INTERNATIONAL JOURNAL OF HIGHER EDUCATION AND RESEARCH
IJHER, Vol., 9(2), 2019, 127-142.
www.ijher.com
IJHER (ISSN 2277 260X)
CODEN:IJHER
ORIGINAL ARTICLE

EFFECT OF CENOSPHERE SIZE AND VOLUME FRACTION ON CELL GROWTH KINETIC OF EPITHELIAL CELL LINE KB EMBEDDED IN TITANIUM -CENOSPHERE FOAM

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ABSTRACT
Titanium foam prepared with various porosities (50% to 80%)by using cenosphere (sizes 90.0±8 to 212±18) as space-holder through powder metallurgy route.The synthesized Titanium foams used as biomaterial to enquire its mammalian cell viability by using KB mouth epithelium cell line supplied by NCCS Pune (Maharastra,India). KB cell line maintained in optimum required medium, temperature and environment by using CO_2 incubator.The influence of micro-porosity and space holder on cell viability has been examined for a time period of 24hrs.,48 hrs. and 72 hrs.It has been observed that foam made with 50%,60% and 70% porosity showed better In-vitro mammalian cell viability as compared to 80% porosity.Before the cell viability experiment on titanium foam all essential examination performed like Micro-structure(through SEM,and FESEM), mechanical property and corrosion resistance.The Optimum cell size and volume fraction of pores which present in Titanium foam for the best cell viability noted to be 70% porosity and 212µm cenosphere size.
Key words :*Titanium foam, Cenospheres, mammalian Cell viability, MTT assay, biocompatibility.*

INTRODUCTION

In the human body, various types of bones do collective works and maintain the body structure. Unfortunately, due to any damage or decay, either through accidental or any disease could occurs.Cure is possible in this era. Right now there are many researches occur in this regard to cure /treatment on the fracture.Some accidental fractures can be curable automatically.Some are curable through the use of implant material. In medical field,use of metal implant is in practice. This research focused on the foam form of the titanium powder. Titanium is a well known bio materials (Prasad *et al*., 2017). The foam of titanium works as a bone-metal integration (Prakasham *et al*.,2017) also known as osseointegration (Manivasagam, Dhinasekaran & Rajamanickam, 2010) when it's attached to the bone. Here, porosity of the foam plays the key role (Saini *et al*., 2015).Through the porous portion of the titanium, live cells of the bones enter and expanded conjointly this interlink performed very well. Researchers have faith in the integration characteristics. Although solid metal is in use, right now, in the medical field, but like the porous material integration not occurred. In this experiment also found the solid metal of titanium showed not good integration with mammalian cell line to compare to porous titanium foam.Experiments on titanium foam with different porosities gave the clue for the right choice of porous bioimplant (Oliveira *et al*.,2008). This is also a big challenge because without the knowledge of the suitable porosity, the correct material cannot be insert into the body (Ozawa & Kasugai,1996). Although, in ancient time, implants was in practice;but through mid 1980's, it is steadily becoming more useful. In this aspect many scientists and research group worked on it (Pal ,2015; Chena & Liuc, 2016; Corrales, Esteves & Vick, 2014).A research group of Germany announced the development of resobone (http://www.ilt.fraunhofer.de/En/press/Press-release-2010/press-release-2010-05-20.html) to replace the all traditional implant for skull. They synthesized the new type of implant by using polyurethane. This substance will be the own part of the human skull (Khatoon & Ahmad,2018). The other subject of such invention they explained that traditional implants takes time to heal and doctors does not permit to heavy work after implantation (Annibali *et al*.,2008). Another doctor named Dr. Jeremy Mao (http://www.prescouter.com/2015/07/growing-new-teeth-in-the-mouth-using-stem-cell-dental-implants/) synthesized the dental scaffold through micro channeled natural material inculcated with a growth factor (Bhola,Bhola & Mishra,2011). He studied in the animal model and got very positive results.He placed this new type implant in recipient mouth and then transferred it to him and he found in its very own tooth like structure causes of stem cell.He also explained his experiment as cell homing based tooth regeneration.Several experiments performed with the Implant materials like Titanium (Xuan *et al*., 2018), Steel (Perren, Regazzoni & Fernandez, 2017) last decade (Mondal *et al*., 2012).Scientists are searching the best hope in this regard.The main reason behind these researches are that all metals are heavy and anyhow can not resist inside the body for a long time, so there is the need for improvement in this field (Jha *et al*., 2013).The implant can reside inside the body without any spoilage and can perform as the part of the body which is the good character of the any implant (Fleck & Eifler, 2010).But,most implants have not this particular quality.In this series, we attempt the synthesis of Titanium syntactic foam through powder metallurgy route (Dolder *et al*., 2003), using cenosphere as space-holder which can be further useful as implant.Prepared titanium foam showed no corrosion characteristic, while the experiment . This foam has the micro hole which was connected with microbaloons to transfer the liquid media (as Biofluid) during the animal cell culture experiment.The optimized foam architecture is identified for giving best cell viability.

MATERIALS AND METHODS

As a primary material, Titanium metal powder (99.9% pure,average size 22±3µm) was used which supplied by Alfa Aesar. As spaceholder various type of cenospheres(average size) presented in Table 1 were used.

Table 1 Average cenosphere Sizes (2r) and cenosphere shell thickness (t) used in this study

Cenosphere Grade	Cenosphere Size (2r) (µm)	Cenosphere Shell Wall thickness (µm)	t/r
C1	90.0±8	4.8±15	0.1066
C2	145.0±11	7.1±1.3	0.09793
C3	185.0±0.15	9.0±1.4	0.09729
C4	212.0±18	10.4±1.8	0.0967

Cenosphere supplied from M/s Cenosphere India showed it microstructure in Figure 1.

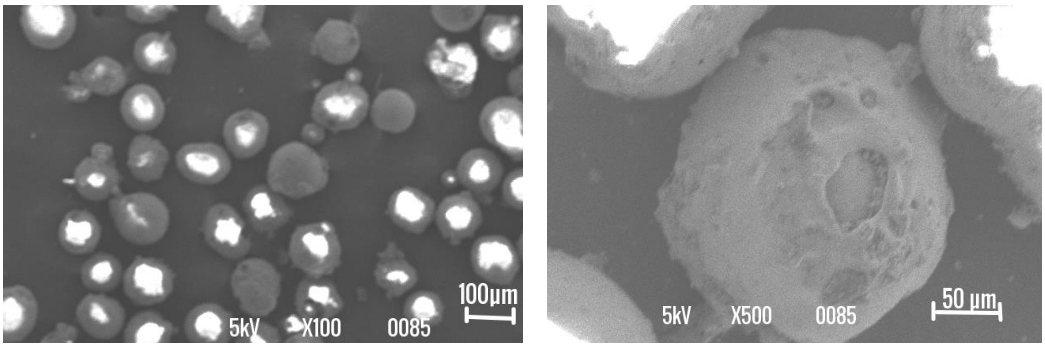


Figure 1 Microscopic Image of Cenosphere

Titanium metal powder mixed with spaceholder,in which organic binder (2 weight % of diluted Poly Vinyl alcohol weight % Ploly Vinyl Alcohol powder and 95% water) was added. Before pouring this titanium and space holder mixture in the die 1 weight % Zinc Stearate powder added during the cold compaction process this powder enhanced the flow ability.Titanium powder and space holder mixed in pre-calculated proportion as per their respective porosity percentage.75 MPa pressure was applied during the cold compaction for two minutes as shown in flow chart 1. A cylindrical dye was used for cold compaction .The flow chart followed for making Ti-cenosphere porous titanium is schematically shown in Figure 2.

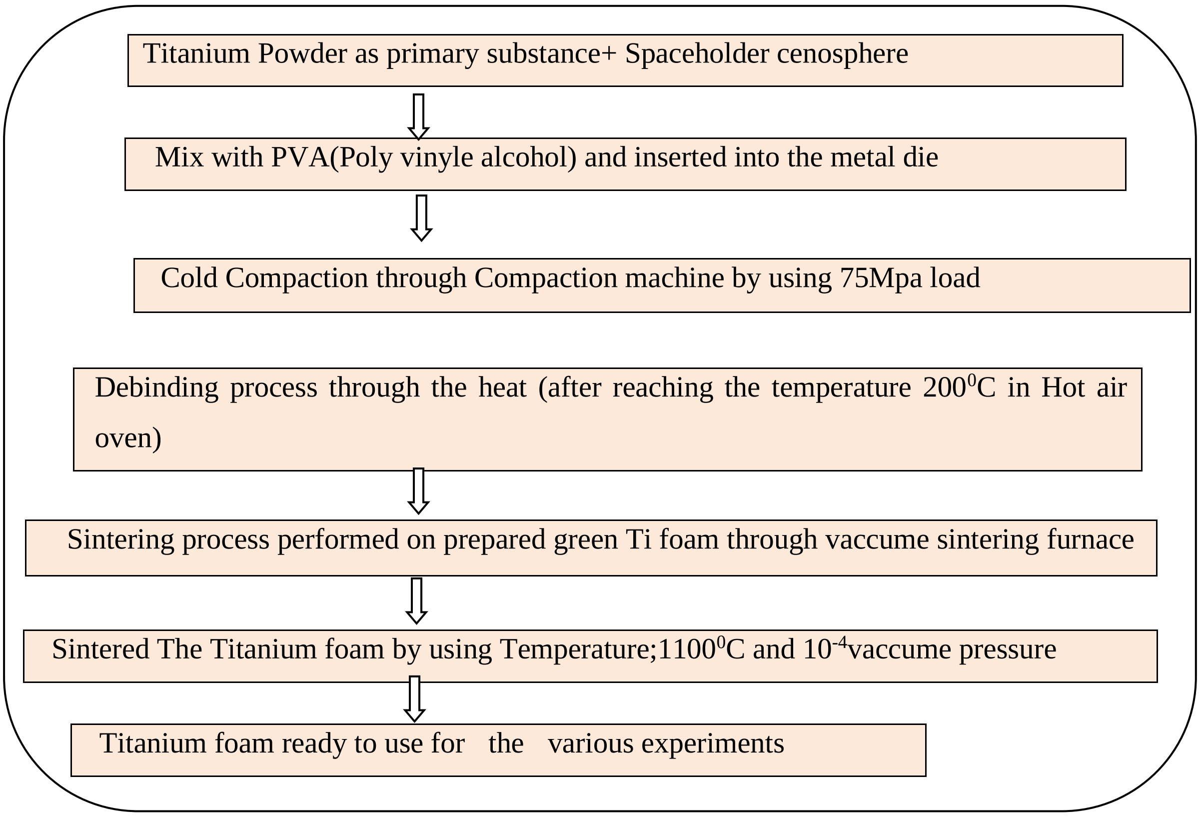


Figure 2 Flow chart 1 To process the Titanium Foam preparation through Powder Metallurgy path

The green sample after cold compaction is placed into the hot air furnace after reaching the temperature 200^0C for two hrs. so that debries can evict from the green sample followed by 2 hrs heating at 400^0C. After that sintering process performed through vaccume sintering furnace (Vaccume Tech, Bangalore) at 1100^0C temperature and 10^{-4}mbar vaccume. At the time of sintering,green samples are placed in the closed stainless steel duct to protect from the graphite evaporation (Villa *et al.*, 2002).The sintered Titanium foam pallets (Figure 3) sliced into 2 mm thickness sample by using slow diamond cutter (LECO VC 50 which wheel size was10 mm plate) for different experiments.

Figure 3 The sintered pallets of Ti -cenosphere foams are shown in this figure.At higher magnification the presence of cenospheres in the pallets are noted additionally,no surface cracks or delamination within these sintered pallets are observed.This also indicate effective sintering

These slices of sintered titanium pellets samples marked for their micro structure,phase constituents,compressive deformation behavior,porosity/density examination.The prepared Titanium foam samples are polished through standard metallographic practice and etched through Keller's reagent(20ml distilled water,70% concentrated HNO_3 20ml, 38% concentrated HCl 20ml HCl and 40% concentrated HF 20ml.The micro structure of titanium foam observed through FESEM (Nova Nano Sem 430).The EDX is carried out through IE Synergy 250,with a detector of $50mm^2$ competent to detect beryllium and above for elemental analysis of the foam.The density of the titanium pellets are determined through Archimedes principle and porosity has been calculated through its theoretical and measured density.The compression behavior of the titanium foam sample was observed by using Universal Testing machine (model, Instron 8801) at a strain rate of 0.01/s at room temperature.Further,corrosion behavior of Titanium foam sample is carried out.We used balanced salt solution i.e. Hank's solution to perform this test. The composition of solution is shown in Table 2 weight loss measurement technique is used to determine corrosion.

Table 2 Composition of the Hank's solution

S.No.	Name of the component	Quantity
1	$CaCl_2$	0.185g/l
2	KCl	0.4g/l
3	KH_2PO_4	0.06g/l
4	$MgCl_26H_2O$	0.1 g/l
5	$MgSO_4H_2O$	0.1 g/l
6	$NaHCO_3$	0.35 g/l
7	Na_2HPO_4	0.48 g/l
8	D-glucose	1.00 g/l
9	Nacl	8 g/l
10	Distl Water	1000 ml

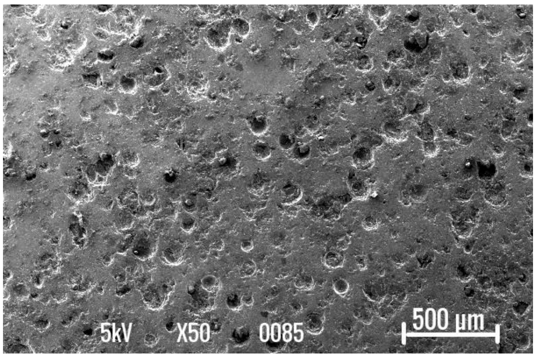
In-Vitro trial

Mammalian cell line named KB by The National Centre for Cell Sciences,Pune,Maharastra India has been used for this study. This cell line is mouth epithelium cell line and is developed in cell culture flask T-25 with media i.e. essential media, MEM (Minimal Essential Media) (supplied from Himedia AT154) supplemented with 10% Fetal bovine serum (supplied from Himedia RM10) and antibiotic 10mg/ml of gentamicin sulfate (supplied from Himedia TC026) incubated in CO_2 incubator (Nuaire, NU5510E). For experiment,the three plates are prepared for 24hrs ,48hrs and 72hrs.In this process 2ml cell line inoculated into the 24 well cell culture plate.These plates are incubated for 24 hrs so that cells can develop ubiquity in 24 cell culture plates. In First well of the cell culture plate,these was only control, in which only cell line was present, while, other wells included the test samples of Titanium foam (2mm in size) with media supplemented with appropriate amount of antibiotic (gentamicin). That the plate after 24 hrs,48 hrs and 72 hrs were observed through an inverted microscope (Magnus Invi). Cell viability of KB cell line examined through aliquot of viable cell line was mixed with equal amount of trypan blue dye and counted through hemocytometer. Colorimetric observation of viable cell performed through MTT Assay(supplied from Himedia CCK003).

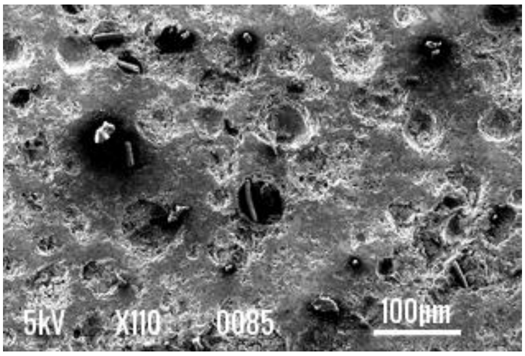
RESULT AND DISCUSSION

Micro-structure

The micro structure of Ti-cenospherems as shown in Figure 4(a) represents the micrograph of Ti-50% cenosphere.It may be noted that the cenosphere are distributed uniformly within the Ti-matrix.Ti powder are fused completely and formed Ti-matrix.The micrograph of Ti-60% cenosphere foam Figure 4(b) also uniform distribution of cenospheres. The cenosphere content increase when one used 80% cenosphere in Ti-cenosphere foam Figure 4(c). At higher magnification it may be noted that Ti-cenosphere bonding its quite sharp and strong Figure 4(d) will be useful for transfer and to obtain higher strength and modulus.



4(a)



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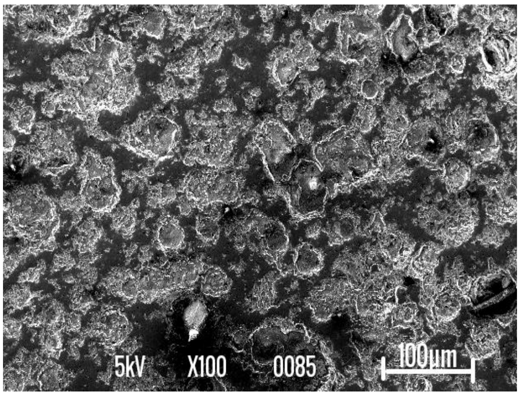

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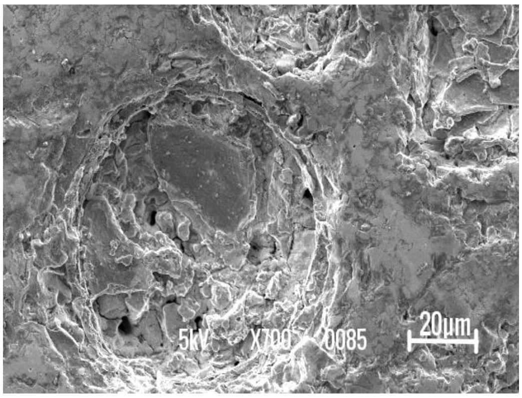


4(d)

Figure 4 Microstructure of Titanium 70% Cenosphere foams:(a) 90.0 ± 8 µm(b)145.0 ± 11µm (c) 212.0 ± 18 µm and (d) Higher magnification micrograph showing micro voids in cell wall and boding of cenosphere with titanium matrix

Mechanical properties

Strength and modulus of Ti foams

The strength and modulus of these investigated foams were determined from compression tests and dynamic elastic property analyses respectively. The yield strength of the foams is reported in Table 3. It is noted that the strength of these foam values in the range of 43.06 MPa to 108.60 MPa and it varies with cenosphere size and cenosphere content. The strength is noted to be decreased with increase in cenosphere size and cenosphere content. However, by controlling the cenosphere size and volume fraction one can make Ti foam which can satisfy sequence strength of equivalent to the bone. Similar a results are observed in case of modulus also.

Table 3 Cenosphere volume fraction, density, peak stress of different Ti-cenosphere foam

Cenosphere size	V_f (%)	ρ_f ±3% (gm/cc)	σ_p ±5% (MPa)
C1	50	2.85±0.0855	74.79±3.73
	60	2.48±0.0744	63.14±3.157
	70	2.26±0.0678	46.59±2.3295
	80	1.65±0.0495	43.06±1.2918
C2	50	2.85±0.0855	131.08±6.554
	60	2.48±0.0744	108.60±5.43
	70	2.26±0.0678	78.95±3.94
	80	1.65±0.0495	30.33±1.51
C3	50	3.05±0.0915	69.08±3.45
	60	2.46±0.0738	52.44±2.6
	70	2.41±0.0723	44.25±2.2
	80	1.99±0.0597	43.88±2.2
C4	50	2.99±0.0897	75.10±3.6
	60	2.8±0.084	43±3.7
	70	2.68±0.0804	68.05±3.4
	80	2.31±0.0693	62.48±3.1

The modulus of foam also varies in the range of 10GPa to 38 GPa which are equivalent to that of cortical bone in different locations (Novitskaya *et al*.,2011). This is because of grater extent of inter particles micro porosities.The size of micropores ranges from 0.3µm to 5µm Fig.4(d). This will help in flow of bio fluid and which can supply the nutrients to the bone cells. In case of Ti foams, the strength and modulus can be controlled by controlling volume fraction of space holder used or the porosity within foams. As cenosphere size increases,stress become localized,interface and cenosphere distance increasing. These causes volume of cenosphere cell at lower load and the Ti matrix deforms more easily. Finally those lead to increasing of strength and modulus.Compressive stress in MPa arranged according to volume fraction and cenosphere sizes mentioned in Table 3.

Corrosion behavior

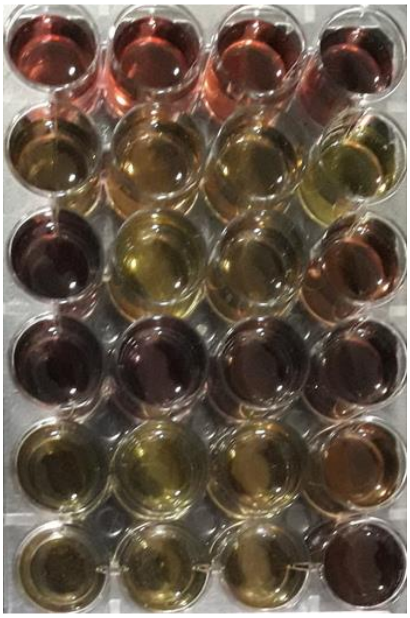
The corrosion behavior of the Ti foams studied by using emersion test of the foams.The foams were examined under simulated bio fluid(Hank's solution,composition mentioned in Table 2). The corrosion rates calculated on Titanium foam with various volume fraction of cenosphere and sizes are reported in Table 4. The corrosion rate on these foams are measured after 30 days of interval upto 120 days. It may be noted that foam samples exhibit very negligible weight loss or gain as mentioned in Table 4. Very marginal deposition is found after 30 days and 120 days in foams with 50% volume fraction of cenosphere.Titanium foam with 70% and 80% cenospheres also observed marginal deposition. This is found due to the formation of calcium hydroxy phosphate on the cell walls.This indirectly indicate that these foam samples are bio- inert and may be used for bone scaffold or bone replacement.In view of this, cell viability on these foams are examined which are stated in subsequent section.

Table 4 Corrosion rate as per the volume fraction of the titanium foam after 30 days, 60days, 90days and 120 days

Cenosphere size	Volume Fraction	Corrosion Rate (mm/Y)			
		30days	60days	90 days	120days
	50%	-0.014±0.001	0.277±0.012	-0.006±0.005	-0.018±0.001
+212μm	60%	0.046±0.003	0.115±0.013	0.023±0.001	0.0029±0.001
	70%	-0.008±0.001	0.282±0.014	0.004±0.003	0.010±0.001
	80%	-0.051±0.004	0.438±0.032	-0.017±0.001	-0.0344±0.002

Cell viability

The cell viability test of the Titanium-foam pellets (2mm thickness) are carried out by using Mammalian mouth epithelial cell line KB (Müller *et al*.,2006)(supplied by National centre for cell science, Pune,Maharashtra,India) followed by the colorimetric test using MTT Assay (Gupta *et a*l.,2006; Moradi *et al*.,2018) Figure 5(a). The cell viability of different Titanium-cenosphere foam samples studied in comparison to control upto 72 hrs at an interval of 24 hrs using 10^5 cells/well. The viable cells cultivated into the Ti-cenosphere foam; images obtained through inverted microscope shown as Figure 5(b), 5(c) and 5(d) at different magnification i.e.10X,20X and 40X respectively after inoculation of MTT assay solution. MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide contained the tetrazolium dye which shows the colour after the reduction .



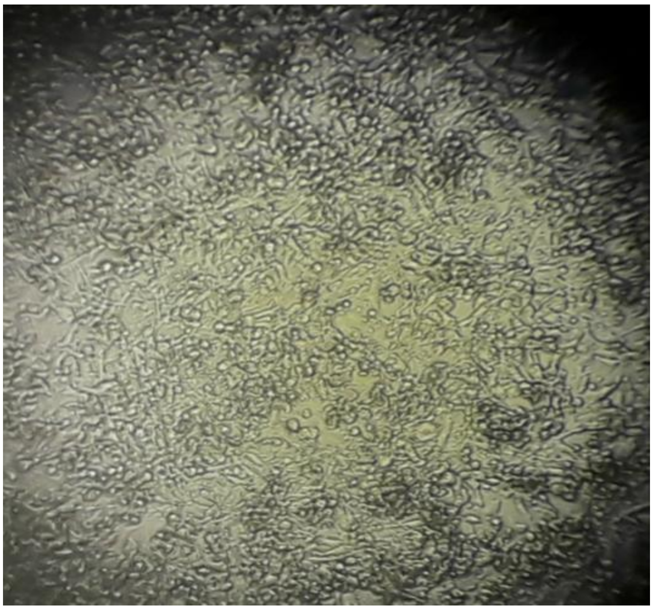
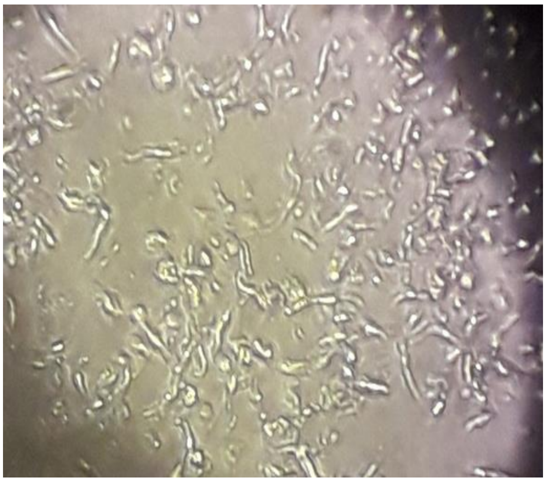
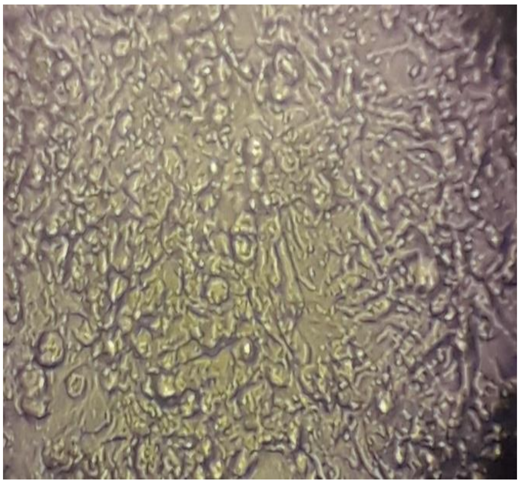


Fig.5(a) 5(b)





5(c) 5(d)

Fig.5 (a) MTT Assay Experiment (b)Microscopic image of viable cells during MTT Assay at 10X magnification (c) Microscopic Image of viable cells at 20X magnification (d) Microscopic Image of viable cells at 40X magnification

This assay works on the concept of the cell metabolic activity. Here, the control is considered as the solution on which only cell lines are put and no foam samples is added. After every 24 hrs, the cell viability on the foam samples were conducted as compared to the control. Table 5 reports the cell viability of foam samples as a function of cenosphere size, volume fraction and exposure time. It is noted that the cell viability after 24 hrs is considerably lower.

But,interestingly after 48 hrs it improved and after 96 hrs the cell viability is quite good. It is further noted that the cell viability is a strong function of cell size (cenosphere size). It may further be noted the best cell viability is observed when the cenosphere size is 100 μm irrespective of volume fraction of cenosphere. Even, in this case, the cell viability is better than that in case of pure and dense Ti. This is suspicious. However, this may be due to the following facts. When foams (porous body) in the solution is added, the cells of the cell lines may be inoculated at the cell walls and thus the effective number of cell in the media is reduced. The surface area of cell walls are quite high. It may be 100 to 200m^2/m^2. As a result, in the initial period the cell density over the surface of specimen or on the solution get reduced. In due course, the cell grows and more cells covers the foam surface as well as come into the media. This lead to higher cell viability after 48 hrs and it continue to grow upto 72 hrs. During this period, the cell grows further and covers much higher surface and cell viability improve significantly.This also indirectly demonstrates cell adhesiveness and excellent cell viability of foam samples. The cells grows into the titanium foam along with cenosphere boundries are shown in Figure 6.

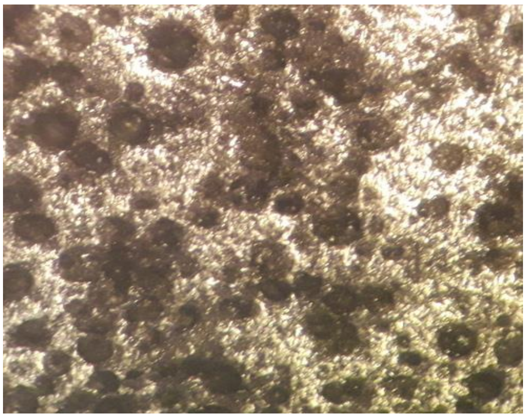


Figure 6 Microscopic image of live cells grows into the Titanium foam

This figure also showed that the cells are adhered well with the titanium foam. But, when the pore size increase the surface area become less.The incubation of cells within cenosphere boundary is reduced and exposed mostly within the media. As a result the cell viability is reduced at coarser cenosphere size and higher volume fraction of cenosphere. The present results demonstrate the fact that Ti-cenosphere foam could be used for bio-implant application. Its corrosion resistance is excellent and some kinds of Calcium-Phosphorus deposition during immersion test is also noted. This indirectly states that these titanium foams l can be helpful in osseointegration damage bone. Further study in this line need to be carried out. From these observations, it may be noted that foams with all four cenosphere size will be suitable for bioimplant. In order to have reasonably good strength and modulus, it is expected to have foams with porosity (60-70%) with cell size of 100µm to be the most suitable.

Table 5 Cell viability as a function of cenosphere size, volume fraction and exposure time

Volume fraction(%)	Cenosphere Size (µm)	Cell Viability (%) after different exposure time (hr)		
		24hr	48hr	72hr
50	+212±18	36.66	38.46	58.64
	185.0±15	39.45	50.50	65.38
	145.0±11	65.63	51.25	65.38
	90.0±8	41.66	59.5	96.15
60	+212±18	38.54	50.54	68.75
	185.0±15	16.66	59.83	63.85
	145.0±11	38.60	57.46	96.30
	90.0±8	14.58	52.37	96.87
70	+212±18	80.20	80.20	86.15
	185.0±15	46.15	50.65	86.15
	145.0±11	65.63	50.17	90.50
	90.0±80	33.33	50.13	96.50
80	+212±18	15.18	50.00	53.85
	145.0±11	45.83	52.08	65.38
	90.0±08	50.22	65.36	96.15
Pure Titanium		50.50	52.37	76.92

CONCLUSION

Cenospheres a thermal power plant waste can be treated and then used for making successfully Ti-cenopshere foams. The strength and modulus of these foams are comparable to that of cortical bones. By varying the cenosphere size and volume fraction the strength and modulus of these foams could be varied and made tailor to different bones. The corrosion rate of these foams in simulated biofluid (Hank's solution) is very low and suitable for bone implant applications. The deposition of phosphate product over the foams may be helpful for bio activeness with bone. The cell viability in these foams are quite good which further confirms its applicability for bone scaffold and bone replacements.

ACKNOWLEDGEMENT

We are thankful to Late Dr. Vinod Singh former HOD & Associate Professor UTD Microbiology Department, Barkatullah University Bhopal (M.P.) also thankful to Dr. Anil Prakash present HOD,UTD Microbiology Department, Barkatullah University Bhopal (M.P.) India.We also thankful to Director, CSIR-AMPRI Bhopal to pursue this project. This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

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