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Surrogate outcome measures of *in vitro* osteoclast resorption of β tricalcium phosphate.

Susan A Clarke*, Joanne Martin, John Nelson, Jean-Christophe Hornez, Marc Bohner, Nicholas Dunne, Fraser Buchanan.

*Corresponding Author: Dr Susan A. Clarke
School of Nursing and Midwifery,
Medical Biology Centre,
97, Lisburn Road,
Belfast,
UK, BT9 7BL
Tel: +44 2890 972171
FAX: +44 2890 972328
s.a.clarke@qub.ac.uk

J. Martin, N. Dunne[#], F. Buchanan: School of Mechanical and Aerospace Engineering, Queen's University Belfast, Ashby Building, Stranmillis Rd, Belfast, BT9 5AH, UK

J. Nelson: School of Biological Sciences, Queens University Belfast, MBC, 97 Lisburn Rd, Belfast, BT9 7BL, UK

J-C. Hornez: LMCPA, Université Valencienne, Maubeuge, France

M. Bohner: Skeletal Substitutes Group, RMS Foundation, Bischmattstr. 12, CH-2544 Bettlach, Switzerland

[#]Present Address: School of Mechanical and Manufacturing Engineering, Dublin City University, Stokes Building, Collins Avenue, Dublin 9, Ireland

Abstract

Introduction of porosity to calcium phosphate scaffolds for bone repair has created a new challenge when measuring bioresorption *in vitro*, rendering traditional outcome measures redundant. The aim of this study was to identify a surrogate endpoint for use with three dimensional (3D) scaffolds. Murine RAW 264.7 cells were cultured on dense discs of β-tricalcium phosphate in conditions to stimulate osteoclast (OC) formation. Multinucleated OC were visible from Day 6 with increases at Day 8 and Day 10. Resorption pits were first observed at Day 6 with much larger pits visible at Days 8, 10 and 12. The concentration of calcium ions in the presence of cells was significantly higher than cell free cultures at Days 3 and 9. Using linear regression analysis, Ca ion release could account for 35.9% of any subsequent change in resorption area. The results suggest that Ca ion release is suitable to measure resorption of a βTCP ceramic substrate *in vitro*. This model could replace the more accepted resorption pit assay in circumstances where quantification of pits is not possible e.g. when characterising 3D tissue engineered bone scaffolds.

Key words: Calcium phosphate, porous, osteoclasts, resorption, outcome measures

1. Introduction

In cases of significant trauma, damage to bone may be too extensive for natural remodelling to occur and surgery is the most likely treatment option, often in conjunction with a bone graft to stimulate healing [1]. Bone grafting using autologous or allograft bone is the 'gold standard' but there are associated limitations; a second surgical procedure with related donor-site morbidity, concerns of immunogenicity and demand outweighing supply [2-4]. This has led to a demand for synthetic bone grafts but to date commercially available synthetic grafts have been unable to match the clinical results seen with autograft [2, 5].

Ideally synthetic bone grafts should be biocompatible, integrate with the bone resorption process and aid new bone ingrowth whilst retaining sufficient mechanical strength. Resorbable materials that can utilise the bone's natural remodelling process to degrade, releasing non-toxic by-products that can be easily metabolised by the body, are very attractive for use as bone graft substitutes. However, some alleged resorbable bone graft substitutes have been detected years after *in vivo* implantation [6, 7]. Innovation of porous scaffolds with an interconnected pore structure has allowed for increased bone ingrowth [8-11] and subsequent increased rate of resorption *in vivo* [12-15].

The introduction of porosity has caused a new challenge for researchers when measuring bioresorption of new materials, rendering the traditional *in vitro* methods insufficient. The traditional methods used to assess resorbability of bone substitutes *in vitro* are OC formation indicated by tartrate-resistant acid phosphatase (TRAP) expression and a cell-based resorption assay, alternatively known as a 'pit' assay, developed by Boyde [16] and Chambers [17]. Initially developed as an assay to investigate OC biology using dentine or bone as a substrate, it is now routinely used to understand biomaterial resorption. OCs are cultured on biomaterial surfaces for specific periods and then detached, at which point the excavated areas (pits) beneath the cells can be analysed by scanning electron microscopy

(SEM) in terms of pit number, pit area or pit volume. The simplest method is to determine the number of pits, which can be quantified using reflected light microscopy (RLM), where staining is not required [18] or by light microscopy (LM) using simple staining techniques [19]. Pit area can be quantified using image analysis software applied to SEM or LM. Ideally, pit volume would be the best method when quantifying resorption as both pit area and depth can be calculated, however, the required equipment is expensive and specialised [20-23]. All of these methods are time consuming, labour intensive and, crucially, do not easily translate to quantification of resorption on porous materials where visualisation of internal structures is difficult. Thus, there is an imperative for an appropriate measure of resorption that can be adopted for both porous and dense calcium phosphate ceramics.

Other methods used to indicate OC resorption are based on their activity, generally assessed using biochemical markers such as the OC enzyme, TRAP, which although not uniquely expressed by osteoclasts is an often used marker [24, 25]. TRAP activity is commonly measured using either a colorimetric method [26, 27] or by using an enzyme-linked immunosorbent assay (ELISA) which uses TRAP specific antigen-antibody reactions to measure TRAP activity [28, 29]. Another commonly used *in vitro* biochemical assay is a colorimetric calcium assay [24, 30], however, there has been no systematic attempt to identify an outcome measure of OC resorption that directly correlates with pit measurements and is transferrable through a broad range of *in vitro* experiments.

The aim of this study was to establish the suitability of several outcome measures as possible indicators of OC resorption *in vitro* in order to identify a surrogate endpoint which could replace pit area. To accurately correlate pit area with alternative outcome measures, the assay was performed on dense beta-tricalcium phosphate (β-TCP) [31], which allowed pit formation and area to be analysed on a substrate free from microscopic imperfections.

2. Materials and methods

2.1 Material Preparation

TCP powder was prepared by an aqueous precipitation technique using a diammonium phosphate solution $(NH_4)_2HPO_4$ (Carlo Erba, France) and a calcium nitrate solution $Ca(NO_3)_{2.}4H_2O$ (Brenntag, France). The solution pH was adjusted to a constant value of 6.5 by continuous addition of ammonium hydroxide. Temperature was maintained at 30°C and the solution was matured for 24 h. After maturation, the solution was filtered and the precipitate dried at 80°C. The precipitate was then calcined at 750°C and the powder was subsequently ground to break up any agglomerates formed during calcination. The grinding step was conducted by ball milling in a high density polyethylene milling jar and Y-PSZ grinding media for 3 h [31].

TCP samples were prepared by a slip casting method. TCP powder (65 wt.%) was suspended in deionised water (dH_20) to form a slurry. To enhance slip stability, a commercial organic defloculant (Darvan C, R.t.Vanderbilt. Co. Inc. USA) was introduced (1.5 wt.% of TCP content). After ball milling for 1 h, the slip was poured into a plaster mould (diameter 3.8 mm x 30 mm), dried and sintered (1100 ^oC for 3 h) with a heating rate of 5 °C/min. Density of the sintered samples, determined by Archimedes' method was >99% [31]. Final cylindrical samples were cut into 3 mm thick discs using a diamond saw (Struers Accutom-50, Struers UK). Each disc was mounted in acrylic resin (Varidur 3000, Buehler, UK) and ground (Buehler Alpha Grinder-Polisher) on one side using silicon carbide papers of decreasing grade (P400, P1200, P2500, P4000) followed by a final polish using a 0.05µm alumina and silicon oxide suspension (Buehler). Polished discs were removed from the acrylic resin using a 48 h soak in chloroform, then washed with 70% isopropyl alcohol (Sigma Aldrich, UK) and sterilised by autoclaving at 121^oC for 30 min in an alkaline

atmosphere. This provided smooth discs with <1% porosity to ensure accurate measurements of resorption pit area.

2.2 Cell culture of RAW 264.7 cells

RAW 264.7 cells (ATCC, UK) were routinely cultured under standard conditions (37^0C, 5% CO_2/95% air) in α-MEM medium supplemented with foetal bovine serum (FBS) (10% v/v), penicillin/streptomycin (1% v/v) and L-glutamine (4mM) (all reagents from Invitrogen, UK). At day 0, cells were seeded onto the polished side of β-TCP discs at a density of 2.5 x10^4 cells/cm^2. To initiate differentiation, Receptor Activator of Nuclear Factor κB Ligand (RANKL, PeproTech EC, UK) was filter sterilised (0.2 μm filter) and added to complete culture medium (20 ng/mL). Culture medium with RANKL was replaced every 3 days. Hydrochloric acid (HCL) (15 mM) (Sigma Aldrich) was added to cultures on days 3, 6 and 9 (including cell free controls) to increase acidification and promote osteoclastogenesis [32]. Cultures were maintained for 12 days. A total of six samples were used per time point for all conditions. Time points were chosen based on our own preliminary studies of the life cycle of RAW 264.7-derived OCs.

2.3 OC identification

At day 6, 8, 10 and 12, cultures were fixed in paraformaldehyde (3.7% w/v) in phosphate buffered saline (PBS) for 10 min, washed in PBS and permeabilised in Triton X-100 (1% v/v) in PBS for 20 min, rinsed again and stained for 20 min with AlexaFluor 488 Phalloidin (Invitrogen) to label cytoskeletal F-actin. Cultures were then washed with PBS and incubated at 37^oC for 5 min with DAPI dilactate (Invitrogen), a nucleic acid counterstain, rinsed again in PBS and air dried. Cultures were imaged under fluorescence microscopy (Leitz-Laborlux D) at x16 magnification. The surface area (SA) analysed per field of view was 1 mm^2. Based on four fields (SA 4 mm^2), 35% of a 3.8 mm disc (SA 11.34 mm^2) was analysed. Actin rings were counted and expressed as mean of all fields. Multiple actin rings

within an OC were counted individually: an OC was defined as having three or more nuclei (Figure 1 a-b).

OC formation was determined using a TRAP staining kit (Sigma-Aldrich). On day 6, 8, 10 and 12, cultures were fixed in citrate/acetone solution for 30 s, washed in dH_20 and air dried for 15 min. Cultures were then covered in a solution containing napthol AS-BI phosphoric acid and fast garnet GBC salt and incubated for 1 h at 37^oC in the dark. Cultures were rinsed with dH_2O for 3 min and allowed to air dry before viewing under LM (Leitz-Laborlux D) at x16 magnification. The method for counting mean number of TRAP positive cells was similar to the actin ring count and required the presence of three or more nuclei (Figure 1 c-d). Using four fields of view, 35% of the total SA of a 3.8 mm disc was analysed.

2.4 OC activity

OC TRAP enzyme activity was measured by the conversion of p-nitrophenyl phosphate (p-NPP) to p-nitrophenol (p-NP) in the presence of sodium tartrate. On days 6, 8, 10 and 12, a separate set of cells were lysed with 100 µL lysis buffer (1M NaCl and 0.1% Triton-X 100) and subjected to a freeze-thaw cycle. Cell lysate (50 µL) was then transferred to an assay plate in duplicate. p-NPP (50 µL of 10 mM) in buffer solution (40 mM sodium tartrate dehydrate, 50 mM Acetic acid 100%, brought to pH 4.8 with sodium hydroxide (NaOH)) was added to the cell lysate and incubated at room temperature for 45 min. The reaction was stopped with 50 µL NaOH (0.2 M) (all reagents were from Sigma Aldrich). Optical absorbance was read at 405 nm on a microplate reader (Genios, Tecan, Austria) and TRAP activity was quantified against a standard curve.

Culture medium during media changes on days 3, 6, 9 and 12, were retained and diluted with dH_2O to a final volume of 10 mL. Elemental concentrations of Ca and P ions were

quantified using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Perkin Elmer Optical Emission Spectrometer, Optima 4300 DV).

2.5 Resorption Assay

Assessment of resorption pits was visualised by SEM at day 6, 8, 10 and 12. To prepare samples for sputter coating, they were transferred into a 24-well plate containing 1ml isopropanol (70%) (Sigma Aldrich) per well and sonicated for 5 minutes. Samples were then cleaned one at a time in a petri-dish containing isopropanol (70%). A small brush was used to remove the cells. Samples were air dried then sputter coated with gold using a Polaron E5150 sputter coater and viewed on a JEOL 6500F SEM (JEOL Ltd., Japan) at 15kV.

Resorption pit area measurements were performed using Image J v1.45 software (National Institute of Health) [33]. A threshold function was used to convert the SEM image into binary mode followed by a particle analysis function to quantify the percentage area resorbed by the RAW 264.7 OC cells. At x100 magnification (SA 1.13mm^2/field of view), 30% of a 3.8 mm disc was analysed. The percentage area resorbed was expressed as mean of the fields (3 fields/sample, n=6).

2.6 Statistics

Statistical Analysis for all outcome measures was conducted using IBM SPSS v.22 software (IBM, UK). Differences between treatment groups were assessed using one-way analysis of variance (ANOVA) with a post-hoc, Bonferroni test. Relationships between each outcome measure were investigated using a Pearson's correlation test. A p-value of less than 0.05 was considered statistically significant. A total of six samples were used per time point for all conditions. Finally, linear regression analysis was used to investigate if the outcome measures were predictive of resorption area and by how much. Using the candidate

outcome measures with the strongest correlations to resorption pit area first, a regression model was built with forced entry of each independent variable.

3. Results

Successful OC formation from RAW 264.7 monocytes was confirmed by multinuclearlity, TRAP expression and actin ring formation (Figure 1). TRAP positive OC were visible from Day 6, increased in number by Day 10 and decreased at Day 12 (Figure 2a). The largest change in OC number was from Day 8 to Day 10 increasing by 87% (15 OC to 28 OC respectively), followed by a 40% decrease from Day 10 to Day 12.

Actin ring formation did not follow the same trends observed with TRAP positive OC count and large standard deviations were observed for all time points (Figure 2a). Fluorescence microscopy indicated that the size and shape of actin rings formed changed with time (data not shown). At Day 6 actin rings were smaller than Day 10 or 12. With various sizes visible at Day 8. This could be due to multiple smaller actin rings being formed by sections of the OC membrane upon first attachment to the substrate, which then merge as the OC either increases in size or forms a sealing zone to begin resorption. To compensate for this, the number of actin rings was analysed as a ratio of OC number. When adjusted, actin ring:OC ratio showed a decreasing trend with time (Figure 2b) with the greatest number of actin rings per OC at Day 6.

TRAP expression, actin ring formation and multinuclearity confirmed the presence of OCs: functionality of the cells was determined by measuring TRAP activity. TRAP activity increased from Day 6 to Day 10 and decreased at Day 12 (Figure 3a) supporting the trends observed for TRAP expression described previously. Cultures of cells without the addition of RANKL expressed similar levels of TRAP activity to cells/+RANKL at Days 4 and 12

indicating that RAW 264.7 monocytes produce a basal level of TRAP enzyme regardless of RANKL stimulation.

For efficient OC formation and activity, an acidic environment is required [32] and this in itself could contribute to resorption of the ceramic substrate therefore pH values were monitored throughout. After addition of 15mM HCL to cultures at Day 3, a marked difference in pH can be observed between cell cultures and cell free cultures (Figure 3b). Cell free culture medium decreased from an alkaline state on Day 3 to near neutral at pH 7.44 on Day 12. Cells +RANKL and cells –RANKL culture medium decreased more rapidly from Day 3 to Day 6 with smaller changes at Day 9 and an increase at Day 12 when OC activity is reduced. In these groups at Day 8, pH dipped to 6.9 which is reportedly optimal for OC activity [32]. Cultures containing cells +RANKL produced the lowest pH values at four of the eight time points measured perhaps indicating that the presence of OC and/or resorption also contributes to pH change.

Mineral ion release into culture medium was time dependent and reflects the trends observed with TRAP activity and OC number however, basal Ca ion concentration in culture medium was approximately 72 mg/L and all conditions produced lower Ca concentrations (Figure 4a) indicating that the substrate had apatite formation on its surface. This apatite is likely to be calcium-deficient hydroxyapatite (CDHA) and could be contributing to the reduction in pH at the early time points. Ca ion concentrations, even in cell free conditions, increased from Days 3 to 9 and decreased at Day 12 (Figure 4a), however the concentration of Ca ions in cultures with cells was higher than cell free cultures at all time points except Day 6 and this was statistically significant at Days 3 and 9 ($p<0.001$). These results suggest that there was some dissolution of the ceramic that was increased in the presence of cells due to active resorption.

P ion concentration showed little change from Days 3 to 9 before decreasing at Day 12 (Figure 4a). Basal P ion concentration in culture medium was approximately 31 mg/L and levels similar to this were found at Days 3, 6 and 9. Statistically significant increases were observed for culture medium with cells compared to culture medium without cells at Days 9 and 12 ($p<0.01$).

Light coloured areas on SEM micrographs represent resorption pits (Figure 4). Resorption pits were circular or lobe-shaped and first observed at Day 6 with much larger pits visible at Days 8, 10 and 12. In the absence of RANKL stimulation multinucleated cells were absent and no pits were visible even at day 12 (Figure 5 a-d). Higher magnification SEM micrographs (not shown) suggest that resorption pits may be deeper with time, as the grains of the underlying ceramic become more visible. Quantification of the resorption pit area showed that there was a significant increase at Day 8 (Figure 4d). At x100 magnification, 30% of the total surface of the β-TCP sample was analysed. Maximum surface resorption recorded was ~20%. There was no significant difference in percentage area resorbed between Days 8 and 12 and standard deviation was large within each time point. This, taken with the SEM analysis, suggests that perhaps OC are excavating deeper with time and not forming new pits.

Statistical analysis showed that all outcome measures were significantly affected by time. Following log transformation of pit area to normalise the data, a Pearson's correlation analysis was performed to determine the relationships between all outcome measures (Table 1). Most outcome measures showed some degree of correlation with all others with the exception of pit area. The strongest and most significant of these were those of TRAP activity correlated with TRAP positive OC count ($r = 0.670^{**}$) and OC count correlated with Ca ion concentration ($r = 0.708^{**}$). Interestingly, Ca ion concentration correlated negatively

with pH (r = -0.740**). Importantly, log pit area had a moderate correlation with TRAP activity, OC count and Ca ion concentration (r = 0.447*, 0.450* and 0.599 respectively).

Using linear regression analysis, the ability of the three candidate outcome measures to predict a change in resorption pit area was assessed in isolation and then a model was built adding the candidate outcome measures stepwise in order of increasing strength of correlation to resorption pit area i.e. Ca ion release, followed by addition of TRAP activity, and then addition of pH (Table 2). The R-squared value indicates how much of the change in resorption area is accounted for by the model therefore a change in Ca ion release would account for 35.9% of any subsequent change in resorption area, compared to 21.9% for TRAP activity and 4.5% for pH. When Ca ion release and TRAP activity are included together, this value increased to 44.8%. Including pH to model as a third predictor only marginally increased this value to 45.5%. For the model to have any meaning the F statistic should be greater than 1 further indicating that the addition of the third variable has limited value. The caveat to this is that the F statistic was not statistically significant for any model however the p value was 0.051 for Ca ion release and given that the resorption area showed such variability, the results could still be indicative of a predictive ability.

4. Discussion

Predicting the *in vivo* resorbability of a calcium phosphate (CaP) based biomaterial is difficult. The propensity of the material to dissolve in cell-free tests at physiologically relevant pH values is not always indicative of its resorbability in the body, particularly with modern materials which can be doped with bioactives designed to directly affect OC behaviour [34]. Therefore, *in vitro* cell based assays remain an important tool for testing novel CaP based biomaterials. The aim of this study was to identify a quantifiable outcome measure of OC resorption that directly correlates with OC pit measurements and is transferrable through a

broad range of *in vitro* experiments, in particular for use with 3D CaP scaffolds. The outcome measures investigated were Ca and P mineral release into cell culture medium, TRAP activity and pH. In order to accurately correlate pit area with the alternative outcome measures, the assay was performed using dense β-TCP as even minimally porous materials were previously found to give inaccurate results.

All measured outcomes varied significantly with time and reflected the natural cycle of OCs in culture as they were formed from monocyte precursors, increased in activity and then became exhausted and apoptosed [35]. OC formation was established by the expression of TRAP enzyme in multi-nucleated cells (>3 nuclei) and the formation of actin rings, however, OC formation is not a guarantee of substrate resorption and indeed, mononuclear cells are also able to resorb substrates [32], so several measures of activity were also included. The first of these was activity of the TRAP enzyme. Although TRAP is not uniquely expressed by these cells, active OCs have been shown to express higher levels of TRAP activity compared to inactive OCs [36]. Reassuringly, the number of OCs formed did correlate with activity of the TRAP enzyme but RAW 264.7 cells without the addition of RANKL displayed similar TRAP activity to cells with RANKL at Days 4 and 12, and this basal level of activity must be accounted for when using TRAP activity as an indication of OC function. The basal level of TRAP activity of RAW 264.7 cells in the absence of RANKL is difficult to establish and has been reported in the literature as 10% of +RANKL levels at day 3 [37], 20% at day 5 [38] and 50% at day 6 [39] but this will depend greatly on the experimental conditions used in each experiment. Given the high levels in the current study, it may be that the βTCP itself is contributing to basal expression of this enzyme making it imperative that baseline level controls are included in every experiment. From Day 6 however, there was a time dependent increase in TRAP activity in the presence of RANKL and the difference between that and the basal activity was significant, so it is possible to detect active OCs using this outcome measure.

When an OC is polarised on a substrate surface a sealing zone forms containing a dense ring of actin. The presence of the actin ring is indicative of the resorptive phase of the OC [40, 41] therefore this could be a further potential measure of OC resorption on 2D substrates *in vitro*. However, actin ring formation did not follow the same trends observed with TRAP positive OC count and TRAP activity results. One may expect to see an increase in actin ring formation with an increase in OC number but this was not the case. Observations from fluorescence microscopy indicated that the size and shape of actin rings changed with time, generally increasing in size. This resulted in a decreasing ratio of actin rings to TRAP positive OC. Although the number of actin rings did correlate with TRAP activity, this was only of moderate strength and the variation in this outcome measure suggests that it would not be suitable for use for *in vitro* biomaterial testing.

The pH required for optimal OC formation is pH 7.35-7.4 [42] and for resorption activity pH 6.95 [32]. Previous experimental work investigating the effects of 15 mM HCL addition to culture medium in the absence of cells recorded pH 7.52 after 8 days in culture (results not shown). It was hypothesised that the metabolic activity of cells, acidic in nature, would further reduce culture medium pH. Certainly OC exhibit a change in pH when they are in their active state although this change is within a localised region beneath the cell delineated by the sealing zone and ruffled border [43-45]. Addition of OC to acidified culture medium did indeed further reduce the pH and coincided with an increase in TRAP activity (from Day 5), OC number (from Day 6) and resorption (from Day 8). Furthermore, a reduction in pH was correlated to an increase in OC number ($r = 0.420$) and an increase in Ca ion concentration in the medium ($r = 0.740$) which might suggest that this could be used as part of a suite of measures to indicate resorption, albeit perhaps an imprecise one. Cell-free conditions showed that the ceramic alone was associated with a reduction in pH with time and this could be due to CDHA formation on the ceramic surface. Although the presence of CDHA

was not confirmed, the phase of CaP that precipitates is determined by the pH of the environment: in the range of 2 < pH <4 dicalcium phosphate (DCPD) will be the preferred phase, 5 < pH < 7 this will be octocalcium phosphate (OCP) and at higher pH values (7 to 9) calcium deficient hydroxyapatite (CDHA will precipitate) [46-48].

Mineral ion release into culture medium was assessed as a potential indicator of OC resorption based on the mechanism of OC-mediated resorption of CaP ceramics [49, 50]. When an OC attaches to CaP, it secretes HCL [44, 45] and enzymes [51-53] to digest the inorganic and organic phases forming a resorption pit under the actively resorbing OC. The Ca and P ions released from the inorganic phase are taken up by the OC, processed and released into the extracellular environment. As the inorganic mineral dissolves, the Ca^{2+} concentration within the microenvironment can increase from 8- 40 mmol/L (320–1600 mg/L) [45]. This local increase in Ca^{2+} concentration increases intracellular Ca, promoting margin retraction and OC cell de-adhesion [54], ceasing resorption. After detachment of OC from the CaP, the accumulated by-products of resorption are released into the extracellular environment. With that, as OC number and activity increases one would expect mineral ion release into culture medium to increase. There was a reduction in Ca ion levels below basal level in the culture medium at Day 3 and 6 in both cell and cell-free conditions that could suggest the formation of CDHA on the β-TCP surface. Similar reductions in Ca levels *in vitro* have been reported by others [55, 56]. Ca ion levels did not return to basal levels during the experiment however, they did increase with time and were higher in the cell conditions compared to cell-free at Day 9, indicating that in addition to dissolution of the ceramic, there was also active resorption. Furthermore, Ca ion release into culture medium reflected the trends observed with TRAP activity and OC number, correlating well with both, and to changes in pH.

P ion release was also not present in greater amounts in medium from cultures with cells compared to without cells until Day 9 and remained at basal levels during this period. At Day 12 there was a reduction in P levels. The mechanism of this loss of P is not clear, especially given that the Ca and P loss in the medium is not stoichiometric, but others have reported similar findings in the presence of bioactive glasses [57, 58]. It is likely that this loss of P is responsible for the surprising finding that P ion concentration was negatively correlated with TRAP activity and resorption pit area.

Each of the outcome measures above are considered as adjuncts to the "gold standard" assay of resorptive capability- the resorption pit assay, and it is to this that other results must be compared. The results showed that the percentage area resorbed significantly increased at Day 8 and the maximum β-TCP surface resorption was ~20%. Although no significant difference in percentage area resorbed was found beyond Day 8 standard deviations were large and SEM analysis suggests the possibility that resorption pit depth increased with time that was not reflected in area measurements. Even given this variability, resorption pit area still showed a moderate correlation with TRAP activity ($r = 0.447$) and Ca ion release ($r = 0.599$) count which provides some evidence for the use of these outcome measures as surrogate endpoints of resorption. This was confirmed by regression analysis which indicated that Ca ion release was the strongest predictor of the three variables considered and could account for more than a third of potential changes in resorption pit area. It was disappointing that this regression model did not quite reach significance but given the variability in resorption pit area measurements this was perhaps not surprising. Volumetric measurements of the resorption pits formed may have been preferable and perhaps would have proved more sensitive and more strongly correlated to other measures.

There are a number of limitations to this study the most important one being the lack of volumetric measurements. Furthermore, the chemistry of calcium and phosphate dissolution,

precipitation and re-precipitation in a small volume static culture system is complex and increasing the bioavailability of ceramic in the substrate has been shown to favour osteoclast formation [59]. Therefore further research is needed to ascertain the reproducibility of these results with other CaP materials, however, the need to answer these questions is clear given our current limited ability to measure resorption of 3D scaffolds reliably *in vitro*. It may also be a consideration that a cell line was used in this study to generate osteoclasts. Our own unpublished pilot experiments suggested that the pattern of results for both the cell line and a primary source of osteoclast precursor cells were similar but that higher numbers of osteoclasts were produced earlier with the cell line. This, and the problems of reproducibility related to inter-donor variability for primary cells, led us to choose the cell line for these experiments. Future development of this model will now require the comparison of resorption profiles for βTCP porous structures with those predicted by 2D results but it will be crucial to consider the effect of geometry on osteoclastogenesis. The effects of pore size and pore characteristics on osteoblasts and osteoprogenitor cells are well established[60] but the effects on osteoclasts are less well known.

5. Conclusion

The results of this study would suggest that Ca ion release is suitable to measure resorption of βTCP *in vitro* and this could be strengthened by the addition of TRAP activity. Caution must be taken however, to control for basal levels of TRAP activity in monocytic cells and to ensure that results are taken during the peak of OC activity in the formation- activity-apoptosis cycle of this *in vitro* assay. If both outcome measures are in accord, this should provide robust evidence that could replace the more accepted resorption pit assay in circumstances where quantification of pits is not possible, for example when determining resorbability of 3D scaffolds *in vitro*.

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Figure Legends

Figure 1. Multinucleated cells in cultures stimulated with RANKL at day 12 showing nuclei counterstained with DAPI (a and c), actin ring formation (b) and expression of TRAP enzyme (d). Key: blue: nuclei, green: actin ring, red: TRAP.

Figure 2: (a) number of actin rings and TRAP positive OC formed, (b) ratio of actin rings to TRAP positive OC per high power field. Mean +/- SD.

Figure 3: (a) TRAP activity of RAW 264.7 cells on β-TCP up to 12 days and (b) pH of culture medium with and without RAW 264.7 cells and RANKL. Mean +/- SD

Figure 4: (a) Ca and P ion concentration in cell culture medium and (b) percentage surface area resorbed by RAW 264.7 OC up to 12 days. Mean +/- SD. Basal Ca ion concentration in culture medium is 72mg/L (▬▬). Basal P ion concentration in culture medium is 31mg/L (▬ · ▬) $^{**}p<0.01$, $^{***}p<0.001$

Figure 5: SEM micrographs of β-TCP resorption by RAW 264.7 OC. Left column with cells, right column following cell removal. (a-d) without RANKL stimulation, (e-l) + RANKL.

Table 1: Correlation statistics between all outcome measures Top figure in each cell represents the Pearson correlation factor (r) showing the strength of correlation and bottom figure indicates significance of the results (p-value). TRAP = TRAP activity, AR = Actin ring count, OC = TRAP+ve OC count, Ca = Ca ion concentration, P = P ion concentration, AR:OC = Ratio of actin ring to TRAP+ve OC, logArea = log of the percentage area resorbed. **Correlation is significant at the 0.01 level, * Correlation is significant at the 0.05 level.

	TRAP	**pH**	**AR**	**OC**	**Ca**	**P**	**AR:OC**	**logArea**
TRAP		-0.225	0.381**	0.670**	0.572*	-0.775	-0.374	0.447*
		0.137	0.01	<0.001	0.013	<0.001	0.079	0.037
pH	-0.225		-0.359*	-0.42**	-0.74**	0.385**	-0.047	-0.212
	0.137		0.015	0.003	<0.001	<0.001	0.83	0.344
AR	0.381**	-0.359*		0.553**	0.084	0.155	0.625**	-0.232
	0.01	0.015		<0..001	0.74	0.54	<0..001	0.3
OC	0.670**	-0.42**	0.553**		0.708**	-0.313	-0.492*	0.45*
	<0.001	0.003	<0.001		<0.001	0.206	0.017	0.036
Ca	0.572*	-0.74**	0.084	0.708**		-0.207	-0.565	0.599
	0.013	<0.001	0.74	<0.001		0.081	0.07	0.510
P	-0.775**	0.385**	0.155	-0.313	-0.207		0.577	-0.783**
	<0.001	<0.001	0.54	0.206	0.081		0.063	0.004
AR:OC	-0.374	-0.047	0.625**	-0.492*	-0.565	0.577		-0.439*
	0.079	0.83	<0.001	0.017	0.07	0.063		0.046
logArea	0.447*	-0.212	-0.232	0.45*	0.599	-0.783**	-0.439*	
	0.037	0.344	0.3	0.036	0.510	0.004	0.046	

Table 2: Hierarchical regression analysis

Model Elements in order of addition	R	R^2	F change	Significance
Ca release	0.599	0.359	5.038	0.051
TRAP activity	0.468	0.219	2.523	0.147
pH	0.212	0.045	0.938	0.344
Ca release + TRAP activity	0.669	0.448	1.287	0.29
Ca release + TRAP activity + pH	0.675	0.455	0.093	0.769

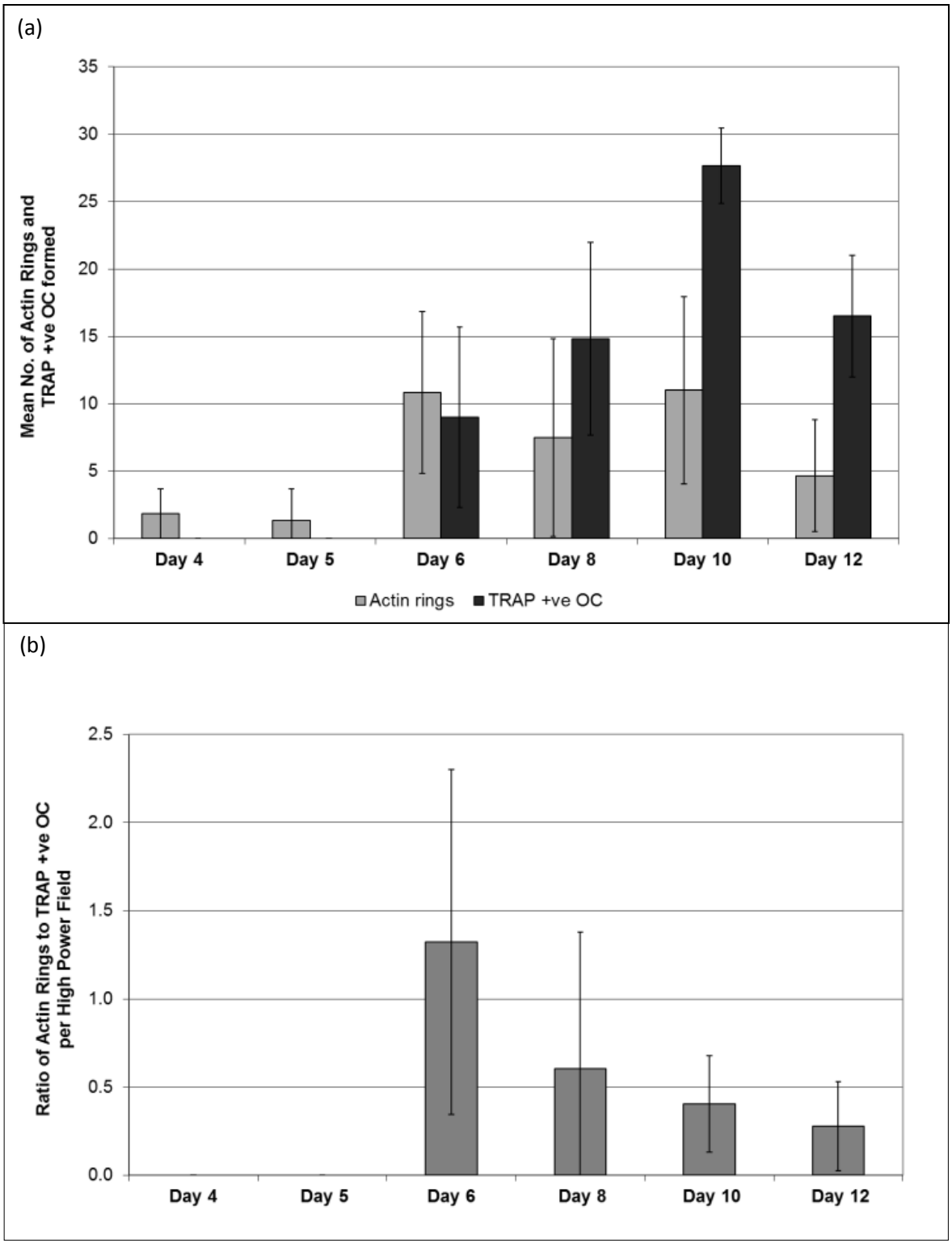
(a)
Mean No. of Actin Rings and TRAP +ve OC formed
35
30
25
20
15
10
5
0
Day 4
Day 5
Day 6
Day 8
Day 10
Day 12
Actin rings
TRAP +ve OC
(b)
Ratio of Actin Rings to TRAP +ve OC per High Power Field
2.5
2.0
1.5
1.0
0.5
0.0
Day 4
Day 5
Day 6
Day 8
Day 10
Day 12

Figure 2.

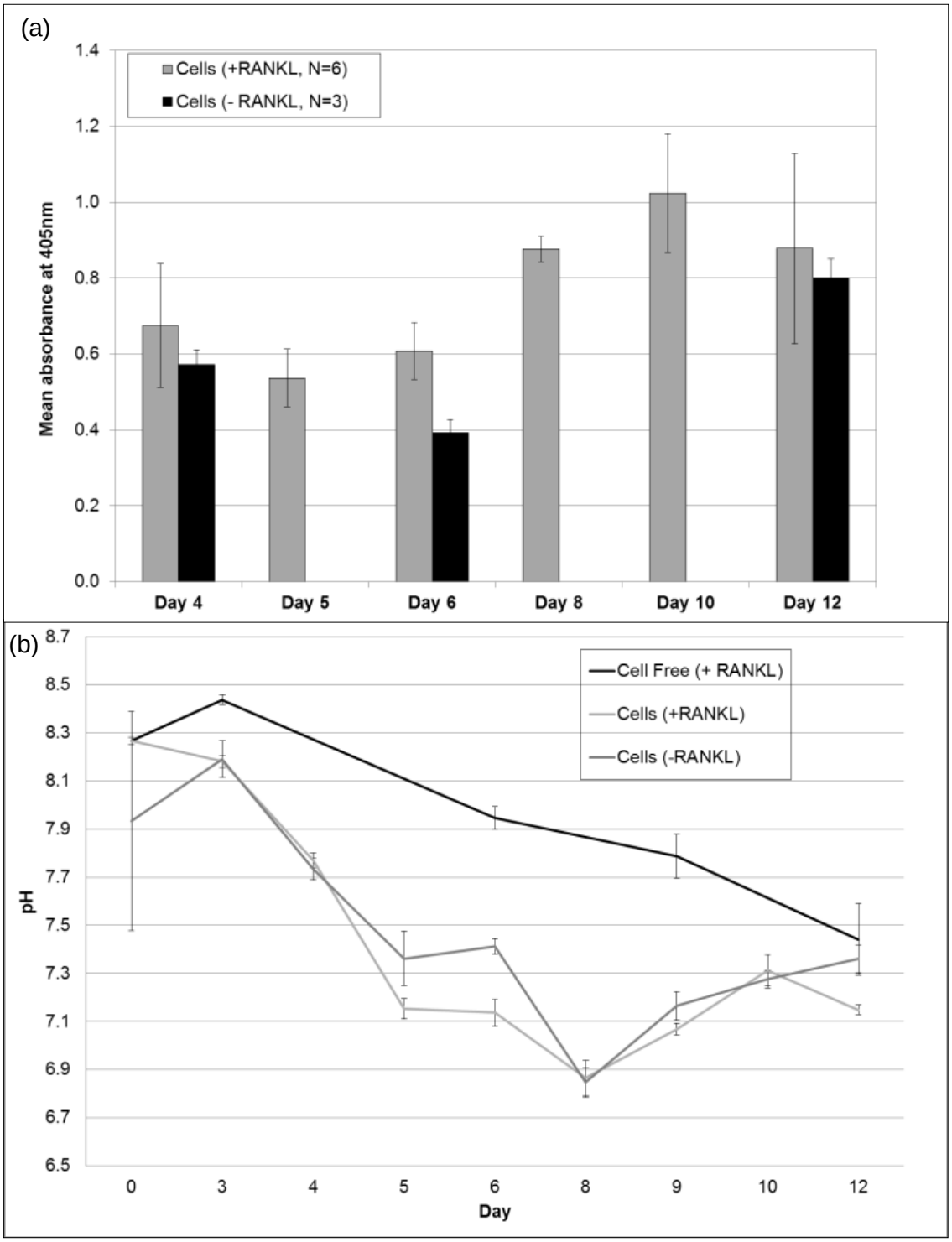
(a)
Cells (+RANKL, N=6)
Cells (- RANKL, N=3)
Mean absorbance at 405nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Day 4
Day 5
Day 6
Day 8
Day 10
Day 12
(b)
Cell Free (+ RANKL)
Cells (+RANKL)
Cells (-RANKL)
pH
8.7
8.5
8.3
8.1
7.9
7.7
7.5
7.3
7.1
6.9
6.7
6.5
0
3
4
5
6
8
9
10
12
Day

Figure 3.

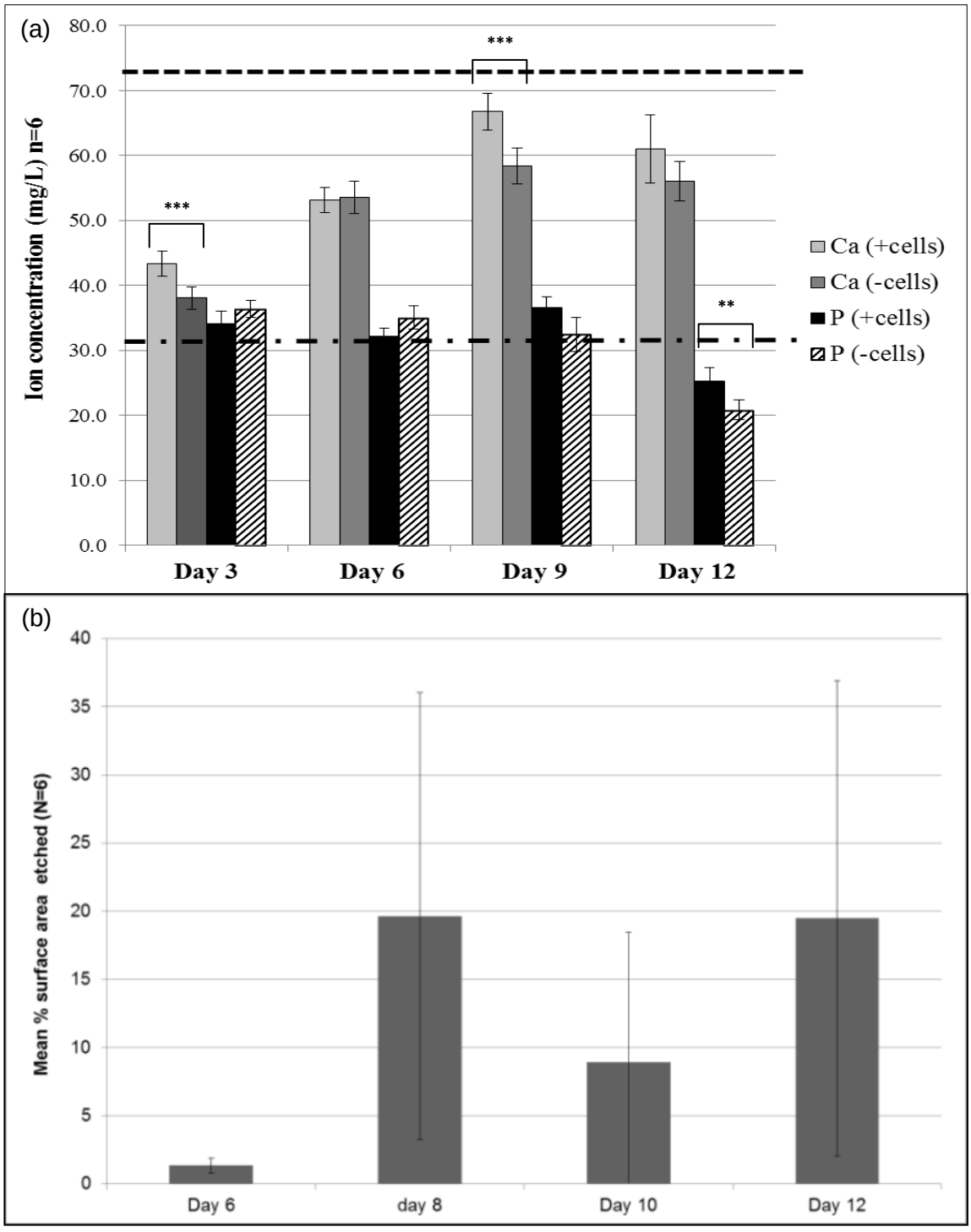
(a)
Ion concentration (mg/L) n=6
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
***
***
**
Day 3
Day 6
Day 9
Day 12
Ca (+cells)
Ca (-cells)
P (+cells)
P (-cells)
(b)
Mean % surface area etched (N=6)
40
35
30
25
20
15
10
5
0
Day 6
day 8
Day 10
Day 12

Figure 4.