In Vivo evaluation of the novel nanocomposite porous 3D scaffold in a rabbit model: hematology and biochemistry analysis

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Abstract

Issues of safety are very crucial with biomaterials and medical devices. Sixteen male New Zealand White rabbits equally into four groups: Group A, rabbits had part of their radial bone (2 cm, mid shaft) and left empty as a control. Group B, rabbits were implanted with scaffold 5211. Group C, rabbits were implanted with scaffold $5211_{GTA+Alginate}$. Group D, rabbits were implanted with 5211_{PLA}. All scaffolds were prepared by freeze-drying method. Blood samples were collected at day 0 and 1st, 2nd, 3rd, 4th and 8th week after implantation. The blood examination included complete hemogram and certain serum biochemical parameters. The results showed that there was no significant difference ($P>0.05$) among each treatment group in comparison with control group (day 0). However, red blood cells, hemoglobin, packed cell volume, mean corpuscular hemoglobin concentration, monocyte, plasma protein, inorganic phosphate, sodium, chloride and urea were significantly increased ($P<0.05$) among treatment groups at week 8. An abnormal architecture of viscera was observed in all animals, thus indicating a form of toxicity related to the degrading scaffold materials. The severity of histopathological lesions in viscera was not coated polymers dependent nor development materials. In conclusion, implantation of 5211 scaffold with or without coated framework has a significant impact on histopathological and certain hematological and biochemical parameters.

Keywords: 3D porous scaffolds, bionanocomposite, coated, biochemical parameters, rabbit
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تقييم فيفو للسقالات الثلاثية الأبعاد المسامية المركبة النانوية الاصيلة في نموذج الأرنب: تحليل الدم والكيمياء الحيوية

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الخلاصة

تعتبر امور السلامة مهمة جدا عند استخدام المواد الحيوية والأجهزة الطبية. تم تقسيم ستة عشر ارنبا ابيضا نيوزيلنديا الى اربعة مجاميع: المجموعة أ، حيث تم قطع 2 سم من جسم عظم الكعبرة وتركها فارغة كمجموعة سيطرة. المجموعة ب، تم زراعة عظم الكعبرة

للارانب بالسقالة 5211. المجموعة ج، تم زراعة عظم الكعبرة للارانب بالسقالة 5211 المغلفة بمادة الالجينيت.اما المجموعة د، فتم زراعة عظم الكعبرة للارانب بالسقالة 5211 المغلفة بمادة بولي لاكتك اسد، حيث تم تحضيرالسقالات بطريقة التجفيف المبرد. تم جمع عينات الدم في اليوم 0 والاسابيع الاول، الثاني، الثالث، الرابع والثامن بعد الزراعة. تضمنت الدراسة فحص شامل لعينات الدم بالاضافة الى المتغيرات الكيموحيوية للدم. اظهرت النتائج عدم وجود فرق معنوي بين المجاميع بالمقارنة مع مجموعة السيطرة. لوحظ وجود زيادة معنوية في عدد كريات الدم الحمراء، هيموكلوبين الدم، حجم الخلايا المتراصة، معدل تركيز جسيمات الهيموكلوبين، خلية وحيدة النواة، بروتين البلازما، الفوسفات غير العضوية، الصوديوم، الكلوريد واليوريا بين المجاميع خلال 8 اسابيع. كما لوحظ وجود افات نسيجية في الاحشاء الداخلية في جميع الحيوانات بعد الزراعة ماعدا مجموعة السيطرة والتي اختلفت في شدتها مما يشير إلى ان السمية متعلقة بتحلل المواد المستخدمة (البوليميرات) في تغليف السقالات وليس المستخدمة في تحضيرها. يمكن الاستنتاج انه زراعة السقالة 5211 المغلفة او بدون تغليف كان لها تاثيرمعنوي في بعض القياسات الدموية والكيموحيوية والنسجية المرضية.

Introduction

Severe fractures leading to nonunion of wounded bone as well as resection of bone linked with the removal of tumor does not heal through natural healing response of the body, it thus needs medical involvement (1). Transplantation is the most widely used treatment to overcome the deficiencies associated with tissue repair. Tissue engineering provides an alternative of transplantation and prosthesis, with the potential to overcome the limitations of using autografts, allografts, and exografts (2,3). Tissue engineering (or regenerative medicine) is defined as the scientific principles applied in the production of living tissues through a use of bioreactors, cells, scaffolds, growth factors, or a combination of them (4). The scaffold can be implanted single-handedly to prompt host cell colonization to the wounded position and tissue restoration, or it can be seeded with cells and/or growth factors and serve to control the release and targeting of these treatments (5-7). Bone tissue engineering shows promise as an alternative to repair critical bone defects. Accordingly, bone tissue engineering was developed in both scope and significance in biomedical engineering. In some way it signifies the linkage of fast growth in cellular and molecular biology and materials, chemical and mechanical engineering in another way (8). The bone tissue engineered eventually invention two major applications, the first one is to give structural support to new tissue and the second one is formation a new bone by promoting movement of mesenchymal stem cells, differentiations and osteoprogenitor cells (9). Biomaterials can offer a solution to these difficulties. Different inorganic biomaterials, for example tricalcium phosphate (TCP), natural coral (NC) and hydroxyapatite (HA) have been broadly utilized as Bone Morphogenic Proteins' (BMPs) carriers (10-12). Bionanocomposite implants is one of the most important ways in the world of orthopedics. Biomaterial is a material that has high biocompatibility (13). Entirely the biomaterials resulting from non-autologous sources can lead to some level of foreign body reaction post-implantation *in vivo* (14). A good bone substitute must be accepted by the host tissue deprived of any rejection response; it ought to encourage formation of bone, possess suitable mechanical strength, be flexible and resorb after fulfilling its purpose (8,15,16). Nanoparticles (NPs) are particles that have at least one dimension between 1 and 100 nm in size (17). This size gives them unique properties from bulk material, thus making them interesting materials in research and applications. Though reduction in their size, NPs dimensions are inversely proportional to their surface/volume ratio chemical reactivity. Cockle shell nanobioceramic material has a similar structure to the bone (18), has high biocompatibility and bioactivity (15,19). In the laboratory, a nanocomposite scaffold that was fabricated from cockle shell-derived $CaCO_3$ aragonite nanoparticles (CCAN), gelatin, dextran and dextrin was used as a basis for tissue engineering. This scaffold is currently being utilized in a number of tissue engineering applications. Gelatin has gotten a reasonable consideration over the past few years due to its outstanding biocompatibility, degradation into physiological end-products and appropriate interaction with macromolecules and cells (20-25). In the setting of bone tissue engineering it is also significant to note that there are different literature view on the association between Glutaraldehyde (GTA) crosslinking and mineralization. It has been recorded that GTA induces calcification in *in vivo* studies (26-30). Normal polymers can offer inherent patterns for cell adhesion, growth and stimulate an immune reaction as a result of their biocompatibility. Similarly, the microstructures of the normal polymers are very organized and contain extracellular substance which performance as provisional extracellular matrix (ECM) for effective bone regeneration. Therefore, natural polymer coating over the ceramic scaffold is a healthier method for making mechanically sound scaffold for orthopedic applications (31). Fabrication of bone scaffolds in tissue engineering field constantly focuses in providing a bioactive scaffold that could represent the actual biomimetic environment of the bone tissue (32). A vital aspect of a scaffold development involves tissue compatibility assessment. The term biocompatibility refers to the measurement of how

compatible a developed product is with a biological system. The evaluation of biocompatibility often is necessary to predict unwanted physiological reactions when its used for intended purposes in which the *in vivo* evaluation acts as a confirmatory step of the end product developed (33,34). Various methods can be employed in order to study the tissue reactions *in vivo* post-implantation. Hematological, serum biochemical and histopathological examination allow qualitative measurements of the scaffolds biological reactions (35-37).

The choice of an appropriate animal model is an important factor should be taken into consideration. Animals having larger structure have to be selected for this experiment for example rabbit because the size-to-weight proportion and the axial loading design of the limbs of rabbits look like those of human being (38).

Non-coated cockle shell-derived $CaCO_3$ aragonite nanocomposite porous 3D 5211 scaffold is a new implant which has characteristics similar to bone rabeculae. In addition, alginate and Polylactic acid (PLA) as coating polymers on cockle shell-derived $CaCO_3$ aragonite nanocomposite porous 3D 5211 scaffold. The *in vivo* testing coated and non-coated cockle shell-derived $CaCO_3$ aragonite nanocomposite porous 3D scaffolds in the radial bone of rabbits have been done. Effect of implantation of coated and non-coated cockle shell-derived $CaCO_3$ aragonite nanocomposite porous 3D scaffolds in rabbits was observed against blood parameters and tissues as scientific information. The aim of this study is to find out the blood profile of the rabbits after implantation with non-coated cockle shell-derived $CaCO_3$ aragonite nanocomposite porous 3D scaffolds and coated by alginate and Polylactic acid (PLA).

Materials and methods

A total of 16 New Zealand white male rabbit aged 8-11months with body weight of 2-4 kg were used in this study. Adaptation of Animals conducted for 2 weeks before treatment to condition all the animals in a healthy status clinically. Rabbits were set in 4 groups; includes the control group rabbits were had a part of their radial bone (2 cm) (mid shaft) removed by a bone cutter and the critical size defect left empty without implantation (Group A). Rabbits of 2nd group implanted with non-coated scaffolds (scaffold 5211) (Group B). Rabbits of 3rd group implanted with coated scaffolds (scaffold 5211 soaked in cross-linking (GTA) and coated with alginate ($5211_{\mathbf{GTA+Alginate}}$)) (Group C), and the final group is treated with scaffolds (scaffold 5211 coated with PLA ($5211_{\mathbf{PLA}}$)) (Group D), all scaffolds prepared by the freeze-drying method.

The study protocol was approved by the Institute of animal care and use committee (IACUC), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) (AUP-R015/2015). The handling of the animals was in adherence to the IACUC guidelines. An overnight (12 hours) food and water deprivation period preceded the surgery. The animals were anesthetized using the ketamine hydrochloride 35mg/kg B.W I/M and xylazine hydrochloride 3-5mg/kg B.W I/M and anesthetic maintenance carried out using Halothane and O_2. Post-operative pain medication was given closely with adequate regular analgesics that comprise Tramadol hydrochloride 5mg/kg B.W I/M and adequate regular of antibiotic that comprise Baytril 5% w/v 1 mL/10 kg B.W subcutaneous.

Blood serum of all groups' samples were collected from the ear vein into plain vacutainer tubes for serum biochemical assays (inorganic phosphate and electrolytes) from pre-implantation and 1st, 2nd, 3rd, 4th and 8th week post-implantation, while (alanine transaminase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatinine, glucose, urea, total protein and albumin) after 8 weeks post-implantation, while whole blood samples were collected into the K2EDTA vacutainer tubes for complete haemogram (red blood cells (RBCs), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white blood cells (WBC), neutrophils, lymphocytes, monocytes, eosinophils, basophils, thrombocytes and plasma protein) before implantation and 1st, 2nd, 3rd, 4th and 8th week post-implantation to determine toxicity.

The all rabbits used were able to tolerate the surgical processes very well, no animal was lost at the surgical process. The animals were euthanized in accordance to the research protocol and the guidelines provided by the central animal laboratory. The animals remain in a good condition during the study (no infections, good appetite, active, etc.), as they are examined daily. Mortality was not recorded throughout the experiment. Following euthanasia at week 8th post-implantation, the liver, spleen, and kidney of the animals were harvested and examined grossly then stored in 10% neutral buffered formalin for histopathological examination.

For light microscopic examination, the internal organs specimens retrieved at week 8th post-implantation and fixed in 10% neutral buffered formalin following gross examination for 7 days at room temperature. Following initial fixation, the specimens processing (dehydration with an increasing concentration of alcohol, clearing in xylene and impregnation (embedding) in paraffin wax) was done automatically using a machine (Leica, Microsystem Nussloch Heidelbergerstr D-69226, Germany). Immediately after embedding, the samples were blocked with paraffin wax and then sectioned using standard histological techniques, exactly 5 µm thick were prepared from the center and margin of each sample using rotary microtome (Leica 2045, Germany). Transverse and

longitudinal sections of liver, spleen, and kidney were prepared (39). The samples stained with hematoxyline and eosin (H&E) for general histopathological observation, existence of inflammatory responses and to examine any possible toxicity effects of the scaffolds. The slides were then immersed in xylene and mounted with coverslip through the use of DPX mounting medium (15-16,40). The slides were examined under a light microscope (Motic, BA410, China) equipped with a camera (Moticam Pro, 285A, China) Plus 2.0 software was to analyze the images before they were being captured to examine the internal organs.

Statistical Analysis

First of all, the quantifiable outcomes were evaluated using Explore for normality of data then one-way analysis of variance (ANOVA) and Kruskal-Wallis test. The results were shown as a mean ± standard error (SE). Post hoc test were calculated for significant values ($P<0.05$) using Tukey's multiple comparison test. All descriptive and inferential statistical analyses were conducted using Excel version 2013 and SPSS version 23.0.

Results

The results comparison was done between day zero (pre-treatment), the control (no treatment, group A) and 1st, 2nd, 3rd, 4th and 8th week post-implantation for three different groups (three different scaffold implants B, C, and D).

Red blood cells count shows a significant increase ($P<0.05$) at 4th week post-implantation in groups C and D, and at 8th week post-implantation in groups A, B and D (Table 1a). The Hb concentration shows a significant decrease ($P<0.05$) at 4th week post-implantation in group C and a significant increase ($P<0.05$) in group D; and a significant decrease ($P<0.05$) at 8th week post-implantation in group A and a significant increase ($P<0.05$) in group B (Table 1a). Moreover, PCV shows a significant decrease ($P<0.05$) at 1st week post-implantaion in group C and at 8th week post-implantation in group A, then fallowed by a significant increase ($P<0.05$) at 8th week post-implantation in groups B and C. The MCV showed a significant decrease ($P<0.05$) at 2nd week post-implantation in group B and at 8th week post-implantation in group D. Whereas MCHC showed a significant increase ($P<0.05$) at 2nd, 3rd and 8th week post-implantation in group D only (Table 1a).

The WBC count showed a significant increase ($P<0.05$) in group A and significant decrease ($P<0.05$) in group B at 1st week post-implantation. Lymphocyte count have showen the significant increase ($P<0.05$) in group A and a significant decrease ($P<0.05$) in group B at 1st week post-implantation then increased significantly ($P<0.05$) and at 2nd week post-implantation in group B. In addition, group D showed a significant increase ($P<0.05$) in monocyte count at 1st, 2nd, 3rd and 4th week post-implantation, at 2nd week post-implantation in groups C, while it shows a significant decrease ($P<0.05$) at 4th week post-implantation in group A and at 8th week post-implantaion in group B.

The eosinophil count showed a significant decrease ($P<0.05$) at 1st week post-implantation in group B. Basophil count was also significantly increased ($P<0.05$) at 1st week post-implantation in group A and at 2nd week post-implantation in group B and a significant decreased ($P<0.05$) at 1st week post-implantation in group C. Plasma protein concentration showed a significant increase ($P<0.05$) at 4th and 8th weeks post-implantation in groups C and D (Table 1b and c).

Serum inorganic phosphate concentration showed a significant increase ($P<0.05$) at 2nd week post-implantation in group B, whilst in group C showed a significant decrease ($P<0.05$) at 2nd week post-implantation. At 3rd week post-implantation, the serum inorganic phosphate concentration showed a significant increase ($P<0.05$) in group A and a significant decrease ($P<0.05$) in group D. Then at 4th week post-implantation, serum inorganic phosphate concnetration showed a significant increase ($P<0.05$) in group A and a significant decrease ($P<0.05$) in group B. Serum ALT concentration showed a significant decrease ($P<0.05$) at 8th week post-implantation in group C. The serum sodium concentration showed a significant increase ($P<0.05$) at 4th week post-implantation in group D. Whilst serum chloride concentration showed a significant increase ($P<0.05$) at 3rd week post-implantation in group D. Serum urea concentration showed a significant increase ($P<0.05$) at 8th week post-implantation in group D (Tables 2 and 3).

Figure 1 shows the liver, kidney and spleen of the four groups were found to be normal with no lesions noticed compare to control group which indicating the absences of toxicity effect caused by the scaffolds degrading by-products. Figures 2, 3 and 4 show the H&E stained sections of the liver, kidney and spleen of the all groups. Group A (control), the liver architecture were found to have mild congestion of the central vein and across the section, the kidney had the normal structure of the renal tissue with normal glomeruli and normal epithelia of the renal tubules, while the spleen had congestion. Group B (implanted by scaffold 5211), the liver architecture showing odema, diffuse degeneration, and necrosis of the hepatocytes, the kidney showing mild congestion in the glomeruli and the spleen had congestion. Group C (implanted by scaffold 5211GTA + Alginate), the liver architecture showing infiltration of inflammatory cells, degeneration, and necrosis of the hepatocytes and Kuppfer cells were visible, the kidney had congestion of the renal glomerulus, interstitial odema, and degeneration and necrosis of the tubular epithelial cells and the spleen had haemorrhage, odema and degeneration and necrosis of histiocytes. Group

D (implanted by scaffold 5211PLA), the liver architecture showing congestion of the central vein, infiltration of inflammatory cells, diffuse degeneration, and necrosis of the hepatocytes and Kuppfer cells were visible, the kidney had severe and diffuse necrosis of the tubular epithelial tissues leaving empty and hallow renal tubules and the spleen had focal congestion, haemorrhage, oedema, and focal necrosis of histiocyte.

Table 1a: Haemogram of treated rabbits during 8 weeks post-implantation, (Mean ± SE)

Groups		Parameter				
		Red blood cells ($x10^9$/L)	Haemoglobin (g/L)	Packed Cell Volume (g/L)	Mean Corpuscular Volume (fL)	Mean Corpuscular Haemoglobin Concentration (g/L)
Group A (Control)	0 D	5.7 ± 0.3	118.8± 5.2	0.37 ± 0.02	65.8 ± 1.3	323.3 ± 4
	1 W	5.5 ± 0.3	112 ± 3	0.35 ± 0.01	63.3 ± 3	323 ± 2
	2 W	5.5 ± 0.2	111.3 ± 4	0.34 ± 0.01	61.3 ± 1	318 ± 2
	3 W	5.5 ± 0.2	114 ± 6	0.34 ± 0.01	62.3 ± 2	331 ± 4
	4 W	5.7 ± 0.1	116 ± 2.3	0.34 ± 0.01	59.3 ± 2	335.3 ± 2
	8 W	5 ± 0.2^{*Z}	103.6 ± 4.3^{*Z}	0.3 ± 0.02 ^{*Z}	60.6 ± 2	339.8 ± 2.7
Group B	0 D	5.7 ± 0.3	118.8± 5.2	0.37 ± 0.02	65.8 ± 1.3	323.3 ± 4
	1 W	5.8 ± 0.3	115 ± 3	0.38 ± 0.01	64.5 ± 3	313.3 ± 13
	2 W	6.1± 0.4	123 ± 7	0.33 ± 0.01	60.3 ± 1 ^{*Z}	328 ± 4
	3 W	6 ± 0.4	118.8 ± 8	0.37 ± 0.01	62 ± 1	324 ± 4
	4 W	5.2 ± 0.3	116 ± 7	0.35 ± 0.02	59.3 ± 1	335 ± 2
	8 W	6.1 ± 0.2 ^{*ZG}	123.8 ± 5 ^{*G}	0.38 ± 0.01^{*G}	59 ± 0.5	330.6 ± 2.6
Group C	0 D	5.7± 0.3	118.8± 5.2	0.37 ± 0.02	65.8 ± 1.3	323.3 ± 4
	1 W	5 ± 0.0	102 ± 2	0.31 ± 0.01 ^{*ZG}	61.7 ± 1	327.3 ± 3
	2 W	5.2 ± 0.0	109 ± 2	0.33 ± 0.01	62.3 ± 2	334.3 ± 0.3
	3 W	5.2 ± 0.1	106 ± 2	0.32 ± 0.01	61.7 ± 0.3	329 ± 2
	4 W	5 ± 0.2 ^{*ZG}	100.5 ± 5 ^{*G}	0.30 ± 0.02	59.8 ± 1.3	338.3 ± 5
	8 W	5.6± 0.1	113.5 ± 2.2	0.79 ± 0.4 ^{*ZG}	56± 4.4	381.5 ± 47
Group D	0 D	5.7± 0.3	118.8± 5.2	0.37 ± 0.02	65.8 ± 1.3	323.3 ± 4
	1 W	5.3 ± 0.1	223 ± 109	0.34 ± 0.01	63.7 ± 3	336 ± 2
	2 W	5.5± 0.1	118.7 ± 5	0.35 ± 0.01	63.3 ± 1	339 ± 2 ^{*ZG}
	3 W	5.8 ± 0.1	121 ± 2	0.35 ± 0.01	60 ± 3	351 ± 8 ^{*ZG}
	4 W	6.1 ± 0.2 ^{*ZG}	123.8 ± 2.1^{*G}	0.35 ± 0.02	60.5 ± 3	343.3 ± 9
	8 W	6.2 ± 0.2 ^{*ZG}	115.7 ± 3.7	0.34 ± 0.01	55.8 ± 2.5 ^{*Z}	350.2 ± 5.7 ^{*Z}

0 D = Day before implantation, W= week, $*^Z$ = shows significant different between zero day and groups at P<0.05, $*^G$ = shows significant different between groups at P<0.05, red label= increasing in level, blue label= decreasing in level, n = 4 rabbits per group.

Discussion

The results of this study revealed that all the rabbits survived after surgical intervention, and sign of inflammation were not seen or adverse tissue response was not detected around the implants. Observations within 8 weeks post-implantation aimed to find the picture of the blood that occurs in the body of the rabbit. Data from the calculation of the number of RBCs in each group treatments showed no significant difference (P>0.05), except in the group D showed a significant increase (P<0.05) at 4th week post-implantation, and at 8th week post-implantation in groups B and D as presented in Table 1a. Nonetheless, the increase in the number of RBCs in the groups B and D still within the range of normal values of rabbit blood (41). Red blood cell function dynamically set the balance of oxygen requirement and distribution nutrients throughout the body and discards the rest metabolic products in the form of CO_2 (42).

Data of hemoglobin (Hb) in all groups also showed a significant decrease (P<0.05) at 4th week post-implantation in group C and at 8th week post-implantation in group A. Moreover, PCV showed a significant decrease (P<0.05) at 1st week post-implantation in group C and at 8th week post-implantation in groups A and B, then followed by significant increase (P<0.05) 8th week post-implantation in group C as presented in Table 1a but still within the range of normal values (41). Hb concentration measurement is

one of the common test carried out as a part examination of blood, dehydration, or hyperhidrosis greatly affect the levels of hemoglobin (43). The process of wound healing involves various functions, one of which is depending on the presence of oxygen. Normally delivery of oxygen by the blood dependent on oxygen bound to the hemoglobin in red blood cells, compared to the arterial oxygen partial pressure (PO_2). This is especially true for muscle tissue, which has a small distance and high intercapillary oxygen consumption (44). It has been recorded that decreasing of Hb concentration in patients induce post-operative complications due to lack of oxygen (hypoxia) which lead to an interruption in the wound healing process (45), prolong the healing process (46) and increased susceptibility to post-operative infections (47). Adequacy percentage of Hb and PCV can speed up the processing time of healing wounds. Oxygen an essential requirement in the process healing wounds as well as to avoid post-operative infection (48-50).

Serum inorganic phosphate concentration showed a significant increase and decrease ($P<0.05$) in all groups during 8 weeks as a result of bone repairing process (46). Serum urea concentration showed a significant increase ($P<0.05$) 8 weeks post-implantation in group D which signified kidney disease (51,52). The changes in haematological and biochemical parameters recorded from this study were related to the cascade of immunological responses caused by materials that used in the development and coating the scaffolds.

Table 1b: Haemogram of treated rabbits during 8 weeks post-implantation, (Mean ± SE)

Groups		Parameter				
		White blood cells ($x10^9$/L)	Neutrophils ($x10^9$/L)	Lymphocytes ($x10^9$/L)	Monocytes ($x10^9$/L)	Eosinophils ($x10^9$/L)
Group A (Control)	0 D	7.6± 1.1	3.8± 0.6	3.7 ± 0.9	2.1± 0.9	0.21 ± 0.07
	1 W	9.86 ± 0.3 ^{*Z}	4.1 ± 0.3	4.6 ± 0.4 ^{*Z}	0.59 ± 0.2	0.23 ± 0.08
	2 W	9.6 ± 0.9	5.4± 0.6	3.5 ± 0.3	0.5 ± 0.1	0.29 ± 0.03
	3 W	9.1 ± 0.9	3.8 ± 1	4.3 ± 0.4	0.5 ± 0.03	0.24 ± 0.08
	4 W	4.8 ± 1.6	2.4± 0.8	2.1 ± 0.5	0.2 ± 0.05 ^{*Z}	0.1 ± 0.02
	8 W	4.7 ± 1.3	1.8± 0.8	2.3± 0.4	1.7 ± 1	0.2 ± 0.06
Group B	0 D	7.6± 1.1	3.8± 0.6	3.7 ± 0.9	2.1± 0.9	0.21 ± 0.07
	1 W	5.4 ± 0.5^{*G}	3.5 ± 0.3	1.8 ± 0.2^{*G}	0.3± 0.03	0.11 ± 0.02 ^{*ZG}
	2 W	9.9 ± 1.3	4.3 ± 0.4	5.1 ± 0.5 ^{*G}	0.5 ± 0.1	0.18 ± 0.02
	3 W	9.7 ± 1	3.8 ± 0.7	4.9 ± 0.9	0.6 ± 0.1	0.17 ± 0.03
	4 W	7.4 ± 0.6	2.6 ± 0.6	3 ± 0.4	0.3± 0.06	0.25 ± 0.05
	8 W	6 ± 1.3	2.3 ± 0.3	3.5 ± 0.6	0.27 ± 0.1 ^{*ZG}	0.1 ± 0.05
Group C	0 D	7.6± 1.1	3.8± 0.6	3.7 ± 0.9	2.1± 0.9	0.21 ± 0.07
	1 W	7.2 ± 0.3	4.4 ± 0.4	2.1 ± 0.04	1.6 ± 1	0.24 ± 0.02
	2 W	8.2 ± 0.7	4.4 ± 0.5	3.1 ± 0.3	4 ± 0 ^{*ZG}	0.27 ± 0.01
	3 W	9.4 ± 1.3	5.5 ± 1	3 ± 0.3	2.8 ± 1.2	0.32 ± 0.09
	4 W	6.2 ± 1.4	3.2 ± 1.1	2.5 ± 0.5	2.2 ± 1.1	0.2 ± 0.05
	8 W	6.8 ± 0.7	3 ± 0.5	2.8 ± 0.3	2.8 ± 0.8	0.2 ± 0.05
Group D	0 D	7.6± 1.1	3.8± 0.6	3.7 ± 0.9	2.1± 0.9	0.21 ± 0.07
	1 W	8.2 ± 0.9	3.3 ± 0.9	3.9 ± 0.1	4 ± 0 ^{*ZG}	0.11 ± 0.02
	2 W	7.6± 1	2.9 ± 0.6	3.7 ± 0.5	4 ± 0 ^{*ZG}	0.22 ± 0.09
	3 W	6.8± 0.2	3 ± 0.34.6	3 ± 0.4	4 ± 0 ^{*ZG}	0.16 ± 0.03
	4 W	7.3 ± 1.1	3.3 ± 1	3.1 ± 0.6	3.1 ± 0.9^{*G}	0.2 ± 0.05
	8 W	5.2 ± 0.8	2.3± 0.6	2.8 ± 0.3	1.04 ± 0.6	0.2 ± 0.05

0 D = Day before implantation, W= week,^{*Z} = shows significant different between zero day and groups at $P<0.05$, ^{*G} = shows significant different between groups at $P<0.05$, red label= increasing in level, blue label= decreasing in level, n = 4 rabbits per group.

Table 1c: Haemogram of treated rabbits during 8 weeks post-implantation, (Mean ± SE)

Groups		Parameter			
		Basophils ($x10^9$/L)	Thrombocytes ($x10^9$/L)	Plasma protein (g/L)	I.I (g/L)
Group A (Control)	0 D	0.17 ± 0.06	251 ± 30	64 ± 1.4	2.5 ± 0.8
	1 W	0.63 ± 0.04 *Z	510 ± 196	69 ± 2.4	2 ± 0
	2 W	0.28 ± 0.1	267 ± 76	67 ± 4	2 ± 0
	3 W	0.29 ± 0.2	343 ± 128	66 ± 0.3	2 ± 0
	4 W	0.2± 0.1	165 ± 74	65.3 ± 5	2 ± 0
	8 W	0.1 ± 0.05	131 ± 48	61.4 ± 3	2.6 ± 0.6
Group B	0 D	0.17 ± 0.06	251 ± 30	64 ± 1.4	2.5 ± 0.8
	1 W	0.15 ± 0.04	435 ± 65	62 ± 2	2 ± 0
	2 W	0.45 ± 0.05 *Z	360 ± 71	66 ± 0.5	2 ± 0
	3 W	0.21 ± 0.06	323 ± 59	67 ± 3	2 ± 0
	4 W	0.2± 0.07	191 ± 44	64.3 ± 4	2 ± 0
	8 W	0.2± 0.1	296 ± 124	61.4 ± 3	2 ± 0
Group C	0 D	0.17 ± 0.06	251 ± 30	64 ± 1.4	2.5 ± 0.8
	1 W	0.023 ± 0.02*G	304 ± 118	66 ± 4	2 ± 0
	2 W	0.21 ± 0.07	339 ± 38	72 ± 3	2 ± 0
	3 W	0.16 ± 0.08	307 ± 11	69± 4	2 ± 0
	4 W	0.25 ± 0.2	220 ± 28	71± 3 *ZG	2 ± 0
	8 W	0.36 ± 0.08	256 ± 32	70 ± 2 *ZG	2 ± 0
Group D	0 D	0.17 ± 0.06	251 ± 30	64 ± 1.4	2.5 ± 0.8
	1 W	0.24 ± 0.07	537 ± 63	65± 4	2 ± 0
	2 W	0.2± 0.04	472 ± 31	65 ± 4	2 ± 0
	3 W	0.3 ± 0.06	286 ± 63	71 ± 5	2 ± 0
	4 W	0.2 ± 0.07	241 ± 37	72 ± 6 *ZG	2 ± 0
	8 W	0.25 ± 0.09	320 ± 83	71 ± 2 *ZG	2 ± 0

0 D = Day before implantation, W= week,*Z = shows significant different between zero day and groups at P<0.05, *G = shows significant different between groups at P<0.05, red label= increasing in level, blue label= decreasing in level, n = 4 rabbits per group.

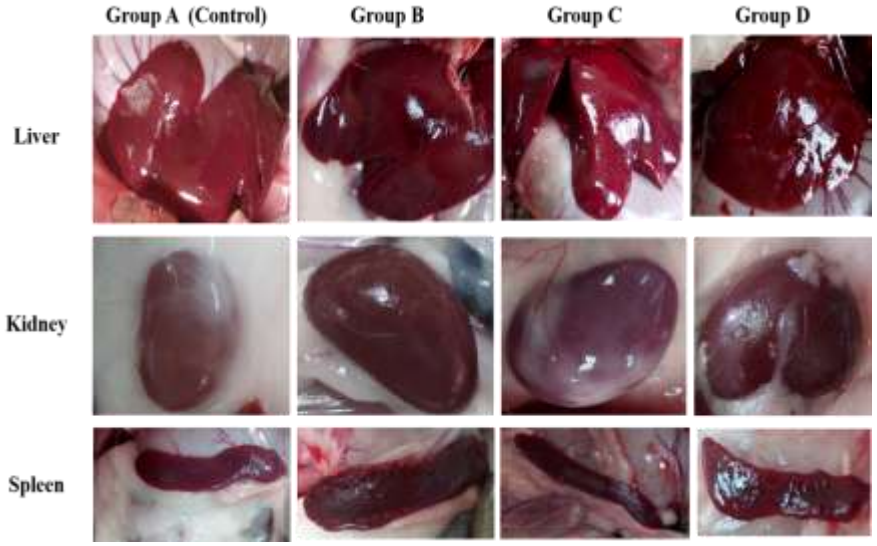

Figure 1: Gross photographs of the liver, kidney and spleen of the fourth groups after 8 weeks post-implantation were found to be normal with no lesions noted as against normal tissues (group A (control)).

Table 2: Serum biochemistry of treated rabbits during 4 weeks post-implantation, (Mean ± SE)

Groups		Parameter			
		Inorganic Phosphate (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)
Group A (Control)	0 D	1.23 ± 0.07	143± 0.9	4.2 ± 0.1	103 ± 1
	1 W	1.33± 0.03	144 ± 0.7	4.8 ± 0.1	102.7 ± 2
	2 W	1.33± 0.03	145± 4	4.3 ± 0.07	108 ± 3
	3 W	1.4 ± 0.09 *Z	141 ± 1.2	4.2 ± 0.2	102.3 ± 2
	4 W	1.5 ± 0.06 *Z	143 ± 0.8	3.8 ± 0.3	101.3 ± 0.9
Group B	0 D	1.23 ± 0.07	143± 0.9	4.2 ± 0.1	103 ± 1
	1 W	1.3 ± 0.2	142 ± 0.9	4.6 ± 0.4	104.3 ± 2
	2 W	1.44 ± 0.1*Z	148 ± 6	5.6 ± 1*G	109.3 ± 5
	3 W	1.32± 0.1	141 ± 2	4.8 ± 0.6	102 ± 2
	4 W	1.1 ± 0.07*ZG	143± 1	4.2 ± 0.5	102.3 ± 2
Group C	0 D	1.23 ± 0.07	143± 0.9	4.2 ± 0.1	103 ± 1
	1 W	0.93 ± 0.2	143 ± 5	3.8 ± 0.07	103.3 ± 2
	2 W	0.90± 0.2 *Z	146 ± 2	3.7 ± 0.2	105. 3 ± 0.3
	3 W	1.1 ± 0.03	145 ± 2	4.2 ± 0.03	106 ± 0.6
	4 W	1.2 ± 0.06	147 ± 1.6	3.8 ± 0.2	105 ± 0.4
Group D	0 D	1.23 ± 0.07	143± 0.9	4.2 ± 0.1	103 ± 1
	1 W	1.1 ± 1	153 ± 4	4.1 ± 0.5	110 ± 5
	2 W	1.2 ± 0.07	145 ± 0.9	4.3 ± 0.3	105 ± 2
	3 W	1 ± 0.06 *ZG	147 ± 0.6	4.5 ± 0.2	107 ± 0.6 *ZG
	4 W	1.24 ± 0.1	153 ± 6 *ZG	4.2 ± 0.2	106 ± 1.4

0 D = Day before implantation, W= week,*Z = shows significant different between zero day and groups at P<0.05, *G = shows significant different between groups at P<0.05, red label= increasing in level, blue label= decreasing in level, n = 4 rabbits per group.

Table 3: Serum biochemistry of treated rabbits during 8 weeks post-implantation, (Mean ± SE)

Parameters	Unit	0 D	Groups (8 W)			
			Group A (Control)	Group B	Group C	Group D
Inorganic Phosphate	mmol/L	1.23 ± 0.07	1.1 ± 2	1.5 ± 0.2	1.1 ± 0.09	1 ± 0.07
Alanine aminotransferase	U/L	74 ± 13	33.7 ± 5	26.2 ± 6	24 ± 5 *Z	53 ± 5*G
Aspartate aminotransferase	U/L	33.5 ± 13	15.5 ± 2	16.3 ± 2	30 ± 6	30 ± 10
Lactate Dehydrogenase	U/L	51 ± 22	84 ± 28	218 ± 107	49 ± 14	71 ± 9
Creatinine	µmol/L	177 ± 34	112 ± 7	112 ± 7	145 ± 15	152 ± 30
Glucose	mmol/L	34.1 ± 3	33 ± 0.5	26.7 ± 1	26.5 ± 4	29.9 ± 3
Urea	mmol/L	4.9 ± 0.7	5.1 ± 0.4	6.4 ± 0.4	6.7 ± 0.6	7.3 ± 0.7 *Z
Total protein	g/L	68.1 ± 3	65.8 ± 3	72 ± 7	63.9 ± 2	64.4 ± 4
Albumin	g/L	41.8 ± 10	31 ± 1.4	37.1 ± 3	34.8 ± 1	35.8 ± 3
Sodium	mmol/L	143 ± 0.9	144 ± 0.4	143 ± 3	143 ± 3	145 ± 0.9
Potassium	mmol/L	4.2 ± 0.1	3.6 ± 0.2	7.1 ± 2	4.3 ± 0.3	3.9 ± 0.2
Chloride	mmol/L	103 ± 1	106 ± 2	109 ± 4	106 ± 0.4	103 ± 3

0 D = Day before implantation,*Z = shows significant different between zero day and groups at P<0.05, *G = shows significant different between groups at P<0.05, red label= increasing in level, blue label= decreasing in level, n = 4 rabbits per group.

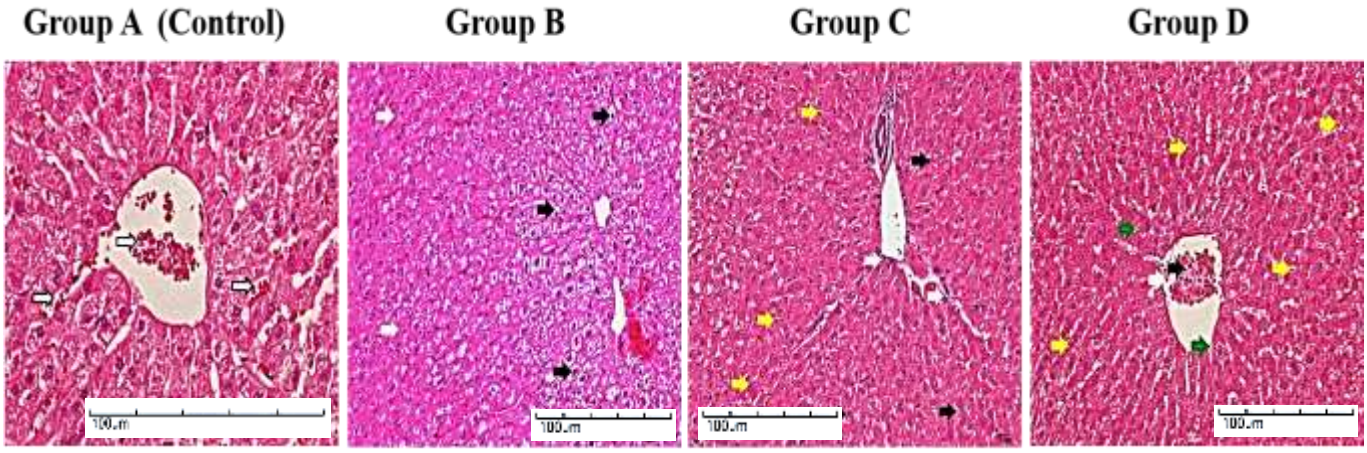

Figure 2: Micrographs of the liver after 8th week post-implantation, group A (control) showing mild congestion of the central vein and across the section (white arrow); group B showing oedema (white arrow), diffuse degeneration and necrosis of the hepatocytes (black arrow); group C showing infiltration of inflammatory cells (white arrow), degeneration and necrosis of the hepatocytes (black arrow) and Kuppfer cells were visible (yellow arrow); group D showing congestion of the central vein (white arrow), infiltration of inflammatory cells (black arrow), diffuse degeneration and necrosis of the hepatocytes (yellow arrow) and Kuppfer cells were visible (green arrow), (H&E, (group A, X400, groups B, C & D, X200)).

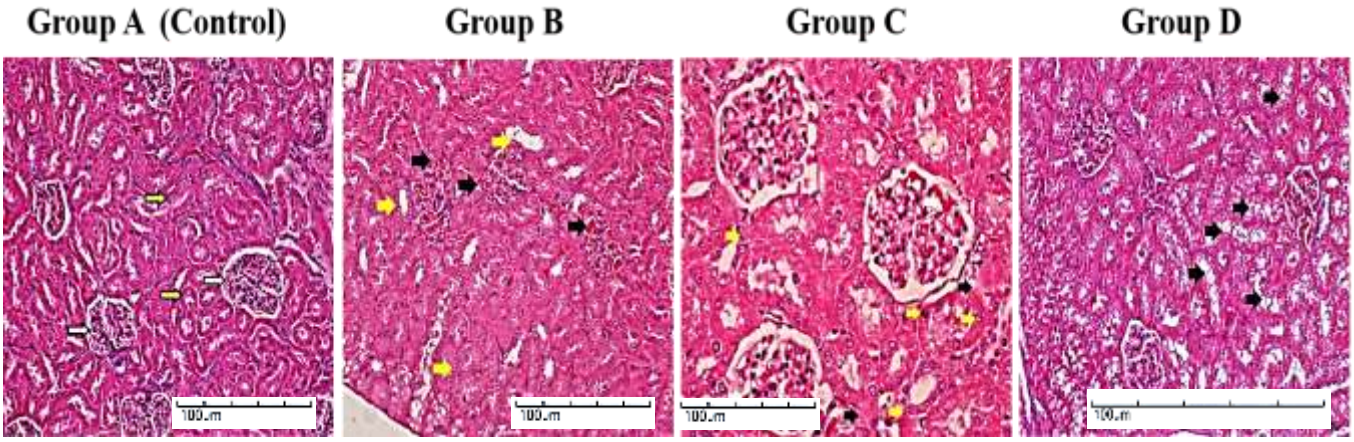

Figure 3: Micrographs of the kidney after 8th week post-implantation, group A (control) showing the normal structure of the renal tissue with normal glomeruli (white arrow) and normal epithelia of the renal tubules (yellow arrow); group B showing mild congestion in the glomeruli (white arrow) ; group C showing congestion of the renal glomerulus (white arrow), interstitial oedema (black arrow) and degeneration and necrosis of the tubular epithelial cells (yellow arrow); group D showing severe and diffuse necrosis of the tubular epithelial tissues (black arrow) leaving empty and hallow renal tubules, (H&E, (groups A, B & D, X200, group C, X400)).

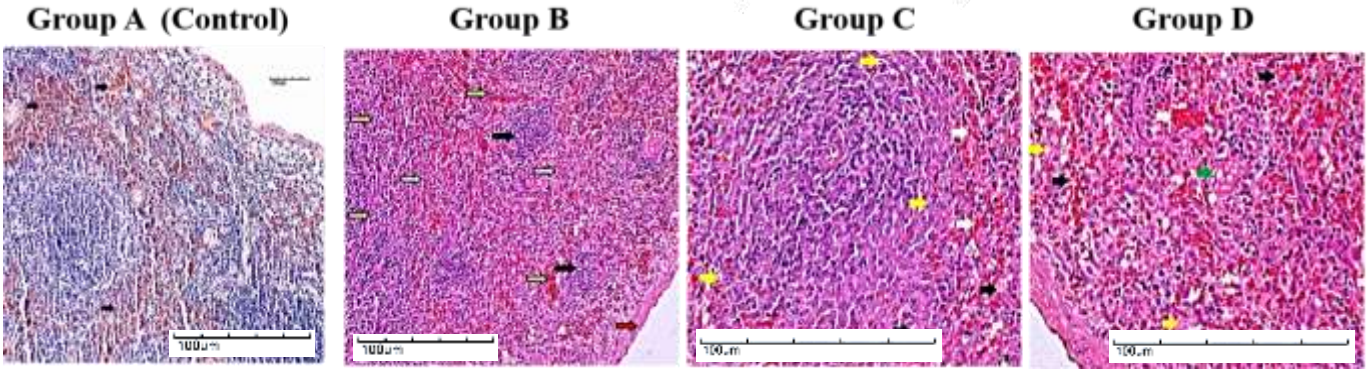

Figure 4: Micrographs of the spleen after 8th week post-implantation, group A (control) showing congestion (black arrow); group B showing the red pulp (white arrow), white pulp (yellow arrow), congestion (green arrow), lymphocytic follicles (black arrow) and splenic capsule (red arrow); group C showing haemorrhage (white arrow), oedema (black arrow), degeneration and necrosis of histiocytes (yellow arrow); group D showing focal congestion (white arrow), haemorrhage (black arrow), oedema (yellow arrow) and focal necrosis of histiocytes (green arrow), (H&E, (groups A & B, X200, groups C & D, X400)).

Gross examinations showed a healthy appearance of the liver, kidney, as well as spleen without signs of inflammation or signs of unwanted tissue reactions which allow the calcification of a developed scaffolds nanocomposite material as an appropriate bone substitution material that facilitates cell attachment and growth (53).

According to the liver, spleen and kidney histological properties, an abnormal architecture was observed in all animals thus indicating the form of toxicity related to the products released from the degrading scaffold materials. The severity of the histopathological lesions in the viscera were coated polymers dependent nor development materials and in agreement with earlier reports by Harlan Laboratories (54) and Aguilar *et al* (55) that documented the absence of treatment-related effects from studies on oral administration of calcium carbonate, Isa *et al* (56) who has demonstrated the biocompatibilities (nontoxicity and nonimmunogenicity) of $CaCO_3$ nanocrystals and making it suitable candidate for nanomedicine and related fields, Hammadi *et al* (57) who proved that $CaCO_3$ nanocrystals to be biocompatible and non-toxic by itself, Jaji *et al* (58) who repoted that $CaCO_3$ is generally safe and the safety of $CaCO_3$ nanocrystals *in vivo* is dependent on its concentration and route of administration, Zuki *et al* (15) and Bharatham *et al* (16) documented the absence of scaffolds materials effects to leak out as they degrade from studies in scaffolds of calcium carbonate, gelatin, dextrin, dextran and alginate, Lee *et al* (28), Gendler *et al* (59) and Wiebe *et al* (60) who reported possibly toxic repercussions of GTA for cells in the direct area; Athanasiou *et al* (51), Xiang *et al* (52) who reported toxicity effect and certain problems, especially pertaining to reduction in cell proliferation. The cytocompatibility of the scaffold materials towards cells has been previously proven through *in vitro* evaluations and it is unlikely for the scaffold materials to cause an adverse toxicity reaction at distant organs of the animals in *in vivo* evaluations.

Conclusion

In the present study, we have introduced the cockle shell-derived $CaCO_3$ aragonite nanocomposite 3D scaffold for tissue engineering. Both the inflammatory response and the toxicity were acceptable after implantation of the cockle shell-derived $CaCO_3$ aragonite nanocomposite 3D scaffolds with and without the coated framework in the rabbits. The final conclusion is that the implant 5211 scaffold with and without the coated framework *in vivo* affect the number of blood and serum parameters but still within the range of normal values in NZW rabbits. Thus, the novel cockle shell-derived $CaCO_3$ aragonite nanocomposite 3D $5211_{GTA+Alginate}$ scaffold may serve as an adequate scaffold material for clinical tissue-engineering approaches.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors read and approved the final manuscript.

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