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BIOCOMPATIBILITY ASSESMENT OF AZ91D/TCP Mg METAL MATRIX COMPOSITE

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The present work explores the novel AZ91D/TCP degradable composite with the inclusion of biologically essential trace minerals aluminum and zinc. The composites were synthesized by stir casting technique along with inert environment created by argon gas .It is estimated that the degradation products were self-possessed elements that have a functional role within human body, also it would be result in entire material reabsorption and supported to biological and enzymatic procedures. The biocompatibility of experimental AZ91D/2Wt% TCP composite is quantitatively and qualitatively assessed. Cytotoxicity effects of major degradation products are measured when used to L-929 cells in In-vitro.

1. Introduction

The main attractive features of biodegradable materials are their facility to serve as a temporary material for biological tissue growth and degrade thereafter [1]. This degradable feature is extremely preferred because it minimizes the necessity of frequent curative post-surgical actions which increases the possibility of patient complications. Presently, numerous metallic and polymeric materials possess a degradative quality appropriate for a biodegradable device. For example magnesium alloy composites, iron alloys and polymeric materials like polyesters for aliphatic hydroxyl acids (PHAs), polyglycolide (PGA), and polylactic-co-glycolide (PLGA) have been revealed to apt for these applications [2-5]. Though, the widespread application of these materials is reserved by natural challenges for each material, such as controlling and profiling degradation kinetics for magnesium alloy composites, iron alloys and polymeric materials [6]. Polymeric materials were originally desirable; however, they have yet to overcome the challenges of producing repeatable and accurate degradation kinetic profiles, long-term performance integrity, minimizing host immune responses, and costly time intensive research and development [5,6].

Past attempts made at developing of magnesium alloys for these applications include Mg-Zn, Mg-Zn-Zr and Mg-3Al-1Z. The addition of ternary elements like Zr, and Al to binary Mg-Zn alloy was made in attempts to control the corrosion rate of the alloys, refine grain structure and improve alloy strength [7, 8]. However, main fall of these alloy compositions is that the alloying elements had no efficient role within the body and are not entirely degradable by endogenous tissues [9]. Moreover, the degradation products of such alloys contain elements that had no practical responsibility within the human body and may possibly entail significant complications to the human excretory system once implanted in-vivo.

The present work explores the novel AZ91D/TCP degradable composite with the inclusion of biologically essential trace minerals aluminium and zinc. It is estimated that the degradation products were self-possessed elements that have a functional role within human body, also it would be result in entire material reabsorption and supported to biological and enzymatic procedures. Earlier studies have been expansively shown that aluminium and zinc has an important role in fighting free oxidative essential elements within the human body, reducing viral expressions, preventing toxicity and plays an important role in tissue growth [10, 11]. Additionally, Tri-calcium phosphate (TCP) has been utilized to treat diseases since the ancient

society of Hippocrates 400 B.C [12]. Calcium phosphate up to eighty percent present in bones and remaining presented in the muscles of human body. It is believed that the reinforcement of TCP particulates in AZ91D Mg alloy to form novel biodegradable composites with more constructive degradation kinetics required for support the tissue healing and growth. The reinforcement of TCP particulates has been shown that no harmful to the living tissues; however, it plays vital role for maintaining proper metabolic and neurological function. Thus, inclusion of only 2 wt% of TCP particulates in design of AZ91D Mg alloy matrix composite.

In current study, biocompatibility of experimental AZ91D/2Wt% TCP composite is quantitatively and qualitatively assessed. Cytotoxicity effects of major degradation products are measured when used to L-929 cells in In-vitro.

2.0 Materials and Methods

2.1.1Sample preparation

The composites were synthesized by stir casting technique along with inert environment created by argon gas. An ISO 10993-5 standard is for biological evolution of medical devices. Square testing specimens were prepared as per ISO 10993 standards having the dimension of 100mm length, 10mm width and 0.9mm thickness by using wire cut EDM. The test sample was mechanically polished with 1200 grit finish using silicon carbide abrasive paper. After mechanical polishing, the samples were cleaned with ethanol and blown by dry air. Material was stored in vacuum incubators for further testing. Before any biological testing, all samples were pre-vacuum sterilize.

2.1.2 MTT Assay

A cell proliferation MTT assay is used to determine the percentage inhibition of cell growth in extract solution of different concentrations. To achieve this, AZ91D/2Wt% TCP composite was immersed in 100µL of Dulbecco's Modified Eagle Medium (DMEM) in 24 hours at 37°C. This process is usually used in the biomedical industry to attain an extract solution in which the major toxic leachable from the material being tested is collected in a useable solution. This procedure simulates the short-term effects of material degradation under dynamic conditions. After 24hours of incubation, 100µL of test item, positive control, negative control and blank were added in duplicates to the plate. Test item is formulated into eight different concentrations (100%, 75 %, 50%, 25%, 12.5%, 6.25%, 3.12% and 1.56%) in DMEM complete medium. Cell lines were exposed to all (eight) concentrations and further incubated for 24h at 37°C, 5% CO_2 with >90% humidity supplement. For these experiments L-929 cells were allowed to reach confluency (70-80%) in T - flask from a semi-confluent monolayer with >90% humidity supplement.

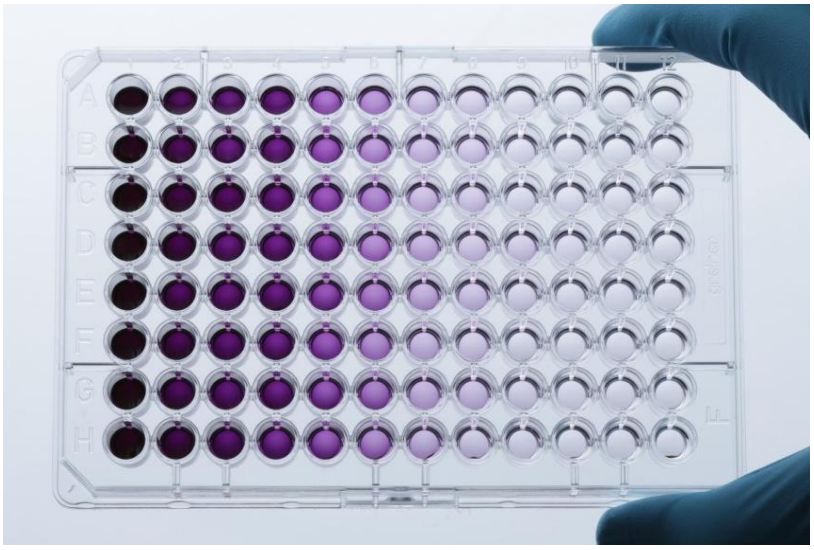


Figure 1 96 well plate for MTT assay

L-929 cells were plated with a density of 2 x 10^3 cells into a 96- well micrometer plate. The plates were incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. After 24hours incubation, cells were examined using phase contrast microscope to check the

morphological changes. Culture medium was removed from the wells, 50µL MTT solution (1mg/mL) were added to each well and the plate were further incubated for 2h at 37±1°C, 5% CO_2 with >90% humidity supplement. MTT solution was descended and 100µL isopropanol was added in each well. Absorbance in terms of optical density was measured at 550nm. The percentage of viability of cells treated with different concentration of AZ91D/TCP Composite Material and positive control were calculated from absorbance of control and respective treated wells.

2.1.3 Cytotoxicity test

The medium from the confluent culture flask was discarded and the cells were washed twice with sufficient volume of Phosphate Buffered Saline (PBS). Appropriate volume of Trypsin-EDTA (0.25%) was added to detach the cells. The cells were re-suspended in 2-5 mL of culture medium to inactivate the Trypsin-EDTA (0.25%) and centrifuged at 1200 rpm for 10 minutes. Test material extract were prepared by adding complete medium to the beaker containing weighed test material under sterile condition with gentle agitation at 37ºC for 24 hours. Preparation of Test material with complete medium is based on the weight of the test item 0.2g/ml. The sample contact surface posses 1.01 cm^2 and thickness of 0.09 cm. After 24 hours period of cell incubation, the specimens were placed on the surface of adherent fibroblast cells. After 24 hours period, the LDH concentration from each well containing cell was quantified using Promega membrane integrity assay.

Further expressly, after 24 hours period, the well plates were centrifuged for 2 minutes using an Eppendorf vacuum concentrator with an orbital speed of 1500 rpm. That was done due to remove any toxin particles resulting from cell lyses as a consequence of contact to the experimental composite. Wells containing pure culture media with cells and pure culture media without cells used as controls. After 24 hours period, lyses solution was added to control well to obtain maximum LDH concentration values, so that cytotoxicity percentages could be calculated. Complete medium alone, which does not have any Cytotoxicity effect on L929 fibroblast cell lines were used as negative control. Four different concentration of positive control Sodium Dodecyl Sulphate (SDS) such as 5mg/ml, 2.5mg/ml, 1.25mg/ml, and 6.25 mg/ml were prepared with complete medium and used. The percentage of cell viability was calculated by using following formula to determine the cytotoxicity percentage of the composite material.

Viability % = 100 x OD550e/OD 550b

Where, OD550e – Mean value of the measured optical density of the test item.

OD550b – Mean value of the measured optical density of the negative control.

Standard deviation (SD) $$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2}, \quad \text{where} \quad \mu = \frac{1}{N}\sum_{i=1}^{N} x_i.$$

Where, x_i = value of each observable
N = number of observables
µ = mean value (Mean = X1+X2+X3…XN/N)

% Coefficient of Variation (% CV) = (SD/Mean OD) x 100

From the direct contact assay, L929 fibroblast cells were scattered at a density of 1.3 x 10^5 cells in 96 well plate with culture medium (DMEM, 5% MTT, 10% Fetal bovine serum) containing the experimental composite. It was performed for characterize the any cellular morphological behaviour from being in direct contact with the experimental composite as well as to determine their cellular adhesion potential. Once the cells were scattered on the composite surface, the cellular growth was monitored for 24 hours period at 37°C with 5% CO_2 humidified atmosphere. After 24 hours period of growth, the samples were observed by Bio-Rad Microplate reder. The percentage of viability of composite was tailored to cover more than 70% in negative control. If the viability is lesser than 70% of the negative control, it shall be considered as cytotoxic potential.

3.0 Results and Discussion

The MTT assay results are presented in Table 1. It was observed that cells maintained 70% cell growth when cultured in eight different compositions extract solution from AZ91D/2% TCP composite. While the cell cultured in composite extract yielded the lowest percentage of cell growth at 100% composition. The AZ91D/2% TCP composite exhibits the lowest percentage of cell viability is 71% when culture in 100% extract solution. There is no statistical difference in percentage of cell viability for cells culture in the extracted solutions observed on the composite.

Table 1 Optical density and percentage viability

ID	Conc. (mg/mL)	OD I	OD II	Mean OD	SD	%CV	%Viability		Mean %Viability
							I	II	
Blank	NA	0.0326	0.0319	0.0323	0.0005	0.0153	NA	NA	NA
NC	NA	0.0941	0.0934	0.0938	0.0005	0.0053	100	100	100
PC1(5mg/ml)	5	0.0388	0.0395	0.0392	0.0005	0.0126	41.23	42.29	41.76
PC2(2.5mg/ml)	2.5	0.0394	0.0399	0.0397	0.0004	0.0089	41.87	42.72	42.29
PC3(1.25mg/ml)	1.25	0.0422	0.0428	0.0425	0.0004	0.0100	44.85	45.82	45.34
PC4(0.62mg/ml)	0.62	0.0548	0.0534	0.0541	0.0010	0.0183	58.24	57.17	57.70
TC1(100%)	100	0.0661	0.0672	0.0667	0.0008	0.0117	70.24	71.95	71.10
TC2(75%)	75	0.0718	0.0711	0.0715	0.0005	0.0069	76.30	76.12	76.21
TC3(50%)	50	0.0732	0.0739	0.0736	0.0005	0.0067	77.79	79.12	78.46
TC4(25%)	25	0.0767	0.0754	0.0761	0.0009	0.0121	81.51	80.73	81.12
TC5(12.5%)	12.5	0.0772	0.0784	0.0778	0.0008	0.0109	82.04	83.94	82.99
TC6(6.25%)	6.25	0.0794	0.0786	0.0790	0.0006	0.0072	84.38	84.15	84.27
TC7(3.12%)	3.12	0.0803	0.0811	0.0807	0.0006	0.0070	85.33	86.83	86.08
TC8(1.56%)	1.56	0.0844	0.0836	0.0840	0.0006	0.0067	89.69	89.51	89.60

Particularly, there were a higher number of cells viable after MTT assay test in different compositions. The tested sample treated cells showed that cell viability decrease when compared to negative control but their percentage of viability is more than 70%. Cell viability of positive control seems to be low when compared to the negative control and their percentage viability was lesser than 70% to negative control.

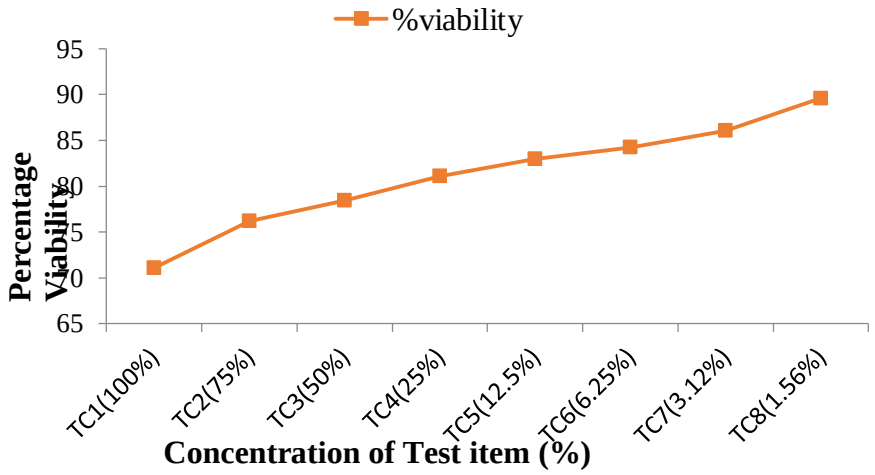


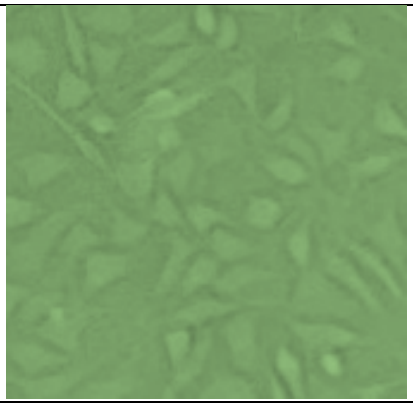
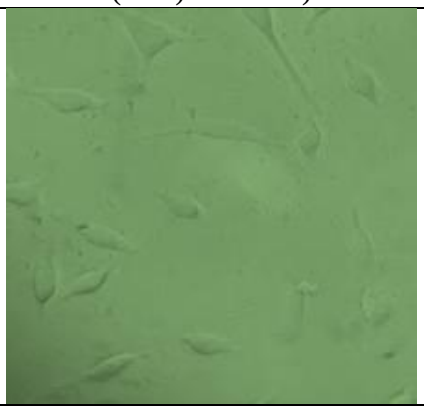
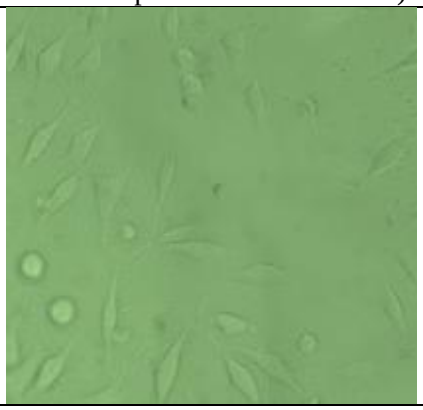
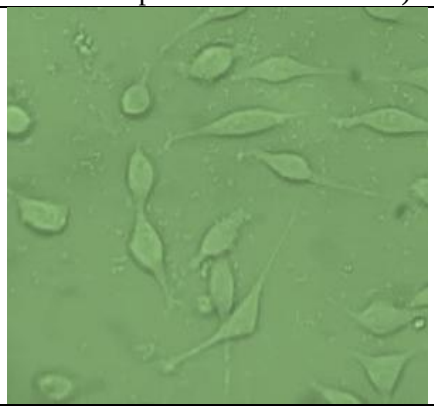
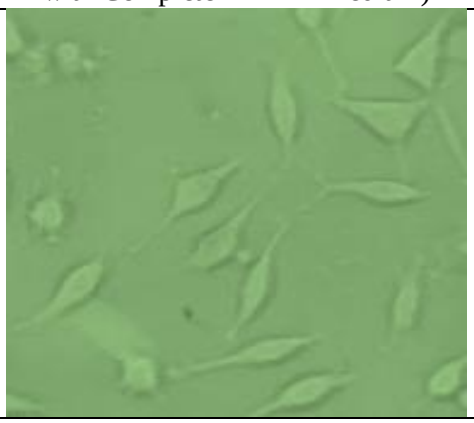
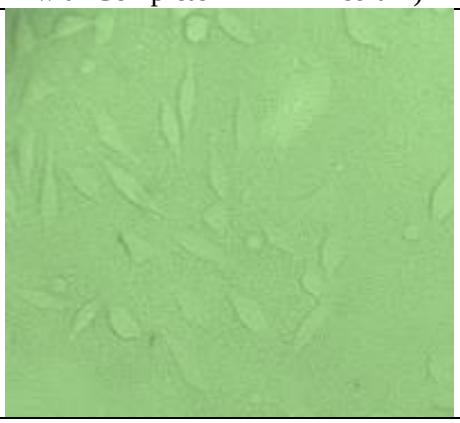
Figure 2 Percentage Viability against eight Concentration of test item.

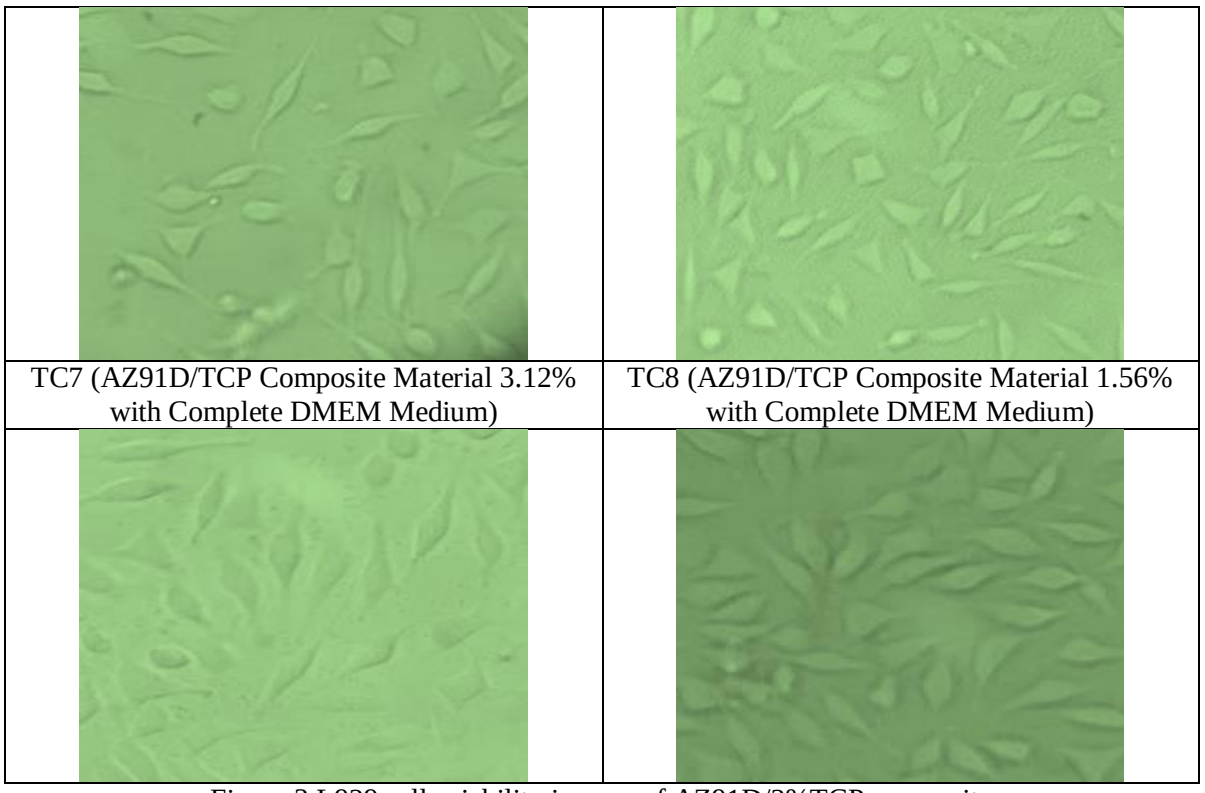
Figure 2 present the cell viability of L929 fibroblast cells when cultured in eight different concentrations after one day of immersion. Results collecting from MTT assay and cell viability was calculated relative to control where fibroblast cells were grown in 100% in media. Cell growth is not statistically different between extract concentration solutions per day.

The cytotoxicity effect of elements leached from the novel biodegradable AZ91D/2%TCP composite was quantified using various In-vitro biological assay tests. Those results were compared with base AZ91D mg alloy which has been exhibit degradation rates suitable for wound healing with attractive biocompatibility with host tissues [13]. Principally, a Promega MTT assay was used to done the experimentation by exposing the L929 fibroblast cells to the degradation products released from the fabricated composites. L929 fibroblast cells were viable in AZ91D/2%TCP composite by the various extracted solutions, while 70% of cell growth obtained by negative control, whereas less than 70% of cell growth was observed in the positive control. The percentage of cell growth can be explained by the presence of Tri-calcium phosphate reinforcement in the matrix material, which can lead to control the toxicity in the living tissues [14]. This effect was augment by nutritional supplement present in the cell culture medium through the addition of fetal bovine serum and penicillin respectively. While the toxicity of TCP reinforcement was limitedly published the necessity of TCP particulates as essential trace mineral serving as an integral role in biological role. Moreover, there is no other alloy composition yielded completely non cytotoxicity when cultured in 100% extract media. The encouraging quality for those experimental degradable magnesium composites, because it have been reported that degradable polymers like polylactide materials were resulted in cellular cytotoxicity because of the dramatic changes in pH caused by degrading polymers [15]. Even though, the use of magnesium composites for orthopedic applications is growing field, the concerns of cytotoxicity is not as great concern while used as biodegradable biomaterials, as research shown that cytotoxicity effects are minimal [16, 17]. However, it should be noted that the lack of cell growth on the degradable composites may make it difficult to achieve periosteum on the surface of the bones.

Cytotoxicity test presented that L929 fibroblast cells culture in straight contact to the AZ91D/2%TCP composite showed the lower percent of cytotoxicity. That means more than seventy percent of the cells in straight contact to those investigational composites survived as show in figure 3. Nevertheless, the data as of the direct contact assay evaluating the cell sticking together on the surface of the materials. Haun and Keim et al., stated that the lack of cell growth on the work surface is common for various types of magnesium alloys and is accredited to the huge amounts of hydrogen evolution from magnesium alloys which noticeably increase the pH levels contained by the instantaneous surrounding area of the magnesium implants which prevents cellular bonding [18, 19]. This is supported those physical observations of cell culture media with the AZ91D/2%TCP composite samples changing color from being red to faintly yellow mostly clear. The color change is possible only if the pH of the solution change from neutral or basic

environment to more acidic environment with a pH less than 6.8. Carrying out tests on the AZ91D/2%TCP composite exhibited that the complete material corroded within the experimental time period, thus no cell bond or toxicity amounts might be quantified.

Negative Control (DMEM Medium alone)	Positive Control (Sodium Dodecyl Sulphate (SDS) treatment)
	
TC1 (AZ91D/TCP Composite Material 100% with Complete DMEM Medium)	TC2 (AZ91D/TCP Composite Material 75% with Complete DMEM Medium)
	
TC3 (AZ91D/TCP Composite Material 50% with Complete DMEM Medium)	TC4 (AZ91D/TCP Composite Material 25% with Complete DMEM Medium)
	
TC5 (AZ91D/TCP Composite Material 12.5% with Complete DMEM Medium)	TC6 (AZ91D/TCP Composite Material 6.25% with Complete DMEM Medium)



TC7 (AZ91D/TCP Composite Material 3.12% with Complete DMEM Medium)	TC8 (AZ91D/TCP Composite Material 1.56% with Complete DMEM Medium)

Figure 3 L929 cells viability images of AZ91D/2%TCP composite

Based on van der valk and Baier et al., studies the material that had low surface energy presents the low cell adhesion and, on the alloy, and composite materials [20]. It is significant that the fabricated materials were conducted In-vitro tests, which is not an exact presentation of an implant material. Of course, a novel material is implanted In-vivo, the fluid environment may not be static, and the fresh nutritional body fluids may create an environment which capable of sustains cell growth and viability.

4.0 Conclusions

1. In-vitro experimentation to assess the biocompatibility of novel biodegradable AZ91D/2% TCP composite.
2. The present work is the first evolution of these biodegradable composites and it has potential to support as orthopedic implant biomaterials.
3. The percentage of cell growth is exposure in this composite examined by MTT assay using L929 fibroblast cells.
4. The percentage viability of AZ91D/TCP Composite Material at all tested concentrations was more than 70% when compared to negative control.
5. AZ91D/TCP Composite Material at tested concentrations was found to be non-cytotoxic in L929 cells in terms of viability under experimental conditions.

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