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Evaluating Physical and Biological Characteristics of Glutaraldehyde (GA) Cross-Linked Nano-Biocomposite Bone Scaffold

(Penilaian Pencirian Fizikal dan Biologi Perancah Tulang Nano-Biokomposit Dipindah Silang dengan Glutaraldehid (GA))

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ABSTRACT

In vivo *stability of biomaterial-based bone scaffolds often present a significant drawback in the development of materials for tissue engineering purpose. Previously developed nanobiocomposite bone scaffold using alginate and nano cockle shell powder has shown ideal characteristics. However, it showed high degradation rate and reduced stability in an* in vivo *setting. In this study, we aim to observe the effect of cross-linking glutaraldehyde (GA) in three different concentrations of 0.5%, 1% and 2% during the fabrication process as a potential factor in increasing scaffold stability. Microstructure observations of scaffolds using scanning electron microscope (SEM) showed all scaffolds crossed linked with GA and control had an ideal pore size ranging from 166.8-203.5 µm. Increase in porosity compared to the control scaffolds was observed in scaffolds cross-linked with 2% GA which also presented better structural integrity as scored through semi-quantitative methods. Tested pH values during the degradation period showed that scaffolds from all groups remained within the range of 7.73-8.76.* In vitro *studies using osteoblast showed no significant changes in cell viability but a significant increase in ALP enzyme levels in scaffold cross-linked with 2% GA. The calcium content released from all scaffold showed significant differences within and between the groups. It can be concluded that the use of GA in the preparation stage of the scaffold did not affect the growth and proliferation of osteoblast and use of 2% GA showed improved scaffold structural integrity and porosity.*

Keywords: Bone scaffold; glutaraldehyde; nanobiocomposite; osteoblast

ABSTRAK

Kestabilan in vivo *perancah tulang berasas biobahan merupakan salah satu kelemahan yang ketara dalam fabrikasi bahan untuk tujuan kejuruteraan tisu. Perancah tulang nanobiokomposit yang telah dibangunkan terlebih dahulu menggunakan alginat dan serbuk nano cengkerang kerang menunjukkan ciri-ciri ideal sebagai bahan pengganti tulang. Walau bagaimanapun, perancah tersebut menunjukkan kadar degradasi yang tinggi dan pengurangan dalam kestabilan struktur dalam keadaan* in vivo*. Kajian ini bertujuan untuk memperhatikan kesan penggunaan glutaraldehid (GA) sebagai bahan pindah silang dalam kepekatan 0.5%, 1% dan 2% terhadap perancah yang dibentuk. Kajian mikrostruktur menggunakan mikroskopi elektron imbasan (SEM) menunjukkan kesemua perancah tulang kajian GA dan kawalan mempunyai saiz liang antara 166.8-203.5* µm *yang bersesuaian untuk tujuan penggunaan. Peningkatan keliangan berbanding perancah kawalan diperhatikan untuk perancah yang dipindah silang dengan 2% GA yang turut menunjukkan peningkatan dalam kestabilan struktur melalui kaedah pemarkahan semi kuantitatif. Nilai pH yang diukur sepanjang tempoh kajian degradasi menunjukkan julat pH berada antara 7.73-8.76 untuk semua kumpulan kajian dan kawalan. Kajian* in vitro *menggunakan osteoblas tidak menunjukkan sebarang perubahan signifikan kepada keviabelan sel tetapi terdapat peningkatan dalam aktiviti enzim ALP untuk perancah yang dipindah silang dengan 2% GA. Perbezaan signifikan pembebasan kalsium juga diperhatikan antara dan dalam semua kumpulan kajian dan kawalan. Secara keseluruhan, didapati penggunaan GA dalam pembentukan perancah tidak memberikan kesan terhadap percambahan dan pertumbuhan osteoblas pada perancah tulang dan peningkatan dalam integriti struktur dan keliangan perancah diperhatikan dengan penggunaan GA pada kepekatan 2%.*

Kata kunci: Glutaraldehid; nanobiokomposit; osteoblas; perancah tulang

INTRODUCTION

Common bone grafting methods such as autografts and allografts have various limitation which includes limited amount of graft supplies, surgical complications, infections and transplant rejections. Current limitations in bone grafting are becoming a driving force for the constant search for new alternative methods and materials often venturing into the field of biomaterial-based grafts for bone tissue engineering. Scaffold fabrications for regeneration of bone tissues are an important application of material sciences in tissue engineering to date. Scaffolds are defined as three-dimensional porous biomaterial used at a defect site to help

increase tissue regeneration rate by providing the needed space and matrix for cell migration, proliferation and differentiation for new tissue formation (Shea et al. 2000).

The primary function of an implanted scaffold is to provide the temporary mechanical integrity needed for cells to grow, proliferate and synthesize cellular matrices in order to accelerate restoration of bone at the defect site (Bose et al. 2012). Thus, a scaffold should possess properties and characteristics that are highly similar to the host bone. Some prominent features of an ideal scaffold include suitable pore size and porosity, high biocompatibility and proper degradation rate in line with tissue generation that does not produce any local or toxic effect (Bose et al. 2012; O'Brian, 2011).

In our previous study, we have developed a novel porous three-dimensional alginate-cockle shell powder nanobiocomposite (Alg-nCP) bone scaffold that has shown ideal characteristics from the aspect of morphology, mechanical strength and also osteoconductivity (Bharatham et al. 2014; Hemabarathy et al. 2017; Nurnadiah et al. 2017). Cockleshell powder from *Anadara granosa* sp. consists of 95%-98% of calcium carbonate ($CaCO_3$) in the form of aragonite (Hemabarathy et al. 2014) that is capable of inducing bone mineral formation such as hydroxyapatite (Kamba & Zakaria 2014). Alginate is used due to its monomer capabilities to cross-link with calcium ion and form an egg box model (Lee & Mooney 2012) that can produce a highly porous structure suited for bone scaffold developments. However many alginate-based materials often possess a risk of reduced structural stability when applied into a fluid based environment thus accelerating its breakage and degradation in an *in vitro* or *in vivo* environment. To address the issue of reduced structural stability, glutaraldehyde (GA), a chemical capable of forming strong bonds within the materials in the scaffold through cross-linking steps (Haugh et al. 2011; Hoffman et al. 2009; Sheu et al. 2001) could be used to increase structural integrity of a scaffold matrix. Despite the presence of many types of cross-linking agents, GA is a prominent choice due to its ability to produce better structural integrity with higher compressive stiffness (Yang et al. 2017). Cross-linking is a technique that is often used to enhance the mechanical properties of a scaffold as proven through various research studies addressing the use of GA in the optimum concentration could increase the mechanical strength of the scaffold (Azami et al. 2010; Ma et al. 2003). Hence, we investigate the effect of cross-linking 0.5%, 1% and 2% GA on altering the characteristics, stability and cytocompatibility of the previously developed Alg-nCP nanobiocomposite scaffold.

Materials and Methods

Alginate-cockle shell powder nanobiocomposite (Alg-nCP) scaffold development

Scaffolds were prepared according to methods of Hemabarathy et al. (2014). The scaffold mixture was prepared in a composition of alginate (Sigma): Nano cockle shell powder of 40:60 (w/v). The mixture was poured into a custom-made cylindrical aluminium mold (5 cm in height, 0.7 cm in diameter) and allowed to freeze at -20°C for 24 h. The mixture was lyophilised using a freeze-dryer machine (Lyolab) at -50°C for 24 h till completely dried. The lyophilised scaffolds were removed from the aluminium mold and soaked in calcium chloride ($CaCl_2$) solution for 20 min for cross-linking purpose. The scaffold was then washed using deionised water thrice and immersed in deionised water overnight to remove any unbound $CaCl_2$. The scaffold was re-lyophilised for another 24 h prior to being used. Fabricated scaffolds were soaked in 0.5%, 1% and 2% GA, respectively, for 24 h at 4°C. GA solutions were prepared from initial 2.5% concentrated glutaraldehyde solution. Treated scaffolds were then washed for five times using deionised water (10 min each) and allowed to freeze overnight at -20°C. Scaffolds were transferred to the freeze-dryer machine for lyophilisation at -50°C for 24 h. The scaffolds were autoclaved and stored in a sterile container.

Morphology studies

Morphology studies were conducted using Scanning Electron Microscope (SEM) (VPSEM-JEOL 1455, Jerman). Scaffolds were cut into smaller sections, coated with gold-palladium and fixed on stubs at various angles for observation of the microstructure. 30 pore measurements from the micrograph taken from three samples of scaffolds from each group at multiple sites were used to measure the average pore size of each scaffold.

Porosity evaluation

Liquid displacement method was used to evaluate the porosity of the scaffolds according to the methods of Zhang et al. (2000). The initial weight (Wo) and volume (V) of the scaffolds were measured before ethanol immersion for 48 h. After 48 h, the scaffolds were weighed again (W1) and the porosity of scaffolds was calculated according to the formula:

$$\text{Porosity } (\%) = \frac{(W1 - Wo)}{\rho V} \times 100\%$$

where ρ is the density of ethanol.

Degradation evaluation

Six scaffolds from each group measuring 0.7 cm diameter and 0.5 cm height were immersed in PBS with pH7.4 for 14 days. The pH values of the PBS were measured daily and the structure of the scaffolds was closely observed daily for semi-quantitative scoring based on the criteria in Table 1.

Scaffold extract preparation for in vitro studies

Pre-sterilized scaffolds were incubated in 20 mL culture media DMEM at 37°C for 24 h to obtain the leachable

TABLE 1. Semi-quantitative scoring criteria

Criteria	Scoring
Completely intact scaffold	0
Slight surface defect	1
Mild breakage	2
Moderately broken	3
Disintegrating scaffold structure	4
Complete loss of structure/Disintegrated	5

extracts. The extract solutions were transferred to sterile falcon tubes and stored at 4°C for further use.

MTT (3-DIMETHYLTHIAZO-2,5-DIPHYNYLTETRAZOLIUM BROMIDE) COLORIMETRIC ASSAY

MC3T3-E1 subclone 4 pre-osteoblasts cells at a density of 5×10^4 cells/well were seeded in 96-well plate. The cells were then incubated at 37°C for 24 h to allow initial attachment. After 24 h, media was removed from each well and replaced with scaffold extracts prior to further incubation for another 48 h. The media was removed after 48 h, replaced with 10% MTT solution and further incubated for another 4 h. Following which the media was removed and 200 µL DMSO was added to each well to stop the MTT reaction by dissolving the formazan crystals. The lysate absorbance was read at 495 nm wavelength using a microplate reader.

CALCIUM RELEASE TEST AND ALKALINE PHOSPHATASE (ALP) ACTIVITY

Calcium release and ALP activity were measured using commercial kits (Abcam Inc., MA, USA) based on manufacturers protocol. Scaffolds seeded with osteoblast were incubated for 3, 5 and 7 days prior to calcium release test from media and ALP activity evaluation from cell lysates obtained from the scaffolds of the tested group. Calcium levels were measured at a wavelength of 595 nm while ALP activities were measured at 415 nm using a microplate reader.

STATISTICAL ANALYSIS

All quantitative results were analyzed using one-way Analysis of Variance (ANOVA). The results were expressed as mean ± standard error mean (SEM). Post hoc tests were done using Tukey's test for significant values ($p<0.05$).

RESULTS AND DISCUSSION

MORPHOLOGICAL STUDIES

Table 2 shows the pore diameter range and average pore size for each group of scaffold quantitated through SEM images. Scaffolds cross-linked with 0.5%, 1% and 2% GA did not show significant changes in pore size. However, increase in pore size was observed with the increase in GA concentration as compared to control group. Figure 1(A)-1(D)) shows the SEM micrograph of control and GA crosslinked scaffolds. The linkage between the alginate monomer and calcium ions from nano cockle shell powder contributed to the formation of the typical egg-box model with the presence of macro and micropores ideal for cellular regeneration (Lee & Mooney 2012). The pore diameter and size observed from this study were consistent with all previous studies conducted on this scaffold material composition (Hemabarathy et al. 2014; Nurnadiah et al. 2017) indicating that cross-linking with GA does not alter the morphological structure of the scaffold. A study by Perez et al. (2016) also reported similar findings in which the use of GA as a cross linking agent on a collagen based scaffold found that GA did not alter the pore size of the scaffold.

POROSITY EVALUATION

Figure 2 shows the porosity of the scaffolds. A significant increase in porosity in scaffolds crosslinked with 2% GA (125.7 ± 11.25%) compared to control scaffold (75.5 ± 4.36%) was observed at $p<0.05$. Increase in porosity could be contributed by the increase in the structural stability of scaffolds cross-linked with 2% GA which indirectly contributes to the preservation of pore structures in the scaffold. Control scaffolds comparatively are likely to have undergone structural collapse and loss of pore structures, which contributes to the reduction in the scaffolds porosity. Porosity is closely related to the mechanical properties, swelling aspect and degradation of a scaffold while playing a crucial role in determining the success of a bone scaffold. A highly porous structure facilitates the migration of cells and vascularisation to create a favourable environment for cell growth to help accelerate bone recovery (Bharatham et al. 2014).

DEGRADATION EVALUATION

The degradation rate of the scaffold is an essential aspect of scaffold application in tissue engineering. The rate of

TABLE 2. Pore diameter range and average pore size for each groups of scaffold

Scaffold	Pore diameter range(µm)	Average pore size(µm)
Control	64.6 - 303.2	166.8 ± 11.55
Treated with 0.5% GA	91.8 - 437.3	178.7 ± 17.87
Treated with 1% GA	75.9 - 397.6	183.6 ± 13.16
Treated with 2% GA	98.1 - 483.6	203.5 ± 16.07

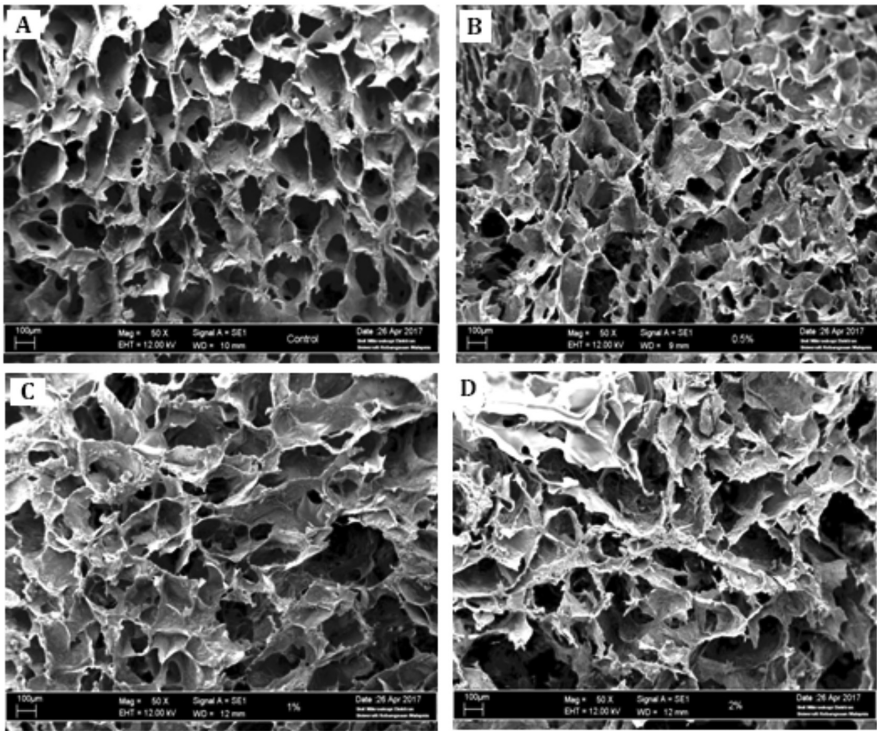
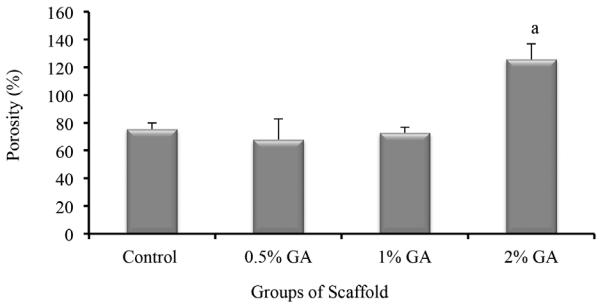


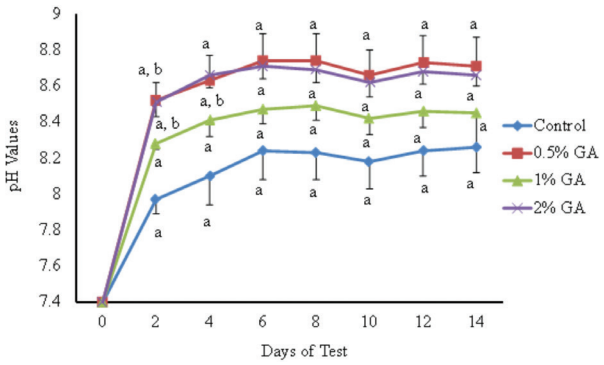
FIGURE 1. SEM micrograph showing the morphology of control scaffold (A), scaffold treated with 0.5% GA (B), 1% GA (C) and 2% GA (D) at 50× magnification



Data were expressed as mean ± SEM, n=6. (a: significant difference from control group at p<0.05)

FIGURE 2. Porosity evaluation for control scaffold and scaffolds cross linked with 0.5%, 1% and 2% GA

degradation should occur concurrently with the reformation of new tissue to allow a smooth transition in load transfer from scaffolds to newly regenerated tissues (Cheung et al. 2007). An unavoidable circumstance of biomaterial-based bone graft degradation is the alteration of pH values in the surrounding microenvironment of a scaffold implanted site. Figure 3 shows the changes in PBS pH values for control scaffold and scaffolds crosslinked with 0.5%, 1% and 2% GA obtained over a period of 14 days. An increase in pH values within the range of 7.73-8.78 were observed within the duration of the study with significant differences between the pH values obtained on day 0 at p<0.05 in all scaffold groups. Significant differences were also observed in scaffolds crosslinked with 0.5% and 1% GA compared to control scaffold on day 2 and 4, respectively. The use of GA as a cross-linking agent has increased the pH values during the degradation phase of the scaffold. pH value effect towards the cell functions is an essential aspect of the principles of tissue engineering. A slight change in pH to an acidic environment would lead to a decline in cell growth thus delaying the regeneration process. It is a known fact that osteoblast is favourable towards an alkaline environment (Kohn et al. 2000) which could be provided by the scaffold in this study as pH values were only found to be increasing in alkalinity during the degradation process. The results of semiquantitative scoring of scaffold structure during degradation study are shown in Table 3 with the highest degradation of structure observed in control scaffolds. The scores were found to be decreasing with the increase in GA concentration used to cross-link the scaffolds structure as noted in Figure 4(a)-4(d). Cross-linking with glutaraldehyde may contribute to structural stability and reduction in breakage between the alginate monomers thus producing better structural integrity to GA crosslinked scaffolds on a concentration based correlation. We support this finding with the increase in pH values of 0.5% GA crosslinked scaffolds comparatively in which higher shift in pH values was obtained with increase breakdown of structure. Contradictorily, higher pH values were also reported in 2% GA crosslinked scaffolds although the structural breakdown of these scaffolds were lesser. This finding can be attributed to the amount of GA present within the scaffold. At a higher concentration it has been previously been reported that GA molecules tend to react



Data were expressed as mean ± SEM, n=6 (a: significant difference from day 0; b: significant difference from control group at p<0.05)

FIGURE 3. Changes in PBS pH values for control scaffold and scaffold cross-linked with 0.5%, 1% and 2% GA within 14 days of degradation study

TABLE 3. Degradation scoring for control and GA cross linked scaffolds

Groups of scaffold	Scoring
Control	4
0.5% GA	3
1% GA	2
2% GA	0

with themselves (Gabrielle et al. 2010) thus forming higher residue molecules that contributes to the increase in pH values.

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

Figure 5 shows the MTT assay for control scaffold and scaffolds cross-linked with 0.5%, 1% and 2% GA. The MTT assay is the most common assay that is used in determining the cytocompatibility of a scaffold in regards to toxicity evaluation of material. A slight reduction in cell viability was noted in cells cultured with leachates from GA crosslinked scaffolds with the lowest level of cell viability noted in 0.5% GA scaffold leachate. Cells grown on leachates from scaffolds cross-linked with 1% and 2% GA showed a similar level of cell viability, however, was found to be slightly reduced as compared to control scaffolds in a non-significant manner. Although GA is known to be cytotoxic, the use of GA as a cross-linking agent in the preparation of bone scaffolds has been previously justified to show good tissue compatibility (Mehdi et al. 2006). The reduction in cell viability observed in 0.5% GA scaffolds compared to 2% GA could be related to the increased structural breakdown of the scaffold. It could be postulated that the reduced structural stability of 0.5% GA crosslinked scaffolds would have increased the pH of the leachate that could have contributed to the reduction in cell proliferation. This could also be supported from

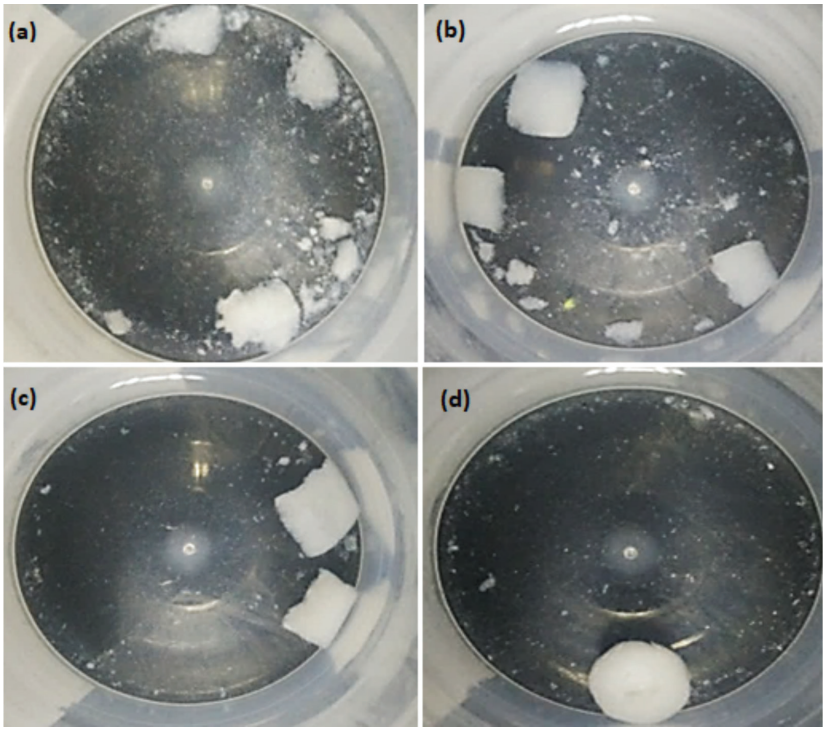
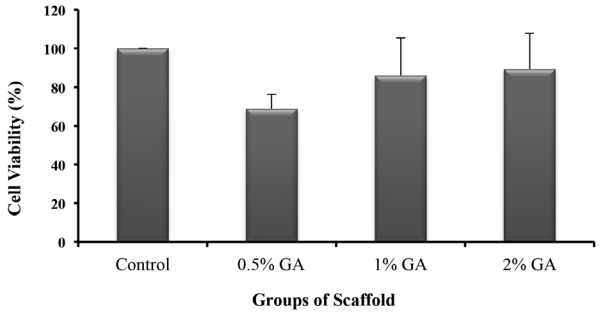
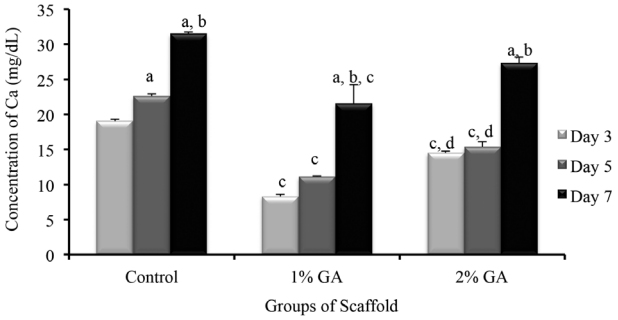


FIGURE 4. Degradation observation of control scaffold (a), scaffold crosslinked with 0.5% GA (b), 1% GA (c) and 2% GA (d) on day 14 of study



Data were expressed as mean ± SEM, n=9

FIGURE 5. MTT assay evaluation for cell viability in control scaffold and scaffold cross linked with 0.5%, 1% and 2% GA



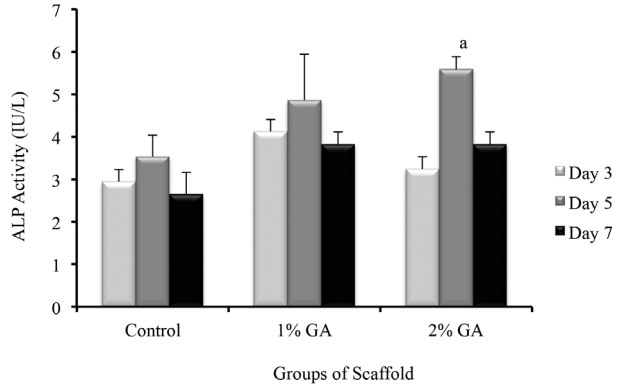
Data were expressed as mean ± SEM, n=6. a: significant difference from day 3 at p<0.05; b: significant difference from day 5 at p<0.05; c: significant difference from control group at p<0.05; and d: significant difference from 1% GA treated at p<0.05

FIGURE 6. Calcium release evaluation for control scaffold and scaffold treated with 0.5%, 1% and 2% GA

the finding from the degradation studies which observed the highest change in pH values occurred in the scaffolds crosslinked with 0.5% GA.

IN-VITRO CALCIUM RELEASE TEST ANALYSIS AND ALP ACTIVITY

Figure 6 shows the results of calcium release for control scaffold and scaffolds cross-linked with 1% and 2% GA. The test was not carried out for 0.5% GA cross-linked scaffolds as the group was eliminated from the study after MTT results showing a reduction in cell viability. An increasing trend in calcium release is observable in all groups from day 3 to day 7 with significant increase noted in control scaffolds at day 7 (31.5 ± 0.26 mg/dL) and day 5 (22.6 ± 0.33 mg/dL) compared to day 3 (19.0 ± 0.3 mg/dL). A significant increase in calcium release at p<0.05 also can be noticed in scaffolds crosslinked with 1% and 2% GA at day 7 compared to day 3 and day 5. A significant difference was also observed between control at day 3 compared and scaffolds crosslinked with 1% and 2% GA as well as between scaffolds crosslinked with 1% and 2% GA on day 3 and day 5. Calcium acts as an important indicator marker for cells proliferation, cells differentiation and reformation of bone tissues (Kamba & Zakaria 2014). Calcium release during cell culture is the marker for osteoblast maturation and closely related to bone mineralisation process (Abu & Mohamed 2011). A common trend observable is the increase in calcium release as ALP activity decreases to facilitate the matrix mineralisation. These findings are supported by the observation of ALP activity as a biological marker for osteoblast growth as shown in Figure 7. There was a general increase in ALP activity in scaffold crosslinked with GA with significant differences noted between day 3 and day 5 in scaffolds crosslinked with 2% GA at p<0.05. From the results of this study, there is an increase in ALP activity on day 5 compared to day 3 followed by a decrease in the activity on day 7 correlating to the increase in calcium release. These results are supported by a previous study by Golub (2009) and the up-down regulation of ALP activity is also reported in studies by Wang et al. (2007) and Zuki et



Data were expressed as mean ± SEM, n=6. (a : significant difference from day 3 at p<0.05)

FIGURE 7. ALP activity evaluation for control scaffold and scaffold treated with 0.5%, 1% and 2% GA

al. (2011). A spike in ALP expression is often noted before mineralisation of bone matrix followed by a decrease when calcification process takes over (Gurumurthy et al. 2016).

CONCLUSION

From the study, it was noted that the use of glutaraldehyde (GA) for cross-linking purpose did not produce an adverse or any cytotoxic effect on the growth and proliferation of osteoblast on the surface of the fabricated bone scaffold and could be concluded as safe to be used at the tested concentrations. However, in this study, an obvious increase in structural integrity and porosity is evident with the use of 2% GA which indirectly provided a better platform for cell attachment and growth suggesting the possible use of GA as a cross-linking agent to increase structural stability for polymer-based scaffolds is feasible.

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