Development of Biomimetic Strontium Incorporated Nanostructured Ceramic Coatings on Cp-Titanium for Orthopaedic Implants

DEPARTMENT OF BIOTECHNOLOGY

BT/PR3171/NNT/28/564/2011, **27.05.2016**

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Objectives

Development of Biomimetic Strontium Incorporated Nanostructured Ceramic Coatings on Cp-Titanium for Orthopaedic Implants

Methodology

Fabrication of TN and TNS material,

Commercially pure titanium (Cp-Ti) cylindrical specimens of diameter 2 mm and length 6 mm was mechanically grinded using 80 to 600 grit silicon carbide paper followed by alkali treatment (10 M) for 24 h to increase surface roughness and to attain a uniform single- and double- layer coatings. The single layer mixed metal oxide in TN was formed by hydrolysis and condensation method. Briefly, the mixed metal oxides of Titanium dioxide (TiO_2) and Niobium Pentoxide (Nb_2O_5) were prepared in a sol state using Titanium isopropoxide (TIP) and Niobium (V) Ethoxide (NE) as precursor's in equal proportion [25.26,27]. Isopropyl alcohol (IPA) was used as the carrier and solvent for the metal oxide preparation. Acetyl Acetone (ACA) was added as an initiator of slow hydrolysis. Polyethylene glycol was added as a stabilizing agent to facilitate gelation. Both the metal oxides were used in equal proportion. The temperature was maintained uniform throughout the reaction at 80°C. The reaction time was for 3 h and allowed for gelation for another 24 h. The stochiometric proportion of TIP:NE:ACA:IPA:PEG is 1:1:1:20:0.5 ratio.

Sr-HAP was prepared by co-precipitation method, briefly the calcium, phosphorous and strontium precursors were calcium nitrate, sodium dihydrogen phosphate and strontium nitrate. The calcium and strontium precursor were taken in a beaker stirred continuously, then phosphorous precursor was added drop by drop, while maintaining the pH at 11 and temperature at 90˚C. The mixture was stirred continuously for 2 h, allowed to rest overnight, washed, centrifuged and dried to obtain Sr-HAP particles.
Both the single- and double-layer coatings were applied using dip-coating method followed by annealing at appropriate temperature. During dip coating sincere precautions were taken to get uniform and thin coatings on the samples. The withdrawal rate for the coating is 1mm / min. The dip coating was done three times with the uniform withdrawal rate to obtain a reasonable thick coating. The samples were sintered at 450°C for 2h.

Analysis of surface morphology

The single (TN) and double (TNS) layer coated samples were subjected to surface morphological studies such as X-Ray Diffraction (XRD), Attenuated total reflectance infrared spectroscopic (ATR-IR) studies and Scanning electron microscopy (SEM) morphological studies.
XRD was carried out using X-ray Difractometer - PANalytical X'Pert XRD System at a scan rate of (2θ) 0.02º over the range of 5º-90º and compared with their respective JCPDS data.
Attenuated total reflectance infrared spectroscopic (ATR-IR) studies were done using Jasco International Co. / Japan, Fourier Transform Infrared Spectrometer Model 6300. The readings were taken using single reflection crystal in a diamond base in the ATR mode, the transmittance frequency between 4000 and 400 cm^{-1}. The sample holder with diamond pin was cleaned with acetone and the prepared material with coatings was placed carefully, so that the tip touches the surface of the coating. The screw was tightened

carefully and the readings were documented. The sample were tested at random positions across the coatings to confirm uniformity and repeatability of the results.
SEM analysis was done using Carl Zeiss MA15 / EVO 18 Scanning Electron Microscope

***In vitro* cytotoxicity**

***In vitro* cytotoxicity by direct contact method**

The cytocompatibility of TN and TNS were assessed by direct contact method using L929 cell line [28,29]. The L929 cells were grown to subconfluency in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS under standard conditions of 37°C and 5% CO_2.The materials TN and TNS and the controls in triplicates were placed carefully in the center and kept for 24h under the same conditions. A blank with L929 cells alone in DMEM and 10% FBS was also included in the assay to compare the morphological changes. The positive control used was Phenol(1.3mg/mL) [29] and the negative control was High- density polyethylene(HDPE).After incubation, the cell cultures were examined for cellular responses by using an inverted phase- contrast microscope (Leica DMC 2900).The morphological changes induced by test materials, TN and TNS were assessed based on ISO10993-5 in comparison with negative and positive controls.

MEM elution assay

MEM elution assay was performed by applying extracts from test materials, TN and TNS to L929 cells [28,29]. Extracts were prepared separately by placing 0.2g of both test materials in 1mL of DMEM with 10%FBSunder standard conditions (37°C,5% CO_2) for 24h. HDPE and phenol (1.3mg/mL) and physiological saline were used as negative, positive, and reagent controls respectively.

Mouse fibroblast cells (L929 cell line) were cultured in DMEM with 10% fetal bovine serum (FBS) and kept at 37°C for 24h to reach subconfluency. The media was removed and the cultured cells were rinsed with Phosphate- buffered saline (PBS). Then the test extracts of TN and TNS at varying concentrations100%, 50%, 25% and 12.5%were added in triplicate to the appropriate wells. The test and control cultures were kept at 37°C for 24h.The cytotoxic affects were analyzed using inverted phase contrast microscope (Leica, DMC 2900). Qualitative parameters of cytotoxicity like cell morphology, cell lysis, and cell growth were studied. The cellular responses were assessed based on ISO10993-5.

Quantitative evaluation by MTT assay

MTT assay was conducted for the quantitative evaluation of cytotoxicity [28,29]. Test extracts were prepared by keeping 0.2g of TN and TNS separately in 1mL of media for 24h at 37°C. The L929 fibroblast ata concentration of 2 x 10^3cells per well was seeded in 96-well plate with DMEM and 10%FBS and incubated at 37°C for24h in 5% CO_2 to 80% confluence. After 24h the media was replaced by material extracts of varying concentrations 12.5%, 25% and 50% and 100%. The positive control employed was Phenol (1.3mg/mL). Cells with DMEM alone served as negative control. After 24h the test and control extracts were removed and 50µL of MTT reagent,(1mg/mL in DMEM without supplements), was added to all the wells. The cells were further incubated for 4h at 37°C in 5% CO_2.The supernatant MTT solution was aspirated out and 150µL of DMSO was added to dissolve the formed formazan crystals. Absorbance was taken at 570nm using an ELISA plate reader. The cell viability was assessed from the Percentage Absorbance ratio of test extracts treated cells and of negative control (representing 100% cell viability) at 570nm.

Hemolytic properties

An *in vitro* hemolytic assay as per ASTM F756-17[30] with some modification in the selection of control samples [31] was carried out to determine the possibilities of hemolysis which can occur due to the contact of materials or its leachants with blood. Both direct contact and Test on extract method was done to assess the extent of hemolysis. Pooled blood (5 mL blood each from three healthy rabbits) with 3.8% tri sodium citrate as anticoagulant in the ratio 1: 9 with blood was collected and plasma hemoglobin and total hemoglobin content were determined using cyanmethemoglobin method. The plasma free hemoglobin content is ensured to be less than 2.0mg/mL. A blood substrate with a concentration of

10.0mg/mL ± 1mg/mL was prepared by diluting with an adequate amount of Phosphate- buffered saline. For extract preparation 30cm^2 of TN, TNS, and negative control (Ultra high molecular weight polyethylene) were taken in 10mL of calcium and magnesium free Phosphate- buffered saline (in triplicates) and kept at 37^{o}C for 72h in an incubator shaker. Positive control used was 10mg/ml of Triton X-100(Sigma).For direct contact method, test materials with a surface area of 21cm^2 was used. The blood substrate was added to the extracts for test on extract method and to test materialsdirectly in direct contact method. All the tubes were maintained at 37^{o}C for 3h in a water bath. After incubation the tubes were centrifuged and the supernatant was examined for free hemoglobin by cyanmethemoglobin method. From this hemolytic index and blank corrected hemolytic index were determined.

Bone implantation study

Animal selection and regulatory aspects

For each material 8 New zealand white Rabbits, 4 animals each for one and four weeks were selected. The body weights of the animals were ensured to be not less than 2 Kg. The animals were obtained from the Division of Laboratory Animal Science, Biomedical Technology Wing, Sree Chitra Tirunal Institute of Medical Science and Technology, Thiruvananthapuram, Kerala. In-bred strain of rabbits was selected for the study and the animal husbandry was well maintained in accordance with ISO 10993-2 [32], Animal Welfare Requirements. Individual animals were identified by tattoo mark on the animal's ear.

Animal cages have labels indicating study number, study name, group, sex, animal number, experiment initiation and experiment completion date. All animals were handled humanely, without making pain or distress. Animal care and management were in compliance with the regulations of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Govt. of India. Institutional Animal Ethics Committee approval (SCT/IAEC-184/January/2016/89) was taken before initiating animal experiments. The bone implantation study designed for the materials TN and TNS was carried out as per ISO 10993 -6 [33].

Implantation in rabbits

The animals were acclimatized for 6 days prior to surgery. Initially, all animals were dewormed using oral administration of albendazole followed by oral antibiotics for the next 5 days. The implantation procedure [32, 33] was carried out under clean and aseptic conditions by a toxicologist in the animal procedure room. All the animals are handled humanely, without making pain or distress and with due care for their welfare. Atropine (0.15mg/Kg body weight) and diazepam (2.5mg/Kg body weight) were given subcutaneously as premedication. The rabbits were anesthetized with intramuscular injection of ketamine (80mg/Kg body weight) and xylazine (5mg/Kg body weight).The skin of the anesthetized animals was gently swabbed with 70% alcohol followed by betadine solution after placing the animal in lateral recumbent position. The cortex region of the femur was exposed and three holes of 2mm diameter was drilled 1cm apart using suitable burr fitted to low speed drill (SURGIC XT NE111, NSK Nakanishi, Japan). Three test samples, TN and TNS were separately implanted on the right femur bone of rabbits, 3 rabbits for each time period (1 week and 4 weeks). Similarly, 3 control samples (Cp Ti) were implanted on the left femur bone. The procedure for implantation is detailed in Figure 1. The body weight of all the animals was taken on the day of implantation and at end of experiment period.

The general physical condition of all the animals was noted throughout the implantation period. The animals were sacrificed at the end of the experiment period by administering an overdose of anesthetic agent, thiopentone. The site of implantation was noted for any evidence of visible tissue reaction. The implanted test and control material along with the femur bone was collected in 10% neutral buffered formalin for histopathological examination. Bone with the implant site was cut cross-sectionally using high precision low speed saw (ISOMET, Buehler). The sections were dehydrated using ascending grades of ethanol starting from 80% to 100%. After embedding in PMMA resin the section were cut using high-speed precision diamond saw (ISOMET 5000, Buehler). Serial sections of 100µm thickness were taken, ground and polished in variable speed grinder–polisher (ECOMET 3000, Beuhler). The sections were stained with Stevenel's blue followed by counter-staining with Van Gieson'sPicroFuchsin. The stained

sections were analyzed using Nikon Eclipse E 600 microscope. Nikon DS-Ri1 digital camera and NiS Elements microscope imaging software were used for capturing and assessing the images.

Results

Analysis of surface morphology

The implant materials TN and TNS were subjected to XRD, ATR- IR and SEM analysis.

X-Ray Diffraction

The X-ray diffraction pattern of TN and TNS is shown in Figure 2a. From the JCPDS No: 28-0317 data the characteristic Nb_2O_5 peaks were confirmed. The crystalline peaks were found at 22.9°, 29.6°, 34.6°, 38.4°, 40.2°, 45.47° and 53.37° corresponds to the (001), (100), (101), (002), (102) and (111) crystalline planes. The shift in the characteristic peak values of TiO_2 and Nb_2O_5 suggests that there is possible interaction between the mixed oxides. Instead of presenting alone, the titanium and niobium metal oxide are in combination. The peak at 70.9° and 77.67° corresponds to the anatase Ti phase of Cp-Ti pristine metal. The fused peaks at around 37° and 50° are indicative of the chemical interaction between the two metal oxides. From JCPDS No. 74-0565 the signature peaks for Sr-HAP has been confirmed. It is clear that all the metal oxide coatings were found to be highly crystalline in nature. The formed coatings were found to have interaction with one another and with the metal surface [34, 35].

ATR-IR

The signature spectrum of TN and TNS samples are given in Figure 2b. In all the four samples the peak at 3500 cm^{-1} is the OH absorption peak due to water molecules present on the surface. The stretching vibration caused due to Ti-O and Nb-O functional groups in the case of TN samples were confirmed using the reported literature [36] and the signature spectrum was found to be at 400 cm^{-1} and at 800 cm^{-1} respectively. Since there is overlap between Ti-O and Nb-O peaks we suggest that the bonding between the metal oxides is strong in the case of TN samples. The phosphate signature peaks of HAP were found at 1030 cm^{-1} and at 550 cm^{-1} for TNS. Apart from the phosphate peak, there is a small broad peak found at 1400 cm^{-1} which the spectrum for carbonated apatite peak. This suggests that the TNS surface is capable of depositing carbonated apatite in *in vitro* and *in vivo* condition. Carbonated apatite is a form of apatite that is formed during bone remodelling process. The proof that the surface is capable of depositing the carbonated apatite layer is beneficial in speeding the implant integration with the natural bone. The signatory peaks for Nb-O and Ti-O is embedded inside the phosphate peak suggesting the bonding/interaction between the first- and second-layer coatings. Further the stretching caused due to Nb-O in TN samples is retained by the TNS samples at 841 cm^{-1}. This even adds to the evidence that the HAP and the mixed metal oxide of niobium have a strong interaction among themselves. This aids in increased adsorption strength of the coating to the metal surface.

SEM Micrograms

From the SEM images **(Figure No. 2c)** it is evident that the TN have uniform sharp planar structure spread throughout the surface. The Sr-HAP coated on the TN sample shows uniform spreading with spherical morphology. The rod-shaped morphology of TN sample was responsible for Sr-HAP to form a uniform coating over the first layer. This suggests that each layer plays a vital role in the builds up of the final implant.

Cytotoxicity by direct contact

Direct contact test provides a qualitative analysis of *in vitro* cytotoxicity. The reactivity grades of cytotoxicity after direct contact test were assessed as per ISO 10993-5 [28]. There was no zone of discolouration around or under the test samples (both TN and TNS) as evidenced from Figure 3a and 3b (Fig 3a, 3b). It was clear that the morphology and cell membrane integrity were retained for both test samples. Hence both materials (TN and TNS) were proven to be cytocompatible [28].

MEM elution assay

In MEM elution test, the degree of cytotoxicity was determined microscopically by assessing morphological changes after exposure oftest material extract to a monolayer of L929 cells [28]. From the

Figure 3c and 3d, it was clear that both thetest material extracts didn't produce any alteration in cell morphology. The cell membrane integrity was maintained with no reduction in cell growth (Fig: 3c,3d), suggesting cytocompatibility of TN and TNS. The positive and negative controls performed as anticipated.

Quantitative evaluation by MTT assay

Quantitative estimation of cell viability was carried out by MTT assay. In the MTT assay metabolism of yellow coloured methyl tetrazolium salt [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide] to purple formazan by mitochondrial dehydrogenase of active cells were assessed colourimetrically. The results are shown in the Fig 4. The values are indicated as mean ± SD, with n=3. The results of metabolic activity by MTT assay suggest that the percentage cell viability with test extracts of both TN (Fig 4a) and TNS (Fig 4b) at all concentrations are comparable with negative control (cells alone). The percentage metabolic activity of negative control is taken as 100%. Hence, both TN and TNS can be considered as cytocompatible based on ISO 10993-5 [28], where the cytotoxic limit is taken as 70% metabolic activity.

Hemolytic Properties

The *in vitro* hemolytic assay is done to determine the hemocompatibility of TN and TNS as hemocompatibility is a critical parameter for all the biomaterials intended to be used as implants. In this assay, the effect of the test materials (TN and TNS) on the integrity of RBC membrane that might contribute to RBC rupture is examined and the hemolytic index is calculated. As per ASTM standards the hemolytic index <2%,2-5% and >5% are graded as non-hemolytic, slightly hemolytic and hemolytic respectively [30]. The hemolytic index obtained by material extract and direct method is given in Table 1. The hemolytic index above the negative control for material extracts of TN and TNS is found to be 0.557±0.16 (n=3) and 0.555±0.42 (n=3) respectively. In direct contact method the hemolytic index above the negative control obtained was 0.099±0.17 (n=3) and 0.393±0.74 (n=3) for TN and TNS respectively. As the hemolytic index produced by material extracts and material alone for both TN and TNS is under permissible limit, the test material TN and TNS meet the requirements of the test as per ASTM 756 and considered as non-hemolytic.

Implantation in rabbits

All the experimental animals were found normal throughout the post implantation period without any abnormality or behavioral changes. There was an increase in body weight for all the animals. There was no evidence of discoloration, hemorrhage, encapsulation, infection or tissue necrosis at the implant site on observation at sample retrieval. Ten test samples for TN, TNS and control (CpTi) were taken for each time period. The test and control slides for one week and four weeks study were reviewed blindly by the pathologist. Better healing response was noted for both the test groups in comparison with the control (Table 2).

Histopathological analysis revealed that at one-week post implantation the implants, TN and TNS were loosely attached with minimal quantity of new woven bone at endosteal surface of bone defect, similar to control (CpTi). New bone formation was not observed at intervening space in both tests and control groups. Mild degree of fibrosis along with a mild degree of mononuclear cell infiltration was noted at the interface in all tests and control groups. There was no degeneration and necrosis in all the groups. The tissue material interface of bone around the TN (5a, 5b), TNS (5e, 5f) and control groups (5i, 5j) is similar at one week. (Fig: 5).

At four weeks post implantation good quantity of new woven bone had formed arising from periosteum and endosteum in tests and control groups. The bone implant interface of all study groups was completely filled by new woven bone with good osteoblastic activity in new bone region. Good apposition between new woven bone and metal implant with no evidence of degeneration, necrosis and inflammation is noted in all study groups. Better healing response is also noted in the groups TN (5c,5d), TNS (5g,5h)and control (Cp Ti) (5k, 5l) with good new woven or primary bone formation at implant interface (Fig: 5).

Conclusion

The biomaterials selected for manufacturing bone implants should be biocompatible with good bone-bonding ability. Pre-clinical safety evaluation must be done before marketing them for clinical use. In the present study, biocompatibility of two bone implants, TN and TNS were assessed. Nanoporous mixed metal oxide coatings of TiO2-Nb2O5 was applied over Ti in TN. An additional layer of strontium incorporated hydroxyapatite layer was applied over TN by dip coating method in TNS. Biocompatibility evaluations of both the implants were done by *in vitro* and *in vivo* methods. *In vitro* cytotoxicity revealed the materials are cytocompatible and both materials are proved to be hemocompatible by *in vitro* hemolytic assay. *In vivo* bone implantation in rabbits and histopathological analysis confirmed biocompatibility and proper functionality of both materials. Hence both TN and TNS can be used safely for orthopaedic applications.

Figures

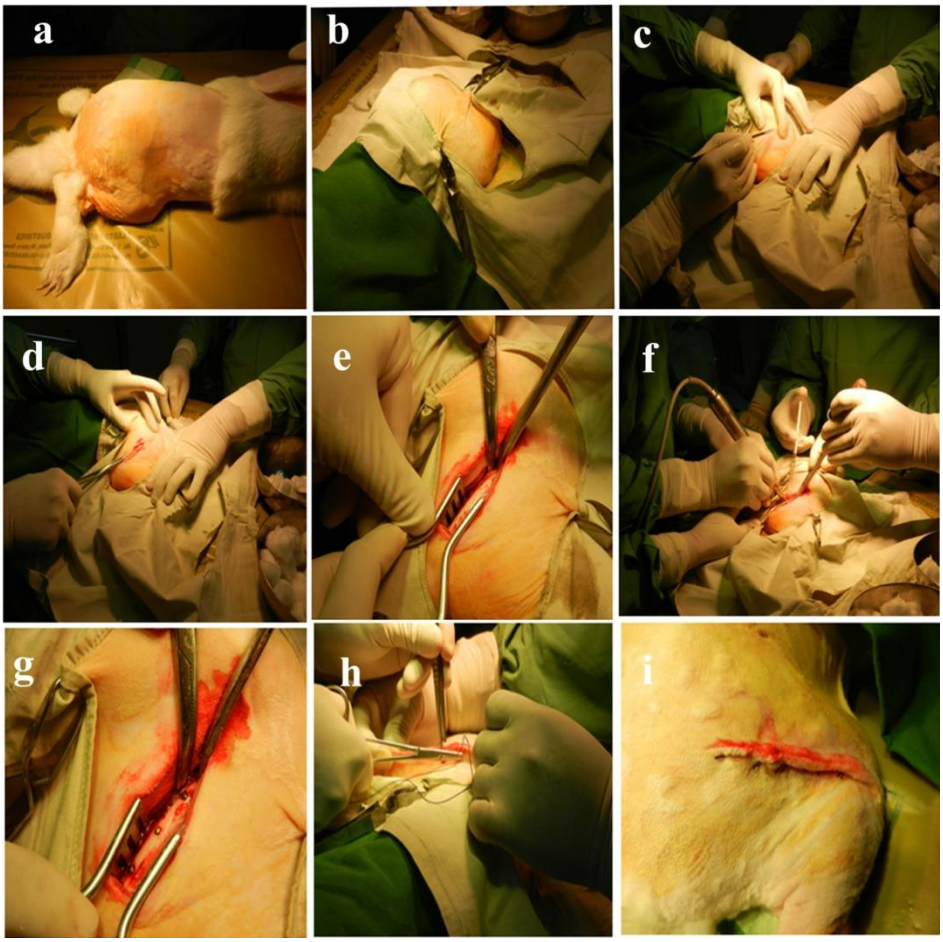

Fig 1: a & b : Animals placed in lateral recumbent position, c & d: cortex region of femur exposed, e: 3 holes of 2mm diameter drilled 1cm apart, f & g: material implanted separately, h & i: closed the site with sterile suture.

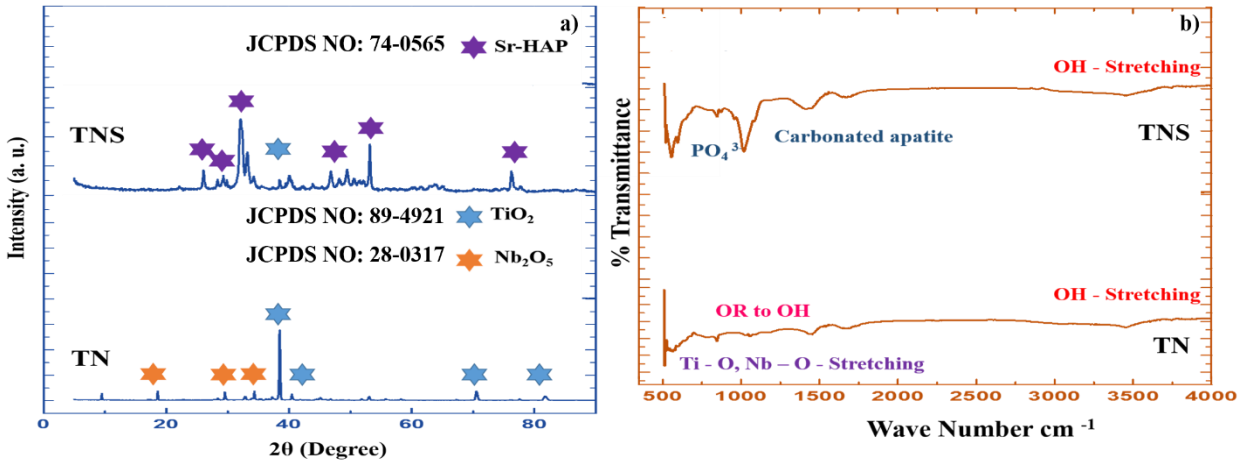

Fig 2: Surface morphological analysis, a) X-ray diffraction patterns of TN and TNS samples, b) ATR-IR spectrum of TN and TNS samples

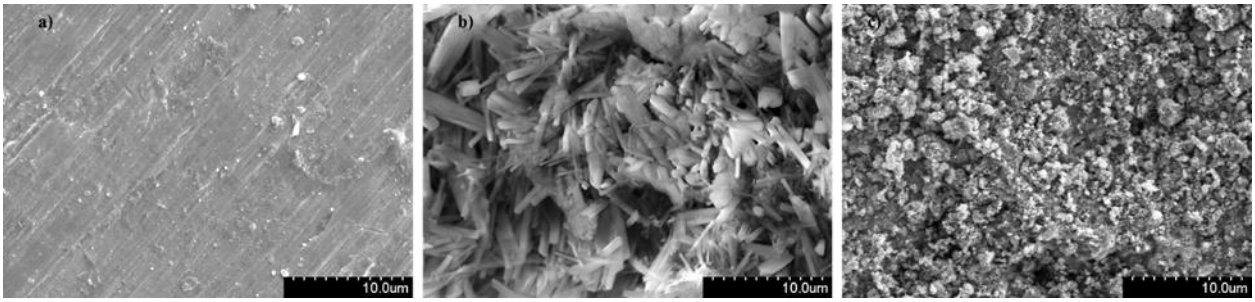

Fig 2c: The SEM micrographs of (a) Cp-Ti, (b) TN and (c) TNS samples

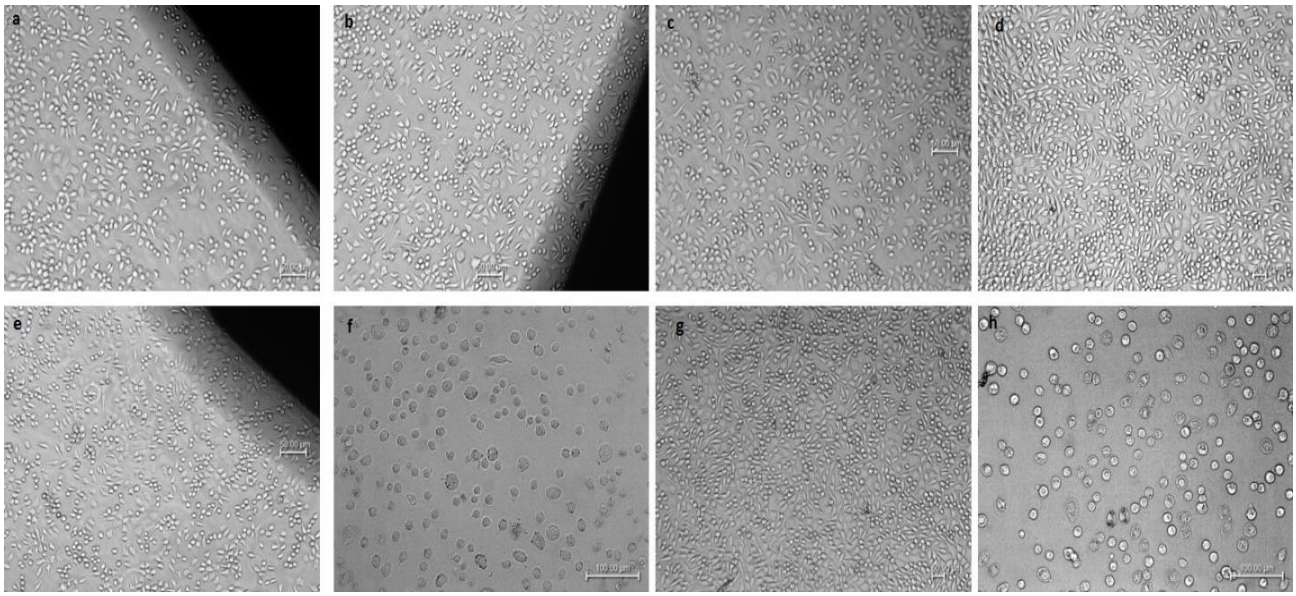

Fig 3:. Direct contact assay using L929 cells for a)TN andb) TNS. MEM elution assay using L929 cells for c) TN and d) TNS. 3e) Negative control (CpTi) and 3f)positive control (phenol) for direct contact assay.3g)Negative control (cells alone) & 3h) positive control (phenol) for MEM elution assay. Magnification:10x

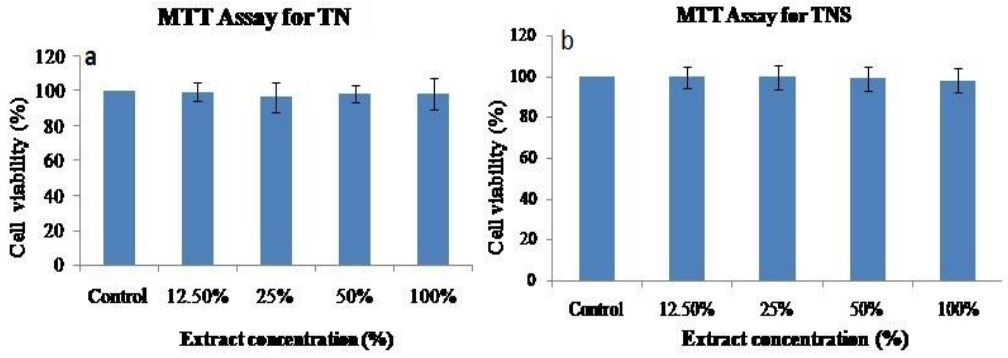

Fig 4:a. MTT assay results for L929 cells exposed to 12.2%, 25%, 50%, and 100% extracts of TNB extract. Cell viability (%) is expressed as mean ± SD (n=3). Metabolic activity of the control (cells cultured in DMEM) is taken as 100%.
b. MTT assay results for L929 cells exposed to 12.2%, 25%, 50%, and 100% extracts of TNS extract. Cell viability (%) is expressed as mean ± SD (n=3). Metabolic activity of the control (cells cultured in DMEM) is taken as 100%.

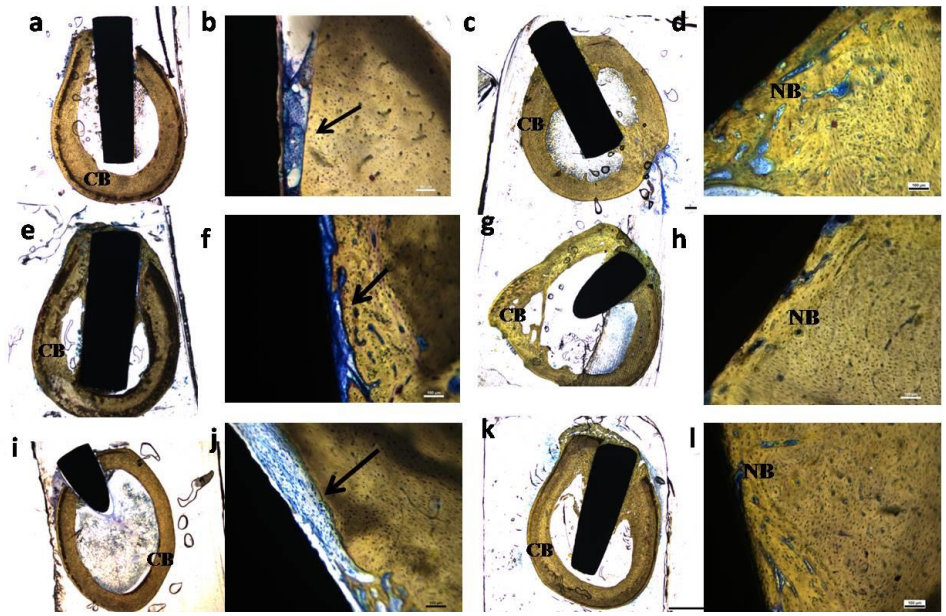

Fig 5. Stevenel's blue (for cell nuclei) and Van Geison (for bone tissue) stained representative photomicrographs of thin histology sections of host implant interface.
a & b: after one-week post implantation of TN, c & d: after four weeks post implantation of TN. e & f: after one-week post implantation of TNS, g &h:after four weeks post implantation for TNS. i&j: after one-week post implantation of control (CpTi), k & l: after four weeks post implantation of control (CpTi). The implant materials can be seen as dark region in all the images. Cell rich woven bone can be seen around both tests and control implants in four weeks images. Magnification: a, c, e, g, i& k - 1x; b, d, f, h &l -10x
CB- cortical bone, NB- newly formed bone

Table 1: Hemolytic index induced by TN and TNS

	TN				TNS			
	Test on extract		Direct contact		Test on extract		Direct contact	
	HI	Blank corrected HI	HI	Blank corrected HI	HI	Blank corrected HI	HI	Blank corrected HI
Blank	10.0±0.01	-	15.9±0.14	-	9.98±0.01	-	15.2±0.24	-
Negative control	10.1±0.14	0.18±0.16	17.1±0.38	1.3±0.40	10.1±0.14	0.18±0.16	16.2±0.25	1.17±0.29
Positive control	120.7±0.25	123.0±0.27	120.7±0.14	124.7±0.17	120.3±0.25	122.5±0.27	120.3±0.25	123.9±0.29
Test material	10.6±0.14	0.74±0.16	17.1±0.14	1.4±0.17	10.6±0.3	0.73±0.4	16.5±0.6	1.5±0.7
HI above negative control	0.557±0.16		0.099±0.17		0.555±0.42		0.393±0.74	

HI –Hemolytic index

Table 2. Summary of histopathological study of TN and TNS after bone implantation

Response	Period of study					
	One week			Four week		
	TN	TNS	CpTi	TN	TNS	CpTi
Degeneration	0	0	0	0	0	0
Necrosis	0	0	0	0	0	0
Inflammation	+	+	+	0	0	0
Fibrosis	+	+	+	0	0	0
Mononuclear cell infiltration	+	+	+	0	0	0

(0) not present; (±) Occassionaly present; (+) present to mild degree; (++) present to a moderate degree; (+++) present to appreciable degree