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Biocompatibility and bone mineralization potential of 45S5 Bioglass®-derived glass–ceramic scaffolds in chick embryos

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Abstract

The aim of the present study was to evaluate the biocompatibility and bone mineralization potential of 45S5 Bioglass®-derived glass–ceramic scaffolds using a chick embryo shell-less (ex ovo) culture system. Chick embryos were divided into two groups: control (C) and experimental (E). Scaffolds were placed on the chorioallantoic membrane (CAM) in embryos of group E at 10 days of total incubation. The 45S5 Bioglass®-derived glass–ceramic scaffolds proved to be biocompatible in terms of the absence of inflammatory response at the implant site (CAM). Moreover, no alterations in the other end-points assessed, i.e. survival, stage of embryonic development and body weight, were detected. However, body length was greater in group E embryos than in group C embryos ($p \leqslant 0.05$). A marked reduction (93%) in Ca content in the scaffolds was evidenced by energy-dispersive X-ray analysis at 5 days post-implantation. Calcium release from the scaffold implanted on the CAM might have been responsible for the restoration of the bone-like phenotype in chick embryonic skeleton of group E as detected by Alcian blue–Alizarin red double staining, as well as by histological and microchemical analyses. Conversely, the control embryos exhibited a chondrogenic phenotype.

Keywords: Biocompatibility; Tissue engineering; Bioglass®; Scaffolds; Chick embryo

1. Introduction

The osteogenic response to bioactive glasses and glass–ceramics has been widely demonstrated in both in vitro and in vivo studies [1–3]. The bioactivity of these silicate and phosphate systems has been attributed to both surface reactions taking place at the material–tissue interface and to the direct effect of glass dissolution products on osteogenesis [1–5]. This large body of results has led to the development of a series of foam-like scaffolds for bone tissue engineering based on bioactive glasses and glass–ceramics [6–10]. One group of highly porous scaffolds recently produced is based on the original 45S5 Bioglass® composition. These scaffolds are fabricated by the foam replication method followed by a densification/crystallization heat-treatment of the glass structure [6]. The microstructure, mechanical properties and surface reactivity of the scaffolds have been investigated [6,8,9]; however, the in vivo biocompatibility has not been evaluated to date.

The need for in vivo experimental models as an alternative to the experimental animal models currently in use has recently been suggested [11]. In this context, several authors have proposed the use of chick embryos as an

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experimental model to study the biocompatibility of materials, given its advantages over mammalian systems [12–17]. Amongst these advantages are universal availability of eggs, relatively low cost, speedy development and growth of the embryos, easy handling, and compliance with the 3Rs principles (replacement, reduction and refinement) on using alternative methods to animal experiments in biomaterials and tissue engineering research [12–21].

Two different techniques have been described for chicken embryo culture, i.e. in ovo or ex ovo (shell-less), and both methods are in regular use. Chick embryos grown in shell-less culture are calcium-deficient by 9 days of incubation [22–24].

Compared with normal controls, calcium-deficient chick embryos exhibit a chondrogenic phenotype that is restored to an osteogenic phenotype upon calcium (Ca) repletion [22–24]. The shell-less chick embryo thus represents a unique experimental system for studying the relationship between Ca-containing biomaterials and skeletogenesis.

The aim of this work was to evaluate for the first time the in vivo biocompatibility and bone mineralization potential of the novel highly porous 45S5 Bioglass-derived glass–ceramic scaffolds [6] using a chick embryo shell-less (ex ovo) culture system as the experimental model.

2. Materials and methods

2.1. Scaffolds

45S5 Bioglass (45 SiO_2, 24.5 CaO, 24.5 Na_2O, 6 P_2O_5 in wt.%)-derived glass–ceramic scaffolds with porosity in the range 90–95% were fabricated using the foam replica technique and sintering at 1100 °C for 1 h, as described elsewhere [6]. Briefly, a polymer (e.g. polyurethane) foam, which serves as a sacrificial template, is coated with a Bioglass slurry, which infiltrates the pore structure, and Bioglass particles (<5 μm in size) adhere on the polymer surface, forming a homogeneous coating. After drying, the polymer foam is burned out slowly at 400 °C to minimize damage to the Bioglass coating. Once the polymer sacrificial template has been removed, the Bioglass scaffold is sintered to the desired density and partially crystallized using a predetermined and optimized heat-treatment schedule [6].

Extensive sintering of the 45S5 Bioglass particles by viscous flow occurs at this temperature, leading to highly densified struts of the foams, as shown in Fig. 1. During the heat-treatment process the glass partially crystallizes and $Na_2Ca_2Si_3O_9$ fine crystalline particles form, which are expected to lead to improved mechanical properties [6]. Fig. 1 also demonstrates the high porosity and pore interconnectivity of the scaffolds which exhibit a pore structure resembling that of cancellous bone.

2.2. Shell-less (ex ovo) culture system

Fertile eggs (*Gallus* sp.) were used. These were incubated in ovo at 37 °C with 60% relative humidity in a standard laboratory incubator for 72 h. The ex ovo embryo development was carried out in a polystyrene container (4 cm × 7 cm diameter, 0.2 mm thick, Huhtamaki Argentina) placed inside another high density polyethylene container with a screw top (8.5 cm × 9 cm diameter, TE&T) containing 50 ml of distilled water for humidification. Neither tissue culture medium nor antibiotics were added to the cultures. All culture chambers were maintained at 37 °C in a humidified air atmosphere for the desired length of time.

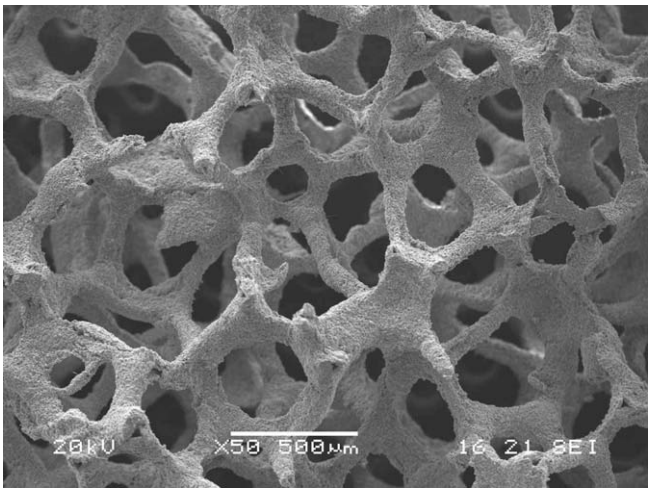

Fig. 1. SEM image showing the pore structure of Bioglass-derived glass–ceramic scaffold fabricated by the foam replica method [6]. (Micrograph courtesy of Ms. D. Mohamad Yunos, Imperial College London, UK.)

2.2.1. In vivo bioassay

Chick embryos were divided into two groups: control (C), and experimental (E). 45S5 Bioglass-derived glass–ceramic scaffolds ($5 \times 5 \times 2\ mm^3$) were placed on the chorioallantoic membrane (CAM) at 10 days of total incubation in embryos of group E (Fig. 2). Each experiment included ten embryos per group and was repeated twice.

The embryos were monitored every 24 h and classified by stage of development based exclusively on external morphological features as described by Hamburger and Hamilton [28]. The embryonic survival was estimated. All embryos were killed at 15 days of total incubation.

2.2.2. Determination of calcium ion content in amniotic fluid

The developing chick embryo exhibits a regular pattern of accumulation and reabsorption of Ca in its extraembryonic fluids [29,30]. For this reason, amniotic fluid was collected from embryos of both groups at 10 days (baseline) and 15 days of total incubation (5 days post-implantation) using a 1 cm^3 syringe with a 23 gauge needle and refrigerated undiluted until used. The concentration of Ca ion was determined by the Arsenazo III spectrophotometric method.

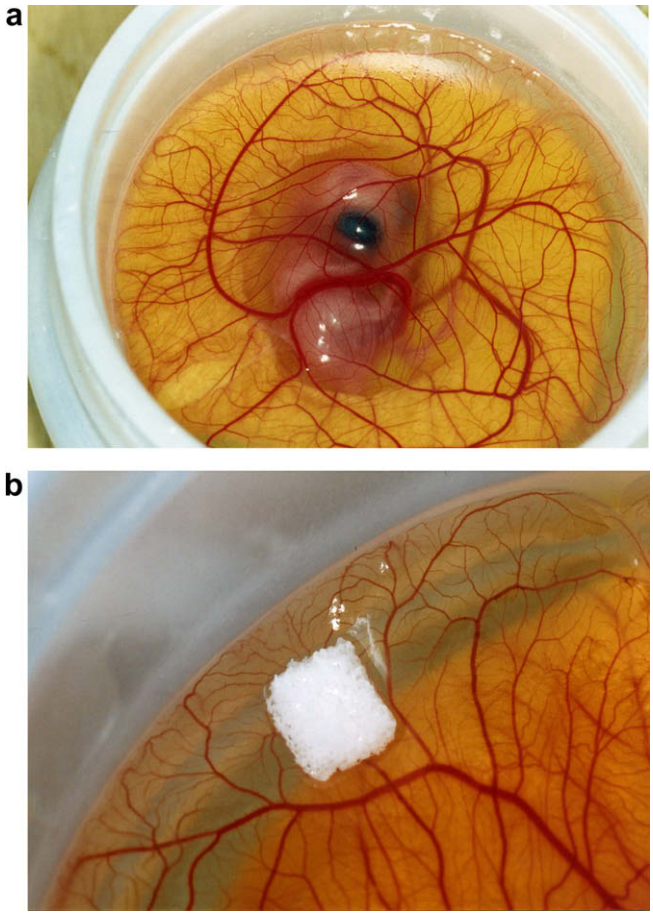

Fig. 2. A shell-less chick at embryonic day 10 (a), the stage when 45S5 Bioglass-derived glass–ceramic scaffolds were placed on the chorioallantoic membrane (CAM) (b).

2.3. Skeletal examination

The embryos were fixed in 10% formalin solution and processed for simultaneous differential staining of cartilage and bone in whole-mount preparations using the Alcian blue–Alizarin red double staining technique [25].

Observation of skeletons was performed under a stereomicroscope (Stemi SV11, Carl Zeiss, Jena, Germany). Chondrification was confirmed by blue staining with Alcian blue and calcification by red staining with Alizarin red. Bone nomenclature was based on that described by Bellairs and Osmond [26].

2.4. Histologic processing

2.4.1. Embryonic bones

Tibiae and femur were dissected. Left bones were decalcified in EDTA, dehydrated in increasing ethanol solutions and embedded in paraffin. Mid-diaphyseal cross-sections were obtained and stained with hematoxylin and eosin for general morphology. Right tibia and femur were processed for embedding in methyl-methacrylate resin. Mid-diaphyseal ground cross-sections were obtained for microchemical analysis.

2.4.2. Scaffolds and surrounding CAM

The scaffolds and surrounding CAM were explanted, fixed in 4% paraformaldehyde in PBS, and processed for embedding in methyl-methacrylate. Ground sections were obtained and stained with silver methenamine [27] for histological evaluation by light microscopy (Zeiss Axioskop 2 MOT, Carl Zeiss, Jena, Germany).

2.5. Microchemical analysis

With the aim of evaluating bone mineralization and potential alterations in the levels of Ca in the biomaterial under study, undecalcified sections of embryonic bones and scaffolds (pre and post-implantation) were carbon-coated in a vacuum evaporator (CAR 001-0045) and examined in a scanning electron microscope (JEOL JSM 6480 LV, Japan) equipped with an energy-dispersive X-ray analysis (EDX) system (Thermo Electron, NORAM System SIX NSS-100).

2.6. Statistical analysis

Student's t-test was used for statistical analysis of the data taking $\alpha = 0.05$ and $\beta = 0.10$. Data are presented as means $\pm$ SD.

3. Results

3.1. Embryo survival

At 5 days post-implantation, embryo survival in group E was $21 \pm 1\%$. No statistically significant differences were observed ($p > 0.05$) compared to survival in group C embryos ($23 \pm 12\%$) at the same incubation time.

At this incubation time (15 days) the embryo development ranged from Hamburger and Hamilton stages 39–41 [28], with no statistically significant differences between groups.

3.2. Weight and length of the embryos

No statistically significant differences ($p > 0.05$) were observed in embryo weight at 5 days post-implantation between the experimental group (616 ± 73 mg) and the control group (687 ± 43 mg).

Group E embryos were significantly ($p < 0.05$) longer (43 ± 2 mm) than group C embryos (39 ± 2 mm) with no biomaterial implanted on the CAM.

3.3. Calcium ion content in amniotic fluid

The embryos in the experimental (E) group exhibited a statistically significant increase ($p < 0.05$) in amniotic fluid Ca ion concentration at 15 days of total incubation (5 days post-implantation) (2.65 ± 0.44 mg dl^{-1}) as compared to the basal value (1.03 ± 0.10 mg dl^{-1}).

3.4. Macroscopic evaluation

Macroscopic evaluation revealed that all biomaterials implanted on the CAM remained in situ. At 48 h post-implantation scaffolds were fully incorporated by the CAM and did not interfere with normal CAM development. No vascular reaction was detectable around scaffolds. There was also no opaque granulomatous tissue surrounding the scaffolds in any of the cases considered (Fig. 3).

3.5. Skeletal examination

As expected, no mineralization was evident in any skeletal element of the day 15 shell-less embryos (group C). However, ossified red regions appeared throughout the whole skeletal system of the day 15 embryos in group E. Ossification was observed, as expected for the embryonic stage under evaluation, in the cranial and post-cranial skeleton, both membrane and cartilaginous (endochondral) bones (Fig. 4).

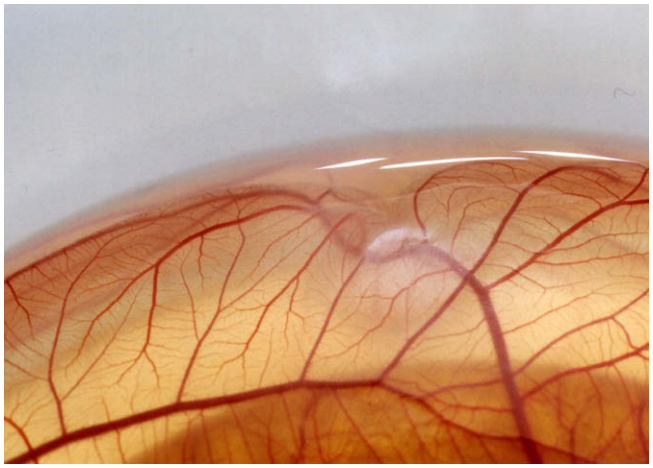

Fig. 3. Image of implanted scaffold showing the scaffold fully incorporated by the CAM at 48 h post-implantation.

Fig. 4. Whole-mount photographs of control (top) and experimental (bottom) chick femur at embryonic day 15 stained with Alizarin red and Alcian blue. Ossification has occurred in group E. Cartilage, blue; ossified region, red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. Histological evaluation and microchemical characterization

3.6.1. Embryonic bones

The examination of paraffin mid-diaphyseal cross-sections showed that there were no detectable differences between group E and C embryos. In both groups the cartilaginous primordium was surrounded by rows of anastomosing trabeculae of bone (Fig. 5). The microchemical characterization by EDX evidenced bone mineralization (Ca/P:1.49 ± 0.10) in embryos of group E. However, no

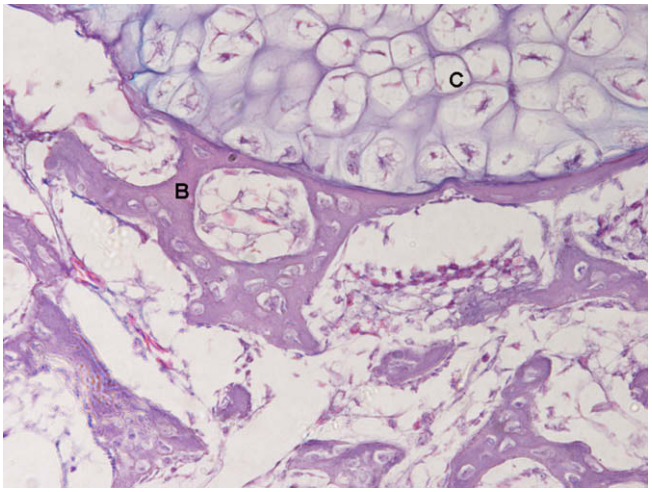

Fig. 5. Photomicrograph of representative cross-section of experimental chick femur at embryonic day 15 displaying a primary ossification center at the level of the mid-diaphysis. Notice the nascent bone collar (B) formation adjacent to cartilage model (C). Hematoxylin and eosin; original magnification 400×.

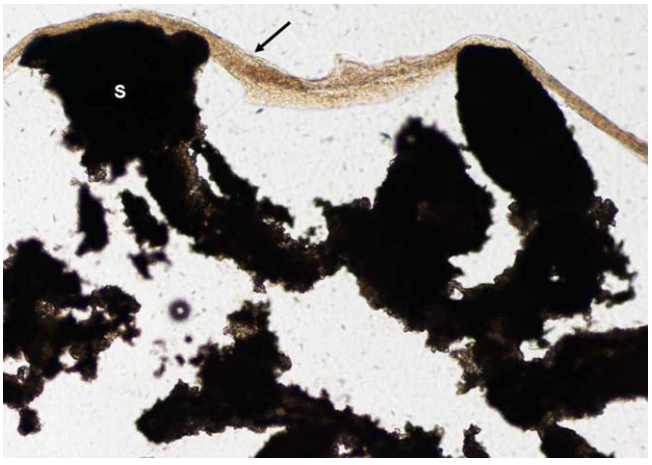

Fig. 6. Photomicrograph of representative ground section of scaffold and surrounding CAM at 5 days post-implantation. Notice that the epithelial tissue of CAM (arrow) had developed a continuous interface all around the implant(s). No tissue penetration in the pores of the scaffold was observed. Silver methenamine; original magnification 100×.

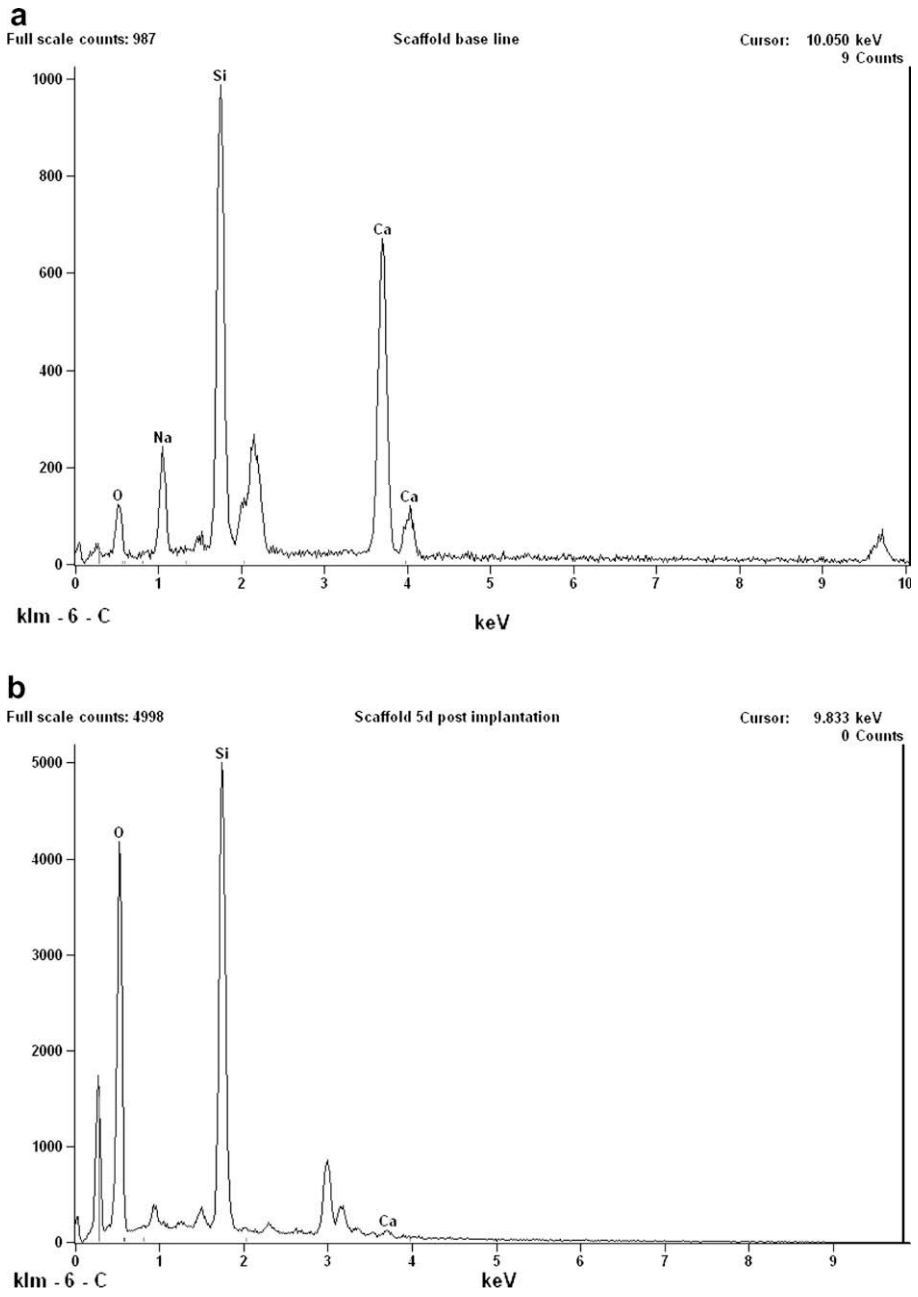

Fig. 7. EDX spectra of 45S5 Bioglass-derived glass–ceramic scaffolds sintered at 1100 °C for 1 h (as-sintered) (a), and 5 days post-implantation on CAM (b).

mineralization of the embryonic bones was detected in group C.

3.6.2. Scaffolds and surrounding CAM

At 5 days post-implantation, histological analysis revealed that the epithelial tissue of CAM had developed a continuous interface all around the scaffold, in intimate contact with the surface of the implant material (Fig. 6). No tissue penetration in the pores of the biomaterial or inflammatory infiltrate was observed. In addition, no angiogenic response was detectable.

At 5 days post-implantation, EDX analysis showed a 93% reduction in Ca content (wt.%) in the scaffold compared to the pre-implantation value ($22 \pm 1\%$ vs. $1.53 \pm 0.40\%$). Fig. 7 shows EDX spectra of 45S5 Bioglass scaffolds as-sintered (a), and following 5 days implantation on CAM (b). The results show that Ca and sodium (Na) content decreased after implantation, whereas silicon (Si) was virtually unchanged.

4. Discussion

To date, the studies that have addressed the issue of biomaterial biocompatibility employing the chick chorioallantoic membrane (CAM) assay only analyzed local response (angiogenesis and inflammation) to different biomaterials

[12–16,18–21]. The present study evaluated, for the first time, the biological response of the CAM and the embryo to a bioactive glass–ceramic material with a three-dimensional porous structure, derived from 45S5 Bioglass [6]. The biomaterial proved to be biocompatible in terms of the absence of inflammatory response at the implant site (CAM) as demonstrated by macroscopic and histological evaluation.

Previous studies have reported the proangiogenic potential of 45S5 Bioglass [31–35]. However, 45S5 Bioglass-derived glass–ceramic scaffolds used in the present work did not produce an angiogenic response. Recently, using several in vitro assays, Leu and Leach showed reduced angiogenic potential with higher masses (i.e.12 mg) of 45S5 Bioglass loaded into absorbable collagen sponges [35]. The evidence in the literature thus indicates that an optimum concentration of bioactive glass may be required to induce angiogenesis. Moreover, evidence gained in previous studies does not exclude the possibility that other factors which play a critical role during angiogenesis may be also responsible for the results observed in the present study. For example, in the CAM assay model, angiogenesis and inflammation have been demonstrated to be affected by altering the porosity of three-dimensional open porous synthetic materials [20]. Additional studies are therefore necessary to clearly identify these different contributions to angiogenesis, which are, however, beyond the scope of the present investigation at this stage.

Moreover, no alterations in the other end-points assessed, i.e. survival, stage of embryonic development and body weight were detected. However, the bodies of embryos submitted to implantation on the CAM were approximately 4 mm longer than control embryos. This could be attributed to the contribution of calcium (Ca) from the Bioglass scaffold.

45S5 Bioglass-derived glass–ceramic scaffolds showed, as early as five days after implantation, in vivo osteogenic potential, with restoration of the bone-like phenotype in chick embryonic skeleton as detected by Alcian blue–Alizarin red double staining in whole-mount preparations, as well as by histology and microchemical characterization of bone by EDX. Conversely, the control embryos exhibited a chondrogenic phenotype.

Previous studies by several authors [22–24], as well as a pilot study from our laboratory, have reported that Ca repletion to the shell-less embryos in the form of two to three 5–6 cm^2 eggshell pieces or a daily addition of $CaCO_3$ suspension (10% w/v in water), applied as several spots, onto the CAM resulted in improved overall growth and increased calcification of the skeletal components, especially the endochondral long bones (femur and tibia). In the chick embryo, the Ca required for skeletal mineralization is mobilized from the $CaCO_3$, in the form of calcite, stored in the eggshell by the CAM [22–24]. In our experimental model, the scaffold was implanted on the CAM at 10 days of embryonic development. It remained in situ until day 15 and exhibited a marked reduction (93%) in Ca content. Furthermore, we observed significant increase (157%) in amniotic fluid Ca content at 5 days post-implantation as compared to basal value, confirming the release of Ca from the 45S5 Bioglass scaffolds and subsequent handling by the embryo. Therefore, the calcium release from the biomaterial implanted on the CAM might have been responsible for the contribution to mineralization of skeletal units in chick embryos of group E.

5. Conclusions

The results of the study reveal the significance of the utilization of the chick embryo shell-less culture system for the analysis of the effect of Ca released from biomaterials on skeletogenesis. It is suggested that the present model may represent a useful preliminary screening procedure to investigate the biocompatibility and mineralization effect of other biodegradable and/or bioresorbable Ca-containing biomaterials providing a reliable alternative method to animal testing in bone tissue engineering research.

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