Evaluating 3D bone tissue engineered constructs with different seeding densities using the alamarBlue™ assay and the effect on *in vivo* bone formation

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Bone tissue engineering using patient derived cells seeded onto porous scaffolds has gained much attention in recent years. Evaluating the viability of these 3D constructs is an essential step in optimizing the process. The alamarBlue™ (aB) assay was evaluated for its potential to follow *in vitro* cell proliferation on architecturally standardized hydroxyapatite scaffolds. The impact of the aB assayed and seeding density on subsequent *in vivo* bone formation was investigated. Twelve scaffolds were seeded with various densities from 250 to 2.5×10^6 cells/scaffold and assay by aB at 5 time points during the 7-day culture period. Twelve additional scaffolds were seeded with 2.5×10^5 cells/scaffold. Two control and 2 aB treated scaffolds were subcutaneously implanted into each of 6 nude mice for 6 weeks. Four observers ranked bone formation using a pair wise comparison of histological sections form each mouse. The aB assay successfully followed cell proliferation, however, the diffusion kinetics of the 3D constructs must be considered. The influence of *in vitro* aB treatment on subsequent *in vivo* bone formation cannot be ruled out but was not shown to be significant in the current study. The aB assay appears to be quite promising for evaluating a maximum or end-point viability of 3D tissue engineered constructs. Finally, higher seeding densities resulted in more observed bone formation.

1. Introduction

The combination of patient own cells and porous ceramic scaffolds to produce 3D hybrid osteogenic constructs is a common theme in bone tissue engineering research [1–4]. Tracking the survival/activity of cells on scaffolds from the time they are seeded until implantation may be helpful to the optimization of these cell/scaffold constructs. The alamarBlue™ (aB) assay (Biosource International, USA) uses an oxidation–reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from metabolic activity. There are several advantages of the aB assay over other methods: The aB assay can assess both proliferation and viability. The assay is also very simple to implement and is not toxic. Therefore, the same culture can be evaluated at several time points and potentially used for additional purposes following aB assay. This method has been used in a variety of proliferation and viability studies [5]. Of specific interest to the current work, aB has been used to monitor the number of melanoma cells adhering to hydroxyapatite beads coated with synthetic peptides [6]. Additionally, the toxicity of tumor necrosis factor alpha (TNF-α) and actinomycin on an osteoblastic cell line (MC3T3-E1) has been investigated by aB [7]. Based upon these data, it can be speculated that the aB assay could be of value in tissue engineering research to evaluate cell growth and viability on scaffolds that can subsequently be implanted. However, so far no data are available on its feasibility and safety in bone tissue engineering using 3D scaffolds. Therefore, the aim of the current study is to determine whether aB can be used to evaluate the proliferation of bone marrow derived cells seeded at various densities onto 3D porous hydroxyapatite scaffolds and to establish if the assay affects subsequent *in vivo* bone production.

2. Materials and methods

Standardized hydroxyapatite (HA) scaffolds with dimensions of $2.8 \times 3.6 \times 5.2\,\text{mm}^3$ and an intersecting network of 400-μm channels were produced using a rapid prototyped negative replica method developed by the

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authors [8]. Briefly, a negative mold of the desired scaffold was designed on a personal computer using computer aided design software (Rhinoceros, Robert McNeel & Associates, USA). This design was then fabricated in wax material using a commercially available rapid prototyping (RP) system (ModelMaker II, Solid-scape Inc., USA). The resulting molds were infiltrated with an aqueous HA slurry and allowed to air dry. The ceramic filled molds were then heated to 1250 °C in an air filled high temperature furnace (Nabertherm 1400, Germany) with a heating and cooling rate of 100 °C per hour. This resulted in complete pyrolysis of the mold material and sintering of the ceramic. Examples of a rapid prototyped mold and the scaffolds produce by such a mold are shown in Fig. 1. A total of 30 scaffolds were produced for this experiment. Twenty-four scaffolds were used for the tissue engineering study with 12 used for the aB assay and 12 serving as controls for subsequent bone formation analysis. The remaining six scaffolds were used to determine if the aB assay is influenced by the HA scaffold material.

Passage one cryo-preserved goat bone marrow cells, obtained from an iliac wing biopsy, were thawed and replated in standard culture medium consisting of *alpha*-MEM (Gibco, Scotland) with 15% (v/v) fetal bovine serum (FBS, Gibco), 0.2 mM L-ascorbic acid-2-phosphate (AsAP, Sigma-Aldrich Chemie BV, The Netherlands), 2.0 mM L-glutamine (Life Technologies BV, The Netherlands), antibiotics (100 U/ml each penicillin and streptomycin) and 1 ng/ml of basic fibroblastic growth factor (bFGF, Sigma). When confluent, the cells were trypsinized and cell suspensions of 1.0×10^6 and 2.5×10^5 cells/ml prepared.

The 12 scaffolds for the aB assay were seeded at various concentrations, with 4 scaffolds seeded at 2.5×10^5 cells/scaffold and two scaffolds each seeded at 250, 2500, 25 000 and 2.5×10^6 cells/scaffold. The 12 control scaffolds were all seeded at 2.5×10^5 cells/scaffold. Seeding of all scaffolds was done in pairs in sterile 10 ml tubes. Each scaffold pair was covered with 2 ml of fluid consisting of the appropriate volume of cell suspension for the desired concentration, made up to 2 ml with standard culture medium as needed. Dynamic seeding for 6 h was conducted by placing the tubes on a roller bank at approximately 2 rpm in an incubator at 37 °C and humidified an atmosphere with 5% CO_2. All scaffolds were sub-cultured for 7 days in 25-well bacteriological plates with 3 ml/well of standard culture medium supplemented with 0.1 mM dexamethasone and 0.5 mg/ml β-glycerophosphate (both Sigma).

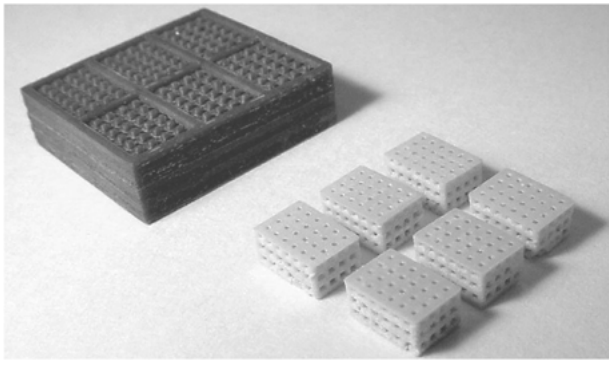

Figure 1 Example of rapid prototyped mold (upper left) and the six hydroxyapatite scaffolds resulting from such a mold (lower right).

On days 1, 2, 3, 5 and 7 after seeding, the aB test scaffolds were replenished with medium containing 10% aB. After incubating for 0 (background), 2, 4 and 6 h, the medium surrounding each scaffold was agitated by repetitive pipetting and 200 µl aliquots withdrawn and placed in a 96 well plate. Additionally, six non-seeded scaffolds were treated and analyzed identically to the seeded scaffolds to determine if the HA material influenced the aB assay. The plate was covered with aluminum foil and placed on the counter for 1 h to equilibrate with room temperature. The plate was then analyzed by fluorometer (Perkin-Elmer, USA) as prescribed by the manufacturer of the aB assay. All scaffolds, including controls, were then refreshed with new medium. Eight days after seeding, these same scaffolds were implanted into six nude mice with two control and two aB treated scaffolds of different seeding densities in each mouse.

Scaffolds were removed after 6 weeks of implantation and fixed overnight in 1.5% glutaraldehyde/0.14 M cacodylate buffer (pH 7.2–7.4). After rinsing in PBS, the samples were dehydrated using a graded ethanol series and embedded in methyl-methacrylate (MMA, Merck, Germany) for undecalcified histology. During histological processing, a control scaffold from mouse one was lost. The embedded samples were sectioned at 10 µm for light microscopy using a sawing microtome (Leica, Germany) and stained with methylene blue (Sigma) and basic fuchsine (Sigma). Sections were digitized using an Eclipse E600 light microscope (Nikon, Japan) configured with a digital video camera connected to a personal computer. Four observers judged *in vivo* bone formation within mice by a blinded pairwise comparison that ranked images from one scaffold as having more, less or similar amounts of bone compared to images from another scaffold. This resulted in 33 comparisons for each observer, three for mouse one (lost scaffold) and six for each of the other five mice. These 33 comparisons consisted of five possible conditions that were tabulated separately: (A) comparison of a control scaffold to an aB treated scaffold with a lower seeding density, (B) comparison of a control scaffold to an aB treated scaffold with a higher seeding density, (C) comparison of two aB treated scaffolds (always different seeding densities), (D) comparison of a control scaffold to an aB treated scaffold with the same seeding density, and finally (E) comparison of two control scaffolds (always the same seeding densities).

Separate statistical analyses of evaluation conditions A, B, C and D were conducted using a Chi-square Goodness of Fit test assuming a uniform distribution of observations. Similarly, the combined results of all observations between scaffolds with different seeding densities, evaluation conditions A, B and C, was also performed. Observations were averaged by the number of observers and a significance of 0.05 was set.

3. Results

Fig. 2 shows the aB results after 4 h of incubation for the different seeding densities over the 7-day test period.

From day 1 to day 7, the aB assay showed an overall decrease in the cellular activity of scaffolds seeded with 2.5×10^6 cells and increase in cellular activity for scaffolds seeded with 2.5×10^5 and 2.5×10^4 cells. Little response was seen at lower seeding densities. By day 7, the aB readings for scaffolds with the two highest seeding densities, 2.5×10^6 and 2.5×10^5 cells/scaffold, were nearly identical. Similar plots were obtained from the 2-h and 6-h incubation times, however, 1 h of incubation did not adequately resolve the increased activity over the 7 day period of the scaffolds seeded with 2.5×10^4 cells. The aB assays of the six non-seeded scaffolds indicated no influence of the HA material.

An example of the histology in shown in Fig. 3. Table I contains scaffold treatments and comparisons made as well as each observers results and the evaluation condition of each comparison. Of the 33 comparisons made, the observers were in complete agreement in 19 comparisons, three observers agreed in eight comparisons and the decision was split in six comparisons. In five of the six split decisions, two observers were in agreement on which scaffold contained more bone and the other two observers indicated that the bone formation was similar. It should be noted that of the 24 scaffolds implanted 22 exhibited bone formation, although the amount was widely varied. The two scaffolds which did not yield bone were the aB treated scaffolds in mouse 4. As a result, all four observers ranked these scaffolds as similar.

The results for each of the five evaluation conditions are contained in Table II and the statistical analyses of these results in Table III. For evaluation condition A, the control scaffolds had higher seeding densities than the aB treated scaffolds. Observers indicated that the controls had more bone formation in 77% of the comparisons. This outcome was statistically significant ($p = 0.015$). Similarly, for evaluation condition B, the aB treated scaffolds had higher seeding densities than the controls and observers indicated that the aB scaffolds had more bone formation in 75% of the comparisons. However, this effect was not significant. When two aB treated scaffolds of different seeding densities were compared, evaluation condition C, observers indicated that the scaffold with the higher seeding density had more bone for 79% of comparisons. This result was statistically significant ($p = 0.044$). However, for evaluation condition D, where control and aB treated scaffolds with the same seeding densities were compared, control scaffolds showed more bone in 54% of observations compared to aB scaffolds showing more bone in 29% of observations. However, this difference was not significant. Finally, all but one observation indicated that there was a difference in bone formation when control scaffolds were compared, evaluation condition E.

The results of all comparisons between scaffolds with different seeding densities, evaluation conditions A, B and C, were pooled to more sensitively evaluate the impact of seeding density on bone formation. Observations were grouped by seeding density, either higher or lower, and averaged by the number of observers. Observers indicated that the scaffold with higher seeding density had more bone 77% of the time. This is statistically significant ($p = 0.001$).

4. Discussion and conclusions

The original goals for this research were twofold: First, to evaluate the potential of aB to observe proliferation of several densities of bone marrow derived cells seeded onto architecturally standardized HA scaffolds. Second, to determine whether aB influences the bone formation in these scaffolds following implantation.

The aB results for the scaffolds with the two highest seeding densities, and possibly even the next lowest density, appear to be converging to a common value. This suggests that the proliferation rate of cells on the scaffolds with the two highest seeding densities was different and that there is a maximum number of viable cells that can be maintained on these scaffolds. In order to determine this with certainty, the time course of the experiment must be expanded. However, when interpreting these results, one must consider the 3D nature of the scaffolds and changes in the diffusion kinetics due to cell propagation and extracellular matrix production over the course of this experiment. The aB assay relies on cellular metabolism of the aB indicator. For 2D cultures and cell suspensions it is

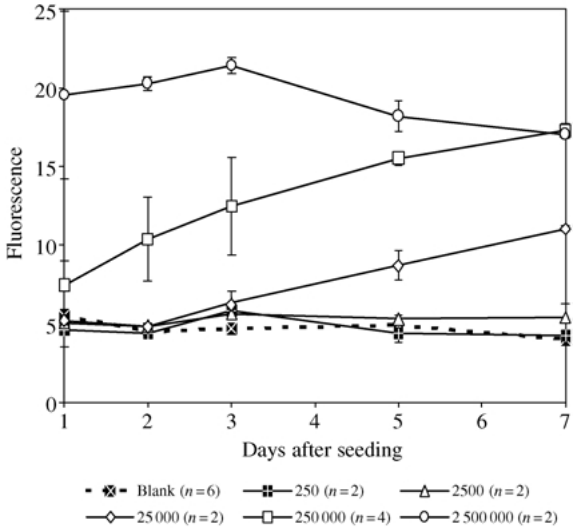

Figure 2 alamarBlue™ assay result for the entire 7-day culture period as a function of seeding density. Incubation time = 4 h. Error bars indicate standard deviation.

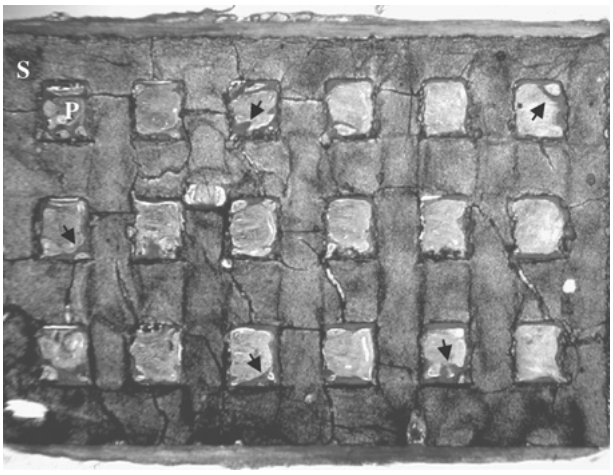

Figure 3 Example of histology. The scaffolds (S) forms a continuous lattice containing a 3 × 6 array of pores (P). Bone is present on the periphery *all* pores. Some of this bone is indicated by the black arrows.

TABLE I Raw data showing scaffold treatments, comparisons made to evaluate bone formation, observer results for the comparisons and the evaluation condition of each comparison

Mouse	Scaffolds compared # (SD, Cond.) to # (SD, Cond)[1]	Scaffold with more bone (S = same)				Eval. Cond.[2]
		Obs. 1	Obs. 2	Obs. 3	Obs. 4	
1	1 (2.5×10^5, CT) to 2 (250, aB)	1	1	1	1	A
	1 (2.5×10^5, CT) to 4 (2.5×10^5, aB)	4	4	4	4	D
	2 (250, aB) to 4 (2.5×10^5, aB)	4	4	4	4	C
2	1 (2.5×10^5, CT) to 2 (25000, aB)	1	1	1	1	A
	1 (2.5×10^5, CT) to 3 (2.5×10^5, CT)	1	1	1	1	E
	1 (2.5×10^5, CT) to 4 (2.5×10^5, aB)	S	1	1	S	D
	2 (25000, aB) to 3 (2.5×10^5, CT)	S	3	S	3	A
	2 (25000, aB) to 4 (2.5×10^5, aB)	4	4	4	4	C
	3 (2.5×10^5, CT) to 4 (2.5×10^5, aB)	4	4	4	4	D
3	1 (2.5×10^5, CT) to 2 (25000, aB)	1	1	1	1	A
	1 (2.5×10^5, CT) to 3 (2.5×10^5, CT)	1	1	1	1	E
	1 (2.5×10^5, CT) to 4 (2.5×10^5, aB)	1	1	1	1	D
	2 (25000, aB) to 3 (2.5×10^5, CT)	3	3	3	3	A
	2 (25000, aB) to 4 (2.5×10^5, aB)	4	4	4	4	C
	3 (2.5×10^5, CT) to 4 (2.5×10^5, aB)	3	3	3	S	D
4	1 (2.5×10^5, CT) to 2 (2500, aB)	1	1	S	S	A
	1 (2.5×10^5, CT) to 3 (2.5×10^5, CT)	3	3	3	3	E
	1 (2.5×10^5, CT) to 4 (2.5×10^5,aB)	1	1	S	S	D
	2 (2500, aB) to 3 (2.5×10^5, CT)	3	3	3	3	A
	2 (2500, aB) to 4 (2.5×10^5, aB)	S	S	S	S	C
	3 (2.5×10^5, CT) to 4 (2.5×10^5, aB)	3	3	3	3	D
5	1 (2.5×10^5, CT) to 2 (2500, aB)	1	1	1	1	A
	1 (2.5×10^5, CT) to 3 (2.5×10^5, CT)	1	1	1	1	E
	1 (2.5×10^5, CT) to 4 (2.5×10^6, aB)	4	4	1	4	B
	2 (2500, aB) to 3 (2.5×10^5, CT)	3	2	2	S	A
	2 (2500, aB) to 4 (2.5×10^6, aB)	4	4	2	4	C
	3 (2.5×10^5, CT) to 4 (2.5×10^6, aB)	4	4	S	4	B
6	1 (2.5×10^5, CT) to 2 (250, aB)	1	1	S	1	A
	1 (2.5×10^5, CT) to 3 (2.5×10^5, CT)	1	1	S	1	E
	1 (2.5×10^5, CT) to 4 (2.5×10^6, aB)	4	1	4	4	B
	2 (250, aB) to 3 (2.5×10^5, CT)	3	3	S	S	A
	2 (250, aB) to 4 (2.5×10^6, aB)	4	4	4	4	C
	3 (2.5×10^5, CT) to 4 (2.5×10^6, aB)	S	4	4	4	B

[1] SD = seeding density (cells/scaffold), Grp = aB for aB and CT for control

[2] There are 5 evaluation conditions: A = Control scaffold compared to aB scaffold with lower seeding density; B = Control scaffold compared to aB scaffold with higher seeding density; C = Two aB scaffolds compared (different seeding densities); D = Control scaffold compared to aB scaffold with same seeding density; E = Two Control scaffolds compared (same seeding densities).

TABLE II Bone formation observations grouped by evaluation condition

Bone formation	Obs. 1	Obs. 2	Obs. 3	Obs. 4	Total
Evaluation condition A:					
Control scaffold compared to aB scaffold with lower seeding density					
Control more	10	10	6	8	34
aB more	0	1	1	2	4
Same	1	0	4	1	6
Evaluation condition B:					
Control scaffold compared to aB scaffold with higher seeding density					
Control more	0	1	1	0	2
aB more	3	3	2	4	12
Same	1	0	1	0	2
Evaluation condition C:					
Two aB scaffolds compared (different seeding densities (SD))					
Higher SD more	5	5	4	5	19
Lower SD more	0	0	1	0	1
Same	1	1	1	1	4
Evaluation condition D:					
Control scaffold compared to aB scaffold with same seeding density					
Control more	4	5	4	2	15
aB more	2	2	2	2	8
Same	1	0	1	3	5
Evaluation condition E:					
Two Control scaffolds compared (same seeding densities)					
Different	5	5	4	5	19
Same	0	0	1	0	1

TABLE III Statistical analyzes for evaluation conditions A, B, C and D as well as the pooled results of A, B and C

A	B	C	D	A + B + C
Summed observations				
34	2	19	15	65
4	12	1	8	7
Averaged observations (4 observers)				
8.50	0.50	4.75	3.75	16.25
1.00	3.00	0.25	2.00	1.75
Chi-square goodness of fit statistic				
5.921	1.786	4.050	0.533	11.681
p-value				
0.015	0.181	0.044	0.466	0.001

reasonable to think that most cells have uniform access to the aB indicator. The aB results for these culture types is, therefore, related directly to cell number. For cultures on 3D porous substrates this assumption may not be so reasonable. This situation is further complicated over long culture periods as cells and extracellular matrix buildup within the pores of the substrate. The diffusion properties of the cell/scaffold construct are likely to change during the culture period and, therefore, the access of cells to the aB indicator and the diffusion of the metabolized product back to the surrounding medium is also likely to change. Consequently, the contribution cells make to the aB assay may diminish the deeper the cell is within the 3D construct.

Exposure of the tissue-engineered scaffolds to aB certainly did not prevent subsequent bone formation *in vivo*. This is substantiated by 10 of the 12 aB treated scaffold, exhibiting bone formation. However, when control and aB treated scaffolds of the same seeding density were compared, evaluation condition D, approximately 54% of observations indicated more bone in control scaffolds compared to 29% of observations that indicated more bone in aB treated scaffolds. Although this difference is not significant $(p = 0.466)$, the influence, if any, that aB has on the amount of bone formation is difficult to ascertain from the observer data. Other researchers have shown that aB is non-toxic and does not appear to affect the function of lymphocytes in culture [9]. In the current experiment the exposure of cultures to aB was very high, approximately 15% of the 7 day culture period. Lowering this exposure by minimizing the number of assay points and/or the incubation time may make aB an effective tool for determining a maximum or end-point viability for 3D tissue engineered constructs. Additionally, when standardized scaffolds are used, as in this study, differences in diffusion kinetics are normalized making this assay very useful for monitoring the influence any parameter has on the vitality of 3D cultures *in vitro*. Continuing research with this assay method will determine its true usefulness for tissue engineering studies.

A consequence of the current experimental design is the possibility to compare bone formation in scaffolds with different seeding densities. The idea that seeding density may influence bone formation in tissue-engineered constructs may seem intuitive, however, little data has been presented to support this conclusion. Yuan *et al*. [10] demonstrated that scaffolds seeded with 50 000 cells showed significantly more bone formation compared to scaffolds seeded with 5000 cells $(p < 0.05)$. In the current study, when scaffolds of different seeding densities were compared, the observation that the scaffold with the higher seeding density contained more bone was significantly more common $(p = 0.001)$ than the converse observation.

To summarize, aB successfully followed cell proliferation on 3D porous HA scaffolds, although, the diffusion kinetics of the constructs must be considered. The assay appears well suited for determining a maximum or end-point viability for 3D tissue engineered constructs. The influence of *in vitro* aB treatment on subsequent *in vivo* bone formation cannot be ruled out but was not shown to be significant in the current study. Finally, higher seeding densities resulted in more observed bone formation.

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