, the positive correlation between the Lekholm and Zarb classification and histomorphometric parameter was found significantly only when compared with BV/TV and Tb.Sp. It suggested that a single variable should not be solely used to define a type of bone. Another study was done in comparison among anatomical quadrants of the jawbone. The posterior maxilla was reported to be the lowest trabecular volume with thinner thickness and lower trabecular number (Tb.N) when compared with other quadrants (15).

In the present study, a large range of deviation demonstrated an overlap between Type D2 and D3 under the Misch classification. This could imply that this classification may not be practical to indicate actual bone density and may not be valid for all four types of bone. Besides the histomorphometric aspects, biological or molecular events also involve bone metabolism which may influence the bone microarchitecture. In this context, bone remodeling process

occurs at discrete sites on cortical and trabecular bone surfaces, and involves sequential actions of osteoclasts and osteoblasts in normal and pathological bone (16).

In conclusion, accurate and thorough measurement of the jawbone density is crucial information to support clinician decision regarding patient selection, implant shape/structure, and surgical technique used. Although many techniques commonly used to determine alveolar bone quality, based on this study, the correlation between radiographic techniques and bone type (L&Z) is the most reliable for evaluating alveolar bone type.

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Reference and Techniques used in Alveolar Bone Classification

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Abstract

The marginal bone loss that usually observed around dental implant has been well documented and expected. It has related with self-reaction to the forensic body of each patient as well as the osseointegrated interface. Bone quantity and quality of the implant site may help to define the implant-bone interface, which in turn affects primary stability of the immediate implant placement. Analysis of bone quality prior to surgery provides vital information during treatment planning for dental implant. Additionally, it helps in predicting postsurgical success. The classification of bone quality, however, is difficult to follow clinically, as tactile assessments are subject to the variation among surgeons. Although imaging techniques, such as computed tomography (CT) or cone bean computed tomography (CBCT), are useful to determine bone quality, the exposure to radiation and its precision, are still of concern. This paper reviews common techniques and reference used in dental bone classification as well as the recent reports from histomorphometric analysis and molecular components. It is well acknowledged that clinical awareness of evaluating the amounts of bone surrounding the implant site by appropriate method is critical for a successful outcome.

Keywords: Alveolar ridge augmentation; Bone formation; Osteointegration; Histomorphometry; Practice guideline

Introduction

The bony structure of human jawbone is irregular in shape and size due to a non-uniform modeling during embryogenesis and early life. The mandible shows a thicker cortical and denser trabecular bone compared with the maxilla while the trabecular in posterior parts in both arch are recognized to have lower density and thickness [1-3]. The prospective clinical studies reviewed that total failure rate of implant stability is associated with bone quality surrounding the implant site [4,5]. These observations emphasize the promise of bone structural analysis in each dental implant surgery, especially in the thinner maxilla, wherein primary stability may be challenging to achieve. Despite studies in the past decades, integration of various tools used in bone type classification remained unclear. Up to now, dental technology and surgical guidelines point to a need for knowledge on bone quality and mechanical behaviour of the bone [6-8]. The stability of implant anchoring in bone crucially ensures implant success and therefore local bone quality and quantity are factors to be considered in routine assessment. The purpose of this review was to elaborate the classification system as well as techniques used in qualification of alveolar bone ridge.

Basic information about bone density in human jawbones

The position of the mandibular and maxillary teeth is commonly divergent to the position of the basal bone. The alveolar bone and basal ridge are varied from each other as more evidence has been shown in the different resorbing patterns following tooth loss [9,10]. The mandible is the largest and strongest bone of the face because of the reception of the lower teeth. It consists of a curved, horizontal portion, the body and two perpendicular portions. Using a computed tomography (CT) scan, Hounsfield unit (HU) value was collected from 4 quadrants of the mouth of over hundreds edentulous sites, both genders with age range of 18 to 89. Based on the number of HU, it has been shown that the densest region is in the anterior mandible>anterior maxilla>posterior maxilla>posterior mandible, respectively [11]. The differences of density in each zone are associated with anchorage loss and clinical situations such as anterior and posterior retraction or molar distalization. These problems brought up a number of studies in trabecular and cortical density in particular sites—i.e. incisor, canine, buccal and palatal premolar, and molar areas. Only within mandible, the buccal cortical bone at incisor demonstrated the lowest density while retromolar shows the highest density. Moreover, the rate of failure of screw implants was reported high in posterior zone of mandible. It was possibly due to excessive heat generation from the dense cortical bone within the area [12].

Using a radiographic scale to determine bone quality, the two highest density of alveolar and basal buccal cortical in maxilla were located in canine and premolar sites whereas incisor and premolar sites caught a similar range of density. Notably, the maxillary retromolar in all regions were categorized only into type III or IV by Misch's classification, which implied that great anchorage loss could be expected in the maxillary posterior teeth. Furthermore, the density of cancellous bone in maxilla is comparable to that of the mandible (incisor, premolar, and molar) except the retromolar and canine which were reported to be of lowest density of all sites in maxilla bone [13].

Reference used in alveolar bone classification

Lekholm & Zarb (1985): The oldest and most frequently used reference in bone classification system is proposed by Lekholm and Zarb (L&Z) [14], which is based on conventional radiograph and histological component [6,7,15]. The classification of each bone type is

described in schematic images as presented in Table (Figure 1). There have been many studies that attempted to relate the Lekholm and Zarb classification with their parameters and techniques [16]. It is still unclear in the radiographic assessment whether Lekholm and Zarb's study was conducted during surgery or prior to surgery. Furthermore, the overall accuracy of this classification reported a low percentage (<50%) when compared with a plain radiography with and without reference image from a trabecular bone morphology. The lowest accuracy percentage (28%) was found when the classification was applied with a sparse trabeculation of mandible [2,17]. All of mentioned above, it is plausible that trabecular volume seen in human mandible was different from L&Z schematic drawing images, suggesting that this feature may not be relevant to human jawbone which is well composed of dense/sparse trabeculations [18].

Reference	Tool used in classification	Type of bone	Images
Lekholm & Zarb (1985)	Plain radiography, Morphology	Type 1: Homogeneous cortical bone	
		Type 2: Thick cortical bone with marrow cavity	
		Type 3: Thin cortical bone with dense trabecular bone of good strength	
		Type 4: Very thin cortical bone with low density trabecular bone of poor strength	
Misch (1990, 1993)	Clinician perception (Hand feel resistance)	D1 = Oak wood	
		D2 = Pine wood	
		D3 = Balsa wood	
		D4 = Styrofoam	

Figure 1: Table representing techniques and reference used in bone classification in dentistry.

Misch (1990- 2008): Following the Misch classification, bone type was defined according to four density groups (D1–D4) in all regions of the jaws based on descriptive morphology and clinician tactile analog (Figure 2). Lately, these data were used to compare with anatomical location and radiographic scale [19].

Classification System	Tool used in classification	Type of bone	Images
Modified UCLA classification, 2008	Clinical Observation (Bone shape and volume)	Type 1: Sufficient alveolar shape for implants	
		Type 2: Insufficient alveolar bone volume on the buccal side	
		Type 3: Knife edge shape with sufficient alveolar bone height	
		Type 4: Insufficient alveolar bone height	

Figure 2: Table representing edentulous bone ridge classification followed three-dimensional (3D) quantity of alveolar bone shape and volume.

D1 bone type represents a homogenous dense cortical, mostly found in anterior mandibles with moderate bone resorption [20]. The cortical lamellar bone in D1 type mostly contributes to the density. This induces healing with little interim woven bone formation, and ensures excellent primary stability next to the implant. In addition, the post-surgical strongest osseointegration in this type is derived from high bone-implant contact. However, the fewer blood vessels and heat generation at the apical portion of the D1 bone may cause some problems with insufficient nutritional supply and delayed bone healing. D2 is a combination of dense-to-porous cortical bone on the crest and trabecular bone from 40% to 60% on the inside, most frequently in the anterior mandible, followed by the posterior mandible. This bone type provides an excellent implant interface healing, and predictable osseointegration.Fair blood supply within the bone tissue allows bleeding during the osteotomy, which in turn is very useful in reducing overheating during surgical bed preparation. D3 is composed of thinner porous cortical bone on the crest and fine trabecular bone within the ridge. The inside portions of the trabeculae are found lesser than 50%. D3 bone is frequently found in the anterior maxilla and posterior regions of the mouth in either arch. However, the D3 anterior maxilla is usually of narrower ridge than its mandibular D3. Not only weaker than D2 bone, the bone-implant contact is also less favourable in D3 bone which cause a higher risk of implant failure. D4 bone has the least trabecular density with little or no cortical crestal bone. It is the opposite structure of D1 (dense cortical bone). The most common locations for this type of bone are the posterior region of the maxilla. It is rarely observed in mandible. The bone trabeculae may be up to 10 times weaker than the cortical bone of D1. In addition, the bone-implant contact after initial loading is often less than 25% as they are sparse bone, initial fixation of any implant design presents a surgical challenge. Implant failure after initial loading reported the highest score in this type. Moreover, it takes the longest time to integrate with the implant after placement. Bone grafting or expansion is often required for improving initial implant fixation as well as incremental loading of the implants over time was suggested to improve stability in this type.

University of California Los Angeles (UCLA) classification: University of California Los Angeles (UCLA) defined a classification of edentulous alveolar bone according to bone volume and shape in three dimensions. The bone volume in the horizontal and vertical dimensions was assessed by clinician observation during the implant placement in the ideal restorative driven position. By degree of deficient ridge volume in apical, horizontal patterns, there were characterized up to 8 classes [21]. Recently, this classification was modified and regrouped into four types, as shown in Figure 2. Type I is a case with sufficient bone in horizontal and vertical dimensions, making it ideal for implant placement. Type II is a case with insufficient bone volume on the buccal side. Type III is a case with knife-shaped like alveolar bone or major deficiency bone volume on the buccal side, but with sufficient heights. Type IV is a case with insufficient alveolar heights and width with all sides of implant, are exposed. Type IV is a complete opposite of Type I in this category [21].

Integration of techniques used in bone quality measurement

The tactile assessment: The tactile assessments are the noninvasive method for evaluating bone quality. They are the most applicable, yet subjective for drilling resistance assessment. These methods are recorded during the surgery or after surgery (i.e, insertion torque, periotest). It has to be taken in early perception that the clinician

experience and implant shape/structure reflecting drilling assessment in each study will be involved in the assessment and outcome. For example, insertion torques assessment, electronic devices offer a computerized programme to simultaneously read resistance torque value for evaluating bone density. To avoid bias during the test, it in genuinely suggested that a single experienced operator should carry out all instrumentations and drill set should be changed every 10 or lesser osteotomy. The insertion torques value is normally recorded every quarter turn as measured by subjective tactile perception during implant preparation. There are many possible scenarios which can occur. For instance, pre-maximum torque and the number of supra-alveolar threads are obtained before complete insertion by hand wrench. On the other hand, full insertion may be completed while the pre-maximum torque is also reached. Lastly, full insertion is completed before pre-maximum torque is reached, thus, the tapered implant may be replaced and remeasured in this scenario [19,22]. The retrospective clinical study determined a strong correlation between the insertion torques values (ISV) and implant stability quotient (ISQ), as well as the mean bone density ($P<0.001$) from over a hundred implants in forty-two volunteers [23]. Furthermore, the correlation between the insertion resistance values and micro CT parameters such as Trabecular number (TbN), Bone volume/Tissue volume (BV/TV), Trabecular bone Pattern factor (TbPf), was reported. In addition, the highest correlation was found between the ISV and bone density variables such as BV and BV/TV. However, ISQ and bone density either from living bone or cadaveric bone did not show any correlation [24,25].

The radiography scale, Hounsfield unit (HU)

Computerized tomography (CT) is a well-established method to evaluate bone density and providing quantitative data for trabecular and cortical bone. In dentistry, CT scan was introduced for pre-operative assessment of dental implant candidates [26]. As a result of three-dimensional anatomic image, it provides a visual set of images in the mesiodistal, buccolingual, and superioinferior dimensions together within jawbone. This technology allows clinicians determine alveolar thickness surrounding the implant bed. The surgical acrylic stents may be a useful tool to apply prior to the CT scan to set accuracy [27]. The scan produces axial images perpendicular to the long axis of the body which creates the images. Axial image has 260,000 pixels and the CT number unit, as defined by the Hounsfield unit (HU), relate to the density within the pixel. HU scale provides a quantitative assessment of bone density as measured by its ability to intensify an x-ray beam. The linear scale can be set into two points from dry air (minus 1000 HU) to pure water (0 HU). Several studies investigated bone density in a living bone or cadaver cortical bone, which showed a scale range of 1000 to 1600 [28]. One of its limitations is the ultimate scale of samples since they are mostly varied from the type of specimens, instrument model, settings, and investigator experience. Data obtained from the CT scan was used to incorporate with the Misch classification [29]. A mean difference of 180 HU at least, was required for clinical call in order to discriminate one level of radiographic density among other levels [30].

Cone beam computed tomography (CBCT) is determined by the individual volume elements or voxels and the dimensions primarily depend on the pixel size of the investigated area. The resolution of the directing area is given in submillimeter for bone quality assessment for dental implants [27,31]. The 3D cubic block of data known as "voxel" represent a degree of x-ray absorption. Voxel values obtained from CBCT images are not absolute values, like the HU values obtained from CT. CBCT generates cone-shaped beams and the images are acquired in one rotation by an image intensifier of flat panel detector [31-33]. The area of interest can be accessed from several different viewpoints as well as from three-dimensional views. During the rotation between 150 and 600 planar projection images sequential of the field of view (FOV) are acquired in a complete or partial arch. The most widely used technique in medical CBCT is maximum intensity projection (MIP) because of its simplicity and user-friendly steps. This technique provides an indication of the maximum available bucco-lingual width (axial view) which is therefore a very useful imaging for implant. CBCT is easily accessed, with proper cost to the patient, and are associated with low levels of radiation dosage. It was reported that the radiation dosages is 15 times lesser than that of conventional CT scans. The dramatically low radiation exposure is preferable to patients who have underlying disorders, as it is thought to be the most important advantage of using CBCT instead of CT. It is important to note that although computer aided implant placement is a common technique; the linear and angular deviation due to arch position can be a major concern for interpretation [33,34].

The subjective hand-felt resistance during forty-two implant surgeries were recorded for comparing with the HU value obtained from CBCT and CT. The mean HU from CBCT shows proportional higher value to those from CT. Bone densities are graded among all bone types (Figure 3). Although the mean HU density from bone Type D2 and D3 obtained from CT are a bit clearer than that from CBCT, the difficulty for distinguishing between D2 and D3 remains in both analyses. This overlap was also reported when it was associated with surgeon hand resistance assessment during bone drilling [35].

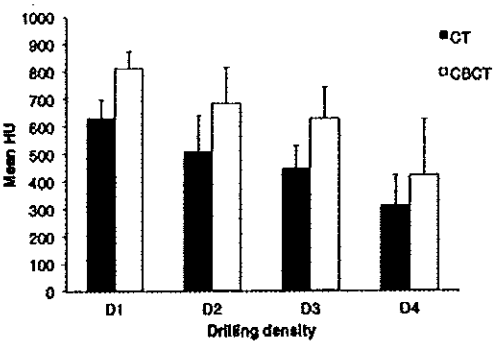

Figure 3: Correlation of drilling density with CT and CBCT analysis: Hounsfield unit (HU); D1 >1250; D2: 850-1250; D3: 350-850; D4:150-350; D5 <150.

Histomorphometrical analysis

Bone histomorphometry has been inferred as a gold standard method for the evaluation of bone microarchitecture, as it allows two-dimensional analysis, inferred estimation of the spatial organisation of the trabecular net configuration measured from a set of histological sections [36]. Histomorphometric parameters were obtained using the formulas proposed by Parfitt et.al 1983 to explain bone compositions [37,38]. In accordance to the bone classification proposed by Lekholm

and Zarb, Type I indicated a homogeneous cortical bone with small marrow cavity while Type III or Type IV are more heterogeneous, mostly composed of the trabeculae portion with a thin layer cortical bone. Thus, the correlations found between BS/BV, Tb.Th, Tb.Sp and the Lekholm and Zarb classification may be not be harmonized. Pereira and colleagues studied a histomorphometrical analysis correlated with the Lekholm and Zarb classification in bone biopsy from 32 implant sites. At least two fields in average were analyzed in each bone specimen. Total bone length, bone area and total tissue (soft and hard) area were measured. The results demonstrated that Type I or II were associated with lower BS/BV, higher Tb.Th, and lower Tb.Sp while Type III and IV were associated with lower Tb.Th, higher Tb.Sp, and BS/BV [39]. Nevertheless, the positive correlation between the Lekholm and Zarb classification and histomorphometric parameter was found significantly only when compared with BV/TV and Tb.Sp. It suggested that a single variable should not be solely used to define a type of bone. Another study was done in comparison among anatomical quadrants of the jawbone. The posterior maxilla was reported to be the lowest trabecular volume with thinner thickness and lower trabecular number (Tb.N) when compared with other quadrants [40]. Mean percentage of histomorphometric bone volume (BV/TV) from biopsies was used to correlate with clinical scoring based on the Misch classification, as shown in Figure 4 [35].

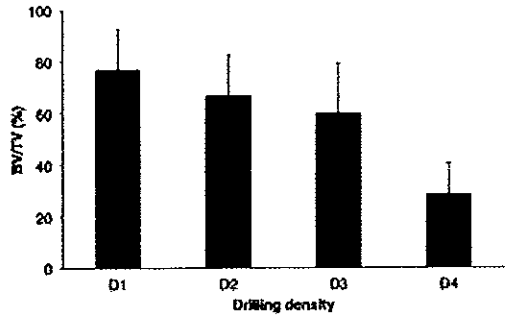

Figure 4: Histomorphometric parameter, mean percentage of bone volume (BV/TV) in different hand assessed scoring.

A large range of deviation demonstrated an overlap between Type D2 and D3 under the Misch classification. This could imply that this classification may not be practical to indicate actual bone density and may not be valid for all four types of bone. Besides the histomorphometric aspects, biological or molecular events also involve bone metabolism which may influence the bone microarchitecture. In this context, bone remodeling process occurs at discrete sites on cortical and trabecular bone surfaces, and involves sequential actions of osteoclasts and osteoblasts in normal and pathological bone [41].

Molecular parameters and immunohistochemistry

Bone multicellular unit integrating in bone remodelling mechanism is regulated by a crosstalk between osteoblasts and osteoclasts. A crucial regulating pathway involves balance of receptor activated nuclear factor κB (RANK), Receptor activated nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG) [41]. These proteins as well as their mRNA expressions were used in the validation of the classification of the bone type. A recent study reported the correlation between clinical-radiographic aspects and molecular parameters of endosseous specimen obtained from implant site in healthy population. RANKL, OPG, and cathepsin K (a cysteine protease expressed in osteoclast) and osteocalcin (a noncollagenous protein mostly found in osteoblasts and odontoblasts) were measured from protein expressions to the level of gene expression in human jawbone specimen. A correlation between the histomorphometric parameters and specific cellular/molecular variables was also observed. The combination of bone relative parameters such as osteocalcin-positive osteocyte density (mm^2), OPG-positive osteocyte density, OPG-positive osteoblast density, and RANKL-positive osteoblast, demonstrated statistical difference relating to the Lekholm and Zarb classification. However, none of these parameters alone could be used to distinguish the four types of bone quality [39]. It is worth to note that the Lekholm and Zarb classification of each bone type in this study were used and given by two surgical evaluations during the osteotomy, thus the possibility of inter-observations error could occur.

Conclusion and Future Prospect

Accurate and thorough measurement of the jawbone density is crucial information to support clinician decision regarding patient selection, implant shape/structure, and surgical technique used. Although many techniques commonly used to determine alveolar bone quality, based on this review, the correlation between radiographic techniques and bone type (L&Z) is the most reliable for evaluating alveolar bone type. Studies on a component of multicellar unit of bone and osteogenic genes or growth regulating factors are increasing [42-44], however, there is no single bone remodeling marker can be used for representing the quality of bone type. It suggested that further studies in molecular analyses associated with alveolar bone quality are required in this field.

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Conflict of Interest

Authors declare no conflict of interest.

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Abstract: Prolactin (PRL) is a hormone involved in the coordination of maternal, extraembryonic, and fetal tissues that remains in high levels during the entire duration of pregnancy. Despite systemic alterations during pregnancy such as hormonal changes which have been associated with periodontitis and tooth loss, PRL function in human periodontal ligament fibroblast (hPDLF) has never been studied. Therefore, the objective of this study was to investigate the role of PRL in the regulation of osteogenesis in hPDLF in conditions that mimicked the pregnant period.
Materials and Methods: HPDLF were cultured in Dulbecco's modified Eagle's medium (DMEM) with supplements, together with a variety of concentrations of PRL that mimicked non-pregnant (10 ng/ml), pregnant period (100 ng/ml), and pathological hyperprolactinemic conditions (600 and 1000 ng/ml). Periostin, Runx2, collagen type I, BMP2, and Sox9 mRNA levels were investigated after the incubation of PRL for 48 h. The osteogenic and chondrogenic characteristics such as mineral nodule formation and proteoglycan accumulation were also examined after long-term incubation with PRL.
Results: The present study found both short and long isoforms of PRLR and its protein expression in hPDLF and hPDL cells that were extracted from orthodontic purpose. The incubation of PRL only at the pathological levels (600 and 1000 ng/ml) modestly decreased hPDLF number. In contrast, PRL at the non-reproductive level (10 ng/ml) and pregnant level (100 ng/ml) significantly upregulated markers of osteogenesis such as Runx2, Bmp2, and periostin but not Sox9. Mineral nodule formation was induced while proteoglycan accumulation was reduced by PRL at the pregnant level suggesting that hPDLF was differentiated toward the preosteoblastic cells.
Conclusion: The present study demonstrated the expression of hPRLR in hPDLF, proposing direct actions of PRL in hPDL and periodontal surrounding tissues. The physiological levels of PRL induced hPDLF differentiation and stimulated osteogenic characteristics supporting the hypothesis that PRL could drive hPDLF to undergo osteogenesis in a high calcium requirement such as during the pregnant and lactating periods.

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Cover Letter
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February 4, 2015

Editor-in-Chief
Archives of Oral Biology

Dear Editor:

Enclosed for your consideration is an original research article, not under publication elsewhere and has been read and approved by all authors, entitled " Osteogenic induction of Prolactin in Human Periodontal Ligament Fibroblasts "

This study was to investigate the presence of PRL receptor (PRLR) and role of PRL in the regulation of osteogenesis in hPDL. The major finding of this study provides the first proof that PRL at level mimicking pregnancy and lactation, decreased hPDL viability while inducing differentiation toward the osteoblast-like cell rather than the chondrogenic characteristic. PRL receptor (PRLR) was expressed in both long and short isoforms in hPDL and primary human PDL which suggests the signaling transduction of PRL in these cells. In our view, this finding suggests a plausible role for PRL to regulate hPDL and other ectomesenchymal cells such as dental pulps, gingival fibroblast, odontoblast or cementoblast.

The results provide new and significant information that high level of circulating PRL during pregnancy, drives the PDL to undergoes osteoblastic differentiation supporting remodeling of alveolar bone and tooth in calcium demanding period. I truly believe this study is well suited for your journal and will contribute valuable new data regarding related physiological alteration.

Sincerely yours,

Dr. Dutmanee Seriwatanachal

e-mail: dutmanee.ser@mahidol.ac.th

*Conflict of Interest Form

Journal: Archives of Oral Biology	**Author name**: Rudee Surarit

Declarations

The following additional information is required for submission. Please note that failure to respond to these questions/statements will mean your submission will be returned to you. If you have nothing to declare in any of these categories then this should be stated.

Please state any conflict of interests. A conflict of interest exists when an author or the author's institution has financial or personal relationships with other people or organisations that inappropriately influence (bias) his or her actions. Financial relationships are easily identifiable, but conflicts can also occur because of personal relationships, academic competition, or intellectual passion. A conflict can be actual or potential, and full disclosure to The Editor is the safest course.

The authors declare no conflict of interests.

Please state any sources of funding for your research

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Please state whether Ethical Approval was given, by whom and the relevant Judgement's reference number

Not applicable

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Not applicable

Abstract:

Background and Objective: Prolactin (PRL) is a hormone involved in the coordination of maternal, extraembryonic, and fetal tissues that remains in high levels during the entire duration of pregnancy. Despite systemic alterations during pregnancy such as hormonal changes which have been associated with periodontitis and tooth loss, PRL function in human periodontal ligament fibroblast (hPDLF) has never been studied. Therefore, the objective of this study was to investigate the role of PRL in the regulation of osteogenesis in hPDLF in conditions that mimicked the pregnant period.

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proteoglycan accumulation was reduced by PRL at the pregnant level suggesting that hPDLF was differentiated toward the preosteoblastic cells.

Conclusion: The present study demonstrated the expression of hPRLR in hPDLF, proposing direct actions of PRL in hPDL and periodontal surrounding tissues. The physiological levels of PRL induced hPDLF differentiation and stimulated osteogenic characteristics supporting the hypothesis that PRL could drive hPDLF to undergo osteogenesis in a high calcium requirement such as during the pregnant and lactating periods.

Keywords: woman oral hygiene, pregnancy, periodontitis, mineralization, bone formation, calcium homeostasis

Highlight: 4 bullets

Title: Osteogenic induction of Prolactin in Human Periodontal Ligament Fibroblasts

- Short and long isoforms Prolactin (PRL) receptor are expressed in Human PDL.
- 600 or 1000 ng/mL PRL (lactating level) markedly decreased hPDL number.
- Osteogenic characteristics were induced in hPDL exposed-high conc. PRL.
- PRL may drive hPDL into osteoblastic differentiation in support of bone remodeling.

- Title page -

Title: Osteogenic Induction of Prolactin in Human Periodontal Ligament Fibroblasts

Short title: Prolactin and hPDL mineralization

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INTRODUCTION

Maternal periodontal disease is associated with several adverse pregnancy outcomes including preterm birth, preeclampsia, low birth weight, and late miscarriage (1-4). A few studies have reported systemic alterations in pregnancy such as chronic inflammation or hormonal changes correlates with moderate to severe periodontitis (5, 6). The ultimate goal of periodontal therapy is to regenerate the periodontal tissues that are damaged due to chronic inflammation. During regeneration, periodontal tissues migrate into the affected area, attach to the root surface, and proliferate and differentiate into the cementum, alveolar bone, and periodontal ligament to reconstitute the lost part (7). The stemness property of the periodontal ligament (PDL) highlighted this connective tissue, which serves to anchor the tooth to the alveolar bone (8-10).

During pregnancy and lactation, hyperprolactinemia, one of the physiological adaptive mechanisms, endures a maternal calcium loss for fetal skeletal development and milk production. Effects of hyperprolactinemia on the bone have been well documented in patients and experimental animals (11-13). Despite a variety of PRL actions in other tissues (14, 15), PRL action(s) involved in the oral ecology has not been well understood. It is possible that elevated plasma PRL levels during pregnancy and lactation (≥100 ng/mL; normal non-pregnant levels ~7–10 ng/mL) could induce human periodontal ligament fibroblast (hPDLF) differentiation in order to prevent the destruction of periodontal tissues from insufficient calcium homeostasis during pregnancy and perhaps induce tooth loss. Furthermore, none of the previous studies attempted to investigate the expression of PRL receptor (PRLR) as well as its

effect on hPDLF. Therefore, the objective of this study was to investigate the role of PRL in hPDLF proliferation and differentiation in the physiological hyperprolactinemia in conditions that mimicked pregnancy.

METERIALS & METHODS

Extraction of Human PDL Cells

Primary human PDL cells were obtained by scraping the tissue from the middle-one-third of the root of premolar or molar extracted teeth in orthodontic purpose, as described previously (16). Briefly, the tissue was placed in a sterile Petri dish, and cut into small pieces in DMEM, supplemented with 10% FBS, 0.25 ug/mL amphotericin B (HyClone Laboratories, Inc., UT, USA), 100 U/mL penicillin G (HyClone Laboratories), and 100 ug/mL streptomycin (HyClone Laboratories). After washing three times, the tissues were explanted and were cultured in 25-cm^2 T flasks with supplemented DMEM. Cells were incubated at 37°C with 5% CO_2 and subcultured every 3 days. The cells obtained from passage 4-6 were used in this study. This study was approved in accordance with the principles and guidelines of the Mahidol University Central Institutional Review Board, (No. MU-CIRB 2007/086), Bangkok, Thailand

Cell Culture

HPDLF (ScienceCell, California, USA); 1 X 10^5 cells/cm^2 were cultured in a maintenance medium containing Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), supplemented with 5% fetal bovine

serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Grand Island, NY, USA). Recombinant human PRL (R&D Systems, Inc., MN, USA) was reconstituted in BSA and HCl following the manufacturer's instruction before being diluted in the medium. Medium freshly supplemented with and without PRL was replaced every 2–3 days.

Osteogenic Induction of hPDLF

HPDLF were then placed in 12-well plates (Corning, NY, USA), each well for 16 h before adding osteogenic induction medium [maintenance medium plus 100 nmol/L dexamethasone (Sigma), 50 mol/L L–ascorbic acid 2-phosphate (Sigma), and 10 mmol/L ß–glycerophosphate (Sigma)]. All cultures were maintained at 37°C in a humidified 5% CO_2 incubator. The specific conditions of the medium were changed every other day according to the experiment.

Cell Viability Assay

HPDLF were propagated in a 96-well culture plate (3000 cells/well). After being incubated with vehicle (1 mg/mL bovine serum albumin in 0.004 N HCl; control group), 10 or 100 ng/mL recombinant human PRL (catalog no. 682-PL; >97% purity, R&D Systems) for 24 and 48 h, culture media were replaced with 3-(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma). After 3-h MTT incubation at 37°C, the absorbance of each well was spectrometrically determined at 540 nm by a microplate reader (model: Epoch; Biotek, Vermont, USA) as described previously. The absorbance of control wells was normalized to 1. The relative values of PRL-exposed cells were presented as fold change compared to the control group.

Immunocytochemistry

HPDLF were cultured on coverslips (50,000 cells/well in 6-well plate) in maintaining medium for 24 h, and fixed in cold 4% paraformaldehyde/PBS. Non-specific binding sites were blocked by 10% FBS for 30 min at room temperature. Thereafter, samples were incubated at 4°C overnight with 1:100 polyclonal rabbit anti-mouse PRLR primary antibody (catalog no. sc-20992; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and then with 1:200 diluted Alexa Fluor 488 conjugated anti-rabbit IgG antibody as a secondary antibody for 1 h at room temperature. Anti-PRLR was digitally captured by using inverted fluorescence microscopy (Zeiss, Germany; Axio observer Z1). Regarding the negative control slides, cells were incubated without primary antibody.

Proteoglycan Assay

HPDLF were cultured in vehicle, 10 or 100 ng/mL PRL-containing differentiation media for 7, 15, and 28 days. Cells were washed twice with PBS and fixed overnight in cold 4% paraformaldehyde. The proteoglycan accumulation was stained on days 15 and 28, respectively. They were then incubated with 1% alcian blue (Sigma) in 5% acetic acid for 2 h to stain proteoglycans. After being destained with 6 mol/L guanidine–HCl, optical density was determined at 650 nm (alcian blue) by standard spectrophotometric method.

Mineralizing Nodule Formation

HPDLF were cultured in vehicle, 10 or 100 ng/mL PRL-containing differentiation media for 7, 15, and 21 days. The 1 mg/mL of alizarin red S solution (Sigma) in distilled water adjusted to pH 5.5, was used to stain mineralizing nodules. After being destained with 5% perchloric acid, the optical density was determined at 540 nm by standard spectrophotometric method as previously described (17).

Fat droplet staining

HPDLF were cultured in vehicle, 10 or 100 ng/mL PRL-containing differentiation media for 7, 15, and 28 days. Cells were washed twice with PBS and fixed overnight in cold 4% paraformaldehyde. Medium was refreshed at every other day. At day 15 and 28 of differentiation, the cells were stained with oil red O to evaluate adipogenesis.

RNA Extraction and mRNA Expression

To investigate the osteogenic action of PRL in hPDLF, cells were cultured in vehicle, 10 or 100 ng/mL PRL-containing differentiation media for 48 h. Thereafter, total RNA was extracted and quantified. One microgram of total RNA was reverse-transcribed by iScript kit (Bio-rad, Hercules, CA, USA). One-microliter aliquots were used for cDNA amplification by MiniOpticon real-time PCR system (Bio-rad) with KAPA SYBR® FAST qPCR (Kapabiosystems, Massachusetts, USA). Amplicon sequencing was performed by the ABI Prism 7900 Sequence and Detection system (Applied Biosystems, Foster City, CA, USA) to detect the alteration of Runx2, BMP2, Periostin and Sox9 mRNA, as described (17, 18).

For detecting endogenous PRLR mRNA expression, hPDLF were propagated in maintaining the medium for 48 h. One microgram of total RNA was extracted and reverse-transcribed by iScript kit (Bio-rad, Hercules, CA, USA). Two-microliter aliquots were used for cDNA amplification by PCR Thermocycler (Model: TProfessional; Biometra, Goettingen, Germany) with Maxima® SYBR Green qPCR Master Mixes (Thermo Scientific, Massachusetts, USA). Sense and antisense primers for all isoform of human PRLR (hPRLR) and GAPDH were specifically designed as described previously (11). The PCR products were visualized on a 2% agarose gel stained with 1.0 µg/mL ethidium bromide under a trans-UV system (model Quantity One 2000; Bio-Rad, Hercules, CA, USA). GAPDH served as a control gene to check the consistency of the reverse transcription and to normalize values between samples.

Statistical Analysis

Results are expressed as mean ± SE. Unless otherwise specified, multiple comparisons were performed by one-way analysis of variance followed by Dunnett's post-test. The level of significance was $p<0.05$. All data were analyzed by GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

PRLR Expression in Human PDL

PRLR expression was first investigated in hPDLF cells and human PDL extracted from orthodontic purpose, to confirm that this cell type could directly

respond to PRL. As depicted in Fig. 1, extracted primary human PDL and hPDLF abundantly expressed PRLR proteins as determined by immunocytochemistry and confocal microscope. The negative control slides (no primary antibody) showed no positive signal (Fig 1B, 1D). Moreover, through an analysis of the PRLR amplicon, hPDLF were shown to express mRNAs of short and long isoforms of hPRLR with a predominant short isoform (Fig. 1E), with consistent gene control, GAPDH. These receptor expressions confirmed that hPDLF were targets of PRL.

Viability of PRL-exposed HPDLF

MTTs were used to determine viability and proliferation of PRL-exposed hPDLF. Exposure to 10 and 100 ng/mL PRL for 24 h did not alter the cell viability while 600 or 1000 ng/mL PRL markedly decreased it (Fig. 2A) compared with the control group. These results indicated that hPDLF responded to PRL in similar action to that reported in human osteoblasts and chondrocytes (17, 18). At 48 h exposure of 100, 600 or 1000 ng/mL, PRL further decreased cell proliferation as shown in Fig. 2B.

Osteogenic Induction in PRL-exposed HPDLF

Since human PDL were reported to comprise stemness property and contain some markers specific for cementoblast, adipocyte or osteoblast (10, 19). In the present study, we investigated the expression of genes responsible for mesenchymal cell differentiation, i.e., Collagen I, Runx2, BMP2, and Sox9. These genes were examined in early differentiation, day 2, according to the entering phase of hPDLF to multipotential mesenchymal lineages (20, 21).

After 2 days of exposure to 10 and 100 ng/mL PRL, periostin a gene predominantly expressed specifically in differentiated PDL tissue was significantly upregulated (Fig. 3A). This result indicated that PRL has direct action on hPDLF differentiation. Moreover, after exposure to 10 and 100 ng/mL PRL, PRL markedly upregulated mRNA expression of Runx2 and BMP2 in HPDLF (Fig. 3B, 3C), while there was no alternation in Sox9 and Collagen I (Fig. 3D, 3E).

Mineralizing Nodule Formation in PRL-exposed hPDLF

HPDLF were differentiated and thereafter were able to form a mineralizing nodule under an osteogenic inducing condition. In this study, the stemness property of hPDLF was confirmed by mineralizing nodule assay as shown Fig. 4A. HPDLF were able to be induced to undergo osteoblast-like cell after culturing in osteogenic inducing medium for 14 and 28 days in this study. The mineral nodule was visualized in hPDLF cultured with osteogenic inducing medium while there was none in those cultured with maintaining medium. The size of the nodules was visually greater in hPDLF exposed with PRL (Fig. 4A). After exposure to 10 and 100 ng/mL PRL for 28 days, mineralizing nodule formation was significantly increased when compared to the corresponding control group (Fig. 4B). On the other hand, we found a reduction of proteoglycan accumulation in PRL-treated hPDLF when compared to that in the corresponding control (Fig 5). The result of oil red O staining showed absence of lipid droplets in differentiated hPDLF (data not shown).

DISCUSSION

The important function of PDL is involved in maintaining the integrity of the periodontium, and in promoting periodontal regeneration. PDL consists of stemness property with heterogeneous cell populations such as fibroblastic, cementoblastic, and osteoblastic characteristics. The factors influencing PDL engineering in periodontal remodeling and regeneration processes in root resorption were reported in many studies (7, 19, 22, 23). However, a number of studies in periodontal regeneration in pregnancy, especially in human oral tissues are still limited. In pregnant rodents, it was reported that the regeneration of PDL and osteoclast mediated alveolar bone resorption were affected by hormonal adaptation in pregnancy during orthodontic tooth movement (24, 25).

During pregnancy and lactation, the mother provides an accelerated bone turnover via bone demineralization and resorption, in order to rapidly supply calcium for fetal growth and milk production (26, 27). PRL has been reported as an important calciotropic hormone that provides additional calcium for fetal growth and milk production during pregnancy and lactation, respectively (28). Our previous studies provided evidence that high physiological levels of PRL (~100 ng/mL) could directly regulate the osteoblast functions with a consequent increase in bone turnover. The increase of RANKL/OPG ratio explained the clinical finding of the hyperprolactinemia-induced high bone turnover and osteopenia (29). Regarding this aspect, our postulation was that the physiological hyperprolactinemia invokes a differentiation of HPDLF to undergo osteoblast –like cell that serves the osteogenic environment for the demineralizing and remineralizing process during root resorption.

PRLR has been well-located in many cell lineages including mesenchymal stem cells, *i.e.*, chondrocyte, osteoblast (11, 17, 29, 30) as well as in tooth bud (31). Together with others, we previously reported the expression of PRLR in osteoblasts from several sources such as primary human osteoblast, murine osteoblast, human pre-osteoblast (SV-HFO), MG-63, and human fetal osteoblast like cell (hFOB). PRL signals through a membrane-bound receptor, member of the class 1 cytokine receptor superfamily. Various isoforms of PRLR have been documented in human and rodents, referred as long, short, and intermediate PRLR. These isoforms are generated by different splicing of the intracellular domain, but remain identical in their extracellular ligand-binding domain (15, 32) . While the signaling of the long isoform of the PRLR has been reported in several pathways, such as Jak2/STAT, MAPK, Src, or phosphatidylinositol 3-kinase (PI3K)/Akt (15), the mechanism of the short isoform of the PRLR remain largely unknown. Moreover, the distribution of each isoform of PRLR can lead to different action of PRL on target cells (33, 34). In this respect, we demonstrated, for the first time, mRNA, protein expression and localization of PRLR in hPDLF and extracted human PDL cell. The distributions of long and short isoforms in hPDLF make a plausible signaling pathway through its heterogenous dimerization. Given our technical limitation, the dimerization of these two isoforms and its transduction signaling remain to be investigated in a future study. In sum, the presence of PRLR in various types of cells in oral cavity hinted the actions of PRL on proliferation and differentiation, as well as function in terms of mineralization in periodontal surrounding tissues.

In this study, we demonstrated that a lower PRL concentration of 10 ng/mL directly induce a number of effects on hPDLF differentiation as indicated by perostin, specific PDL differentiating marker involved in the remodeling and metabolism of ECMs during tooth development. Furthermore, osteogenic genes such as Runx2 and BMP2 were upregulated in similar fashion with that of periostin suggesting an action of PRL on osteogenic inducing in HPDLF. It is worth noting that concentration of 10 ng/mL of PRL is comparable to the basal plasma levels of male and non-pregnant female adult rodents (35, 36). This could explain the role of basal level in circulating PRL to promote periodontal ligament differentiation that is preferable for osteogenic induction in normal mammals. Higher PRL concentration of 100, 600, and 1000 ng/mL significantly reduced a number of hPDLF cells in our study. This inhibitory effect of PRL on cell proliferation and apoptosis has been shown previously in other mesenchymal cells (11, 17, 18, 29, 37, 38). After short-term exposure of PRL at 100 ng/mL, the osteogenic genes, *i.e.*, Runx2 and BMP2 were significantly increased while the chondrogenic-inducing gene, SRY (sex determining region Y)-box (Sox)-9 was unchanged. We further investigated an ostegenic characteristic and the mineral nodule formation in hPDLF after long-term exposure of PRL. The analysis of mineral positive staining showed higher intensity in the hPDLF exposed with PRL at concentration of 100 ng/mL than that of the control group. In human osteoblast derived from fetal bone, PRL similarly induced bone formation by upregulating osteoblast differentiating marker, osteocalcin, while reducing the RANKL/OPG ratio (29). These results suggested that PRL promotes the differentiation of hPDL

toward the osteogenic characteristic rather than the chondrogenic characteristic. This result was confirmed by the reduction of proteoglycan accumulation in high PRL-treated HPDLF that indicates that the extracellular matrix formation sequentially occurred after chondrocyte differentiation. It may be possible that a high level of circulating PRL drives the PDL cells to undergo osteoblastic differentiation in order to support a remodeling of avelolar bone and tooth in calcium demanding periods such as during pregnancy and lactation.

In conclusion, the present study demonstrated the effect of PRL on hPDLF proliferation and differentiation, which can explain the possible role of PRL in maintaining human periodontal ligament differentiation in male and non-pregnant female mammals. We found that high PRL concentrations (P100 ng/mL during pregnancy and lactation) ceased proliferation of hPDLF while it also induced differentiation toward the osteoblast-like cell and mineral nodule formation, both of which are sequential processes leading to cementum and alveolar bone regenerations. This study shed a light on PRL actions in hPDLF, one of the ectomesenchymal cells, which make plausible that PRL may also regulate other cell types such as dental pulps, gingival fibroblast, odontoblast or cementoblast.

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FIGURE LEGENDS

Figure 1. Immunofluorescent analysis of hPRLR protein expression in hPDLF and extracted human PDL cells (n=3) **(A, C).** Negative controls were performed by incubating hPDLF and extracted human PDL cells in an absence of anti-PRLR **(B, D)**, respectively. HPRLRs were labeled in green. Expression of human hPRLR mRNA (semi-qRT-PCR) of all isoforms (hPRLR-A), long isoforms (hPRLR-L), and short isoforms (hPRLR-S). GAPDH was a housekeeping gene in cultured hPDLF **(E)**.

Figure 2. Dose response study of hPDLF viability after exposure to 10–1000 ng/mL PRL for 24 h **(A)** and 48 h **(B)**, as determined by MTT (n=10 per group), $*p<0.05$, $**p<0.01$ compared with the control group.

Figure 3. Relative mRNA expression of genes responsible for PDL differentiation and osteogenic differentiation, *i.e.* **(A)** Periostin, **(B)** Runx2, **(C)** BMP2, **(D)** Sox9, and **(E)** Collagen type I after being incubated with 10 or 100 ng/mL PRL (n=3 per group). GAPDH was a housekeeping gene for normalization. $*p<0.05$, $**p<0.01$ compared with the corresponding control group (open bar).

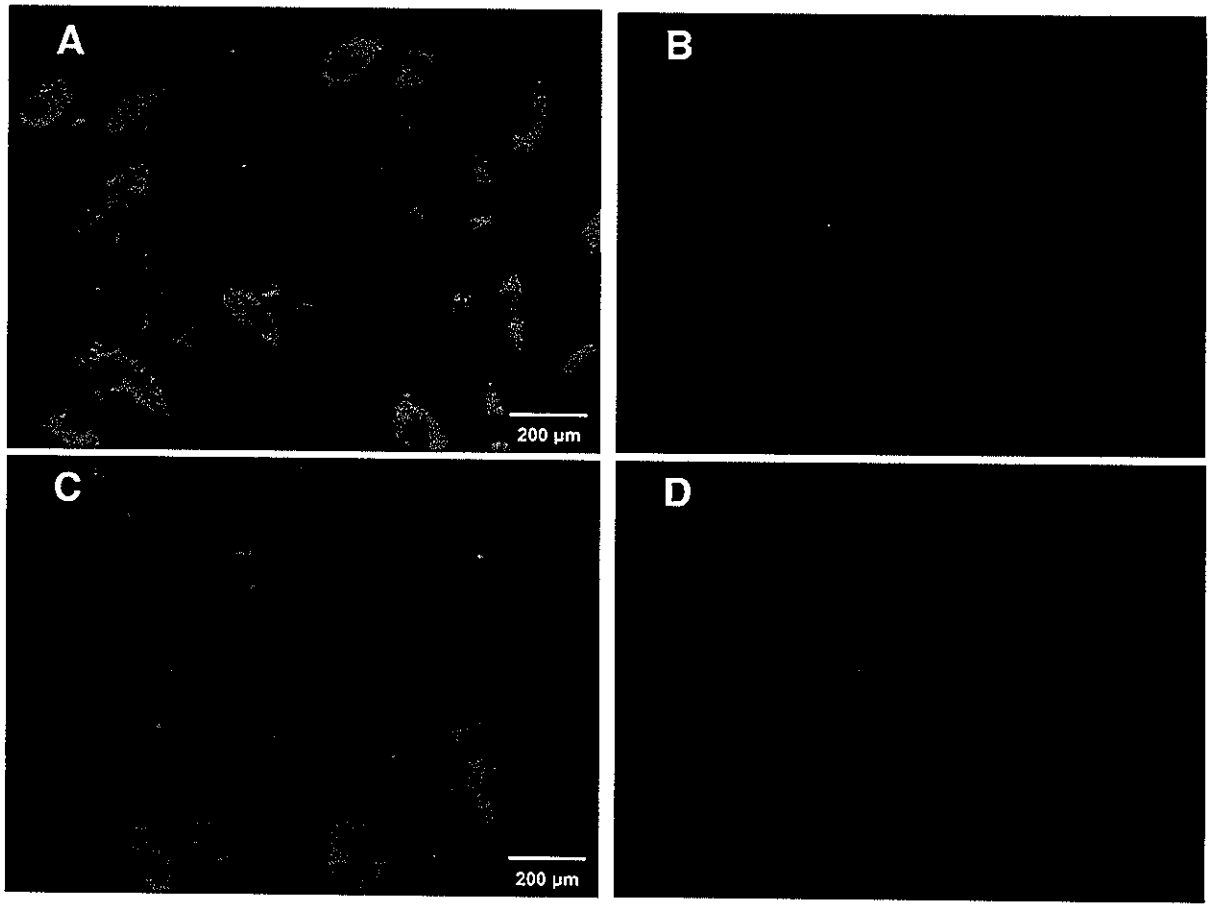
Figure 4. Mineralizing nodule formation of hPDLF (day 28 of PRL treatment; alizarin red S staining) in maintaining medium (non-diff) and differentiating medium (diff) containing 0, 10, and 100 ng/mL PRL (n=3 per group).

Representative culture plates **(A)** and spectrophotometric optical density (OD) 540 nm **(B)**. $^{\dagger}p<0.05$ compared to non-diff group (open bar) (student t-test), $^{*}p<0.05$, $^{**}p<0.01$ compared with the corresponding control group.

Figure 5. Proteoglycan accumulation of hPDLF (day 28 of PRL treatment; alizarin red S staining) in maintaining medium (non-diff) and differentiating medium (diff) containing 0, 10, and 100 ng/mL PRL (n=3 per group). Representative culture plates **(A)** and spectrophotometric optical density (OD) 650 nm **(B)**. $^{\dagger}p<0.05$ compared to non-diff group (open bar) (student t-test), $^{*}p<0.05$, $^{**}p<0.01$ compared with the corresponding control group.

Figure(s)

Figure1



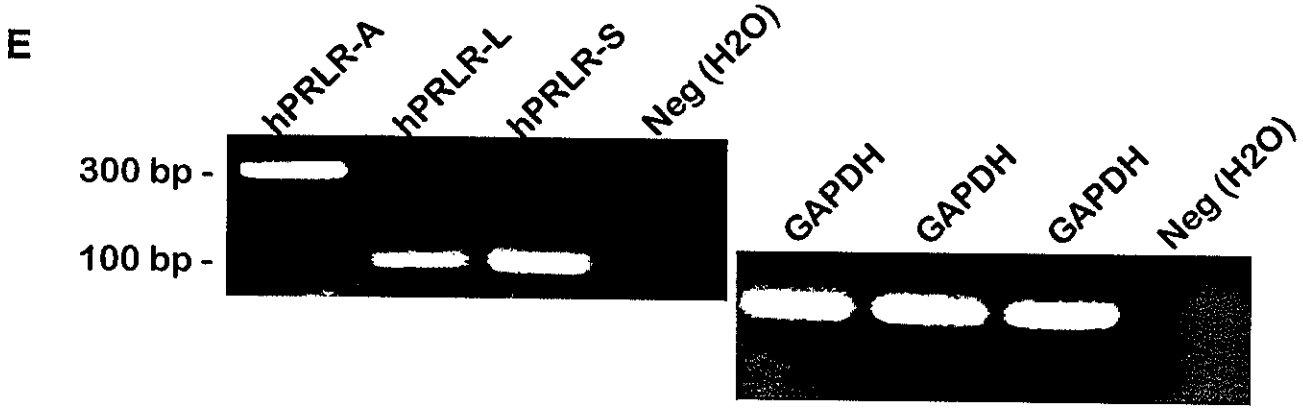


Figure2

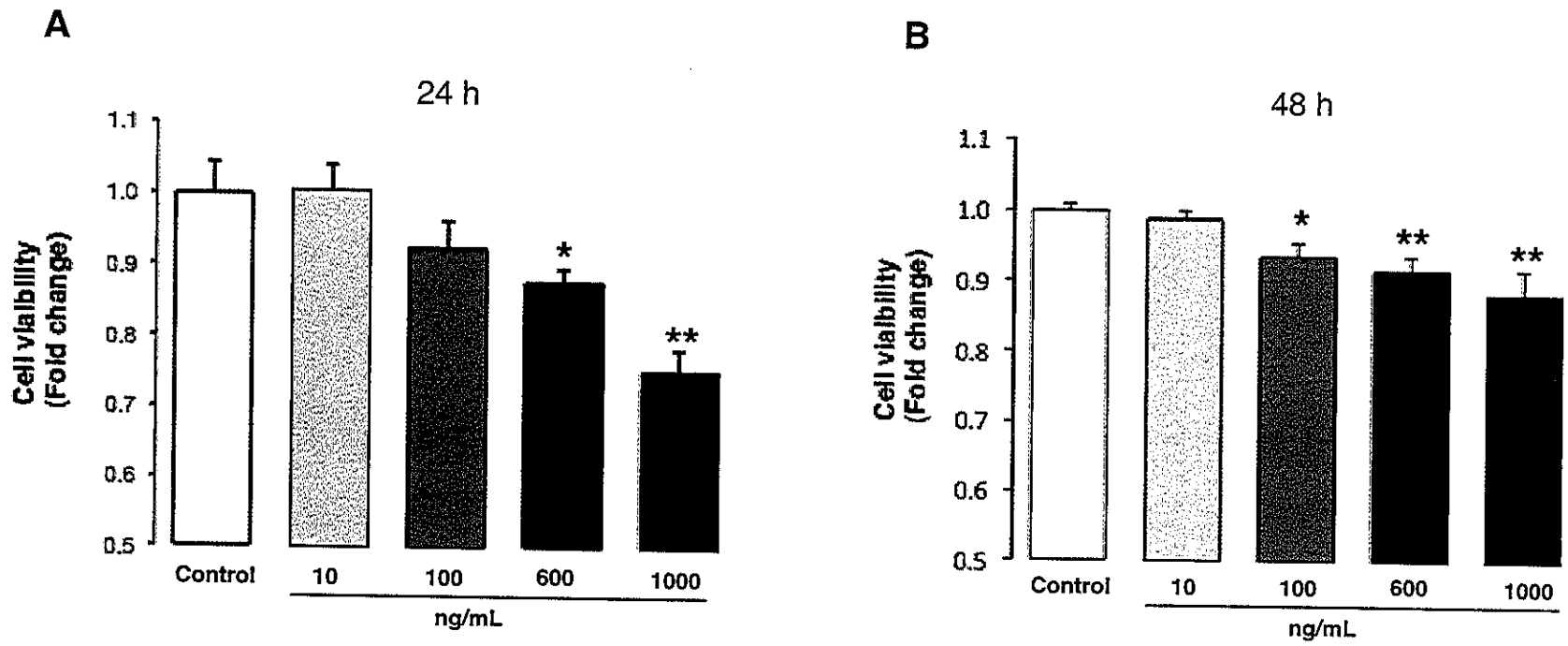

A
24 h
Cell viability
(Fold change)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
*
**
Control
10
100
600
1000
ng/mL
B
48 h
Cell viability
(Fold change)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
*
**
**
Control
10
100
600
1000
ng/mL

Figure3

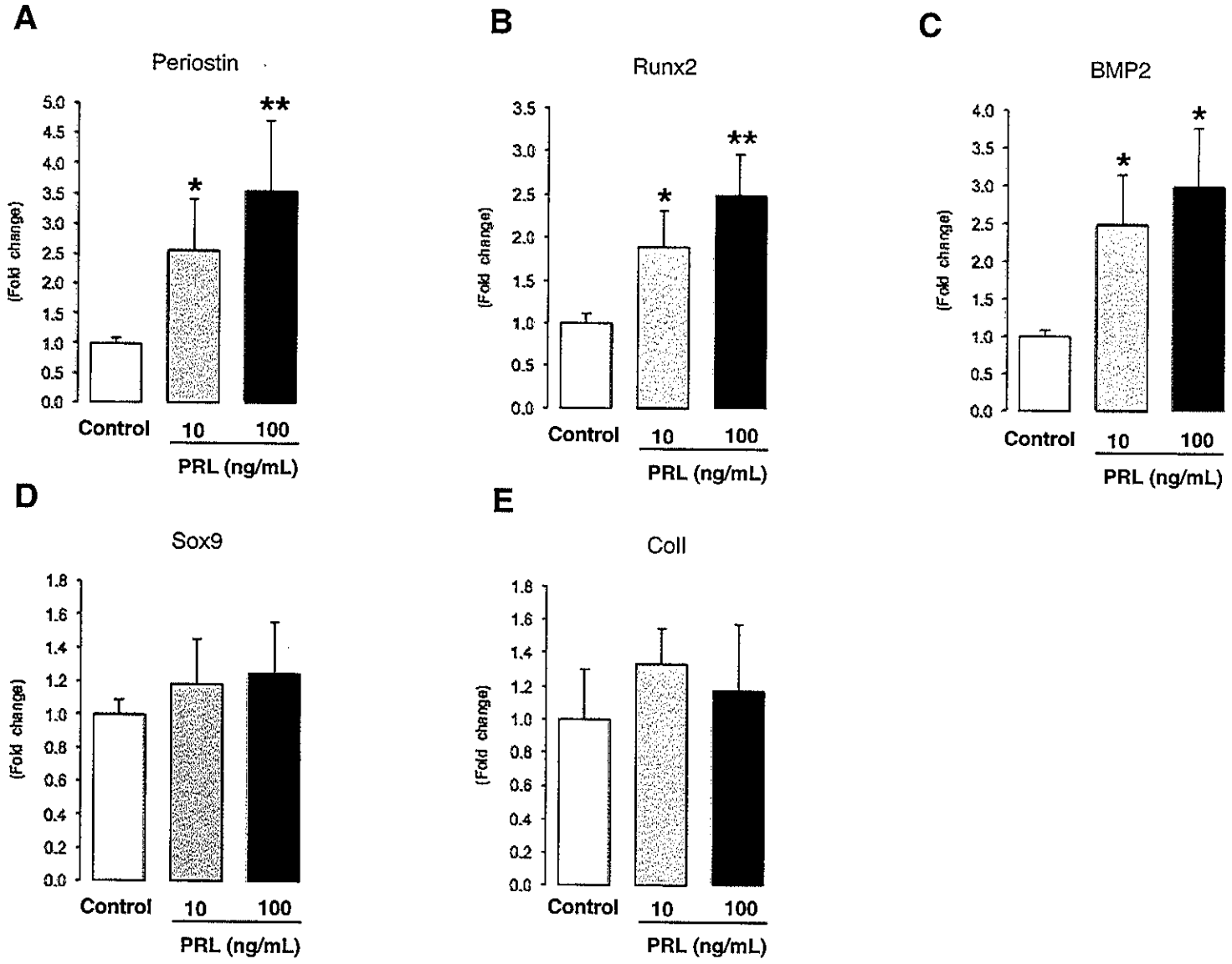
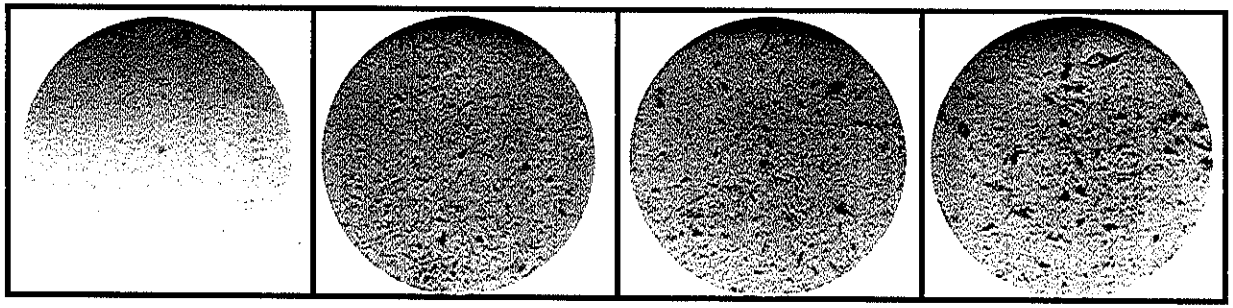

A
Periostin
(Fold change)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Control
10
100
PRL (ng/mL)
B
Runx2
(Fold change)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Control
10
100
PRL (ng/mL)
C
BMP2
(Fold change)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
10
100
PRL (ng/mL)
D
Sox9
(Fold change)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
10
100
PRL (ng/mL)
E
Coll
(Fold change)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
10
100
PRL (ng/mL)

Figure4

A

Alizarin Red staining



B

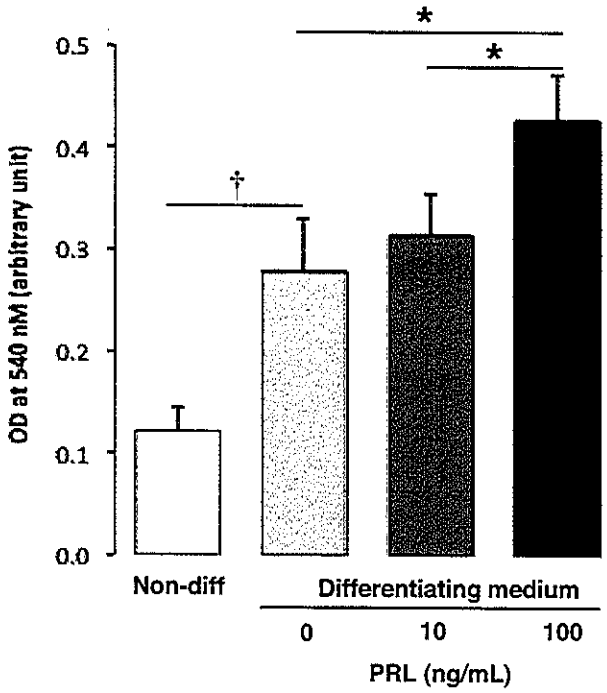
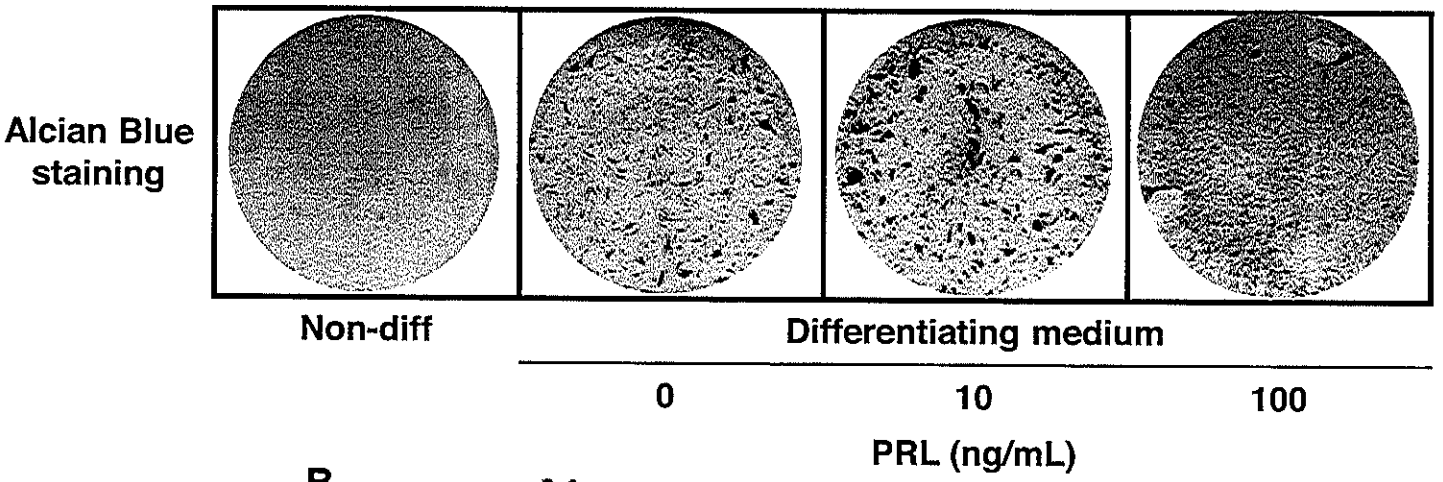
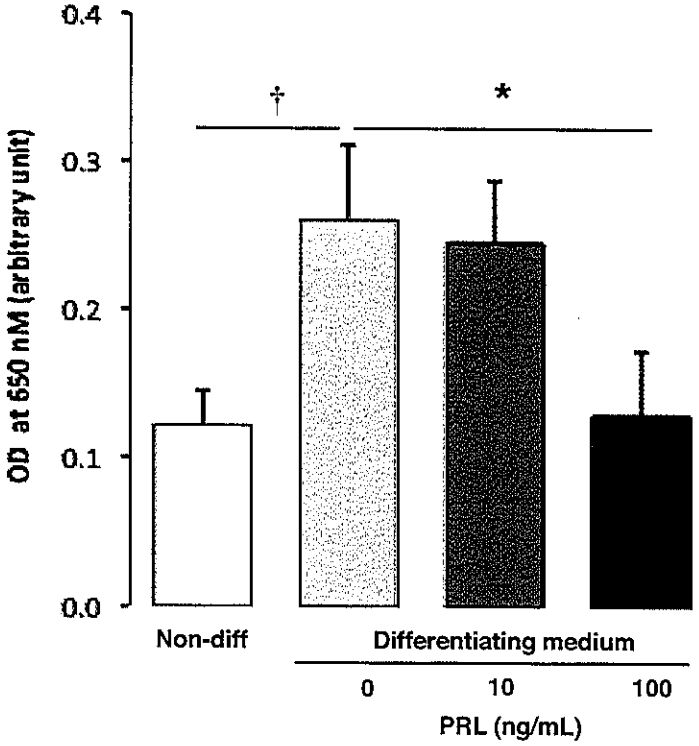


Figure5

A


Alcian Blue staining
Non-diff
Differentiating medium
0
10
100
PRL (ng/mL)

B


OD at 650 nM (arbitrary unit)
0.4
0.3
0.2
0.1
0.0
†
*
Non-diff
Differentiating medium
0
10
100
PRL (ng/mL)