IN VITRO PLACENTA STEM CELLS ISOLATION AND IN VIVO DIFFERENTIATION ON POROUS HYDROXYAPATITE BLOCK IN RABBITS MANDIBLE

Ali Abass Ajeel [*], Kasim Obaid Hussein Ali [**] , Karima Akool Al-Salihi [*]

[*] College of Veterinary Medicine, University of Al Muthanna, Al Muthanna, Iraq

** College of Medicine, University of Al Muthanna , Al-Hussein Teaching Hospital. Al Muthanna, Iraq.

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Correspondening author :*kama_akool18@yahoo.co.uk,*

ABSTRACT

This study intended to isolate placenta stem cell (PSCs) and, to assess its ability to differentiate into osteoblasts on the porous hydroxyapatite block (PHAB). In addition to evaluating the bone formation around PHAB graft introduced in the mandible defect area using light (LM) and scanning electron microscopy (SEM). A protocol was developed for isolation of PSCs, and osteogenic differentiation medium (ODM) was used for in vitro differentiation into osteoblasts. Four New Zealand male rabbits were use to place the PSCs-PHA blocks (PSCs-PHABs) in the created defect on the body of the right mandible. The left mandible was use as control where PHABs-without cells (PHABs-WCs) were introduced on the created defected area. The implants retrieved at 60 days and processed for SEM and histological investigations. No bone formation was seen in the control (PHABs-WCs) implants. Both LM and SEM revealed adherence of PSCs to PHABs in vitro after seven days of the incubation period. Moreover, SEM verified the attachment and growth of the cells on the surface accompanied by invading in varying depths of PHABs matrix. Macroscopically, PSCs-PHABs retrieved implants revealed vascularization and bone-like tissue. The mature bone development was seen in the histological slices, while, multiple layers of cells were seen in SEM accompanied by layers of collagen that covered the surface of PHABs. In conclusion, this study approved the isolation of PSCs and its differentiation into osteoblasts.

Moreover, PSCs can be considered as an easily reachable supply of multipotent stem cells for prospect trials and tissue engineering clinical purposes for the reconstruction of bone in the presence of appropriate scaffold material.

INTRODUCTION

The undifferentiated stem cells donate unlimited promising for clinical reconstruction applications because various daughter types cells may be originated from these cells (1). Embryonic stem cells (ESCs) derivation is a breakthrough achievement in science (2, 3, 4). ESCs considered as pluripotential cells that have the ability to form more than one type of body tissue. These cells can only be isolated from the inner cell mass of early embryos associated with scientific, religion, ethical and technical conflict issues that lead to hinder its accessibility (2). Adult stem cells (ASCs) also have a potential capability to differentiate to various tissues of different germ layer (5). Various tissues such as placenta, adipose tissue (6), bone marrow (7, 8) and tooth pulp (9) considered as a possible origin of stem cells. A placenta exists as temporary tissue with the possibility to donate the ASCs (10). During pregnancy, placenta maintains fetus requirements such as nutrition and oxygen. Moreover, it is a feto-maternal structure, molded by both maternal and fetal tissue. Additionally, its successful development is a critical process in embryogenesis. The normal growth and function of the placenta are essential to the well-being of the fetus. Naturally, the placenta is discarded (postpartum /Medical waste) after achieving its necessary purpose of conserves the embryo and fetus (11). A placenta comprises a large, wealthy resource of ASCs; also, they can differentiate into multiple diverse cells types. PSCs authorize to use in cell therapy-based treatment because their immunological features, simplicity in obtaining from the donor and no ethical issues d for its use. Adult stem cells have a wide range of plasticity to differentiate into various cells and could offer therapeutic impact in a range of applications. The differentiation ability of adult stem cells is depended on its progenitor nature and the micromilieu in which it exists. Also, many factors are affected on the differentiation mechanism such as tiny particles, drugs, cytokines, and growth elements (12). Osteoblasts and its progenitors are the cellular components of the bones and its ability to form the bone is affected by certain biomaterials and the micromilieu (13). Limited bone function and poor outcome occur from inappropriate healing and regeneration mechanisms in many diseases, although the regeneration process of the bone occurs after injuries. Different techniques can approach alveolar bone regeneration, and autogenous bone graft remains the preferred

reconstructive method. However, this source has some limitations such as increased operative time for bone graft harvesting, graft resorption, donor site morbidity, and limited availability. Therefore, there has been attention in the development of new grafting materials using allogenic, xenogeneic and synthetic bioimplants for reconstructive bone damages, and have been applied as a substitute for autogenous bone grafts (14, 15, 16, 17). Tissue engineering is developed successfully using a different combination of genetic factor, protein and cell therapy and considered as an efficient and safer treatment system for soft and hard tissue regeneration (18). Placenta stem cell had been accepting as an abundant source and an ethically unproblematic multipotent cell that has multi-lineage differentiation and immunosuppressive properties (19). Moreover, it has been approved as an easily existing in the in vitro model for elderly women osteoporosis treatment, and its osteogenic differentiation ability might have a potential therapeutic effect in osteoporosis treatment (1). The crystalline phase of natural bone is analogous to porous hydroxyapatite (PHA) that have been used as bone grafts in different forms (blocks and granules) in orthopedic, dental or craniofacial construction (20, 21). Besides, previous reports approved that PHA is filled with newly formed host bone when implanted in vivo. Furthermore, seeding PSCs with appropriate ODM micromilieu stimulated differentiation of these cells into osteoblasts (6, 21, 22).

The publications concerning isolation and differentiation of placental stem cells into osteoblasts and bone tissue engineering are scarce in Iraq. Consequently, this study designed to isolate and differentiate PSCs. In addition, to assess its ability to differentiate into osteoblasts on PHABs and to evaluate the bone formation around grafts introduced into created non-healing mandible defect.

MATERIALS AND METHODS

This study was officially permitted from animal and research ethical committees (College of veterinary medicine / Al-Muthanna University). The gaining of the placenta from the donor was done according to signed informed agreement formula at the labor room / Child Hospital / AL Muthanna province. A complete medical examination have been done for the donors to exclude cancer and some infectious diseases such as Hepatitis and HIV. In this study, 3 full term-pregnancy placentae (38-40 weeks' gestation) were collected, and the PSCs were isolated according to methods described by (2). Initial, the placenta was drainage from the umbilical cord blood and splashed several times by PBS (pH 7.2). Then, each placenta was

dissected and minced to small pieces and washed again with PBS. Later on, it treated with DNase I and trypsin (0.01% and 0.25%) for one hour at 37 ^{o} C for removing trophoblasts. Metal sieve (250 µm) was used for sample filtration. Collagenase I (0.1%) was used for digestion the remnant's tissue for 1 hour at 37 ^{o} C and the undigested fragments were also eliminated by passing via a 250 µm sieve then 100 µm cell nylon membranes. Centrifugation was done for ten minutes at 300 g, where cells were gathered and mixed with a solution containing 155 mmol/L NH4Cl and 20 mmol/l Tris for five minutes for lysis red cells. Later on, centrifugation was done for the cells at 300 g for 10 minutes. The cell's pellet was cultured in a basal medium containing a Dulbecco's modified medium complemented with Penicillin/ streptomycin (each 100 U/ ml), 20% fetal bovine serum (FBS) and 5 ng/ml basic fibroblast growth factor and 1mM/L-glutamine. Cell cultures were kept in a humidified chamber containing 5% CO2 at 37 ^{o} C. The medium of the cells was change once or twice per week. The non-adherent cells were removed from the culture after 24 hrs, and fresh medium was added. After 7 days and upon reaching 80% confluences, the cells were trypsinized by trypsin/ EDTA solution (0.25%/ 0.02%). PHA was cut into blocks size (1.5 X1.5X1.5 mm) and sterilized. The cells were counted and seeded on the PHABs in ODM (23) that consists alpha-MEM culture media supplemented with 10% FBS, kanamycin (60 µml-1), ascorbic acid (0.2mM phosphate – ester form), 1mM ß-glycerophosphate and dexamethasone (10 nM). The cells were put in the 8-well cell culture plates at a density of 10^{6} cells cm^{2}, while control PHAB was cultured identically in the plate without cells. The plates were maintaining in a 5% CO2 humidified chamber at 37 ^{o} C for 7 days, and the medium was changed every 48 hours. All blocks were examining under an inverted phase contrast microscope. Two blocks (with and without cells) were harvested and fixed with 2.5 % glutaraldehyde for SEM investigations.

In vivo implantation of PSCs-PHABs

Four, six months old, white male rabbit (New Zealand) were sedated using xylazine and ketamine (10mg and 100 mg/ kg). The mandible was reached after dissecting the muscles. Defects were created on both sides of the mandible. PSCs-PHABs placed on the right side, while the left side was used as control and implanted with PHABs-WCs. The blocks were retrieved at 60 days. The retrieved blocks were processed for ground histological sections and SEM investigations. Every collected specimen was split into two portions. One part was kept for 72 hrs in 10% formalin buffered solution. Later on, the samples were prepared as undecalcified sections and stained by methylene blue, H&E (Hematoxylin and Eosin) and

Masson's trichrome stain. The other parts fixed by 2.5% glutaraldehyde solution and post-fixed with 0.5% osmium tetroxide (OsO4) solution for scanning electron microscope investigation. Then, the samples were dehydrated throughout different concentration series of alcohol, absorbed three times in hexamethyldisilazane for ten minutes and air-dried at room temperature. Finally, sputter-coated with Gold was done for each sample The samples were examined using SEM at 10 KV (Cambridge S360/ Leica). (Figure.1).

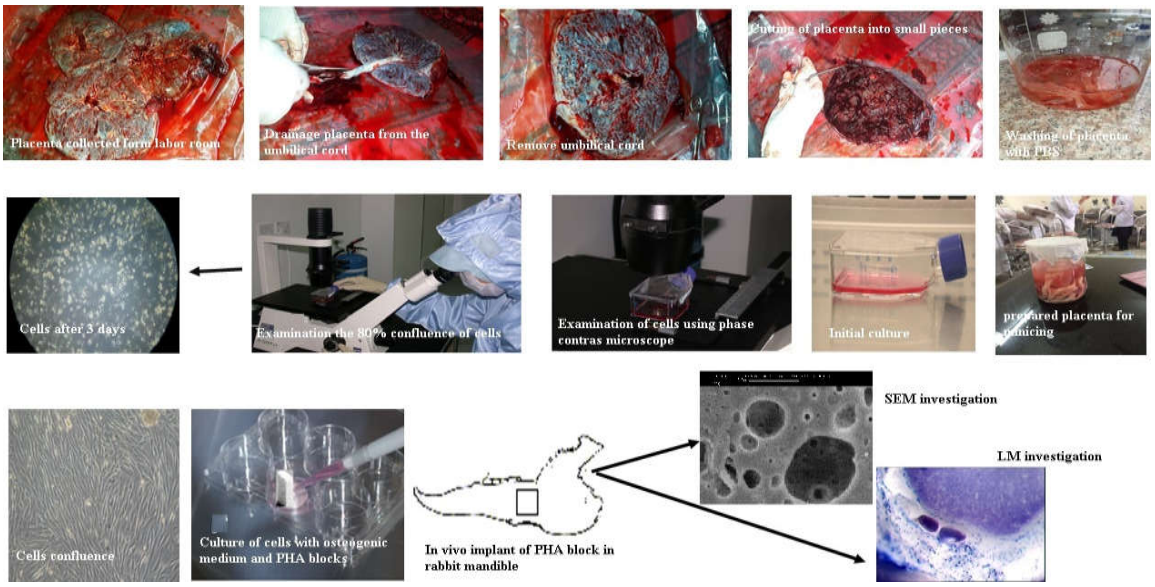

Figure.1: Describes the in vitro procedures for the isolation and differentiation of PDSCs and in vivo implantation of PHA-cells blocks in rabbit mandible.

RESULTS

In vitro PSCs properties

Placenta stem cells observed as fibroblast-like adherent cells at primary culture. Two types of cells appeared at the beginning; the floated (non-attached) epithelial-like cell that was eliminated and the second type cells were the large fibroblast-like cells, which were attached and reached 80% confluence after 7 days (Figure. 2 A, B& C).

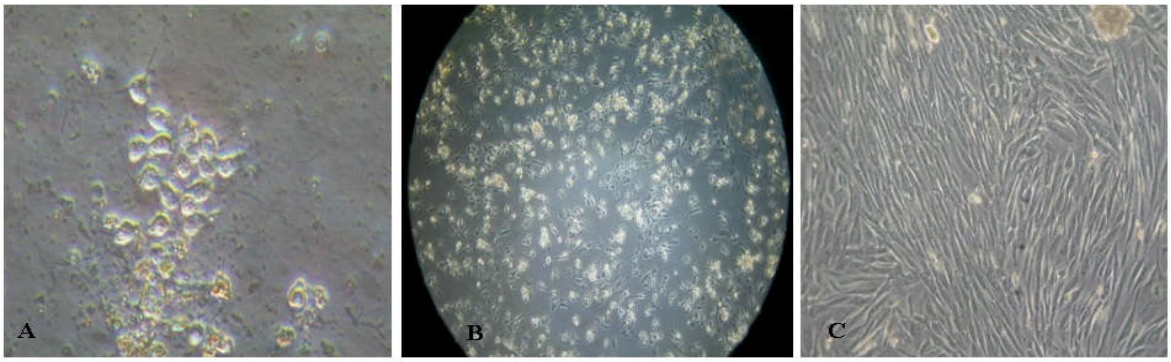

Figure.2: A& B: The primary culture of PSCs (epithelial like cells and fibroblasts like cells); C: Fibroblasts like cells that show confluence at 7 day of incubation.

PSCs-PHABs complex properties

The inverted phase contrast microscope of PSCs-PHABs complex cultured in the ODM at seven days showed the attachment of the cells on the surface of PHABs as well as inside the pores (Figure. 3 A&B).

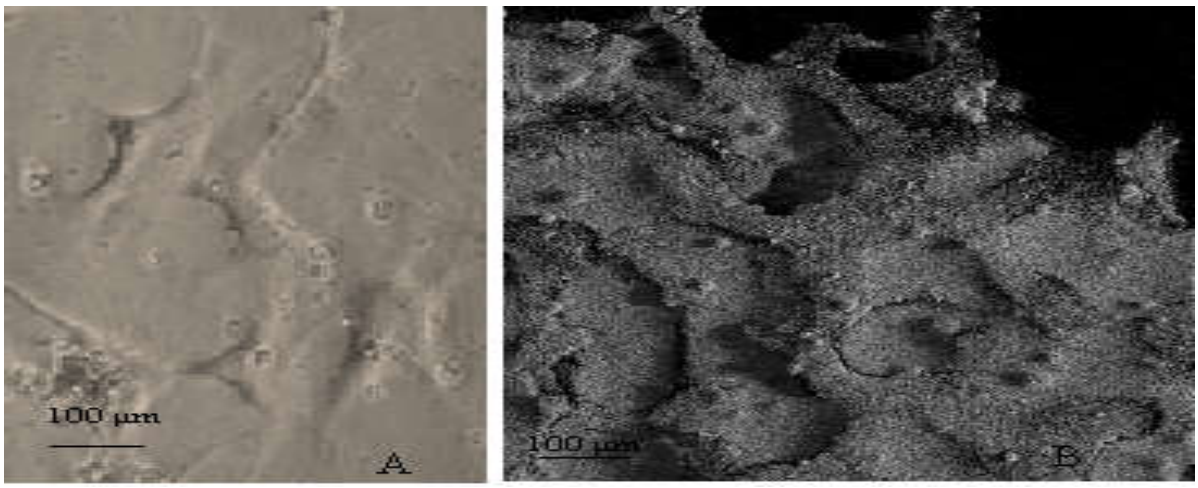

Figure.3 A&B: Shows the sections of PSCs-PHABs complex in the osteogenic medium at 7 days. A. Spindle like cells attached on the surface of PHA. B. Attachment of the cells on the surface as well as inside the pores.

The SEM investigation shown attachment, spreading and proliferation of the cells on the PHABs. The 3-dimensional view demonstrated the attachment, proliferation and well organization of spindle-like cells PDSCs on the surface of PHA. Moreover, PHABs pores were filled with cells that migrated and produced multilayered filopodia in different direction. A large synthesis of cells extracellular matrix was given a rough appearance to the surface of the implanted blocks (Figure. 4 A& B).

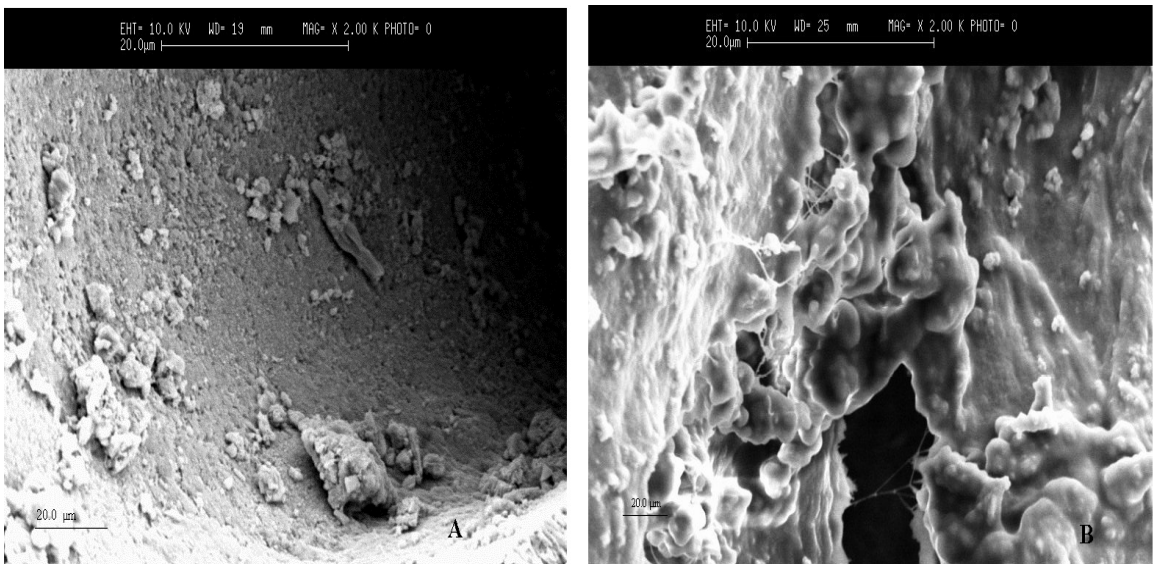

Figure.4 A&B: Shows SEM micrograph of PSCs-PHABs complex in the ODM at 7 days. A. Attachment and spreading of the cells inside the PHABs, moreover the adhesion of the cells presented the granular appearance of PHABs surface. B. The cells produced multilayered filopodia in different direction. and synthesis of extracellular matrix on PHABs surface

In vivo PSCs-PHABs complex implant

Neither infection nor inflammatory reactions were found at the PSCs-PHABs complex implant as well as in the control blocks. Vascular-Like bone tissue was observed in macroscopical examination of the PSCs-PHABs complex implants, whereas PHABs- WCs (control) were revealed incompletely absorbed coarse surface. Mature, plentiful aggregates of dense bone formation were seen in undecalcified sections that prepared from PSCs-PHABs (Figure. 5). An active stripe of secretary osteoblasts accompanied with active seam was also seen on the PSCs-PHABs. The pores of the PSCs-PHABs were revealed abundant neovascularization, besides, multinucleated giant cells that appeared adjacent to the surface of PHABs. The new bone formations appeared to resemble the intramembranous bone formation, majorities of PHABs disappeared, and only a few remnants deformed dispersed fragments of PHABs remained in few regions inside the bone furthermore in adipose- fibrous tissue (Figure. 5 A, B & C).

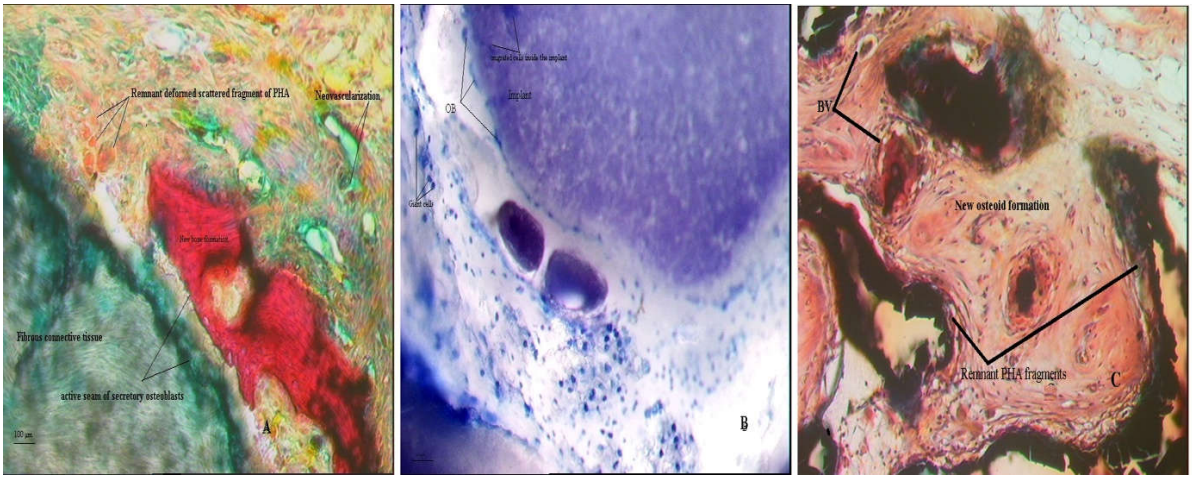

Figure. 5. A, B & C: The Undecalcified sections of PSCs-PHABs complex implants. A. shows aggregate of condensed bone formation. A strip of osteoblasts appeared in the PHABs and secretory osteoblasts that appeared as active seam (Masson's trichrome stain). B. Osteoblasts (OB) surrounded the implant material (Methylene blue stain). C. Shows new osteoid formation and abundant neovascularization and few remnants deformed scattered fragments of PHABs remained in regions within adipose- fibrous and bone tissue (Hematoxylin & Eosin stain).

The SEM investigations revealed well integration between implant blocks and the surrounded bone (Figure. 6). The bone formation was profuse on the surface as well as inside the implant. Moreover, collagen fibers, cellular matrix, and cells were occupied the PSCs-PHABs complex accompanied with replacing of pores by bone like osteon. (Figure.7 A, B & C). Some non-uniform erosions accompanied by resorption of PHABs-WCs with residue pores occupied by the fibrous connective tissues were seen in the control blocks. In addition, PHABs-WCs revealed no features of bone formation in SEM observations.

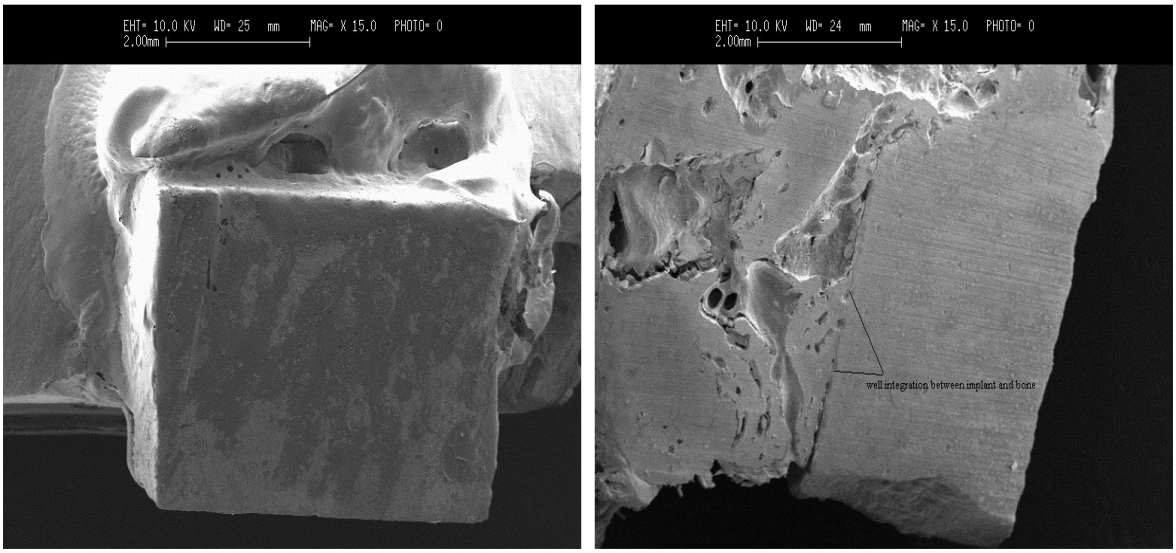

Figure. 6. The scanning electron microscope micrograph shows well integration between implant blocks and the surrounded bone.

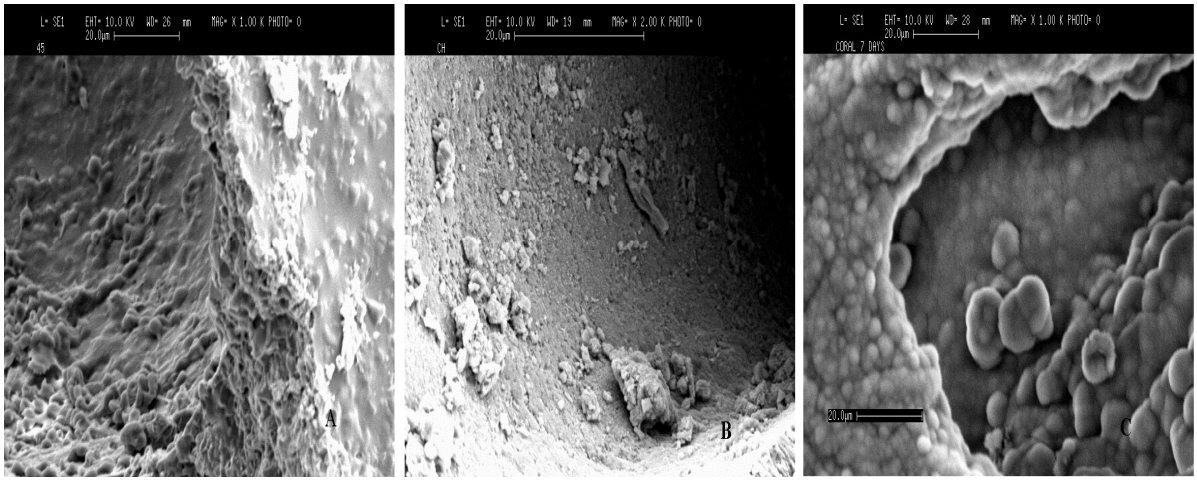

Figure. 7. A, B & C: A& B. profuse bone formation on the surface and inside the implanted blocks accompanied by well attachment of the cells in the depth of the PHA. C: Bone formation and proliferation of the cells inside the PHA pores led to narrowing of the porous.

DISCUSSION

Stem cell possesses self-renewal features and abilities to differentiate into diverse body tissue that donates the stem cells a huge potential impact for clinical applications (24, 25). Conversely, ethical issues and biological limitations harass ESCs. Thus, alternative sources have approached, and groups of ASCs were isolated from placenta. These cells owned the capability to differentiate and produce different kinds of cells, comprising an osteoblast (1). The advantages of placental stem cells in comparison with other multipotent cells resources

are the absence of ethical issues and available broadly. Dissimilar to bone marrow-derived mesenchymal stem cells, PSCs can be obtained without aggressive techniques and possess important immunosuppressive properties like several types of non-hematopoietic stem cells. Thus, these cells should be taken into account as the best candidates for prospect therapeutic applications. The collection of the placenta is less invasive and has no side consequences for both mother and neonate. In the current study, PSCs were differentiated into osteoblasts using ODM as have been indicated in prior studies emphasizing their pluripotency potential (1, 26, 27, 28, 29). The PSCs revealed typical mesenchymal stem cells features and formation of single, separated fibroblastoid colonies. In this study, PSCs were seeded in vitro on PHA blocks into ODM, and bone formation was recognized in the PSCs-PHABs that introduced in the rabbit's mandible. PHABs seeded with PSCs improved some features of the implant such as low absorbability, osteogenic activities, and biomechanics, though, the control PHABs-WCs did not lead to bone formation. In this study, well attachment cells were appeared on the surface as well as inside the pores of PHABs that incubated with PSCs in ODM at seven days of incubation under inverted phase contrast microscope. This result reflexes the typical initial cellular differentiation and interaction with biocompatible implant materials (7, 8). Similar results were also observed by other researchers (30) in the cell morphology, assumed that the primary point of contact between the cells and the substratum was to be a random process, and mostly recognizing by the distribution of adhesion proteins that adsorbed onto the surface of the biomaterials. The results of the SEM investigations also showed the spreading of the firmly attached cells on PHABs. Moreover, 3-dimensional proved the attachment, proliferation and well organization of spindle-like PSCs on the surface of PHABs. The PSCs migrated inside block pores accompanied with massive production of multilayered filopodia that emerged in diverse direction with extracellular matrix synthesis on PHABs surface. These results indicated that PHABs encourage the PSCs to grow and differentiate to osteoblasts in ODM. Subsequently, the cells were rapidly distributed on the PHABs surface as well as inside the pores. The current study's result is compatible with former findings that found PHA as a biocompatible scaffold that acted as a background for cell growth and differentiation. The perfect scaffold biomaterial would facilitate proliferation and attachment of the cells accompanied by suitable biodegradability and biocompatibility (31). The results of the current study showed no inflammatory reactions at the PSCs-PHABs complex implant. Moreover, vascular-like bone tissue was seen in the macroscopical examination of PSCs-PHABs complex implant, whereas PHABs-WCs (control) showed incomplete resorption with a coarse surface. Additionally, light microscope investigations of in vivo implanted PSCs-

PHABs shown numerous lakes of newly bone formation that resemble intramembranous bone creation. and demonstrated the osteoconductivity and bioactivity properties of PHA. These results are compatible with previous studies, that explained the occurrence of direct initial bone formation within implant against the surface and expressing the bioactivity of PHA then osteoconductivity due to the proliferation of the cells on the implant surface (7, 8). Meanwhile, the PHA pores revealed scarce chondroblasts. Hence, this activity is closely analogous to membranous than osteochondral bone development (7, 8). Tissue factors were release from the borders of the wound during the routine bone healing. These factors act to differentiate the cell into osteoblasts and produce the mineralize extracellular matrix and bony lakes. These lakes offered a scaffold with new bone growth. The histological sections also revealed active osteoblasts stripe on the border of PHABs and active seam of secretory osteoblasts accompanied with abundant neovascularization that was seen in the pores as well as surrounded the implant. These results are compatible with previous studies that mentioned the importance of vascularization as an essential requirement for bone production without chondrogenesis mechanisms in particles of porous hydroxyapatite seeded with bone morphogenetic protein as a holder, but not for cartilage formation (32). The in vivo bone formation mechanism occurs probably due to quickly blood vessels formation via interconnected pores in the porous particles of hydroxyapatite, that prevent the hypoxic micromilieu necessary for cartilage formation. The researcher found that in vivo bone formation need higher oxygen (35%) and nutrient for osteoblasts. Therefore, in this study, the bone formation in PSCs-PHABs complex implanted in the mandible of the rabbit showed excessive blood vessel formation (vascularization) suggesting the superior quantity of oxygen and nutrient in the porous implant that encourages osteogenesis. Moreover, the bone development inside the PSCs-PHABs complex is a type of osteogenic production via seeded cells and probably can be due to the differentiation of PSCs into osteoblasts by modifying ODM.

Conclusions

In brief, this study approved the isolation of PSCs from the placenta. Moreover, it showed its ability to differentiate into osteoblasts in ODM in vitro and to regenerate bone tissue in vivo in the presence of osteoconductive bioactive biomaterial. In addition, this study approved that PSCs may be an appropriate source of the cell for maxillofacial and alveolar bone regeneration. The results of this study offer the principle of further future studies to use the placenta as an available and wealthy source of stem cells and to demonstrate the PSCs multipotency to differentiate into another cell lineage.

Conflicts of interest

All authors declare that they have no conflicts of interest.

عزل الخلايا الجذعية المشيمية في الزجاج وتخصصها للخلايا العظمية على وسادة الهيدروكسي ابيتيت في الفك السفلي للارانب

علي عباس عجيل ،قاسم عبيد حسين ،كريمه عاكول الصالحي

كلية الطب البيطري ، جامعة المثنى ،المثنى ،العراق.

الخلاصة

تهدف هذه الدراسة الى عزل الخلايا الجذعية من المشيمة وكذلك تقيم قابليتها للتخصص الى الخلايا العظمية في وسادة الهيدروكسي ابيتيت، وتقيم تكوين العظم حول الزرع المغروس في عيب الفك السفلي الغير قابل للشفاء في الارانب باستخدام المجهر الضوئي والالكتروني. لقد تم تطوير بروتوكول خاص لعزل الخلايا الجذعية المشيمية وتخصصها للخلايا العظمية باستخدام الوسط الزرعي الخاص لنمو الخلايا العظمية في الانبوبة. تم استخدام اربعة ارانب ذكورنيوزيلندية لوضع وسادات الهيدروكسي ابيتت المزروعة بالخلايا الجذعية في عيوب تم احداثها في جسم الفك السفلي الايمن، في حين تم زرع وسادات الهيدروكسي ابيتيت الخالية من الخلايا في الفك الايسر كسيطرة. تم استخراج الوسادات المزروعة بعد ٦٠ يوم وتم تحضيرها بالطرق النسيجية التقليدية وكذلك للمجهر الالكتروني المسحي. اظهر كل من المجهر الضوئي والالكتروني نمو والتصاق الخلايا الجذعية المشتقة من المشيمة على سطح وسادات الهيدروكسي ابيتيت في الانبوبة في اليوم السابع من الحضن في المختبر. بالاضافة لذلك فان المجهر الالكتروني اظهر التصاق ونمو الخلايا على سطح الوسادة وبدرجات مختلفة من العمق. جميع الوسادات المزروعة اظهرت درجات عالية من نمو الاوعية الدموية والانسجة الشبيهة للعظم. اظهرت المقاطع النسيجية المحضرة من الوسادات المزروعة تكون العظم المتكامل النمو، بينما اظهر المجهر الالكتروني وجود الطبقات المتعددة الغنية بالكولاجين والذي غطى جميع سطح الوسادات المزروعة. في الخلاصة فان هذه الدراسة اثبتت عزل الخلايا الجذعية من المشيمة وامكانية تخصصها للخلايا العظمية. بالاضافة لذلك، تثبت هذه الدراسة سهولة الوصول للخلايا الجذعية المشيمية لاستخدامها في تطبيقات الهندسة النسيجية لترميم العظام وبوجود الوسادات الزرعية المناسبة.

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