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Original Research Article

An in-vitro medical device material biocompatibility study using primary cell cultures of rat osteoblasts

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ABSTRACT

Background: We questioned if simple chemical methods could be applied as a possible biocompatibility test for biomaterials.
Methods: In a qualitative experiment, osteoblasts cells harvested from newly born (3-5) day old sprague-dawley rats were cultivated in growth medium in a controlled environment in the presence of titanium (Ti) (American Elements®), cobalt-chromium (Co-Cr) (Nobilium®), bio-activated rattan wood, orthopaedic bone cement (CEMEX®), implant fixtures (Astra TDC), and resorbable suture material (Poly-Gly-Lac) (VICRYL®). Sample aliquots were withdrawn periodically over 16 days. One and two-dimensional polyacrylamide gel electrophoresis (PAGE) as well as isoelectric focusing (IEF) produced spots that were subjected to enzyme digestion and molecular weight determination. In a follow-up quantitative experiment, the same samples, except for those containing CEMEX and VICRYL, were prepared. The alkaline phosphatase (ALP) activity was monitored continuously using the colorimetric Stanbio® kits. The ALP activities at days 2, 4, 8, 12, and 16, designated as a longitudinal variable, TIME, were analysed, using SPSS V.22, as a mixed ANOVA model with TIME as a repeated measure and the material type as an independent factor.
Results: The IEF and second dimension PAGE produced an additional spot for Ti at pH(I) of 5-6. This was identified as IQUB_RAT IQ, a ubiquitin-like domain, molecular weight 2.6 kDa. This method was able to find finite differences in osteoblast activity after initial exposure to a foreign body. Both the ALP activity changes from one day to the next for all materials and the TIME-material type interaction effects were significant (p=0.000).
Conclusions: This technique is suitable for use with human cell lines or clones. Experiments like these reduce the need for animal testing.

Keywords: Osteoblast, Bioactive wood, Biomarkers, Medical device testing, Alkaline phosphatase

INTRODUCTION

The three basic tests for biocompatibility are the cytotoxicity, sensitization and irritation tests.[1,2] Sensitization and irritation tests are performed on animals after proving the device is not cytotoxic to cell lines for example MG-63, MC3T3-E1, SaOs-2 and mouse L929 fibroblasts.[3-6] Up to a few years ago there were approximately 3,600 cell lines from over 150 different animal species.[7] This poses a serious and daunting challenge when looking for human relevance. The protocols for testing medical devices are given in the ISO standards ISO13485.[8] Both animal and in vitro testing are detailed in the ISO10993 series.[9]

New products concurrently come with an increase in animal testing. At the same time there is also a concerted effort to reduce animal testing.[10] In terms of using animals for biocompatibility studies, applying the '3R' principal of replacement, reduction and refinement improves the quality of science.[11,12] In the early 70's two high performance methods of separating proteins were available. The first was described by Laemmli.[13] With this method, molecular weight was used to separate proteins. The second method by Grownow and Griffith involved iso-electric focusing (IEF), in which the net charge of the protein was used.[14] However, the identification of proteins was limited by the detection range of the coomassie dye stain.[15] Development of electronic equipment as well as software analysis has improved the method. The current procedure involves the sample preparation, IEF, SDS-PAGE, staining, and molecular weight determination of spots using mass spectrometric analysis. The method used here was adapted from standard protocols of the 2D SDS-PAGE technique.[16,17] The Ti, Co-Cr, orthopaedic bone cement and resorbable sutures are materials currently used in various forms in medicine. Bio-activated wood was included in the study because of the recent attention given to wood as a possible raw material for fabrication of artificial bone.[18-20]

METHODS

Reagent used for cell cultures

One litre of phosphate buffered (PBS) saline was prepared using sterile distilled water (dH_2O) containing 8.0 g NaCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, and 0.2 g KCl (10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl). This solution (PBS stock) was adjusted to pH 7.4 using 10 M NaOH and concentrated HCl, then cold filtered into sterile bottles under vacuum using the Nalgene® 20 µM bottle top filter, and stored at room temperature. Hank's balanced salt solutions (HBSS) were made up with and without calcium and magnesium using sterile dH_2O containing 8.0 g NaCl, 0.358 g Na_2HPO_4, 0.6 g KH_2PO_4, 0.4 g KCl, 1.0 g glucose, 0.185 g $CaCl_2$ and 0.0978 g of anhydrous $MgSO_4$ (HBSS stock). The pH was adjusted to 7.4 using 10 M NaOH and concentrated HCl, then cold filtered and stored in the refrigerator at 4ºC in dark bottles. Both solutions were tested for fungal/bacterial growth by incubating small aliquots in the humidified CO_2/Air incubator for 2 weeks. The complete growth medium consisted of a cocktail of the following chemicals: basal cell media 199 or minimum essential media (MEM), purchased from sigma-Aldrich; 10% foetal calf serum (FCS); and 0.2% antibiotic (100 U penicillin/100 µg/ml streptomycin and 0.25 µg/ml amphotericin B), also purchased from Sigma-Aldrich. Collagenase enzyme solution was prepared fresh using HBSS. Collagenase dry powder was purchased from Fisher bioreagents. It was made into solution which contained 0.25% collagenase, 0.5 mM $CaCl_2$ and 0.5 mM $MgSO_4$ freshly made up to 5.0 ml. Trypsin-EDTA solution, without calcium and magnesium, was purchased from Sigma-Aldrich. This was stored in aliquots of 5 ml between 4ºC and 6ºC in a refrigerator. The solution consisted of 1.0 mg/ml Trypsin in PBS pH 7.4. Vials were quickly equilibrated to 37ºC prior to use. Tyrode's solution, Ca^{2+} and Mg^{2+} free, containing 5 mM EDTA was also purchased from Sigma-Aldrich and stored between 4ºC and 6ºC in a freezer. This reagent was thawed and equilibrated to 37ºC prior to use.

Reagents for electrophoresis

The first dimension IEF consumables were purchased as a kit from BioRad®. The rehydrating buffer (buffer I) consisted of 8 M urea, 4% CHAPS or 3-[(3-cholamidopropyl) dimethylammonio] -1-propanes ulfonate, 0.2% Pharmalyte 3-10, 50 mM Dithiothreitol (DTT) and 0.0002% Bromophenol blue made up in 25 ml double distilled water (ddH_2O). The solution of 8 M urea was prepared immediately before use. The Pharmalyte® and 10 mM DTT were added to the rehydration buffer just before loading the sample for the first step of the rehydration process. Bromophenol blue powder was obtained from Sigma. A 1% stock solution was prepared dissolving 100 mg Bromophenol blue in 10 ml ddH_2O. The final concentration used in the rehydration buffer was 0.0002%. The equilibrating buffer (buffer II) was used to bind the proteins separated in the first dimension run to SDS (sodium dodecyl sulfate). This buffer consisted of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS and Bromophenol blue. This was prepared by adding together: 10 ml of Tris-HCl, pH 8.8, 72.02 g urea, 69.0 ml glycerol, 4.0 g SDS, 400 µl Bromophenol blue and making up to 200 ml using ddH_2O. There were two additional buffers prepared and used prior to the second-dimension run. DTT equilibrating buffer (buffer III) consisted of buffer II with 3% DTT. This was prepared by dissolving 70 mg of DTT in 25 ml of buffer II. It was prepared immediately before use.

The last buffer that was used (buffer IV) consisted of buffer II to which iodoacetamide was added. This buffer was made up using 25 ml of buffer II which contained 2.5% of iodoacetamide. The electrode SDS running buffer used in the second-dimension run was also used to prepare the agarose sealing solution. This buffer was diluted 5X by combining Tris base with other reagents described below. Agarose sealing solution was prepared using 0.5 g agarose, 200 µl Bromophenol blue that was added to 100 ml of the electrode SDS running buffer. This mixture was gently warmed in a 500 ml Erlenmeyer flask until the agarose dissolved. The IPG strips used were 7 cm long by 3 mm wide. The pH range used was 3-10. The first-dimension gel electrophoresis was performed on a flatbed or horizontal iso-electric focusing system. The total volume loaded with the IPG strips (sample with rehydrating buffer I) was 125 µl. The strips were incubated for 12 hours in the presence of sample solution plus the rehydrating buffer I.

Several reagents, which had to be prepared in preliminary steps, were utilized in the formation of the second-dimension gel. First, 100 ml of acrylamide/bis (sigma/aldrich) (30%), prepared using distilled water (dH_2O), was filtered through Nalgene® bottle-top filters non-surfactant cellulose acetate membrane 0.2 µm pore size, under vacuum and collected into sterile dark glass bottles. This solution was kept at 4ºC in the dark until used. A solution (300 ml) of tris-HCl (sigma/aldrich), concentration of 1.5 M, adjusted to pH 8.8, was prepared using distilled water and also stored at 4ºC. Another 100 ml of tris-HCl solution, at a concentration of 0.5 M and adjusted to pH 6.8, was prepared and stored in a similar manner. SDS was prepared at a concentration of 10% using deionized H_2O (diH_2O) and made up to 100 ml. The sample buffer was made up to 8 ml using the following reagents: 3.8 ml distilled water, 1.0 ml of 0.5 M tris-HCl, pH 6.8, 0.8 ml glycerol, 1.6 ml of 10% SDS, 0.4 ml 2 β-mercaptoethanol and 0.4 ml of 0.05% bromophenol blue. The electrode SDS running buffer was prepared by combining Tris base (15 g/l), glycine (72 g/l) and SDS (5 g/l). This was made up to 300 ml of diH_2O and the pH adjusted to 8.3. This was stored at 4ºC. Before using, it was warmed to 37ºC in the water bath and diluted to 1/5 of the original concentration (300 ml in 1200 ml diH_2O).

The concentration of acrylamide used was 12%. It was prepared by combining the following reagents, 33.5 ml dH_2O, 25.0 ml of 1.5 M tris-HCL, pH 8.8, 1.0 ml of 10% SDS and 40.0 ml of 30% acrylamide stock. It was de-gassed for approximately 15 minutes before use. In the last step of the procedure, 500 µl of freshly prepared 10% ammonium persulfate (Sigma-Aldrich) and 50 µL of tetramethylethylenediamine (TEMED), from thermo scientific pierce, were added before the resulting liquid was poured into 7.3×8 cm gel electrophoresis plates, separated by a spacer of 0.5 mm.

The stacking gel that formed the comb or wells to hold the samples on top of the separating gel contained a lower concentration of acrylamide. The concentration of acrylamide used in the preparation of this gel was 4%. It was prepared using the following reagents, 6.1 ml dH_2O, 2.5 ml of 0.5 M tris-HCl, pH 6.8, 100 µl of 10% SDS and 1.3 ml of 30% acrylamide/bis which was again degassed for 15 minutes. In the last step of the procedure, 50 µl of freshly prepared 10% ammonium persulfate and 10 µl of TEMED were added before the resulting liquid was poured on the top of the separating gel in the glass slabs. A 10-well spacer of 0.5 mm thickness was inserted immediately after the stacking gel was introduced. This effectively formed the wells or sample chambers on the top of the gel slab.

The staining solution used consisted of a 0.1% Coomassie blue R-250 solution made up in 40% methanol and 10% acetic acid, both of which were also purchased from sigma-aldrich. The de-staining solution was made up with 40% methanol and 10% acetic acid. The potential was set at 120 V and the running time was kept at 1-1/2 hours. Two 24-well cell culture plates were used for the six materials plus one for cells only and a blank. Each of these was set up with replicates of 4.

Protein determination and sample loading

A calibration curve of the sample absorbance at 595 nm vs concentration of protein allowed for the determination of protein in the cultures. A stock solution of bovine serum albumin (BSA) (sigma aldrich) was made up by dissolving 1.0 mg BSA in 1 ml of 0.15 M sodium chloride. The sequential dilution of BSA resulted in solutions ranging from 0.0 µg (pure dH_2O) to 400 µg (2.5 X dilution) and was done in duplicate. The Bradford 5x reagent was diluted 1 to 1. The calibration curve and unknown protein quantitation was done at the same time. Following this, an equal amount of protein was loaded onto the gels which run in the first dimension. The amount of protein loaded for the first dimension was 150 µg. A volume of sample containing 150 µg was added to the corresponding volume of rehydration buffer to make up a total volume of 125 µl. This was introduced into the well after which the IPG strips were placed gel-side down to start the rehydration process.

Preparation of test materials

All the test materials used were shaped as discs of comparable dimension and fitted into wells of a 24-cell culture template. The exception was the resorbable suture material (Poly-Gly-Lac) (VICRYL®). The entire length (16 mm 3-0 Coated Vicryl TM, Ethicon®) was rolled into a circle 8.0 mm in diameter and fitted into the well of the experimental template. Titanium discs used were purchased from (American elements, Merelex Corp.). The Co-Cr alloy was purchased as ingots (Nobilium Inc.) and casted into discs. The PMMA used was CEMEX® (Tecres® lot. AA9382). This is a type of orthopedic bone cement. It was obtained from a local hospital. It was sold as a packet containing 20.0 g powder along with and separate from 8.35 ml of a liquid component. The powder and liquid were mixed according to the manufacturer's instruction and molded into discs before complete polymerization. The bio-activated wood was prepared using the protocol given for phosphorylation of microcrystalline cellulose Patent: US2759924.[21] The activated wood discs and other test materials were all preconditioned before use. This was done by incubating them in the growth medium in a humidified incubator (5%/95% CO_2/Air at 37ºC) and checked for bacterial/fungal growth within 2 weeks.

Animal model

Sprague dawley rats were obtained from Charles Rivers Laboratories® USA. Newly born rats were used to obtain osteoblast cells. The method used here involved the sequential enzymatic digestion of the calvaria as previously described.[22] The details of weight and sex of

the rats used are given in Table 1. The harvested cells were collected as a pellet and re-suspended in 10.0 ml of growth medium by gentle vortex. The growth medium consisted of MEM containing 10% FCS and 0.2% antibiotics (100 units of penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B). Freshly re-suspended cells were then distributed equally into 24 wells containing titanium, cobalt-chromium, PMMA and wood. A blank with growth medium only was included. A cell count (using Trypan Blue solution) was done to estimate how many cells were introduced into each experimental well. The average number of cells introduced in each well was 6.25×10^5.

Table 1: The weights and sex of the 6 rats used in the 2-D SDS-Page Experiment.

Rat ID number	1	2	3	4	5	6
Sex	F	F	F	F	F	M
Weight (grams)	5.0	4.8	4.4	5.1	4.5	4.8

Cell culture template and sample preparation

Four replicates of 1.0 ml culture medium were used for each test material. These were pipetted into 1.8 ml Nalgene® cryogenic vials then frozen in the refrigerator. After 2 hours they were removed and placed in liquid nitrogen. The ALP assay was performed manually. The vials were removed from the cryogenic chamber and thawed on the bench top for 10 minutes. An aliquot of 500 µl from each replicate was pipetted into 2.0 ml Eppendorf tubes and centrifuged at 1000 g for 10 minutes. After centrifugation 20 µl was pipetted from each replicate and added to clean test tubes containing 1.0 ml buffer reagent from the Stanbio® kit and incubated at 37°C in the water bath for 5 mins. To this 250 µl of substrate was added and the timer started. This liquid was transferred to the optical cuvette and the absorbance at 405 nm was recorded every minute for 4 mins. The liquid was then discarded and the procedure repeated for all samples. The ALP measurements were done on days 2, 4, 8, 12 and 16 days at which time the experiment was discontinued. Samples were also collected in a similar manner on the 9th day for the 2D experiments.

Statistical analysis

Quantitative data from the ALP activity measurements for different materials on each of five days after incubation were analysed with SPSS V.22, using a mixed model analysis of variance (ANOVA) routine with 'time after incubation' of the biomaterial (with or without the cell cultures) designated as a repeated measure (TIME) and the type of biomaterial as an independent predictor (Material). ALP activity was the outcome (dependent variable) used for the effectiveness of biocompatibility under the different experimental combinations of the categories of TIME and Material. This SPSS routine for repeated measures ('within subjects') ANOVA offers three corrections, for situations when the assumption of sphericity is not met. All of these corrections involve adjusting the p value upwards so that it becomes more difficult to meet the inference of significance ($p<\alpha$). These are the Greenhouse-Geiser, the Huynh-Feldt, and the lower bound corrections. The significance level was set at $p<0.05$. Least significant difference (LSD) *post hoc* tests were conducted to do pair-wise comparisons of effectiveness among the biomaterials used.

RESULTS

The separation of proteins found in media using one dimensional polyacrylamide gel electrophoresis

The images of the various gels were photographed and uploaded onto the computer. The software program SynGene® was used to identify the presence of bands and relative molecular weights based on the standard wide molecular weight range Sigma® (No: 4036) molecular weight ladders. There is a limited number of inferences which can be drawn for qualitative experimental techniques. That being said, when all materials were matched on one gel at day three, for the implant fixture, wood, Ti and VICRYL® there was no protein band in the 51-59 kDa molecular weight range. The media which contained heat-cured PMMA, cobalt-chromium, and cells only produced similar numbers of protein bands with equally matching molecular weights. At day 7, there was greater uniformity in protein distribution seen for all materials. However, the number of bands appearing in the gel for each material dropped from 5-6 bands at day 3 to 4 bands for each material tested. At day 10 the pattern remained essentially consistent with that at day 7 in four systems -the medium only, PMMA, Ti and VICRYL®. Four bands were seen at approximate molecular weights in the range 70-126. kDa. The samples of culture medium taken at day 10 from wells containing cobalt-chromium, wood, and the implant fixture no longer showed a protein band corresponding to 24-25 kDa. Similar observations were found in the gels ran for samples of medium, extracted on different days from wells with individual test materials. This was true for all the materials. For example, in the gels where samples from the wells containing the implant fixture, wood, Ti and VICRYL® were analysed individually, there was an absence of protein bands in the 51-59 kDa range on day three, as reported above when all the materials were matched on one gel. At days 7 and 10, the protein bands produced were also comparable to those found in gels when all materials were matched on one gel.

Two-dimensional gel electrophoresis, ALP activity and mass spectrum analysis

The gel, which ran samples of medium (day 9) in the cells containing Ti, showed one additional spot at the isoelectric pH, pH(I) 5-6. This was in a different or

unmatched position when all the gels were compared together. The spot was excised and subjected to mass spectrometric analysis. The molecular mass was estimated to be 2.6 kDa. The online mass spectrum software MATRIX SCIENCE® was used to upload the data that was generated for this protein. The software program estimates that a protein score of greater than 52 is a match. The software identified this protein as IQUB_RAT, IQ and ubiquitin-like domain-containing protein OS=Rattus.

Analysis of the ALP activity on days after incubation

The ALP activity was recorded after 2, 4, 8, 12, and 16 days. The statistical output from the repeated measures ('within subjects') ANOVA is given in Table 2. This estimates the significance of ALP activity changes with TIME for all materials taken together and of the TIME X Material interaction. Mauchly's test of sphericity, not shown, was not significant. This implies that the variances of the ALP activity differences between all possible pairs of times were statistically equal so the uncorrected p value, namely that for sphericity assumed, was used to assess significance instead of one of the three corrections. That said, all four omnibus multivariate tests were significant (p=0.000) for the effects tested.

Table 2: P values for significance tests of within-subjects' effects for ALP activities.

Source	Sphericity assumed	Greenhouse-Geiser	Huynh-Feldt	Lower Bound
ALP Activity	0.000	0.000	0.000	0.000
ALP Activity X Material	0.000	0.000	0.000	0.000

The p values, for the tests of total ALP activity and for ALP X Material contrasts between each measurement day and a reference, are given in Table 3 and 4. Two references were used in the contrasts. First, each ALP activity was compared with that for the 2-day sample. Then each was compared with that for the 16-day sample. Inferences can be made from these comparisons, which give the significance of the differences in ALP activities between days, and from Figure 1, an interaction plot (TIME X Material) which shows the ALP activities for the different biomaterials and for cells only on different days after incubation from 1 (2 days) to 5 (16 days).

Material comparison

In Table 5 are given confidence intervals for mean differences in ALP activity, obtained from pair-wise comparison of the materials using LSD *post hoc* tests. Only the confidence intervals for significantly different means are quoted in Table 5 and all of these pairwise tests have a p value=0.000.

Table 3: P values for significance tests of within-subjects' contrasts against a reference sample of ALP activity at 2 days.

Source	4-day	8-day	12-day	16-day
ALP Activity	0.001	0.000	0.000	0.000
ALP Activity X Material	0.325	0.000	0.001	0.000

Table 4: P values for significance tests of within-subjects' contrasts against a reference sample of ALP activity at 16 days.

Source	2-day	4-day	8-day	12-day
ALP Activity	0.000	0.000	0.331	0.000
ALP Activity X Material	0.000	0.000	0.000	0.175

Table 5: Confidence intervals (at the 0.001 level) for mean ALP activity (U/l) differences (reference – material).

Reference material	Ti	Co-Cr	Wood
PMMA	4.35– 7.52	3.25-6.42	3.47-6.65
Cells only	3.56– 6.73	2.46-5.63	2.68-5.85

Ti: titanium, Co-Cr: Cobalt-chromium, PMMA: Polymethylmetacrylate,

DISCUSSION

None of the materials tested was bio toxic. The IQUB_RAT, IQ and ubiquitin-like domain-containing protein, is usually found in all eukaryotic cells.[23] This protein was found in titanium cultures at 9 days and was absent from the gels on which other test materials were run. This is a 76 amino acid protein with a molecular weight of 8.5 kDa, which is found in diverse animal groups.[24] The amino acid sequence is identical in humans, the house mouse, the common street rat and the fruit fly (Homo sapiens, Mus musculus, Rattus norvegicus, Drosophila melanogaster).[25] Ubiquitin performs a regulatory role in protein breakdown and repair, cell-cycle control, stress response, DNA repair, growth-factor signaling, transcription, and gene silencing.[25] Also, ubiquitin-binding domains (UBDs) are a collection of modular protein domains which non-covalently bind to ubiquitin.[26]

The proteins are added and removed from the ubiquitin by an activating enzyme, conjugating enzyme, and a ligase termed E1, E2 and E3.[27] Any disorder involving this process can result in cancer, nerve, cardiovascular, and/or immune diseases.[28,29] As a result of this, there is a great interest in finding out more about this process and trying to rationalize what happens in a disease state.

In this study, the analysis of ALP activities for all biomaterials on different days after incubation shows that

there were real changes in ALP activity with TIME and the pattern of change was different for the different materials (the interaction effect of ALP activity over TIME X Material was significant)- Table 2. The mean ALP activity decreased significantly with TIME for each material and for the compartment with the cells only.

Activities on all measurement days were significantly lower (p=0.000) than that for the first measurement at 2 days after incubation (Table 3). ALP activities at 2, 4, and 12 days were also higher than that for the final time period at 16 days (Table 4). However, the ALP activity at 8 days, which is the lowest for all materials (except Ti), as shown in Figure 1, is not significantly different from that observed after 16 days (p=0.331) (Table 4).

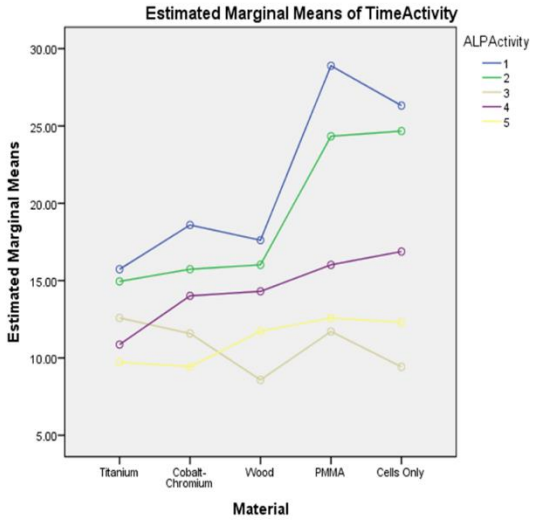

Figure 1: SPSS V.22 interaction plots of estimated marginal means of ALP Production for different test materials at different times of 1 (2 days), 2 (4 days), 3 (8 days), 4 (12 days) and 5 (16 days).

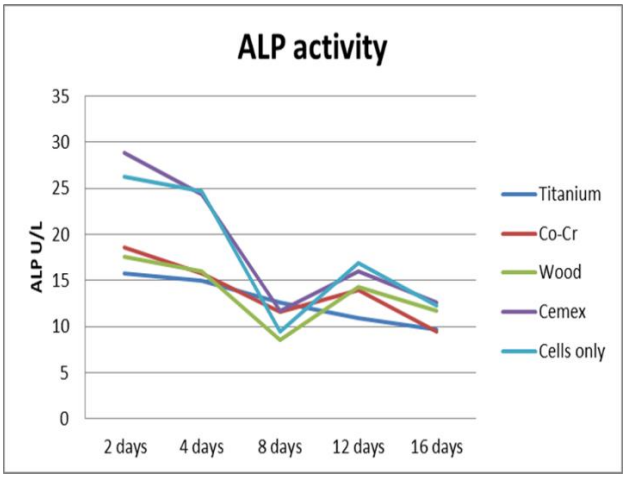

Figure 2: The ALP activity (U/l) of the cells measured over the period of 16 days shows the materials have generally the same pattern and no material including bioactive wood is particularly cytotoxic.

The interaction effect, TIME (ALP activity) X Material, essentially measures how ALP activity changes with TIME from one biomaterial to the next OR from one day to the next for different materials. At days 2 and 4 the patterns of ALP activity differences among biomaterials are the same (p=0.325, Table 3). The same is true for days 12 and 16 (p=0.175, Table 4). This is seen clearly too in Figure 1 for days 2 and 4 but is not as obvious for days 12 and 16. On the other hand, the pattern of ALP activity differences between the biomaterials is different on day 8 from that on any other day. This is seen in Figure 1 and shown by significant p values (p=0.000) for the contrasts of the interaction effects for days 8 and 2 in Table 3 and for days 8 and 16 in Table 4.

ALP activities for Ti, Co-Cr, and wood are statistically equivalent and each of these is associated with an ALP activity value which is significantly less than that for either PMMA or cells only. The ALP activity associated with PMMA, although it 'looks' larger in Figure 1, is statistically the same as that for cells only. Figure 2 shows this more clearly capturing the similar activities for CEMEX (PMMA) and cells only on each measurement day. The highest activities were obtained for PMMA and for cells alone (Figure 1 and Table 5). The ALP activities for these are significantly higher than those for Ti, Co-Cr, and wood discs, these last three of which are statistically equivalent (Table 5). The ALP activities in the compartments with the PMMA and with cells only are almost identical on the graphs shown in Figures 1 and 2.

CONCLUSION

Primary cell cultures, although harder to obtain, are more attractive to researchers since the cells encompass a behavior more representative of the live animal. This gives more weight to predicting pre-clinical outcomes. The use of human clones or embryonic cells may offer an opportunity to remove some of the limitations of immortalized cell lines. It would produce data more relevant to humans.

There is as yet no 'one-test' from which multiple inferences can be obtained. Also, not all test methods are well developed. This standard analytical technique could also be applied to identify novel markers for new infectious diseases.

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