Journal of Hunan University

(Natural Sciences)

Vol. 51 No. 12

December 2024

Available online at

http://jonuns.com/index.php/journal/index

Open Access Article

https://doi.org/10.55463/issn.1674-2974.51.12.2

Culture and Characterization of Osteoblasts from the Parietal Bone of Neonatal Wistar Rats

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Article History:

Received: October 12, 2024
Revised: November 10, 2024
Accepted: November 19, 2024
Published: December 30, 2024

Abstract: The proposed protocol is a simple, economical, and efficient method for primary osteoblast culture, demonstrating greater stability and yields than traditional approaches. Osteoblast in vitro cultures are widely used as a tool for studying bone formation and regeneration. However, protocol execution is one of the most challenging and least standardized steps in osteoblast culture. Objective: to present a modified, useful, and simple protocol for osteoblast culture from the parietal bone of Wistar rats. Methods: Parietal bone from 11-day-old Wistar rats was used; the sample was fractionated for histochemical analysis and cell culture, while the human osteoblast cell line (NHost) was seeded as a control. Phenotypic characterization of osteoblasts in both cultures was performed by immunostaining for alkaline phosphatase and osteocalcin. Primary cultures were stained with alizarin red for morphological and calcium deposition characterization. Results: Parietal bone was found to undergo intramembranous ossification with higher levels of osteoblasts in the suture periosteal area. Positive immunostaining for the three markers was observed in both primary and control cultures. After culture confluence, osteoblasts form three-dimensional structures that resemble bone spicules. Conclusions: Preservation of the periosteum and sutures during bone collection is crucial because these regions contain abundant osteoblastic progenitors. The proposed model can be a valuable tool for tissue engineering and bone biology research, particularly in low-resource settings where

the high costs of more complex techniques may be a barrier.

Keywords: Calvaria; cell culture; osteoblast; periosteum; Wistar rat.

新生威斯塔大鼠顶骨成骨细胞的培养及表征

摘要：所提出的方案是一种简单、经济、有效的原代成骨细胞培养方法，与传统方法相比，其稳定性和产量更高。成骨细胞体外培养被广泛用作研究骨形成和再生的工具。然而，方案执行是成骨细胞培养中最具挑战性和最不标准化的步骤之一。目的：提出一种改良、实用且简单的 Wistar 大鼠顶骨成骨细胞培养方案。方法：使用 11 日龄 Wistar 大鼠的顶骨；将样品分馏以进行组织化学分析和细胞培养，同时接种人类成骨细胞系 (NHost) 作为对照。通过对碱性磷酸酶和骨钙素进行免疫染色，对两种培养物中的成骨细胞进行表型表征。用茜素红对原代培养物进行形态学和钙沉积表征。结果：发现顶骨发生膜内骨化，骨缝骨膜区域的成骨细胞水平较高。在原代培养物和对照培养物中均观察到三种标记物的阳性免疫染色。培养汇合后，成骨细胞形成类似于骨针的三维结构。结论：骨采集过程中保存骨膜和骨缝至关重要，因为这些区域含有丰富的成骨细胞祖细胞。所提出的模型可以成为组织工程和骨生物学研究的宝贵工具，特别是在资源匮乏的环境中，更复杂的技术成本高昂可能是一个障碍。

关键词：颅盖骨；细胞培养；成骨细胞；骨膜；威斯塔大鼠.

1. Introduction

Osteoblasts, the architects of bone tissue, play a pivotal role in osteoid synthesis and maturation and mineralization through a repertoire of proteins and signaling factors secreted into the extracellular matrix [1, 2]. These cells maintain intricate communication networks not only among themselves, but also with other bone tissue constituents (osteocytes, osteoblasts, osteoprogenitor cells and osteoclasts-which together form the cellular functional unit of bone. This unit is essential for the regulation of bone formation, growth, remodeling, and regeneration [3-5].

The journey of an osteoblast begins with its differentiation from a mesenchymal stem cell through a process called osteoblastogenesis. Once fully differentiated, osteoblasts can become osteocytes embedded in the calcified matrix, transform into bone lining cells, or undergo apoptosis [6, 7]. This differentiation is tightly regulated by transcription factors such as Cbfa1/Runx2 and Osterix, with Runx2 playing a critical role in directing cells toward the osteochondroprogenitor lineage and serving as an early marker of osteogenesis, particularly in pre-osteoblasts, which are characterized by the expression of alkaline phosphatase (ALP), an enzyme critical for calcium salt precipitation [4, 8].

Osterix is a critical transcription factor for the maturation of osteoblastic precursors into fully differentiated phenotypes. Mature osteoblasts synthesize key components of the organic extracellular matrix, such as type I collagen, osteocalcin (OCN), bone sialoprotein (BSP), and osteopontin (OPN), which are essential for mineralization and bone structural integrity of bone [9, 10]. The phenotypic characterization of mature osteoblasts, especially in vitro, often uses markers such as alkaline phosphatase (ALP) and OCN [11, 12].

Cell culture models are invaluable tools for investigating the factors that influence osteoblast formation, differentiation, and maturation, as well as extracellular matrix secretion and mineralization. These models provide a controlled environment free of confounding variables, facilitating precise analysis [3, 8]. They are also essential in tissue engineering, allowing the evaluation of new biomaterials for biocompatibility and osteogenic potential and the generation of autologous grafts from osteoblastic populations. The protocols for these cultures vary based on sample extraction, processing methods, and the specific culture media used to promote the osteoblastic phenotype [11, 13, 14].

Several approaches are used to obtain osteoblasts for cell culture, including the use of established osteosarcoma cell lines such as UMR-106, ROS 17/2, ROS 17/2.8, SaOS, TE-85, OHS-4, MG-63, and MC3T3-E1. Alternatively, primary and organ cultures, which are often derived from bone fragments or bimodal structures, provide a direct source of osteoprogenitor cells and osteoblasts [15]. Commonly used samples for these cultures include long bones, such as the tibia and

femur, and cranial bones, such as frontal and immature parietal bones (calvaria) [16]. In particular, neonatal rat parietal bones are a highly efficient source for the isolation and study of osteoblasts [17, 18]. A comparative study [19] demonstrated that cultures derived from neonatal rat calvaria exhibit superior growth, proliferation, and accelerated matrix maturation and mineralization compared with cultures derived from human trabecular bone or long bone explants.

Primary cultures of rat calvarial osteoblasts have greatly advanced our understanding of their molecular and cellular properties. In addition, these cultures allow the use of genetically modified bimodal cells and provide a robust platform for assessing the effects of specific factors, gene products, or small-molecule inhibitors on osteoblast function. These cells are capable of proliferating and forming a mature, mineralized collagen matrix, typically over a period of 21-28 days, providing a reliable system for assessing the effects of different experimental conditions on osteoblast maturation and activity [20].

Although cell culture is an invaluable tool for studying osteoblast biology, it has inherent limitations. The expression of a specific cell phenotype in culture is highly dependent on multiple variables, including the source of the biological material, methods of sample extraction, handling procedures, and the conditions of the culture itself, such as medium composition, growth duration, and the presence of factors that influence proliferation and differentiation [8, 21]. Furthermore, these cultures typically contain a heterogeneous population consisting of osteoblasts at different stages of maturation, as well as potential contaminants such as fibroblasts and periosteal progenitor cells. Nevertheless, under optimized conditions, these cultures efficiently synthesize mineralized bone matrix through the upregulation and secretion of extracellular matrix marker genes, rendering them robust in vitro models for bone formation [20].

Despite the availability of various methods for isolating bone cells, significant differences between protocols make it difficult to establish a standardized approach for obtaining osteoblasts with consistent activity. Many of the existing literature relies on classical protocols that often lack detailed descriptions of extraction and culture techniques, as well as comprehensive characterizations of sample composition and histological structure. This study aimed to provide a detailed, simplified, and effective protocol for the isolation of osteoblasts from the parietal bone of Wistar rats, tailored to the needs of basic research.

2. Methods and Materials

2.1. Biomodel

This study was approved by the Animal Ethics Committee of the Universidad del Valle, Faculty of Health (approval code: 004-016, 16 June 2016). All procedures followed the standards outlined in the National Research Council's Guide for the Care and Use of Laboratory Animals [22] and complied with the ARRIVE guidelines 2.0 for reporting in vivo experiments [23].

Eleven five-day-old neonatal Wistar rats obtained from the Intermediate Laboratory of Preclinical Research and Biotherium (LABBIO) of the Universidad del Valle were used in this study. The animals were maintained under controlled environmental conditions, including a 12-hour light/dark cycle, a temperature range of 22-23°C, and relative humidity between 45% and 70%. Both male and female neonates were included to ensure balanced representation of the sexes. The number of animals used was determined based on practical considerations. The protocol included several key steps: extraction of bone fragments, histochemical processing, cell culture and subsequent immunohistochemical characterization and mineralization assays.

2.2. Bone Fragment Extraction

Biomodels were euthanized using an overdose of 100% isoflurane according to the AVMA Guide for the Euthanasia of Animals [24]. After euthanasia, they were thoroughly cleaned with 10% iodopovidone and immersed in 70% ethanol for four minutes. Dissection was performed in a sterilized laminar flow cabinet where the parietal bones were carefully extracted. The bones were then placed in a Petri dish containing phosphate-buffered saline with antibiotics (PBSa) prepared from 500 mL of 0.1 M PBS (1.31 g sodium phosphate monobasic monohydrate, 5.75 g anhydrous sodium phosphate dibasic, 4.45 g sodium chloride) with 2% amphotericin B. Muscles and blood vessels were carefully removed during dissection. The periosteum, dura mater, and sutures were either completely removed or preserved, depending on the requirements of the study. The bone samples were then divided: one portion for histological analysis and the remainder in a Falcon tube containing PBSa for subsequent cell culture.

2.3. Histological Processing

For histological examination, samples were fixed in Bouin's solution (750 mL 1.9% picric acid, 250 mL 37% formaldehyde, 50 mL glacial acetic acid; pH 2.2) for five hours, followed by three washes in 70% ethanol for 20 minutes each. Post-fixation was carried out in 10% formalin buffer, and the specimens were embedded in paraffin after dehydration through increasing concentrations of alcohol and clearing in xylene. Thin sections, 4 μm thick, were cut using a Leica RM 2135 rotary microtome (Buffalo Grove, Illinois, USA) and stained with hematoxylin-eosin for microscopic evaluation.

2.4. Cell Culture: Establishment of Primary Culture

Primary culture was established at the Cytogenetics and Developmental Biology Laboratory of the Universidad del Valle. Bone fragments were subjected to two consecutive enzymatic treatments to facilitate cell dissociation. First, a digestion solution (7mL) containing 15 mL trypsin-EDTA solution (0.1% EDTA in 50 mL sterile PBS with 0.25 mL trypsin) and 9.6 mg type II collagenase was added to the fragments. The mixture was incubated in a shaking water bath at 37°C for 20 min to promote enzymatic digestion. After incubation, the supernatant was removed, and the digestion process was repeated to ensure optimal dissociation. After the second incubation, 2 mL of fetal bovine serum (FBS) was added to neutralize the enzymatic activity. The suspension was gently mixed and the supernatant was discarded.

The fragments were rinsed with Dulbecco's modified Eagle's medium (DMEM), and 1 mL of the supplemented medium was added to resuspend the fragments. The supplemented medium consisted of 1% penicillin (10,000 U/mL), streptomycin (10,000 µg/mL), 0.5% amphotericin B (250 µg/mL), ascorbic acid (100 µg/mL), and 10% FBS, all diluted in a 1:1 mixture of DMEM and osteogenic growth medium (OGM). The fragments were evenly distributed into five 25 cm² tissue culture flasks using a micropipette. An additional 1 mL of the supplemented medium was added to each flask. The culture medium was refreshed and cell growth was monitored three times a week to assess cell proliferation and viability.

2.5. Positive Control for Osteoblastic Phenotype NHost Cell Line Culture

To validate the osteoblastic phenotype in primary culture, the NHost human osteoblast cell line (C2538, LONZA Inc., Walkersville, USA) was used as a positive control. The crystal fluid containing the cell line was decontaminated with 70% ethanol and partially immersed in a 37°C water bath until only a thin layer of ice remained. At this point, the crystal tissue was removed and 1 mL of the cell suspension containing approximately 500,000 cells was transferred to a 75 cm² cell culture flask containing 10 mL of osteoblast growth medium (OGM) supplemented with 20% fetal bovine serum (FBS). The cells were gently resuspended by pipetting, and 5 mL of the suspension was transferred to another flask, distributing approximately 250,000 cells per flask at a density of 3,333 cells/cm². The medium was refreshed three times a week. The initial number of cells was determined according to the manufacturer's protocol and not by the study authors.

Subcultures of both primary and NHost cell lines were prepared for subsequent phenotypic characterization. The process began by aspirating the culture medium, rinsing it with phosphate-buffered saline (PBS), and adding 3 mL of trypsin solution to each flask. The flasks were incubated at 37°C for six minutes to ensure complete cell detachment, which was then stopped by adding 5 mL of Dulbecco's modified Eagle's medium (DMEM) and 1 mL of FBS. The cell suspension was centrifuged at 1500 rpm for 10 min, and the resulting cell pellet was resuspended in 1 mL of the supplemented medium. Cell counting was performed using a Neubauer chamber to determine the total number of cells.

For the characterization assays, cells were seeded into 24-well plates containing 12-mm round glass slides. Approximately 42,000 cells per well (37,168 cells/cm²) were seeded for the primary culture, following the recommended seeding density for osteoblast cultures (Merck, 2024). For the NHost cell line, approximately 3,700 cells per well (3,274 cells/cm²) were seeded, reflecting the total cell yield. After 1 month of cultivation, the cells were fixed in an ascending alcohol dehydration series (70%, 80% and 95% ethanol) to ensure proper preparation for further analysis.

2.6. Immunocytochemical Characterization

To characterize the cultures immunocytochemically, specific immunolabeling was performed for Alkaline Phosphatase (TNAP N18, SC-23430, goat polyclonal IgG, 200 µg/mL; Santa Cruz Biotechnology, Dallas, Texas, USA) and Osteocalcin (Osteocalcin V-19, SC-18319, goat polyclonal IgG, 200 µg/mL; Santa Cruz Biotechnology, Dallas, Texas, USA). The permeabilization process used PBS-T (PBS with 0.5% Tween 20) for 45 min, followed by a 10-min block of endogenous peroxidase activity using 0.3% hydrogen peroxide. To prevent nonspecific antibody binding, Ultra V Block (TA-125-UV) from the Lab Vision™ Ultra Vision™ LP Detection System: HRP Polymer/DAB Plus Chromogen kit (ThermoFisher, Waltham, Massachusetts, USA) was applied for 5 min.

The primary antibodies were incubated with the cultures at a 1:50 dilution in phosphate-buffered saline (PBS) for 72 h at 4°C to ensure optimal binding to their targets. The secondary antibody, Donkey anti-goat + horseradish peroxidase (SC-2020; Santa Cruz Biotechnology, Dallas, Texas, USA), was used at a 1:500 concentration and incubated for 90 min at room temperature to achieve effective signal amplification. Chromogenic detection was developed using 3,3'-diaminobenzidine (DAB) with hydrogen peroxide for 10 min, producing a visible precipitate at the sites of antigen localization. Counterstaining was conducted with Harris hematoxylin for 30 s to provide cellular contrast. The samples were then subjected to a graded dehydration series, including three 30-s washes in 95% ethanol, 100% ethanol, and xylene, before being mounted for microscopic examination. This meticulous

procedure ensured the reliable visualization of osteoblastic markers and confirmed the phenotypic characteristics of the cultured cells.

2.7. Mineralization Assay

To evaluate mineralization in the primary culture, an assay was performed to detect calcium deposition over time. After the subcultured cells reached more than 75% confluence (referred to as day 0), they were fixed by alcoholic dehydration at three different intervals: 14, 21, and 39 days after confluence. The mineral deposits were stained using a pH 4.2 alizarin red solution (prepared by dissolving 1 g alizarin red in 50 mL distilled water), which was applied to each well for 10 min. After staining, the wells were thoroughly rinsed with running water to remove excess dye and then counterstained with Harris hematoxylin for better contrast. The samples were then subjected to a dehydration series of 95% ethanol, 100% ethanol, and xylene for optimal preservation and mounting.

To further examine the cytoskeletal architecture and morphology, α-actin filaments were visualized using a phalloidin-labeled immunofluorescence technique. Cell fixation was achieved using a series of graded alcohol baths (70%, 80% and 95% ethanol) for 5 min each to ensure structural preservation. Permeabilization was performed using PBS-T (PBS, 0.1M with 0.5% Tween 20) to facilitate antibody penetration. After two 3-min rinses with PBS, the cells were incubated with phalloidin conjugated to tetramethylrhodamine B isothiocyanate at a 1:200 dilution for 1 h at room temperature. The coverslips were then mounted onto microscope slides using ProLong Gold antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI) to counterstain the nuclei, thereby ensuring clear nuclear localization. The stained specimens were stored in the dark to prevent photobleaching of the fluorescent labels, ensuring optimal visualization of both mineralized deposits and cytoskeletal structures.

2.8. Data Analysis

The histochemical, immunocytochemical, and alizarin red-stained preparations were analyzed by light microscopy (Leica DM750) equipped with an integrated camera (Leica DFC295). Image acquisition was performed using LAS V3.8 software (Leica Application Suite) to maintain consistent light parameters across all images for each specific characterization assay (Leica Biosystems, Buffalo Grove, Illinois, USA).

Qualitative comparisons were performed to evaluate the morphological characteristics of both primary and NHost cell line cultures, focusing on the expression of osteoblastic markers, specifically alkaline phosphatase and osteocalcin. A detailed descriptive analysis was performed to assess variations in cell morphology and calcium deposition in the primary culture. For higher resolution and more accurate imaging, additional images were taken using a Zeiss LSM 700 laser scanning confocal microscope and LSM ZEN 2009 software to visualize and document the cytoskeletal organization and mineralization processes in greater detail.

2.9. Reagents

The reagents used in this study were purchased from reputable suppliers to ensure the consistency and reliability of the experimental results. Enzymes and media included trypsin (Catalog No. 17-16E) and EDTA (Catalog No. 51201), both of which are critical for cell dissociation processes. Fetal bovine serum (Catalog No. 14-502F) was used as a supplement to cell culture media to provide essential growth factors. Amphotericin B (Catalog No. 17-836E) was used as an antifungal agent, whereas penicillin/streptomycin (10,000 U/mL penicillin and 10,000 μg/mL streptomycin, Catalog No. 17-602E) was used to prevent bacterial contamination.

Osteoblast cultures were prepared using ascorbic acid (70 mM, Catalog No. CC-4398Y), osteoblast growth medium (OGM) supplemented with single quotients and growth factors (Catalog No. CC-4193), and Dulbecco's modified Eagle's medium (DMEM, with 4.5 g/L glucose and L-glutamine, Catalog No. 12-604F) from LONZA Inc. of Walkersville, USA. Collagenase type II (0.5-5.0 FALGPA units/mg solid, Catalog No. C6885-100MG), which is required for enzymatic digestion of the extracellular matrix, was purchased from Sigma-Aldrich (St. Louis, MO, USA.

Anesthesia during the procedures was achieved using isoflurane USP (Baxter International Inc. (Deerfield, Illinois, USA). Iodopovidone (polyvinylpyrrolidone iodine, 0.1 g/mL), an antiseptic agent, was provided by Laboratorios ECAR, Medellin, Antioquia, Colombia. These reagents were selected for their high quality and relevance to the experimental protocols, ensuring that all critical factors for successful cellular and biochemical assays were adequately addressed.

3. Results and Discussion

Histological examination of the samples, particularly those in which sutures and external bone surfaces had been removed, revealed significant histomorphological differences compared with samples in which the bone surfaces were preserved (Figure 1). In specimens in which the periosteum, sutures and suture-adjacent areas were removed, there was a marked reduction in cellularity within the bone matrix, with sparse cellular presence along the bone surfaces (Figure 1C). Notably, no cultured cells were obtained from these samples, indicating a significant compromise in cell viability.

In contrast, bone fragments with intact surfaces showed maturing parietal bone (H.lib) characterized by a two-layered compact bone structure. This structure consisted of an outer bone surface (upper part of the

cranial vault) and an inner bone surface (adjacent to the encephalon). The medullary cavity, which was fully developed between the two surfaces, was populated by red bone marrow. Within this medullary region, small trabeculae were particularly prominent near the internal surface. The compact bone of the outer surface exhibited aligned osteocytes and cement lines parallel to the surface architecture. The ongoing calcification process was incomplete, with areas of the organic matrix exhibiting basophilia. The outer surface of the periosteum is remarkably thin with no discernible osteogenic layer. Conversely, an osteogenic layer rich in osteoblasts was observed between the internal bone surface and the dura mater, which exceeded the cellular activity observed on the external surface (Figure 1A).

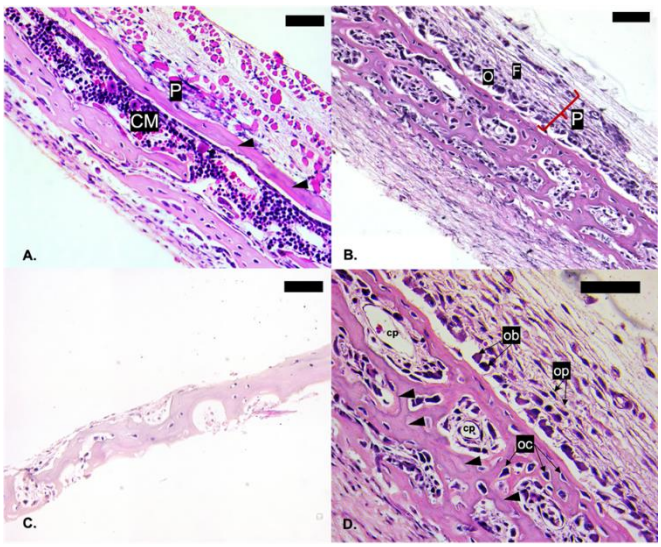

Figure 1. Histology of neonatal calvaria. A. Parietal bone ×10. Periosteum (P) with few osteoblasts and osteoprogenitor cells. Medullary cavity (CM) between two layers of lamellar bone. Arrowheads: basophilic regions running parallel to the lamellae. **B.** Parietal bone part of the suture zone, magnification 10x. Periosteum with two layers, fibrillar layer (F) and osteogenic layer (O), with abundant osteoprogenitor cells and osteoblasts. **C.** Parietal bone with mechanical removal of periosteum and dura mater 10x. No osteogenic layer is observed on any of its surfaces, no formation of bone marrow or lamellar bone layers. Low density of osteocytes compared to the parietal bone of the suture zone. **D.** Detail of the F and O layers in B at 40x. Osteoblasts (ob), osteoprogenitor cells (op), capillaries (cp). Arrowheads: basophilic regions show parts of extracellular matrix without mineralization. Bar in A, B and C 200µm, in D 50µm. (Authors' photo)

In the bone region corresponding to the suture area (H.sut), the confluences of spicules interspersed with capillaries and connective tissue were identified. Osteocytes in this region were both prominent and abundant, indicating active bone formation. There was also clear evidence of an early-stage mineralization process (Figure 1D). The periosteum in the outer bone region exhibited a pronounced osteogenic layer, whereas in the region contacting the dura mater, only a sparse distribution of osteoblasts and progenitor cells was observed (Figure 1B). This indicates differential osteogenic activity depending on the anatomical location within the bone**.**

3.1. Primary Cell Culture Analysis

The primary cell culture showed robust cell attachment to the culture surface, accompanied by active proliferation, intercellular junction formation, and secretion of extracellular matrix components. Approximately 15 days after seeding, the culture reached >75% confluence, indicating a substantial expansion of the cell population.

Initially, the cells detached from the calvarial fragments exhibited an elongated and slender. As the culture matured, the cells underwent significant morphological changes, with an increasingly prominent nucleus containing one or more nucleoli. The cytoplasm expanded significantly and developed multiple extensions that facilitated further intercellular interactions. As cell proliferation progressed, the distinction between individual cells became increasingly blurred, reflecting the high-density monolayer characteristic of a confluent culture (Figures 2 and 3).

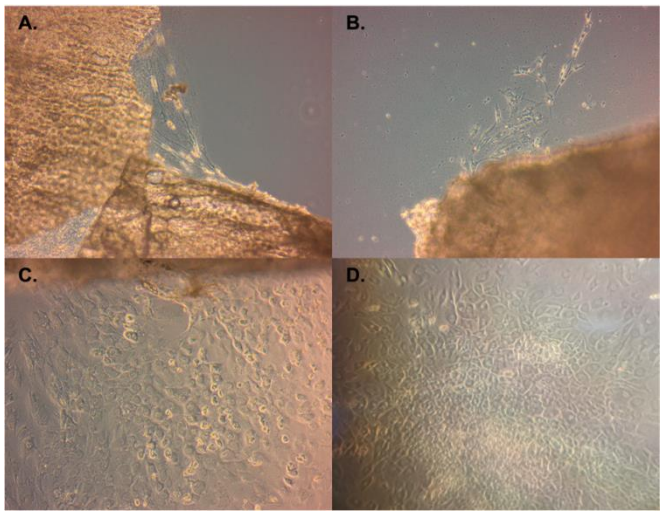

Figure 2. Primary culture of calvaria 10x. A. A calvarial bone fragment with some cells adhered to the surface. **B.** Migrating cells from the bone fragment adhere to the surface of the cell culture flask, their morphology is spindle-shaped. **C.** Confluence of the culture greater than 95%, almost all cells have a polyhedral and irregular morphology. **D.** Cells in culture grouped together to form a cluster (Authors' photo)

Immunofluorescence analysis provided further

insight into the cytoskeletal organization within these cells. Stress fibers composed of actin filaments were clearly labeled with phalloidin, highlighting the structural integrity and tension within the cells. In addition, nuclei were clearly delineated using DAPI staining, allowing precise visualization of nuclear morphology (Figure 3). These findings highlight the dynamic nature of cultured cells and their capacity for growth and structural organization in vitro.

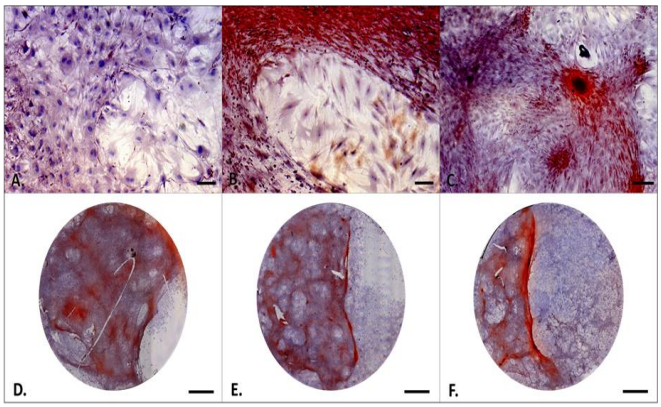

Figure 3. Filaments of α-actin in osteoblastic cell culture. A and **B** show the different morphologies acquired by the cells in culture. **C** and **D** show how the cytoplasm of the cells can expand widely and form connections with neighboring cells. A 40x; B and C 100x; D 150x. (Authors' photo)

3.2. Advanced Culture Development and Osteoblastic Differentiation

Approximately 60 days after subculture, the primary cell culture showed the emergence of highly prominent nodules, which subsequently gave rise to complex three-dimensional structures organized in circumferential arrays (Figure 4). These nodules marked an important stage in culture maturation, indicating advanced cellular differentiation and tissue organization.

Within the regions of direct cell-to-cell contact, small deposits of calcified matrix were observed. These deposits were successfully identified using the alizarin red staining technique, which confirmed the presence of mineralized calcium in the extracellular matrix (Figure 4A). As the culture progressed, these structures began to fold, forming distinct stain-positive borders. Notably, a new layer of cells was observed within the free spaces created by these folds, suggesting ongoing cellular proliferation and matrix deposition beneath the established layers (Figures 4D, E and F).

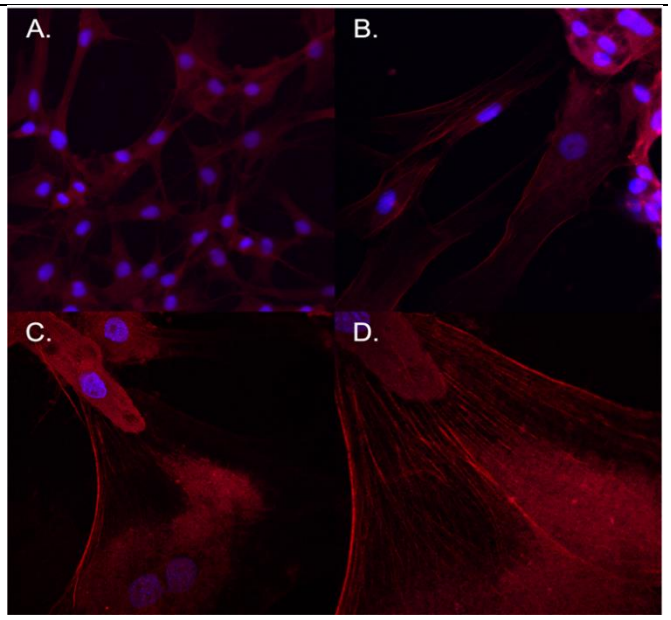

Figure 4. 10X: Alizarin red. Assay of mineralization and calcium deposition in primary culture of neonatal calvaria. **A**. At 30 days of culture. Small calcium deposits are observed stained dark red. **B** and **C** 60 days in culture. Three-dimensional conformation of clustered cells, abundant deposits of calcified matrix and red-stained nodules. **D, E** and **F** 60 days in culture, different times of cell culture collection. As the culture is folded, the remaining free spaces begin to populate with cells. Bar in A and B 100μm; C 200μm; D, E and F 2mm). (Authors' photo)

Immunocytochemical analysis confirmed the osteoblastic phenotype of cultured cells. The cells showed positive staining for key osteoblastic markers, including alkaline phosphatase and osteocalcin, confirming their osteoblastic identity and their role in the observed mineralization process (Figure 5). This progression indicates the successful differentiation of the primary culture into a mature osteoblastic lineage capable of forming and mineralizing bone-like structures in vitro.

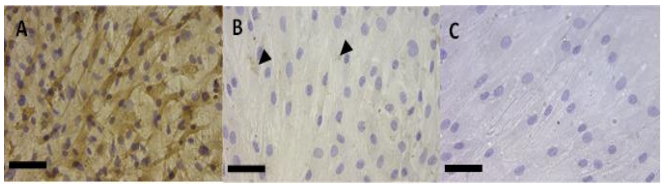

Figure 5. Immunocytochemical characterization of cell culture 40x. Primary culture from neonatal calvaria. A Alkaline phosphatase, **B** Osteocalcin, **C** Negative control. Immunolabeling was mainly at the intracellular level. Arrowheads: extracellular labelling. Human osteoblast cell line cultures were used as a positive control. Bar 50μm. (Authors' photo)

3.3. Behavior of the Human Cell Line

The human cell line showed a distinct growth pattern

compared to the primary culture. After 45 days in culture, the cell line reached approximately 50% confluence. In contrast to the ordered growth observed in primary cells, these cells aggregated into irregular clusters and nodules without any discernible pattern. These clusters contained both cells and extracellular matrix, reflecting a more disorganized structure.

Despite the lack of a defined arrangement, the cells were confirmed to express key osteoblastic markers, including alkaline phosphatase and osteocalcin, consistent with their expected phenotype. However, the cells maintained an elongated and slender morphology throughout the culture period, with no significant morphological changes observed. This persistent cell shape suggests a stable but atypical differentiation trajectory compared with primary cultures, indicating potential differences in the cellular behavior or maturation capacity of this human cell line (data not published.

Osteogenic stem cells are central to the repair and regeneration of craniofacial structures, particularly for treating small fractures and craniofacial pathologies. Their potential extends beyond these applications, offering a promising alternative for fracture repair beyond critical size, as highlighted in [25] and [26]. Furthermore, the isolation and culture of these cells provide invaluable insights into mineralization processes, the intricacies of hormonal interactions [28], and the synthesis of bone-specific proteins [29, 30]. This study presents an efficient and cost-effective protocol for deriving osteoblasts from neonatal rat calvaria, which may significantly advance research efforts in bone biology.

In the field of regenerative medicine, the extraction of osteogenic stem cells has traditionally focused on mesenchymal stem cells derived from the bone marrow of long bones [31]. However, recent research suggests that the flat bones of the skull, particularly the frontal and parietal bones, harbor a unique reservoir of osteogenic stem cells within the sutures, periosteal regions, and dura mater. These cells exhibit distinct characteristics that distinguish them from those routinely harvested from long bones, making them a viable and effective source for osteoblast extraction and subsequent studies [32-34].

In particular, as demonstrated in [35], neonatal mouse calvarial bone exhibited faster growth, higher proliferation rates, and a more rapid onset of maturation and matrix mineralization compared with cultured human humeral trabecular bone and rat humeral long bone. These results highlight the advantages of using calvarial cells in osteogenic research.

While there are structural and mechanical differences between human and rodent bone—for example, the presence of Haversian systems in human compact bone, which are absent in rodents due to their thinner bone composition—the basic histological components (cells and bone matrix), signaling pathways, and responses to mutations and pharmacological agents are remarkably similar across species [36]. This similarity reinforces the utility of the mouse model as a robust platform for preliminary investigations in bone research, providing critical insights that can be translated into human studies.

One of the key variables influencing these protocols is the developmental stage of the models. The selection ranges from 15 to 16 days post coitus (dpc), which coincides with the onset of ossification in the parietal bones, to neonatal stages extending up to 21 days postnatal [37]. The calvarial bones, which develop by direct or intramembranous ossification bypassing the osteochondrogenic precursors involved in endochondral ossification, provide a unique opportunity to study osteogenesis directly from mesenchymal cells [38].

The selection of sample age is critical because it reflects the level of osteogenic development and differentiation within the calvarial bone. Samples are typically selected before the completion of osteogenic development to ensure the presence of mesenchymal stem cells, which are critical for studying early osteoprogenitor behavior. However, it is important to avoid using samples from very early developmental stages because the presence of more pluripotent, uncommitted mesenchymal progenitors increases the risk of contamination and reduces the specificity of the osteogenic lineage.

The developmental timeline of the rat neurocranium provides further insight into the selection process. Complete formation of the neurocranial elements occurs at approximately 34 days postnatally, by which time the cranial vault has reached approximately 93% of adult size [39]. This developmental milestone marks the near-completion of cranial ossification, suggesting that earlier stages are more suitable for obtaining osteoprogenitors that are still actively involved in bone formation.

However, our results show notable differences compared to the seminal work of Binderman et al. [27]. In their study, the periosteum from adult and fetal rat calvaria was cultured using a medium composition strikingly similar to that used in our protocol. Although they achieved comparable times to confluence (8 to 15 days) over a slightly larger culture area, they experienced significantly longer periods before matrix mineralization occurred – approximately five weeks longer than in our study, irrespective of the age of the bimodal.

This discrepancy highlights the presence and persistence of osteoprogenitor cells within the periosteum, even up to 21 days postnatally. However, it also highlights the challenge of rapidly committing these cells to the osteogenic lineage and progressing them to

osteoblastic maturity, as evidenced by their delayed ability to produce a mineralized matrix. The extended timeframe observed by Binderman et al. [27] suggests that while progenitor cells are indeed present, the process of differentiation and maturation is significantly influenced by both the microenvironment and the specific cellular sources used.

In our study, we processed parietal bone fragments with and without the periosteum, dura mater, and suture-associated regions. The removal of these structures was harmful to cell growth, highlighting their critical role in osteoblastic activity. In contrast, when these bone-associated structures were retained, not only was contamination by other cell types effectively prevented, and robust osteoblastogenic growth was observed (Figure 5). This was evidenced by the presence of a significant population of osteoprogenitor cells and mature osteoblasts, as confirmed by histological analysis (Figure 1).

These findings, in conjunction with the seminal work of Binderman et al. [27], highlight the osteogenic potential of these structures and emphasize the importance of their preservation during bone fragmentation. Therefore, preservation of the periosteum, dura mater, and suture regions is critical for successful osteoblast culture because these areas serve as vital reservoirs of osteoprogenitor cells that contribute to the robust formation of osteoblasts.

One notable point of divergence from our study can be found in the work of Zheng et al. [35], whose study, which included media conditioned with supplements such as beta-glycerophosphate and dexamethasone, resulted in a growth rate 15 times higher than ours. However, certain supplements, particularly when used in isolation, may be inadequate or problematic. For example, beta-glycerophosphate alone does not induce mineralized nodule formation, so additional supplements are required, which increases the cost and complexity of the culture process. In addition, although effective, dexamethasone is associated with undesirable adipogenic effects in bone cell cultures, potentially compromising the osteogenic focus of the study [41, 42].

In contrast, our study showed that the use of ascorbic acid alone was sufficient to support the growth of osteoblastic cells capable of producing a mineralized extracellular matrix. These findings suggest that a simplified supplementation strategy may be effective in reducing both the cost and potential side effects associated with more complex or aggressive supplementation regimens. Our findings support a more streamlined approach to osteoblast culture, highlighting the preservation of critical bone-associated structures and the judicious use of supplements to optimize osteogenic outcomes. This approach not only improves the efficiency and reliability of osteoblast cultures and provides valuable insights into the refinement of protocols for bone tissue engineering and regenerative medicine.

Culturing of the NHost cell line served as a positive control to ensure the purity of our culture and, as expected, showed strong positivity for osteoblastic phenotype markers. This result is very similar to that obtained by culturing bone fragments with the periosteum and suture areas preserved and supplemented with ascorbic acid. In particular, the latter method offers a much more cost-effective alternative to the use of established cell lines [21, 43].

Although cell lines such as NHost offer the advantage of highly purified culture, they are associated with significant financial and logistical burdens. The high costs associated with these cell lines arise not only from their initial purchase but also from the need for specialized culture media, which are proprietary and significantly more expensive than those used in primary cultures. In contrast, our approach using bone fragments with preserved periosteum and sutures, coupled with the relatively simple addition of ascorbic acid, provides comparable osteoblastic outcomes at a fraction of the cost. This method offers researchers a practical and economical alternative, particularly in contexts where budget constraints are a significant concern.

By demonstrating that a more affordable protocol can achieve results similar to those obtained using expensive, commercially available cell lines, this study highlights the potential for optimizing resource use in osteoblast research. This not only makes advanced research more accessible but also encourages the continued refinement of cost-effective methods without compromising the integrity or efficacy of experimental results.

4. Conclusion

To summarize, this study presents a well-developed, cost-effective protocol that significantly reduces production costs while remaining highly efficient, making it a practical and accessible option for laboratories, especially in resource-limited settings. The method successfully establishes primary cell cultures from neonatal flat bones, with a particular focus on preserving key regions such as the periosteum, dura mater, and suture, unlike other approaches that discard these structures. Our findings confirmed that this method reliably produces cells expressing osteoblastic markers, as demonstrated by the phenotypic characterization. The potential for broad application in biomedical and biomaterials research is evident, positioning this protocol as a valuable tool for advancing osteoblast-related studies while overcoming the financial challenges typically associated with more expensive cell culture techniques. Further investigation and validation of its long-term applicability in various research contexts are encouraged.

Declarations

Author Contributions

Conceptualization, A.M.S.E. L.S.M., M.A.O., and M.C.P.; methodology, A.M.S.E. L.S.M., M.A.O., and M.C.P.; software, A.M.S.E. L.S.M., M.A.O., and M.C.P.; validation, A.M.S.E. L.S.M., M.A.O., and M.C.P.; formal analysis, A.M.S.E. L.S.M., M.A.O., and M.C.P.; investigation, A.M.S.E. L.S.M., M.A.O. and M.C.P.; resources, A.M.S.E. L.S.M., M.A.O., and M.C.P.; data curation, A.M.S.E. L.S.M., M.A.O. and M.C.P.; writing—original draft preparation, A.M.S.E. L.S.M., M.A.O. and M.C.P.; writing—review and editing, A.M.S.E. L.S.M., M.A.O., and M.C.P.; visualization, A.M.S.E.; supervision, A.M.S.E.; project administration, A.M.S.E. L.S.M.; funding acquisition, A.M.S.E., L.S.M. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data presented in this study are available on request from the corresponding author.

Funding

Funding information is not available, but The Universidad del Valle, Colombia, is committed to providing financial support for this research project. As the sponsoring institution, Universidad del Valle will provide the necessary resources and oversight to facilitate the successful completion of the study.

Institutional Review Board Statement

The animal study protocol was approved by the Institutional Review Board of the Universidad del Valle, Cali, Colombia.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this manuscript. In addition, ethical issues including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies were completely observed by the authors.

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