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Nukbone® promotes proliferation and osteoblastic differentiation of mesenchymal stem cells from human amniotic membrane

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ABSTRACT

Bovine bone matrix Nukbone® (NKB) is an osseous tissue-engineering biomaterial that retains its mineral and organic phases and its natural bone topography and has been used as a xenoimplant for bone regeneration in clinics. There are not studies regarding its influence of the NKB in the behavior of cells during the repairing processes. The aim of this research is to demonstrate that NKB has an osteoinductive effect in human mesenchymal stem cells from amniotic membrane (AM-hMSCs). Results indicated that NKB favors the AM-hMSCs adhesion and proliferation up to 7 days in culture as shown by the scanning electron microscopy and proliferation measures using an alamarBlue assay. Furthermore, as demonstrated by reverse transcriptase polymerase chain reaction, it was detected that two gene expression markers of osteoblastic differentiation: the core binding factor and osteocalcin were higher for AM-hMSCs co-cultured with NKB in comparison with cultivated cells in absence of the biomaterial. As the results indicate, NKB possess the capability for inducing successfully the osteoblastic differentiation of AM-hMSC, so that, NKB is an excellent xenoimplant option for repairing bone tissue defects.

1. Introduction

The treatment of bone defects, such as fractures with bone loss, bone infections, hormonal changes, or tumours is an enormous challenge for tissue engineering. Several natural or synthetic biomaterials (as allograft, demineralised bone matrix, hydroxyapatite, calcium phosphates, among others), have been studied because of their osteoconductive effect in bone regeneration. However these biomaterials are capable for inducing migration, adhesion and proliferation of osteoblastic cells [1–3], but few of them exhibit osteoinductive activity in the absence of exogenous soluble differentiation factors [4]. In spite of the osteoconduction is necessary for generating and maintenance of the new bone after an injury, meanwhile the osteoinduction capability is a major desirable property because induce the osteoblastic differentiation of progenitor cells [5]. In this context, the Nukbone® (NKB) is a human bone biomimetic material from the bovine distal femoral condyle that preserves its physiological topography after a gentle method of scaffold recovery (ionic detergents, proteases and soft overheating). This processing for the obtaining of NKB allows maintain both, the collagen and the mineral phases [6] and its manufacturing process is following the directions of the international and national standards for human medical devices, ISO-137779 and the Mexican Standard NOM-059-SSA1-1993. Previous clinical studies are showing that NKB was completely osteo-integrated without inducing any immunological reaction at the site where it was implanted [7] and this demonstrated that NKB was involved in the whole bone repair. The aim of the present work is to demonstrate that NKB has an osteoinductive capacity on human mesenchymal stem cells from amniotic membrane (AM-hMSC) after evaluating its effect on proliferation and osteoblastic differentiation of these cells. These findings are contributing to the goal of the tissue engineering.

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2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT, USA) Dulbecco's Modified Eagle Medium (DMEM), antibiotic–antimycotic solution, and trypsin–EDTA solution were obtained from Life technologies (Grand Island, NY, USA). Type II collagenase, ascorbic acid, dexamethasone and β-glycerophosphate were supplied by Sigma Chemical Co. (St. Louis, MO, USA). alamarBlue® Cell Viability Reagent and TRIzol reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA).

2.2. Characterization of Nukbone

The morphology and macrostructure characterization of NKB was done using a Stereo Microscope (Stemi 2000-C Zeiss, Germany) meanwhile its microstructure was observed with a Scanning Electron Microscope (SEM) DSM-950 (Zeiss, Germany) at 15 kV. Mean pore diameters were estimated through SEM-micrographic after measuring 100 pores. Estimations of the thermal behavior of NKB were performed by thermogravimetric analysis (TGA) and differential thermal analysis (DTA) using a Thermogravimetric Analyzer Q600 (TA. Instrument, NC, USA), under nitrogen atmosphere and heating intervals from 25 °C to 700 °C with a heating rate of 10 °C/min.

2.3. Nukbone disks preparation for culture cells

The NKB disks (diameter 12 mm; thick 2 mm) were kindly provided by Biocriss, S. A. de C.V. (México City, México). NKB and polystyrene disks (used as experimental controls) were UV-sterilized and pre-moistened in high-glucose Dulbecco's Modified Eagle Medium supplemented with 1% antibiotic-antimycotic and 10% heat-inactivated fetal bovine serum (H-DMEM) and incubated for 24 h in a humidified atmosphere of 5% CO_2 in air, at 37 °C, and used for subsequent experiments.

2.4. Isolation and culture of amniotic membrane human mesenchymal stem cells

Caesarean-delivered term placentas were collected from healthy donor mothers, previous informed consent. This research was performed in accordance with the World Medical Association's Declaration of Helsinki regarding ethical conduct of research involving humans and approved by the Research Ethic Board of Facultad de Medicina, UNAM (project number 101-2012). Amniotic membrane was mechanically separated from underlying chorion by blunt dissection, cleaned and submitted to enzymatic digestion with type II collagenase for AM-hMSC obtaining according to methodology previously described by Leyva et al. [8]. The AM-hMSC obtained, were cultured in H-DMEM under standard cultured conditions. All non-adherent cells were removed with the medium change after one week, while adherent cells were reached 90% confluence and recovered by trypsinization, then, seeded into 24-well cell culture plates at a density of 5×10^5 cells on pre-moistened NKB as well as polystyrene disks and incubated overnight according to Fassina et al. [9]. Later, disks were placed in a new 24-well cell culture plate (time zero) in three conditions: (1) positive condition (+OS), AM-hMSC seeded on polystyrene disk with H-DMEM supplemented with 82 μg/mL ascorbic acid, 100 nM dexamethasone, and 10 mM β-glycerophosphate (H-DMEM-OS); (2) negative condition (−NKB), AM-hMSC cultured on polystyrene disk in H-DMEM and, (3) AM-hMSC with H-DMEM seeded on NKB disk as experimental condition (+NKB). Cell cultures were maintained for 21 days and recollected at 0, 7, 14 and 21 day for subsequent studies.

2.5. Cell morphological analysis

On the third day of culture, the cell-disk samples were rinsed twice with PBS 1X and fixed with 4% (v/v) glutaraldehyde solution in 0.1 M Na-cacodylate buffer (pH 7.2), and prepared for SEM observation as previously reported by Rivera-N et al. [10]. Observation of samples was performed in a SEM DSM-950 (Zeiss, Germany) at 15 kV.

2.6. Cell proliferation assay

AM-hMSC cultures in three conditions previously mentioned were incubated with 10% of alamarBlue (AB) reagent according to manufacturer's guidelines. The AB color turnover is an indicative of the cell proliferation and was quantified by measuring the absorbance in a SUNRISE-reader (TECAN, Germany) at 570 nm with a reference wavelength of 600 nm. Measurements were performed immediately after the addition of AB (0 h) and each 24 h until reaching 7 days of cell culture.

2.7. RNA extraction and reverse transcription-polymerase chain reaction

The cells seeded on +NKB, −NKB and +OS conditions were recovered and lysed by adding 500 μL of TRIzol reagent, broken up and gently homogenized with a pipette tip. Then, RNA-homogenate was extracted following the manufacturer's protocol. Total RNA concentrations was measured by the optical density of the samples at 260 nm using NanoDrop 1000 (Thermo Scientific, USA). Subsequently, 500 ng of total RNA was reverse-transcribed via the First cDNA synthesis Kit (One Lambda, Inc.). The polymerase chain reaction (PCR) was performed using the KAPA2G Robust PCR Kit (KAPA BIOSYSTEMS, USA) according to manufacturer's guidelines in a Gene Amp 9700 PCR Systems (Applied Biosystems). Amplification conditions for cDNA samples and primer sequences for core binding factor (CBFA)-1 and osteocalcin (OC), were performed under conditions reported by Kang et al. [11] and Seebach [12]. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. The PCR products were analyzed using 1.5% (w/v) electrophoresis agarose gel with ethidium bromide staining. The relative quantification was normalized to GAPDH via Quantity One 4.6.9 gel-photo documentation system (Bio-Rad). Integrity of RNA was confirmed by denatured-gel; whilst cycles curve profiles were produced for each target gene in order to confirm the specific amplification time (data no showed).

2.8. Statistical analysis

Experimental procedures were repeated with $n = 3$ biological replicates for each sample and time. Data were analyzed by one-way ANOVA to determine statistical significance ($p < 0.05$) between groups; the values were reported as means ± standard deviation.

3. Results

3.1. Structure and thermal characteristics of Nukbone®

In order to better understand to structure characteristics of NKB, the thermal and macrostructure of NKB were analyzed. Stereo micrograph showed the surface topography of material, where is

possible to observe detailed the porosity (Fig. 1A-a) and its interconnectivity was assessed by SEM observations (Fig. 1A-b) in which measurements of diameters were from 200 to 2000 μm (measurements were obtained from similar images and they were calculated from 100 determinations). Inside of the pore, it was observed the presence of lacunas (Fig. 1A-c) and inside of them, with a higher augmentation; it was observed that the fibrillar collagen structure was preserved (Fig. 1A-d).

The thermal behavior of NKB was found to present two events (Fig. 1B) where the first one indicated in the TGA curve corresponds to a mass loss of 23.3% of the initial sample (24.9 mg) and it can be associated with the loss of surface water molecules

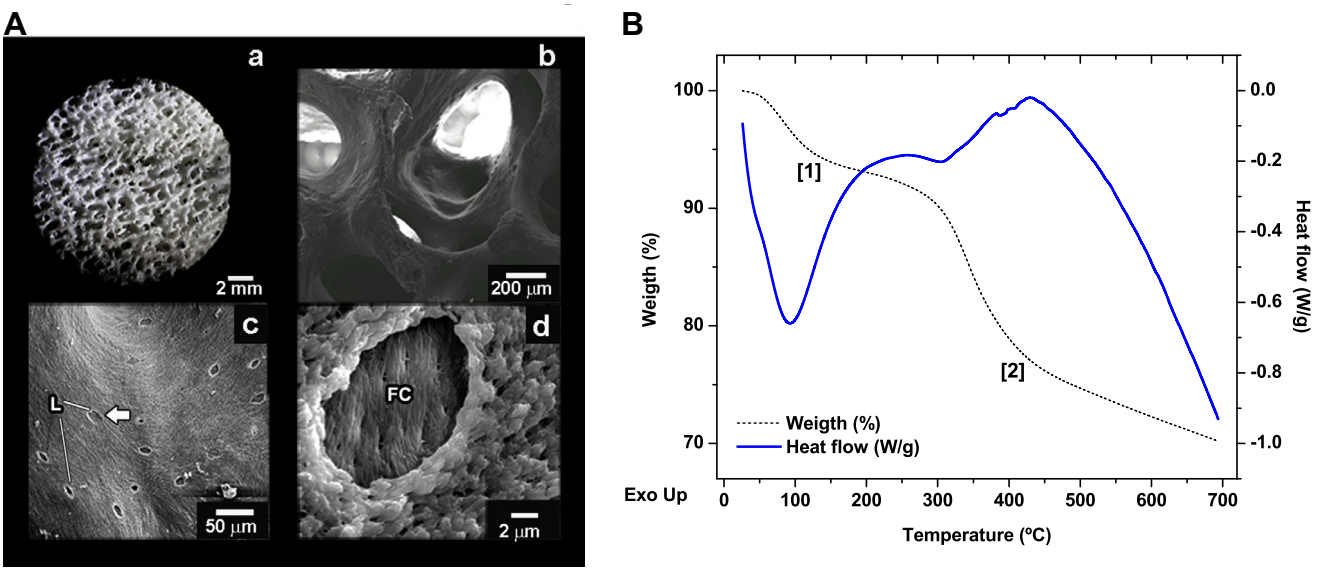

Fig. 1. Characterization of NKB. (A) Morphological characterization. (a) NKB disk macrostructure observed trough a Stereo Microscope, (b–d) Scanning electronic micrographs of NKB microstructure; in b is possible to estimate the shape, diameter and the interconnectivity of the porous. In c, inside of a NKB pore, there are several lacunas (L). Magnification of a lacuna, signalled by an arrow in c, is seen amplifying in d. Inside of the lacuna is observed a pattern of fibrillar collagen (FC). (B) Thermal behavior of NKB. Quantization of mineral and organic NKB residues measured by TGA (% of Weight) shown as dotted line, while that DTA analysis is represented as solid line and evaluate the thermal behavior as W/g heat flow. TGA analysis is showing that there are two major events: one corresponding to the loss of surface water and two, which corresponding to the loss of organic components. Exo up = exothermic events indicated upwards.

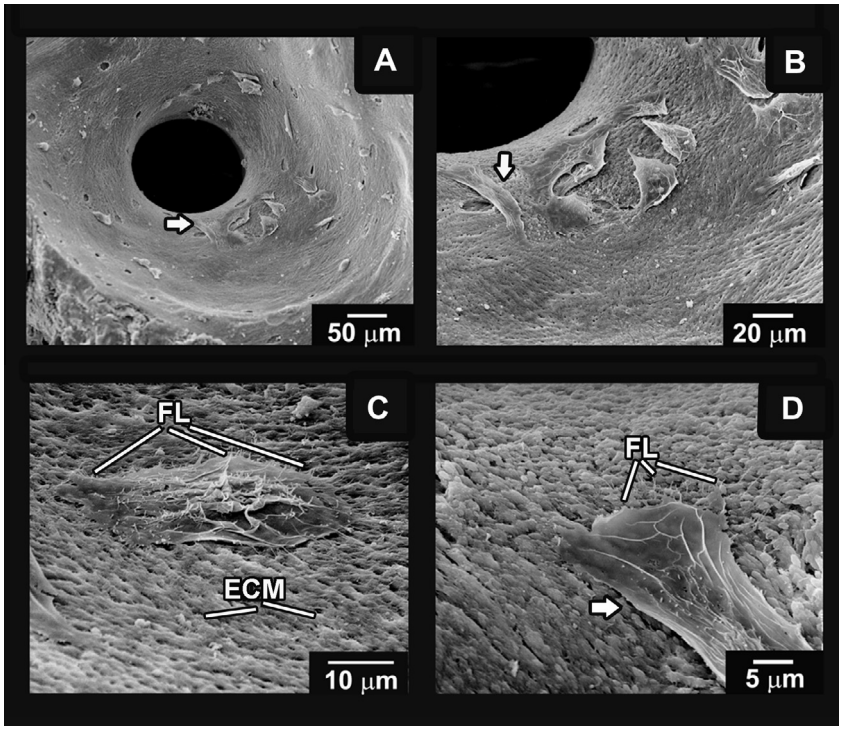

Fig. 2. Adhesion of AM-hMSCs to NKB. Series of SEM images, from lower to higher magnifications (A–D) are showing (after 3 culture days) the attachment of the cells to one pore through the production of extracellular matrix (ECM). (A) Panoramic vision of a NKB pore with several cells adhered to the biomaterial. (B) Amplification of the pore, shown the interaction of several cells with NKB surface. (C and D) Amplifications of a cell that is showing during their spreading they are in a process of interaction with NKB trough filopodial processes (FL). The arrow shows the cell in all the magnifications.

because it occurs at 90.9 °C. The second mass loss, corresponds to 22.9% of the initial mass and occurs at 429.9 °C, which correlated with the structure destabilization of the biomaterial and may be attributed to the loss of organic material (collagen) as has been reported by Pietrucha et al. [13].

3.2. Attachment of AM-hMSCs on Nukbone® scaffolds

As scanning micrographs shown, the AM-hMSCs behavior at third day after layering on the NKB, the surface of biomaterial was covered of some spreading cells apparently attached to the NKB surface. At higher magnification, the spreading cells appeared to exhibit a close contact via filipodial processes (FL) (Fig. 2).

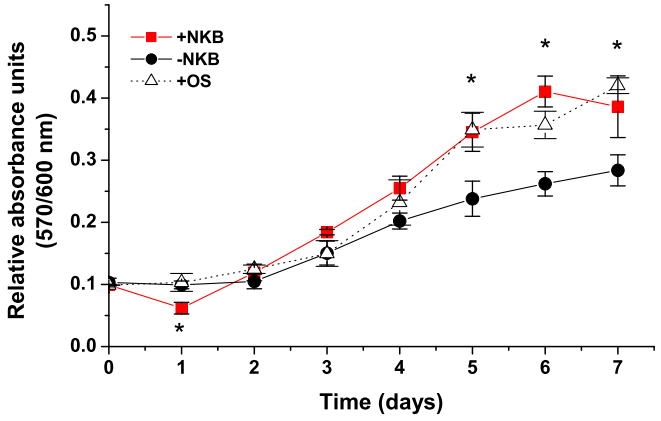

Fig. 3. Effect of NKB on AM-hMSCs proliferation. Cell proliferation was evaluated by AB reduction and expressed in relative absorbance units along 7 days of cultivation. Results are shown the influence of NKB (+NKB) in AM-hMSCs proliferation similarity to cells incubated with osteogenic inducers only (+OS). There is no significant difference between the results, however, incubation with Nukbone (+NKB) was statistically different in significance ($^*p < 0.05$) in comparison with the NKB absence (−NKB).

3.3. Effect of Nukbone® on AM-hMSCs proliferation

Cell proliferation was measured as relative absorbance units (RAU) using AB assay during 7 days of AM-hMSCs culture on +NKB, −NKB and +OS conditions. The results of the AB assay showed a small decrement in relative absorbance of NKB induction (+NKB) after 1 day of incubation, compared with negative and positive control. Then, starting on fourth day, the presence of NKB induces a similar kinetic cell proliferation as positive condition (+OS), and dissimilar behavior regarding the condition without NKB (Fig. 3).

3.4. Nukbone® participation on osteoblastic differentiation of AM-hMSC

In Fig. 4A, shows that gene expression of CBFA-1 in presence of NKB was up-regulated at 7 day, and then it was slightly down-regulated at 14 day and maintained until 21 day. In contrast, the CBFA-1 expression in positive condition (+OS) was observed only at 7 day and was down-regulated at 14 and 21 days (Fig. 4B). Furthermore, OC expression on NKB cultures was up-regulated from 7 day until 21 day the same manner as the positive condition (Fig. 4C). There were not gene expression osteogenic markers in the negative control along the performed studies.

4. Discussion

The most basic function of a bone substitute is to act as a physical substrate, producing a three-dimensional environment, for the attachment of the cells and favouring the organization of cells as providing signals for cellular differentiation [14]. However, this is not the main objective of a biomaterial during bone regeneration, it is to induce the regeneration trough its osteoconductive and/or osteoinductive capacities. In this context, NKB exhibits the required properties for being used as a bone regeneration

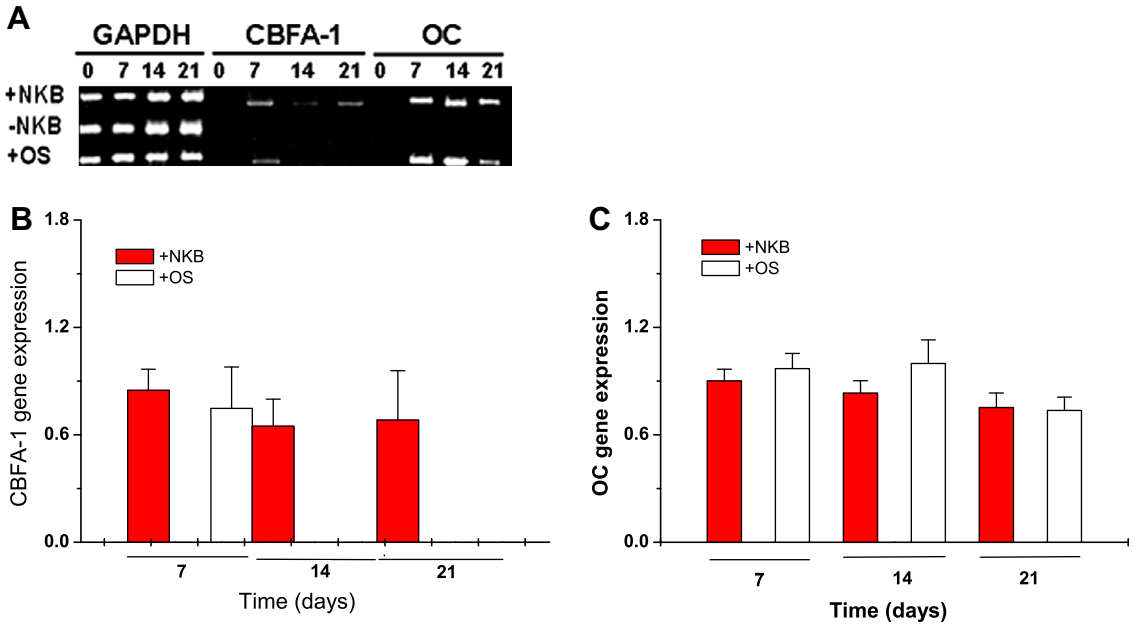

Fig. 4. Osteoblastic differentiation induced by NKB. Along 0, 7, 14 and 21 days of cultivation it was established, by RT-PCR analysis, the gene expression of the house keeping GAPDH and osteogenic CBFA-1 and OC genes (A). Variations were found in the expression of the osteogenic genes induced by the absence (−NKB) or the presence of osteogenic inductors (+OS). Notably, the induction of the expression of genes was similar with NKB only and osteogenic inductors. B and C are representing the quantitative determinations (normalized to GAPDH) of the expression of osteogenic genes in the presence of NKB or osteogenic inductors. Red column represents +NKB while white column without NKB (+OS). Data are presented as mean ± SD of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biomaterial; contains mineral and collagen components, optimal pore sizes (200–2000 μm) and porous interconnectivity that, as physiologic human cancellous bone, represents an optimum structure for distribution of vessels and cell proliferation [13,14]. These are properties that create a suitable topography and environment for cell adhesion, proliferation and differentiation necessaries for osteoinductive activity [9,15]. Also, NKB topography offers a suitable environment for cell adhesion, as showed by spreading cells seen at SEM images of Fig. 1A, where AM-hMSCs cultured on NKB adapt its morphological characteristics in response to surface topography (roughness, porosity, interconnectivity and chemical composition) as have been reported for bone scaffolds [16,17]. Likewise, the successful of *in vitro* AM-hMSCs proliferation is an indicative that the NKB properties affect the cellular process for bone regeneration; because proliferative cells are precondition for osteoconduction and/or osteoinduction effects. In this context, by itself the NKB showed a significant positive influence on cellular proliferation as compared with the negative control without NKB and osteogenic soluble factors. Clearly, NKB led an increased AB reduction for AM-hMSCs, over the observation of the 7th day and this is a comparable result to that found for other ceramic-biomaterials where there is a similar trend of proliferation [16,18,19]. Moreover, the osteoinductive capability of NKB had been demonstrated by osteoblastic differentiation induction of AM-hMSCs. It is possible that NKB might activate, by itself, any kind of signalizations because it is not requiring soluble osteogenic factors for activating the cells as seen for the increase of CBFA-1 and OC gene expression in AM-hMSCs. Probably, the NKB-cell interactions promote changes on gene transcription and synthesis of new proteins related with osteogenesis. In addition, the presence of mechanical stimuli (associated to the sensitiveness of the cell to the extracellular environment as the surface chemistry and topography of NKB) could be factors that possibly the stem cell differentiation [20–22]. Consistent with this hypothesis, several signalling molecules have been demonstrated to play a key role in mechanical stimuli-initiated signal transduction; among them, are the MAP kinases. It has been demonstrated that those enzymes are activated by mechanical stresses and may link the effects of mechanical stress to the biochemical responses and gene expression [23]. Other reports suggesting that MAP kinases pathways, play critical roles in directing MSC commitment to the osteogenic lineage. In conclusion, our research shows that NKB has capabilities for being a successful scaffold for cell adhesion and proliferation; furthermore this material induced osteoblastic differentiation of AM-hMSCs and, according with these, NKB is an excellent option as a xenoimplant for bone tissue engineering. Regardless the present studies, more experiments need to be performed in order to elucidate which mechanisms are involved in the intracellular stimulation of NKB.

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