

Artikel Asli/Original Articles

Cytotoxicity and Oxidative Stress Evaluation of Alginate/Cockle Shell Powder Nanobiocomposite Bone Scaffold on Osteoblast (Penilaian Kesan Sitotoksiti dan Tekanan Oksidatif Perancah Tulang Nanobiokomposit Alginat/Serbuk Kulit Kerang terhadap Sel Osteoblas)

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ABSTRACT

Biocompatibility and growth of osteoblast on bone scaffolds play an important role towards their therapeutic application. The presence of oxidative stress generated by bone scaffolds highly influences osteoblast growth and its functional performance. In this study in-vitro interaction of developed Alginate/Cockle Shell powder nanobiocomposite bone scaffold on osteoblast with regards to cytotoxicity and oxidative stress are evaluated. Cytotoxicity studies using MTT assays revealed a significant increase in viability of cultured osteoblast in the presences of the scaffold extracts. The growth of osteoblast on the scaffold were not deterred with the presence of any major oxidative stress factors as determined through oxidative stress profile studies using SOD, GSH and ROS assays. The nanobiocomposite scaffold evaluated in this study shows promising use in regards to facilitating osteoblast proliferation, growth and viability.

Keywords: Nanobiocomposite bone scaffold; oxidative stress; osteoblast; cytotoxicity; Cockle shell powder

ABSTRAK

Biokompatibiliti dan pertumbuhan sel osteoblas pada perancah tulang memainkan peranan yang penting terhadap aplikasi terapeutiknya. Kehadiran tekanan oksidatif aruhan perancah amat mempengaruhi pertumbuhan sel osteoblas dan keberkesanan fungsi perancah tersebut. Dalam kajian ini, interaksi perancah tulang nanobiokomposit Alginat/Serbuk kulit kerang terhadap sel osteoblas dari segi sitotoksiti dan aruhan tekanan oksidatif dikaji secara in-vitro. Kajian sitotoksiti yang dijalankan menggunakan assai MTT menunjukkan peningkatan signifikan kadar viabiliti sel osteoblas yang dikultur dengan ekstrak dari perancah. Pertumbuhan sel osteoblas pada perancah juga didapati tidak dipengaruhi dengan kehadiran sebarang tekanan oksidatif ketara seperti yang dianalisis dengan menggunakan profil assai tekanan oksidatif seperti SOD, GSH dan ROS. Perancah nanobiokomposit yang dibentuk menunjukkan potensi dalam membantu meningkatkan viabiliti dan pertumbuhan sel osteoblast seperti yang ditunjukkan dalam kajian ini.

Kata kunci: Perancah tulang nanobiokomposit; tekanan oksidatif; sel osteoblas; sitotoksiti; serbuk kulit kerang

INTRODUCTION

Studies on cockle shell powder has shown promising use of the powder as potential biomaterial for the development of bone scaffolds. Malaysia has a rich source of cockle shell that is easily available as a low cost biomaterial. The cockle shell has been shown to consist as high as 95-98% of calcium carbonate in the form of aragonite polymorph (Abdullahi & Zuki 2014) and these powders obtained from the shells nacreous materials are shown to possess high similarities with coral exoskeletons (Awang-Azmi et al. 2007). Molluscan shells calcium carbonate compound are known to exist in three different forms namely calcite, aragonite and vaterite. The denser nature of aragonite compared to the others proves to be an attractive feature for its application in the field of material engineering. The purely aragonite form of calcium carbonate polymorph, which is denser in nature gives it an added advantage to be incorporated, resolved and then replaced by bones over

time compared to the other forms of calcium carbonate polymorphs (Stupp et al. 1997). The conversion of the cockle shell powder to nano particles could easily be achieved using bio-mineralization catalyst through a simple chemical method as described by Nurul et al. (2012). The ultra fine structure of nano particles helps provide larger surface area for stronger bonding property and improves mechanical stability. This characteristic are currently being widely used for the purpose of tissue engineering application to facilitate bone tissue healing through fabrication of bone scaffolds using nano particle cockle shell powder as shown from studies by Nurul et al. (2012) and Hemabarathy et al. (2014).

A scaffold fabricated for the intentions of being used as a bone substitute material, should be produced from a highly biocompatible material with an adequate physical and mechanical properties without eliciting an immunological or clinically detectable foreign body reaction. The fabrication of this nanobiocomposite scaffold

is achieved with the use of alginate, a naturally occurring polymer that provides the underlying matrix for the scaffold. Alginate from the brown algae is a polysaccharide that dissolves in water and is generally regarded safe by Food and Drug Administration (FDA) (Wong 2011). Due to its non-toxic, non-immunogenic natures as well as its biocompatibility and biodegradation properties (Mooney & Lee 2012), alginate has found a wide range of applications in the medical field.

The previously fabricated nanobiocomposite scaffold (Hemabarathy et al. 2014) using nano cockle shell powder and alginate, produced a three dimensional structure that exhibits excellent morphological and compatibility properties suited for facilitation of osteoblast attachment and growth. However, studies on the oxidative stress induction by the scaffold in an in-vitro setting are still lacking. Oxidative stress has been shown to have a strong influence on osteoblast by inhibiting the bone cells differentiation (Liu et al. 2011). Oxidative stress occurs when there is an imbalance between antioxidant and free radical production (Mody et al. 2001). It is not known whether the degradation of the scaffold in an in-vitro setting may cause free radical production that could hinder the osteoblast activity. Thus, this study is vital to determine the effects of the fabricated nanobiocomposite on osteoblast viability and oxidative stress balance. Performance of the scaffold as an implant material was studied by evaluating the levels of antioxidant and oxidative markers such as glutathione (GSH), superoxide dismutase (SOD) and reactive oxygen species (ROS) generated from the scaffold.

MATERIALS AND METHODS

ALGINATE/COCKLE SHELL POWDER NANOBIOCOMPOSITE BONE SCAFFOLD DEVELOPMENT

The nanobiocomposite scaffold was fabricated according to methods of Hemabarathy et al. (2014). Briefly, alginate nanocockle shell powder scaffolds (NS) was developed by mixing preweighed nano powder into prepared alginate hydrocolloid solution under constant stirring on a homogenize stirrer machine at 600 rpm until a homogenized smooth slurry is obtained. The scaffold mixture was prepared in composition of alginate: nanocockle shell powder in a ratio of 40 : 60 (w/v), poured into customized molds, frozen at -20°C for 24 hours prior to lyophilization. The lyophilized scaffolds were then removed from the molds, cross-linked with calcium chloride (CaCl₂) solution and re-lyophilized prior to storing in a sterile container. For comparative purposes, purely alginate containing scaffolds (AS) was prepared in similar manner to serve as an experimental control.

SCAFFOLD EXTRACT PREPARATION

Extract in the form of leachables from the developed scaffolds were obtained according to the methods of Peter

et al. (2010). Scaffolds sterilized through autoclaving and UV irradiation for 20 minutes were incubated in 20 mL of DMEM culture media in separate flasks at 37°C for 24 hours in order to obtain the scaffold extracts. The leachables from the scaffolds were then collected into individually labeled sterile falcon tubes for *in-vitro* study.

OSTEOBLAST CULTURE

Osteoblast which were differentiated from sheep mesenchymal stem cells (MSC) were donated from the Centre of Tissue Engineering, Medical Centre of Universiti Kebangsaan Malaysia (PPUKM). Cells from a single vial were cultured into T75 flasks containing Dulbecco's Modified Eagle Medium (DMEM: E15-810 PAA) and added with 10% fetal bovine serum (FBS : Sigma F4135) and 1% penicillin/streptomycin (PAAP11-010) prior to incubating at 37°C with 5% CO₂. The culture medium was changed every 2-3 days and cells were routinely segregated 1:2 every 7-8 days at 80% of confluency.

MTT ASSAY (3-DIMETHYLTHIAZO-2,5-DIPHENYLTETRAZOLIUM BROMIDE)

MTT assay was performed according to methods of Peter et al. (2010) with slight modifications on scaffold extracts from nanobiocomposite scaffold (NS) and alginate scaffold (AS) groups. Osteoblast were seeded in a 96 well plate with a seeding density of 5×10^4 cells/well. The cells were incubated under standard culturing conditions for 24 hours to allow initial attachments. After 24 hours, the medium was removed from each well and replaced with medium containing the scaffold extracts and incubated further for 1, 3, 5 and 7 days. Following the incubation period, the medium with scaffold extracts were removed from each well and replaced with fresh medium containing 10% of MTT solution. The cells were then incubated at 37°C for additional 4 hours in order to perform the standard MTT assay. After 4 hours the medium was removed and the reaction was terminated with the addition of 100 µl dimethyl sulfoxide (DMSO) as a solubilization buffer to each well to dissolve the formazan crystals. The lysate absorbance was then read using a microplate reader at 570 nm. One row from each plate was designated for control and was cultured with normal culture medium.

CELL LYSATE PREPARATION

Cell lysate or homogenates were prepared prior to conducting oxidative stress profiling assays. 1 ml of cell suspensions from cells cultured at density of 5×10^4 cells/ml at day 1, 3, 5 and 7 is obtained from cells cultured in scaffold extracts (NS and AS) and cells cultured in culture media (control). The cells were then washed once with 1 ml of chilled phosphate buffered saline (PBS) with two times centrifugation. Supernatant was discarded and cell pellet was treated with cell lysis buffer that contained 0.1% of triton-X 100 and were centrifuged again at high speed of

12000 rpm for 15 minutes at 4°C. The cell lysates obtained was used immediately for testing.

DETERMINATION OF CELLULAR GLUTATHIONE (GSH) LEVEL

GSH assay was performed according to modified methods of Ellman (1959). Briefly, 50 µl cell lysate from scaffold extracts (NS and AS) and control was added with 40 µl of reaction buffer (pH8) and 10 µl of DTNB (5,5'-Dithiobis 2-nitrobenzoic acid) reagent and mixed by resuspension. The plate was incubated for 15 minutes at room temperature prior to be measured at 415 nm. The GSH level is determined using the equation formed by the standard graph in unit of nmol/mg of protein content that was determined by Bradford Protein Assay.

DETERMINATION OF CELLULAR SUPEROXIDE DISMUTASE (SOD) LEVEL

SOD levels in cells grown with scaffold extracts were determined according to the methods of Beyer & Fridovich (1987). Cell lysate obtained at day 1, 3, 5 and 7 were pre-diluted by mixing 50 µl of each cell lysate with 50 µl of distilled water in Eppendorf tubes. Subsequently, 1 ml of freshly prepared substrate solution was added into each tubes prior to 20 µl of PBS-EDTA. The reaction was then initiated using 10 µl of freshly prepared riboflavin that was added into each tube. Immediately after adding the riboflavin, mixtures in the tubes were homogenized with vortex mixer and exposed to 18 W Sylvania lamps for 7 minutes. After 7 minutes, the nitroblue tetrazolium (NBT) reduction by SOD enzyme was spectrophotometrically determined by reading the absorbance at 560 nm. SOD activity was then calculated using a standard formula.

DETERMINATION OF CELLULAR REACTIVE OXYGEN SPECIES (ROS) LEVEL

ROS assay was performed according to methods of Li et al. (2002). ROS levels were determined at day 1, 3, 5 and 7 in cell suspensions obtained from cells cultured in scaffold extracts with a density of 5×10^4 cells/ml. The cells were centrifuged at 2500 rpm for 5 minutes at 4°C and the resulting supernatant was discarded. Remaining cells were re-suspended in 1 ml of 37°C pre-warmed DMEM without fetal bovine serum (FBS) prior to incubation with 1 µl of hydroethidine (HE) (10 mM) in the dark for 15 minutes. Following the incubation period, the cells were centrifuged at 2500 rpm for 5 minutes at 4°C and the supernatant was discarded. Remaining cell pallets were re-suspended in 300 µl of chilled PBS before transferring it into Falcon tubes for flow cytometric assessment.

STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) was used to compare element concentrations in percentages. Results were expressed as mean \pm standard deviation (SD). Post hoc test were done for significant values ($p < 0.05$) using Tukey's multiple comparison test.

RESULTS

MTT ASSAY (3-DIMETHYLTHIAZO-2,5-DIPHENYLTETRAZOLIUM BROMIDE)

Figure 1 shows the MTT assay findings for cells cultured in the extracts of scaffolds and control for 7 days. An increase in cell viability was noted from day 1 to day 7 in all groups. Viability of cells was found to be significantly higher

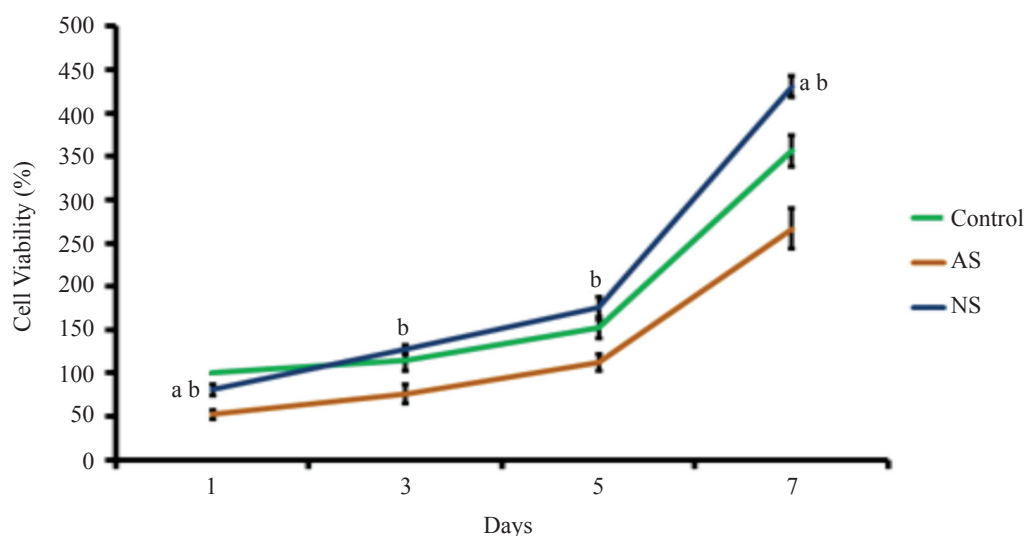


FIGURE 1. MTT assay of NS extract, AS extract and Control. ^a $p < 0.05$ vs Control, and ^b $p < 0.05$ vs AS. Data are presented as mean \pm SEM.

(AS: Alginate scaffold, NS: Nanobiocomposite scaffold)

($p < 0.05$) in cells cultured with NS extracts compared to AS at day 3, 5 and 7 as well as with the control group on day 7. No significant differences was observed between cell viability of AS group compared to control which showed higher percentage of viability comparatively.

However within group analysis showed a significant increased in GSH level on day 3 and 5 in AS group and day 3, 5 and 7 in the control group compared to day 1. The GSH levels in NS group was found to remain constant for the 7 days of culturing period.

GSH EVALUATION ON OSTEOBLAST

Figure 2 shows the GSH level of cells cultured with extracts of scaffold and control. Significant differences in the GSH level are observed at day 1 between NS and control group at $p < 0.05$. There were no significant difference observed between NS and AS group throughout the culture period.

SOD EVALUATION ON OSTEOBLAST

Level of SOD activity for cells cultured in scaffold extracts and control is shown in Figure 3. SOD levels were found to be significantly ($p < 0.05$) increased at day 1 in NS group as compared to control. There is no significant difference ($p < 0.05$) in SOD activity observed between AS

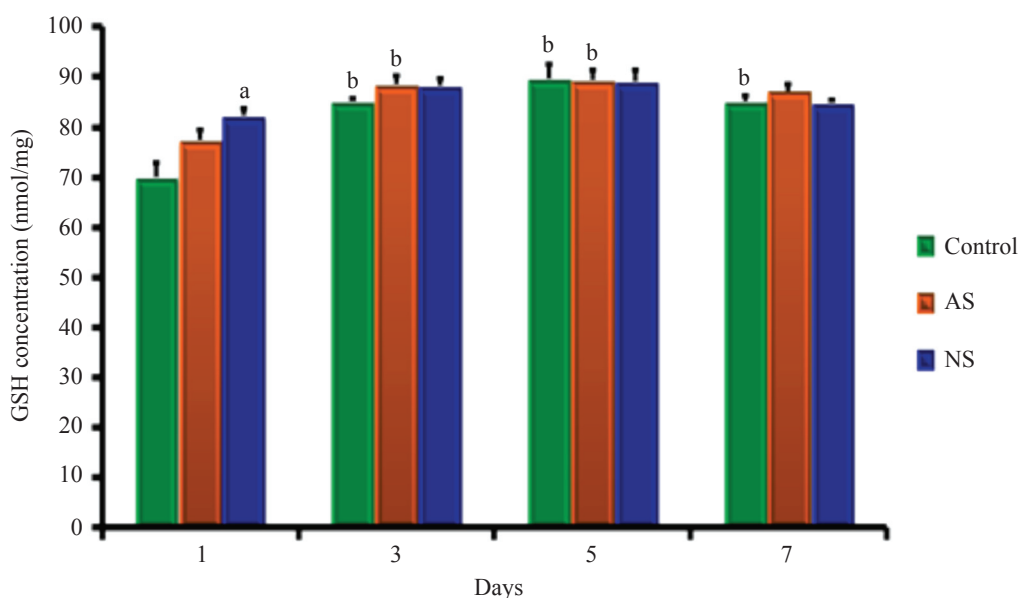


FIGURE 2. GSH Evaluation. ^a $p < 0.05$ vs Control and ^b $p < 0.05$ vs day 1. Data are presented as mean \pm SEM. (AS: Alginate scaffold, NS: Nanobiocomposite scaffold)

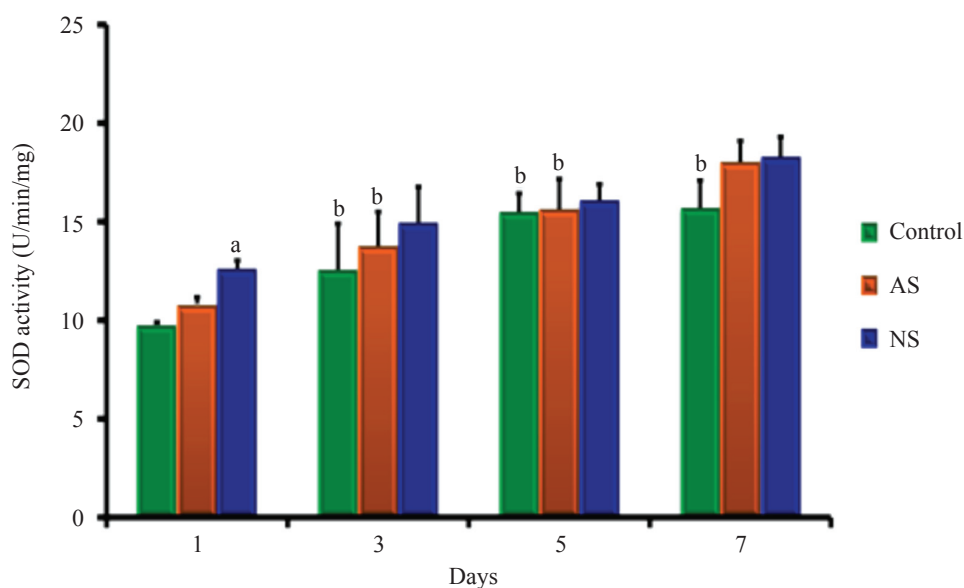


FIGURE 3. SOD Evaluation. ^a $p < 0.05$ vs Control and ^b $p < 0.05$ vs day 1. Data is presented as mean \pm SEM. (AS: Alginate scaffold, NS: Nanobiocomposite scaffold)

group compared to NS and control groups throughout the culture period. However, within group analysis showed a significant increase in SOD activity in AS group on day 3 and 5 compared to day 1, while SOD activities in control group showed a significant increase on day 3, 5 and 7 compared to day 1.

OXIDATIVE STRESS EVALUATION ON OSTEOBLAST

Figure 4 shows ROS levels in cells cultured in scaffold extract. Significant difference at $p < 0.05$ was observed in AS group which showed higher ROS levels compared to both NS and control group at day 1 and 3. Within group analysis

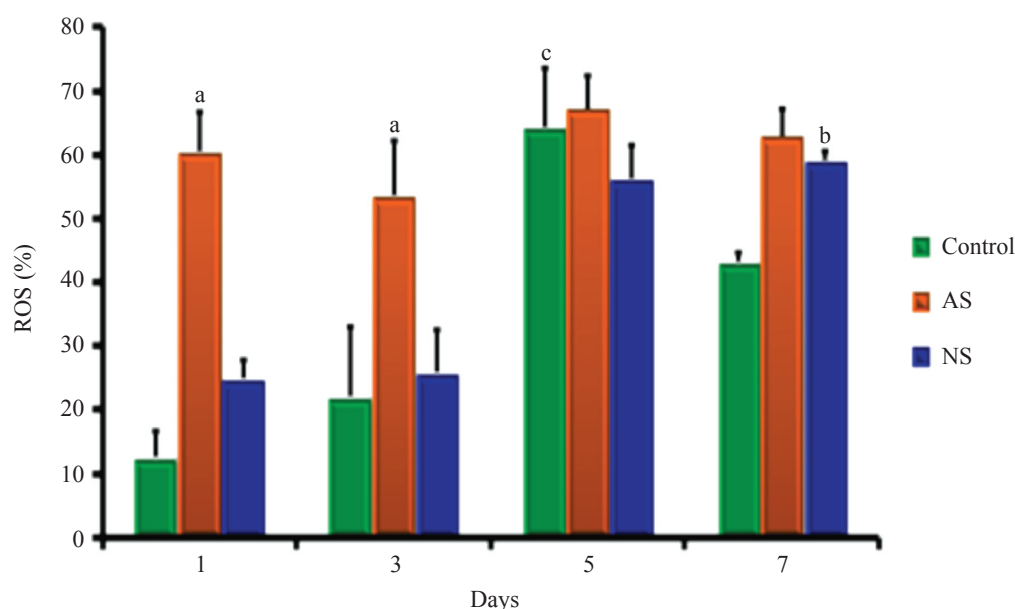


FIGURE 4. Oxidative Stress Evaluation. ^a $p < 0.05$ vs Control and NS, ^b $p < 0.05$ vs day 1 and ^c $p < 0.05$ vs day 1. Data are presented as mean \pm SEM.

(AS: Alginate scaffold, NS: Nanobiocomposite scaffold)

revealed an increase in ROS levels in NS group on day 7 and in control group on day 5 compared to day 1. ROS levels in all groups were found to gradually increased from day 1 to 5 prior to decreasing in day 7. However AS group showed higher levels of ROS for all 7 days comparatively.

DISCUSSION

Bones are complex structures that play an important role for attachment of muscles and tendons as well as to support and protect the delicate internal organs (Czekanska et al. 2012). Study by Shea et al. (2000) stated that damage bone tissue can be repaired by implanting porous bone scaffolds at the defect site in order to facilitate new bone cell migration and differentiation. The use of natural product as a source of biomineral in scaffold designing has been taking center stage for the latest development in bone tissue engineering. Powder obtained from cockle shells are one such biomineral that is currently being studied for the purpose of bone tissue engineering. The high calcium carbonate content in the form of aragonite polymorph has been shown to be favorable in bone tissue applications (Zakaria et al. 2004).

In this current study, the previously developed nano biocompostie Alginate/Cockle shell powder bone scaffold

was evaluated for its cytotoxicity and oxidative stress generation. MTT assays performed proved the absence of cytotoxic effect from the use of the scaffold as indicated by an increase in cell proliferation rate on cells cultured with the extracts of the nanobiocomposite scaffold. The presence of high calcium content from the cockle shell powder (Zakaria et al. 2004) which were used in the scaffold are a favorable characteristic that helps facilitate osteoblast growth and differentiation. Studies show that osteoblast relies on the presence of calcium minerals in order for mineralization process to take place. The newly developed nanobiocomposite scaffold provides sufficient source of calcium minerals as shown by the exponential increase in osteoblast proliferations.

The use of scaffolds in an in-vitro setting is expected to generate free radicals during the cell scaffold interaction phase. It has been shown that oxidative stress is a major contributing factor that could hinder osteoblast growth and proliferation (Liu et al. 2011). Therefore, it is important that the scaffold is evaluated for oxidative stress in order to determine if the scaffold does generate free radicals that could affect the growth of bone cells on the scaffold as well as its potentials to provide an antioxidative protection during cell proliferation and growth. Antioxidants such as GSH and SOD play an important role in defending the cells from free radical damage (Kaplowitz 2000).

These antioxidants protect cells from the toxic effects of superoxide radicals (McCord & Fridovich 1998) and could act as good markers for oxidative stress evaluation. From this study GSH and SOD levels were found to be high and constant that correlates with the levels of ROS which indicates the absence of any major oxidative stress from the usage of the bone scaffold in an in-vitro setting. Studies by Ramkumar et al. (2012) previously showed that calcium carbonate is known to act as antioxidant catalase for GSH and SOD that could indirectly help in protecting the cells from damaging effects of any free radicals generated during the degradation of the scaffold material. A significant increase in the proliferation of osteoblast could be linked with the oxidative profile of the scaffold which showed the absence of any significant oxidative stress as osteoblast proliferation and growth was not hindered but was found to be accelerated with the use of the scaffold. The nanobiocomposite scaffold was found to help in promoting osteoblast growth in-vitro without eliciting any major oxidative stress and providing antioxidative protection towards the growing cells. The presence of calcium carbonate from cockle shell powder in the nanobiocomposite scaffold is most likely to have provided the antioxidative catalase properties to the scaffold thus indirectly counter balanced the generated oxidative stress during scaffold degradation and osteoblast proliferation.

CONCLUSION

The study showed that screening for primary antioxidant properties and oxidative stress generation by the developed bone scaffold is an important aspect that helps in determining the successful use of the scaffold in regards to bone tissues regeneration. There is no evidence that oxidative stress inhibited the growth of osteoblast in the in-vitro study performed. The presence of calcium carbonate in the nanobiocomposite scaffold showed a general improvement in the antioxidant function, however further studies are required in order to evaluate the ability of the scaffold to facilitate new bone growth in an in-vivo setting.

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