HIGH RESOLUTION FILMS FOR BONE REGENERATION EVALUATION

María V. Jammal[1], Erika B. Territoriale[1], Carlos M. Abate[2], Liliana R. Missana[1,2]

[1]Oral Pathology Department, Dentistry Faculty, Tucumán University.
[2] Industrial Institute of Microbiology and Biotechnology Process (PROIMI-CONICET). Tucumán, Argentina.

ABSTRACT

Diagnostic imaging techniques (DIxT) seem to be a useful tool for evaluating bone formation in both human and animal models. There is little evidence on the use of Soft X-Rays (sXR) with high-resolution films for studying the healing process in critical bone size defects (CSD). The aim of this study was to evaluate the ability of soft X-Ray – High Resolution Films (sXR) to distinguish bone regeneration in CSDs. A CSD was created in each of 16 Wistar rat calvariae. The animals were euthanized at 1, 3 and 6 weeks after surgery. The samples were submitted to cXR (conventional X-rays), sXR techniques and histological procedures (HP). Bone formation was observed at CSD edges at all periods of time. At 6 week, there was also new bone in the central area. The CSD was not fully regenerated after any period of time. Histometric results were 0.16%; 0.75% and 0.89% new bone formed at weeks 1, 3 and 6 respectively; radiometric results at cXR were 0% in all samples. Evaluation of sXR shows 0.4%; 0.50% and 3.64% bone at weeks 1, 3 and 6. Mean and Standard Deviation were calculated. The data were submitted to statistical analysis using the Pearson product-moment correlation coefficient test. The r value was 0.581. Under these experimental conditions, sXR was found to be a suitable method for detecting new bone formation, based on the positive correlation between sXR and HP during the bone healing process of CSDs in rat calvaria. Furthermore, the sXR technique allowed us to obtain samples with appropriate spatial orientation.

Key words: bone regeneration, histology, radiology.

EVALUACIÓN DE REGENERACIÓN ÓSEA CON PLACAS RADIOGRÁFICAS DE ALTA RESOLUCIÓN

RESUMEN

Las técnicas de diagnóstico por Imágenes (DxI) han demostrado su utilidad para evaluar formación ósea en situaciones de salud y enfermedad. Son utilizadas tanto en humanos como en modelos animales; aunque la técnica de rayos X blandos en placas de alta resolución (rXb) ha sido escasamente aplicada. El objetivo de éste trabajo fue evaluar la capacidad técnica de los rayos X blandos en placas de alta resolución (rXb) para distinguir la neoformación ósea en defectos óseos críticos (DOC) en calotas de ratas, durante el proceso de regeneración ósea. En 16 ratas Wistar hembras (150 ± 50 g), se realizaron DOC circulares en calota. Los animales fueron eutanasiados a la 1º, 3º y 6º semana post-quirúrgica. Las muestras experimentales (MEx) recibieron rayos X convencionales (rXc), rayos X blandos (rXb) y luego fueron procesadas histológicamente (TH). Se realizaron estudios histométricos y radiométricos; utilizando soft Image J (NIH). Los resultados histológicos demostraron presencia de tejido de granulación en el área del DOC a la 1° semana y se observó tejido fibroso desde la 3° semana. En todos los periodos de tiempo, se observó formación ósea en los bordes del DOC, mientras que a la 6° semana, fue evidente en el área central del mismo. No se evidenció regeneración ósea en ningún período estudiado. Los resultados histométricos fueron 0,16%; 0,75% y 0,89% a la 1º, 3º y 6º semana respectivamente. Los resultados radiométricos obtenidos utilizando placas radiográficas convencionales (rXc) fueron de 0% en todos los casos; mientras que en placas de alta resolución con rayos X blandos (rXb) fueron 0,4%; 0,50% y 3,64% a las 1º, 3º y 6º semanas respectivamente. Se calcularon la media y Desvío Estándar a la 1°, 3° y 6° semana. Además se utilizó el coeficiente rho de Pearson, para estimar la correlación existente entre rXb y TH; obteniendo un valor r de 0,581. En las condiciones experimentales utilizadas, podemos concluir que la técnica de rXb fue un método apropiado para la detección de neoformación ósea, ya que demostró una correlación positiva con la TH, durante los periodos de tiempo estudiados; además de facilitar la orientación de las MEx durante su procesamiento histológico.

Palabras clave: regeneración ósea, histología, radiología.

Introduction

Various animal models have been developed in the field of bone research for evaluating therapeutic strategies. Some of them most outstanding are the critical bone defect models, which compare new bone formation in treated and untreated defects. Hollinger et al. (1986) introduced the concept of critical sized defect (CSD), defined as the smallest size defect that will not heal without treatment, in a percentage of bone not greater than 10% of the total area[1,2]. Bone defects can be performed on different bones, such as calvaria, tibia, femur, maxilla, etc. The CSD model in rat calvariae has

been widely used for evaluating bone fill materials, bone anabolic agents and other treatments. It is based on morphological and embryonic similarity of maxillofacial region bones[3,4]. Various methods have been used to evaluate capacity for promoting bone healing in critical bone defects, including clinical observation, macroscopic, microscopic and mechanical tests[5]. There are also diagnostic imaging techniques (DxI), such as conventional X-rays (cXr), micro-computed tomography (mCT), quantitative roentgen densitometry (qRd) and soft x-ray–high-resolution film (sXr). Conventional X-rays are a useful tool, quick and inexpensive, however when used for bone defects, the information they provide is poor and unreliable[6]. Micro-computed tomography is efficient to evaluate CSD regeneration based on similarities found with histological studies. However, it is only a future prospect for developing countries like ours[7,8,9]. Quantitative roentgen densitometry uses high-resolution X-ray equipment that allows quantify bone density and detect small differences in mineral content[10,11]. There are also soft X-ray (sXr) techniques, used for specific purposes such as microcalcification in mammary pathology, which provide sharper images with greater contrast[12]. In bone pathology, few studies have used this technique. It has been applied in humans to determine bone changes in the hands of hyperparathyroid patients[13]. In animal models it has been used to evaluate xenografts in rabbit tibias and rat calvariae, to determine the role of the dura mater during bone regeneration[14]. As the film has such high sensitivity and definition, we decided to evaluate the technical capacity of soft X-rays on high-resolution film (sXr) to identify new bone formation during the bone regeneration process in critical bone defects (CSD) of rat calvariae.

MATERIALS AND METHODS

Sixteen (16) female *Rattus norvegicus* var wistar were used (150 ± 50 g, approximately 9 weeks old).

Surgical procedures

All animals under anesthetize, received an unilateral full thickness osteotomy over calvaria. Each of them were made by *ad hoc* manual punch (5 mm in diameter) non-serrated, avoiding dura mater damage[3,4]. The animals were sacrificed at weeks 1, 3 and 6, respectively. The samples (skin, subcutaneous cellular tissue, muscle, parietal bone and brain) were fixed in 20% buffered formalin for 24 hours. The animals were divided in two groups. Group A: ten (10) animals were submitted to soft X-rays (sXR) and decalcified histological studies (DHP). And Group B: six (6) animals were study by conventional X-rays (cXR), soft X-rays (sXR) and undecalcified histological procedure (UDHP).

Conventional X-ray technique

The specimens were X-rayed by conventional technique with periapical Kodak Insight film and dental X-ray equipment (DSJ brand). The focus-plate distance was 20 cm and exposure time 0.35 seconds. The samples were placed on films at antero-posterior direction, recorded individually, and following the film upper right-hand corner as reference.

Soft X-ray technique

The experimental samples were subject to soft X-rays using high-resolution Kodak film (mammography type, 18 x 23 cm). GBA Mamograf HF Digital brand equipment was used. The focus-plate distance was constant, at chassis contact. The exposure time was 0.8 seconds, 27.5 Kv and 7.0 mA. All experimental samples were placed in a row, 6 per plate. They were antero-posterior placed, with the CSD on the right parietal bone, following the film upper right-hand corner as reference.

Decalcified Histological Technique

Samples were decalcified by modified Morse Solution and submitted to routine histological processing. Serial sections were cut (7 μ) through the defect center to reach the maximum diameter (5 mm), and then stained by hematoxylin and eosin.

Undecalcified Histological Technique

Samples were dehydrated and embedded in methyl methacrylate. The sections were made by grinding (10 μ), and stained with the modified Masson-Goldner technique, which allows the osteoid tissue to be observed.

Morphometric study

Morphometric studies were performed using conventional and high-resolution radiographic images (cXR and sXR), digitalized with an HP Scanjet 4070 scanner. They were analyzed using ImageJ software (National Institute of Health, USA). The parameters selected were: 1) radiolucent and 2) radiopaque areas. For histometrical studies, microphotographs were taken from each slides using a Sony digital camera adapted to an Olympus CH30 microscope. The pic-

tures were taken using Soft Pinnacle Studio 9.4 with 116.7 X magnification. The parameters selected were newly formed bone periosteal, endosteal, and at CSD edges and center. Newly formed bone was measured as a percentage.

Statistical analysis

Mean (X) and Standard Deviation (ô) were calculated for weeks 1, 3 and 6. Data were correlated according to Pearsons *rho* coefficient.

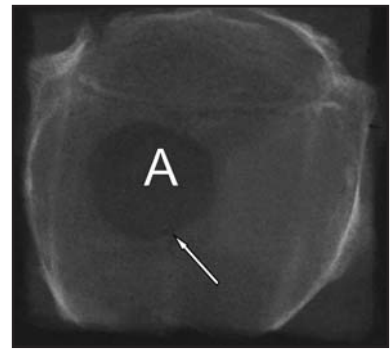

Fig. 1: Radiographic image. Conventional X-rays. CSD 5 mm across in rat calvaria (A). No radiopaque areas are observed. Post-surgery week 6. Magnification: 30x.

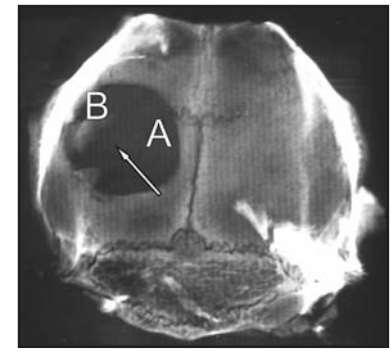

Fig. 2: Radiographic image. Soft X-rays. CSD 5 mm across in rat calvaria (A). Irregular radiopaque areas at the edges of the defect (B). Post-surgery week 6. Magnification: 30x.

RESULTS

Conventional X-ray technique (cXR)

At weeks 1, 3 and 6, there was a circular radiolucent area corresponding to CSD; no radiopaque area was found at any period of time (Fig. 1).

Soft X-ray – high-resolution film technique (sXR)

At week 1 there was a rounded radiolucent area with sharply defined edges on the parietal bone, corresponding to the defect area, containing a few small radiopaque dots. At week 3, there were many, large, irregular shaped radiopacities on the defect area, compared to week 1. At week 6, the radiopacities on CSD were also irregular, but larger than those from the first two periods. Nevertheless, they were not able to regenerate the defect (Fig. 2).

Histological results

In CSD area, granulation tissue was observed at week 1. From week 3 to 6 there was fibrous connective tissue. New bone and osteoid were formed on CSD edges, from 1 to 6 weeks. Also, bone formation was founded in the CSD center at week 6. Osteoid was observed only in undecalcified samples.

Morphometric results

The radiometry from cXR showed a 0% volume on radiopaque areas, and 100% volume on radiolucent areas at all time periods. The values from sXR showed a radiopaque areas amount of 0.4%; 0.50% y 3.64% at weeks 1, 3 and 6 respectively. Histomorphometric measurements demonstrated a new bone formed (calcified bone and osteoid) of 0.16%; 0.75% and 0.89% at weeks 1, 3 and 6; obtained from CSD edges and center.

Statistical results

Mean and Standard Deviation were calculated (Table 1). In addition, the correlation between sXR and HP was estimated using Pearson's *rho* coefficient test (normal value -1 and + 1), which produced a value for *r* of 0.581 (Fig. 3). The cXR observations are equal to zero, and therefore cannot be analyzed statistically.

Table 1: Mean ± Standard Deviation Values at weeks 1, 3 and 6 for Soft X-ray-high resolution film technique (sXR) and histometry (HP).

Week	HP	sXR
1 (n=5)	0.16 ± 0.11	0.40 ± 0.56
3 (n=5)	0.75 ± 1.05	0.51 ± 0.90
6 (n=6)	0.86 ± 0.48	3.64 ± 3.97

r value: Pearson's *rho* coefficient test recorded a strong positive correlation (r<1) r = 0.581
Conventional X-ray technique (cXR) values are not included because they were negative.

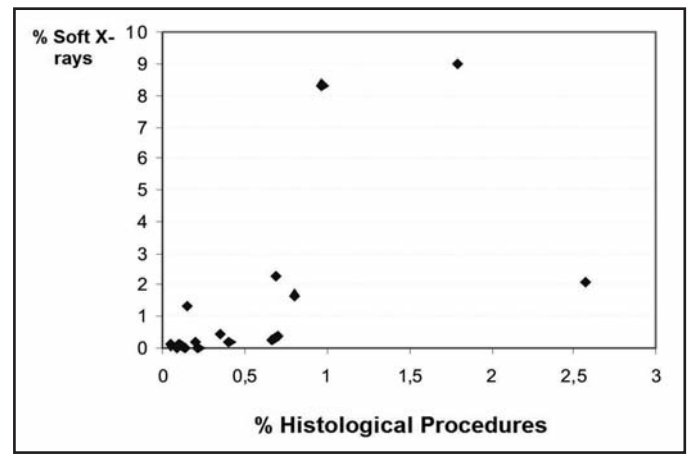

Fig. 3: Dispersion values diagram of bone regeneration obtained from histological procedures vs. soft X-rays at weeks 1, 3 and 6 showing positive correlation.

DISCUSSION

The need to find a valid, efficient, simple method to demonstrate bone regeneration in animal models with critical bone defects has led researchers to seek and develop new methods. In this study, we have evaluated the use of soft X-rays–high-resolution film as a means for detecting new bone formation in experimental models. There are few papers in which this technique is used to determine bone formation[10,14], although our results encourage us to use it, particularly due to its high correlation with histology. It is important to highlight that the measurement planes for areas on sXR and histological sections are orthogonal projections. The film provides an actual frontal view of bone formation, while the histological section is plane, obtained from a sample cut on angles 90°. The sXR images clearly showed the difference between the defect radiolucent area and radiopacities of new bone formed. However, the cXR technique yield a low level of accuracy, based on our 0% radiopacity results. Pryor et al.[6] (2006) also determined the validity of cXR in rat calvariae CSD models as providing unreliable information[5,6]. These results may be conditioned by the thinness of the bone as well as the size and distribution of silver halide granules on both sides of the radiographic plate[16]. Moreover, the fact that the equipment used could not change Kv and mA affected radiation penetration. Related to our histological results from decalcified and undecalcified obtained from CSD samples, were agree with other authors[1,4]. We have considered using them, like Pryor et al.[6], as a control group, but in order to provide greater validity for our results, we also performed non-decalcified histological studies with the aim of comparing the amount of bone obtained with both techniques. Our results showed that the differences are not statistically significant ($p = 0.1904$). The statistical results of comparing the two study groups show positive correlation, i.e. there is association between areas of radiopacity (sXR) and the new bone tissue observed in the CSD area. We can thus say that the use of soft X-rays–high-resolution film is an appropriate tool for studying new bone formation in CSDs on rat calvaria. The method is simple, the equipment needed is available and low-cost, and the method provides precise information on the evolution of the bone regeneration process. We therefore consider that our study lays the foundations for the use of sXR in the field of bone research.

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CORRESPONDENCE

Prof. Dra. Liliana Missana
Pje Leopoldo Lugones 80. B° Ojo de Agua.
(T4000MFB) Tucumán, Argentina.
e-mail: missli@arnet.com.ar

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