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In-Vitro Evaluation of the Cytotoxicity of Binders and Powder Used in 3D Colour Printing of Maxillofacial Soft Tissue Prostheses

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Abstract:
Purpose: *The purpose of this study was to evaluate biocompatibility of the binders 'ZB58' and the starch powder 'ZP15e' used by the Z510 colour printer for printing soft tissue facial prostheses.*
Materials and Methods: *Biocompatibility was investigated by testing four binders and the powder at three concentrations on two cell lines (ROS - osteoblasts and L929 - fibroblasts). The binders and the powder were exposed to the cells for 3, 5 and 7 days, after which the cells were treated with 10% Alamar Blue. Binder and powder toxicity was measured as a comparison against the uninfected media only cell control.*
Results: *The Alamar Blue assays for the ROS and L929 cell line for all days no significant toxic effects were observed at lower binders and powder concentrations. However there was evidence of reduction in all cell vitality at higher concentrations and it appears to be more pronounced at seven days exposure*
Conclusion: *The Alamar Blue assay highlighted the potential toxic effect of the four coloured binders and at less extent the powder used in the manufacture of soft tissue facial prostheses when used in high concentrations when applied on two types of cell lines used in this study (ROS and L929). This effect was most pronounced for the clear binder at seven days exposure.*
Clinical significance: *Further work will need to be carried out to determine if the binder is able to leach out of the soft tissue prostheses at concentrations that may cause a local tissue reaction.*
Keywords: *Biocompatibility, cytotoxicity, Alamar Blue, ZB58, ROS, L929.*

I. Introduction

Biocompatibility analysis is performed to determine the capability of an element to function normally and coexist with convenient host response in a certain situation [1]. Biocompatibility test is performed for implant materials that are either placed in contact with the skin or implanted inside the body to assess how the body reaction to these materials that is usually elicit in diverse ways according to their location within the body and materials used. Essentially, implant materials should be tested for their biocompatibility and their ability to coexist with human tissues at an early stage of development [2] Biocompatibility/cytotoxicity testing is performed by cell culture assay in order to investigate the toxic effect of medical devices and implant materials on human cells by applying one or more of the following methods; scanning electron microscopy, enzyme assay, cytokine expression, MTT, LDH and Alamar Blue assay [3].The Alamar Blue assay is designed to determine cell line proliferation for different types of living cells. This assay displays the cytotoxic level of an agent among different chemical classes [4]. It is a straightforward cell vitality assay, a rapid, reliable and non-radioactive assay which requires a minimum of 80 cells to show positive results [5]. The Alamar Blue assay provides the essential data for predicting the cytotoxic effect of a novel agent by comparing these data with known *in-vitro* data [4].

Several models of 3D printers have been developed and commercialized by the Z-corporation; the Z510 is one of these printers that utilize a natural polymer such as cornstarch and water based binder for printing 3D parts. However, the powders and the binders employed by the printer are used for industrial rather than medical applications [6].

Recently a research team at the University of Sheffield with the help and support Fripp Design and Research, a Sheffield based Industrial Design Company; have been able to manufacture soft tissue facial prostheses using additive manufacturing with the use of 3D colour printing [7]. This has been achieved by layered fabrication of a starch powder held together by an aqueous binder containing a resin and inks and then infiltrated with a medical grade silicone polymer. Earlier work has shown that the prosthesis thus produced does not cause any toxic reaction. However, the powders and the binders employed by the printer used for industrial

rather than medical applications. To date, these powders and binders have not been investigated for use in medical applications, therefore the aim of this study was to evaluate the biocompatibility of the binders 'ZB58' and the powder 'ZP15e' used by the Z510 colour printer for printing soft tissue facial prostheses.

II. Materials and Methods

The biocompatibility of the binders was tested on two types of cells stored in liquid nitrogen: the rat osteosarcoma derived cells ROS 17/2.8 [8] and L929 cells, derived from an immortalized mouse fibroblast cell line [9]. Three concentrations (0.1%, 0.5% and 1.0%) of the four binders (clear, yellow, cyan and magenta) and the powder (0.025, 0.05 and 0.1 mg/ml) were used separately for the cell vitality assay.

II-1 Growth medium preparation and cell culture procedure:

500 ml of Complete growth medium was prepared by adding 50 ml of bovine calf serum (BCS), 5 ml of glutamine and 5 ml of P/S (penicillin and streptomycin) to 440 ml of DMEM (Dulbecco's Modified Eagle's Medium).

ROS cells and L929 cells were obtained from stocks stored in liquid nitrogen, after recovery cells were cultured in a Complete growth medium and incubated at 37ºC in a humidified atmosphere of 5% CO_2 and 95% O_2 for 72 hours. After the incubation period the flasks were washed in 5 ml phosphate buffered saline (PBS). 2 ml of Trypsin-Ethylenediaminetetraacetic acid (Trypsin-EDTA) was added to the flasks and the flasks were incubated for 3-4 minutes to allow the cells to split. The cell suspension was transferred to a 15 ml tube, and centrifuged at 1000 RPM for 5 minutes. Then, the content (supernatant) was discarded leaving the cells densely attached to the bottom of the tube. 10 ml of growth medium was added to the tube and the tube was shaken gently in order to achieve homogeneous cell suspension.

II-2 Cell counting procedure:

A haemocytometer and a manual counter were used to count the live cells in four selected squares. The total number of cells in these squares divided by four to consider the average cells number then number of cells in 1 ml was calculated by applying the following formula

Cells/1ml = n $x10^4$, Where n is the average cell count per square

Cell density was calculated using the following formula:

Cell density (µl /ml) = (1.25 / n) x 1000 (µl /ml)**,** Where 1.25 is the dilution factor.

II-3 Cell vitality assay:

A 12 well plate was used for each binder (clear, yellow, magenta and cyan) and $1.75x10^4$ of each cell (ROS and L929) in medium was added to each well. The plates were incubated at 37ºC for 48 hours at 5% CO_2 and 95% O_2. After the incubation period the medium was discarded and the cells were exposed to three concentrations (1.0%, 0.5% and 0.1%) of each of the four binders (clear, yellow, magenta and cyan) and the powder (0.025, 0.05 and 0.1 mg/ml) separately in growth medium, then the plates were incubated at 37ºC for 3 days, 5 days and 7 days. Control groups (cells and medium) also were set for each type of cells.

At day 3 and following 3 days exposure to the different concentration of the dyes and the powder, unattached cells were removed by washing the monolayer with PBS twice, then one ml of 10 % Alamar Blue (Invitrogen, UK) in medium was added to each well, and the plates were incubated for 2 and 4 hours. After the incubation periods 200 µl for each test and control samples for the powder and binder were placed into 96-well plates separately (Greiner Bio-One, Germany). The fluorescence intensity was measured using a fluorescent plate reader (Infinite® 200 PRO, Tecan, Reading, UK) at an excitation wavelength of 570 nm and an emission wavelength of 600 nm.

At day 5 and day 7, as on day 3 the cells in the 12 well plates were washed with PBS. Then, 1 ml of 10% Alamar Blue in medium was added to each well and the plates were incubated for 2 and 4 hours. After each incubation time, 200 µl samples were placed into a 96-well plate in order to measure the fluorescence intensity.

III. Results

III-1The binders:

A colour shift from blue to red after incubation time was detected in both control and the majority of the test plates for both elements applied in this study, the binders 'ZB58' and the powder 'ZP15e'. This colour shift indicates cell activity and vitality in the medium. Cell growth in the medium reduces the non-fluorescent blue dye resazurin to fluorescent red dye resorfurin [10-11]. The detailed information of the Alamar Blue assay for the binder 'ZB58' is presented in Table 1 for ROS cells and Table 2 for L929 cells; the Tables demonstrate the effect of the 3 concentrations of the four binders on these cells at days 3, 5 and 7.

Table 1: Alamar Blue assay for the binders on ROS cell lines.

Concentration	Control and Test Samples –Fraction				
	ROS - Day 3				
	Control	Clear	Cyan	Magenta	Yellow
0.1%	100	88.4	102.8	100.1	93.1
0.5%	100	90.4	99.9	100.2	106.9
1.0%	100	58.0	97.6	101.2	102.1
	ROS - Day 5				
0.1%	100	98.4	100.6	96.2	97.5
0.5%	100	69.6	83.7	86.5	85.5
1.0%	100	69.2	80.9	77.3	81.8
	ROS - Day 7				
0.1%	100	111.2	110.6	118.3	109.8
0.5%	100	9.6	59.9	100.1	59.3
1.0%	100	0	12.7	33.2	36.5

At day 3, cell vitality assay showed normal cell growth for both cell lines ROS and L929 for the three concentrations of the four binders compared to their control groups as both Tables 1 and 2 shows. Furthermore, photographs were prepared for the different cell lines under light microscopy for the four binders at different concentrations and for the control wells, some of these photographs for ROS cells and L929 are demonstrated in Fig. 1and 2 respectively.

At day 5, all cell line growth for the four binders and for the two cell types ROS and L929 had shown a similar level of growth and cellular activities at all concentrations. However, there was a slight reduction in cellular activities for both types of cells after 5 days exposure to 0.5% and 1.0% concentrations of the four binders.

The detailed data for 7 days - exposure of the cell lines to different concentrations of the four binders are presented in Table 1, Table 2 and Fig. 3. At day 7, the picture was different especially with the higher concentrations of the binders for ROS cell lines. At 0.5% and 1.0% concentrations of the binders results showed a gradual reduction in cell activities. Exposure of ROS and L929 cell lines to a concentration of 1.0% of the clear binder showed no cellular activity, which indicates that these cells had died. However, the yellow binder and the magenta showed considerable cellular activity, with less toxicity of all concentrations on ROS cell lines and L020 cell lines. Furthermore, light microscopy revealed no evidence of live cells for the clear binder at 1.0% concentration and only a few cells were detected for the cyan binder at 1.0 % (Fig. 3).

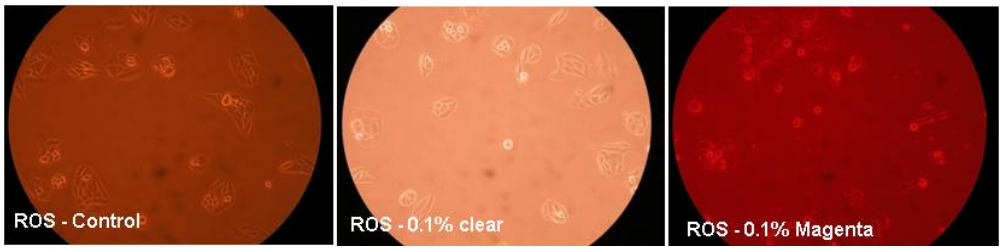

Figure 1: Light microscopy of ROS cell lines at day 3 for control well and 0.1% clear binder and magenta

Table 2: Alamar Blue assay for the binders on L929 cell lines.

Concentration	Control and Test Samples –Fraction				
	L929 - Day 3				
	Control	**Clear**	**Cyan**	**Magenta**	**Yellow**
0.1%	100	92.9	95.4	92.0	93.6
0.5%	100	94.2	96.1	94.1	94.0
1.0%	100	87.9	99.2	98.4	101.7
	L929 - Day 5				
0.1%	100	98.4	100.6	96.2	97.5
0.5%	100	69.6	83.7	86.5	85.5
1.0%	100	69.2	80.9	77.3	81.8
	L929 - Day 7				
0.1%	100	65.41	81.5	74.0	106.6
0.5%	100	36.3	56.2	75.3	82.3
1.0%	100	0	39.1	104.6	41.1

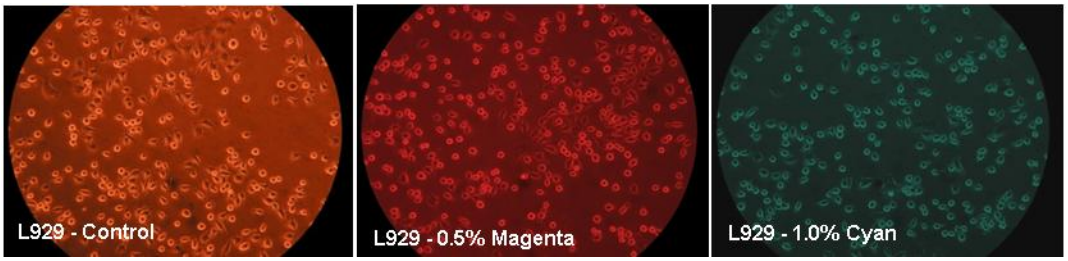

Figure 2: Light microscopy of L929 cell line at day 3 for control well and the binders at two different concentrations.

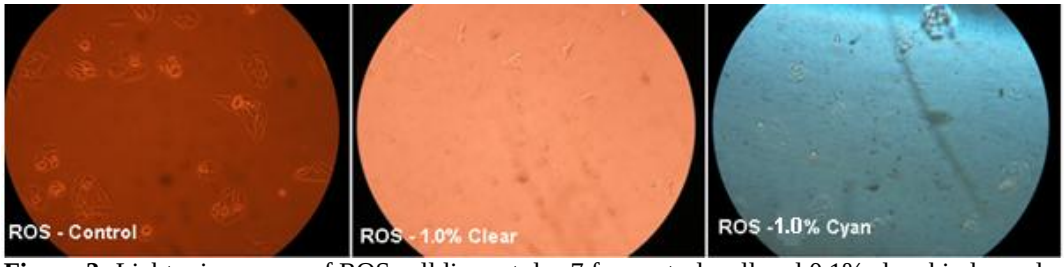

Figure 3: Light microscopy of ROS cell lines at day 7 for control well and 0.1% clear binder and cyan.

III-2 The powder:

Detailed information on the Alamar Blue assay is presented in Table 3 for the cytotoxic effect of three concentrations of starch powder 'ZP15e' (0.01 mg/ml, 0.005 mg/ml and 0.0025 mg/ml) on two cell line growth (ROS and L929) after 3 days, 5 days and 7 days exposure to these concentrations.

At day 3 no extensive effect of the three concentrations of starch powder used in this study on cell line growth of ROS and L929 to control groups 'cells only' was detected, however at day 5 and day 7 considerable reduction in cells activity particularly for the higher concentration (0.1) was recorded for both cell lines ROS and L929 as shown in table 3.

Table 3: Alamar Blue assay for 3 concentrations of starch on ROS & L929 cell lines at days 3, 5, & 7.

Days	Samples- ROS - Alamar Blue 4 hours			
	Control	0.01 mg/ml	0.005 mg/ml	0.0025 mg/ml
3	100	84.8	102.2	129.8
5	100	38.8	80.5	105.6
7	100	49.9	78.4	76.3
Days	Samples- L929 - Alamar Blue 4 hours			
3	100	93.8	111.4	119.1
5	100	62.8	81.4	83.7
7	100	61.7	88.6	80.2

IV. Discussion

The Alamar Blue assay was performed to determine the level of toxicity of the binders and the starch powder on two cell lines (ROS and L929) at different concentrations. These cells are derived to simulate osseous tissues and fibroblasts respectively and they are commonly used for Alamar Blue assay for evaluation of the cytotoxic effects of implant materials on human cells. L929 mouse fibroblasts have been widely used for biocompatibility assessment of dental materials because of their reproducible growth rate and biological responses [12] [13-14].

Alamar Blue is reduced by living cells through a mechanism whereby as cells start to grow, chemical reduction takes place in the growth medium and then the non-fluorescent dye 'resazurin' is changed to a fluorescent dye 'resorfurin'. Continued cell growth sustains a reduced environment and alters the oxidation reduction 'REDOX' indicator from an oxidized non-fluorescent blue colour to a reduced, fluorescent red colour [10-11]. The fluorescent signal is observed under 530-560 nm excitation wavelength and 590 nm emission wavelength, while the absorbance is monitored at 570 nm and 600 nm and the generated signals usually are comparative to the number of living cells in the specimen [15].

The result of Alamar Blue assay after 3 and 5 days exposure of the cell lines to three concentrations of four binders did not show toxic effects to any concentration of the four binders on the 2 cell lines used compared to the control cell cultures. However, some growth inhibitions were found at day 7, particularly for the clear binder and cyan. The reduction in cell vitality was dose-dependent and also correlated to the exposure times: the higher concentrations - 0.5% and 1.0% of cyan and clear binders showed the greatest reduction in vitality on ROS and L929 cell line growth at day 7. There was no evidence of cell activity/growth for 1.0 % clear binder at

day 7 for either ROS or L929 cells. However, no toxic effect for 0.1 % of all binders on both cells at the three timeframes was detected.

The printing binders are water based [16] and the infiltrants are maxillofacial SP, as medical grade silicone elastomers have already been used for decades in the manufacture of soft tissue prostheses; however, some traditional maxillofacial SPs have in some cases caused a degree of inflammation and irritation to the skin varying from mild to moderate [17-18].

Furthermore, starch powder has caused some irritation when used in other medical practices, such as lubrication of surgical gloves. Varying degrees of inflammatory reaction were reported in surgical wounds as a result of contamination of those wounds by the powder used on the surgical teams' gloves [19].

Several studies in vitro have investigated the ability of 3D printing technology to produce external and internal designs of scaffolds for tissue engineering and bone regeneration purposes using different types of starch based polymers and biodegradable powders combined with a water based ink [6] [20]. However, the main concern of the researchers was binder cytotoxicity, especially in the event of not all the binder being removed during post processing. Therefore, it can be assumed that any new material, or combination of materials, should be at least as biocompatible.

The above results can be summarized as follows: the investigations of Alamar Blue for the assessment of the binders and the starch powder revealed a slight toxicity at the higher concentrations after prolonged contact between the test material and the cell line growth. However, lower concentrations and shorter contact time between the test materials and the cell lines revealed normal cell activities.

The biocompatibility testing is a risk assessment, and the toxicity testing of these materials was done to determine if these binders are likely cause toxic response to the patient. Exposure of these materials to cells provided us with some information about the level of their toxicity and most materials at some concentrations produce a toxic response, no product is ever 100% safe. The question is it ever likely that the patient would be exposed to those source of levels or concentrations of the ingredients to cause a toxic response? It is unlikely that the patient will actually be exposed to the level of binder as used in these experiments was started causing toxic response. The binder is mostly binding to the starch particles so the chance to release from these particles is very low and even if it leaches out, it has to leach through the silicone. Therefore, the chances of coming out is very small, and it hasn't come out in the amount to cause a toxic reaction. Moreover the printed model is encapsulated in the silicone [21] it is unlikely to be in contact with the skin unless the materials start disintegration and tearing of the prosthesis takes place and in that case the prosthesis should be replaced anyway. Therefore, we believe that the risk of causing any toxicity to the patient is low. However, it is probably to carry out some experiments to reveal the amount of binder leaches out from the prostheses or doing a contact experiment with the skin by using skin patch test hold in contact with the skin over 24 hours time or more.

V. Conclusion

The binders 'ZB58' and the starch powder 'ZP15e' used by Z510 colour printer for production of soft tissue prostheses are found to be toxic only at higher concentrations and prolonged contact with the cell line growth. However, no evidence of toxicity was observed at lower concentrations and shorter exposure of the binders and the powder to the cell line growth.

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