CHAPTER 2

MATERIALS AND EXPERIMENTAL METHODS

2.1. Introduction

This chapter describes the details of three types of stainless steels used in the present study. It presents their chemical compositions and heat treatments used for the study. It includes the specimen geometry and sample preparation for the mechanical, corrosion and biocompatibility testing. It also describes the various characterization tools and techniques like X-Ray Diffraction (XRD), Optical Microscopy, Scanning Electron Microscopy, Transmission Electron Microscopy, surface roughness measurement, mechanical testing (tensile, hardness, fatigue), electrochemical corrosion and biocompatibility.

2.2. Materials

In this study, three different types of austenitic stainless steels are used. The 316L stainless steel has been used as reference material to compare its various properties with two other austenitic stainless steels with very low content of nickel, stabilized by manganese and high nitrogen. One of the two nickel-free steels was without molybdenum (Mo) and the other one contained Mo. The stainless steel without Mo is designated as HNS and that with Mo is designated as HNS-Mo. The 316L and HNS stainless steels were supplied by M/s Jindal stainless Ltd., Hisar, India, in the form of hot-rolled plates. The Chemical compositions of all these three austenitic stainless steels are given in **Table 2.1**.

The dimension of the plates of 316L was 300 mm x 300 mm x 18 mm, whereas that of HNS was 300 mm x 300 mm x 16 mm. They were given a standard solution treatment at 1050 °C for one hour and quenched in water. The HNS-Mo steel with Mo was designed

by us and developed with the help of M/s Mishra Dhatu Nigam Limited, Hyderabad. It was rolled into rods of 18 mm diameter and solution treated as mentioned above.

Table 2.1. Chemical compositions of the three austenitic stainless steels (wt%).

Material	C	Cr	Mn	Ni	Si	Mo	N	Cu	Fe
HNS	0.057	18	22	0.10	0.33	0.03	0.65	0.05	Balance
316L	0.024	16.3	1.2	10	0.25	2	0.034	0.4	Balance
HNS-Mo	0.034	19.4	19.8	0.08	0.23	0.74	0.63	0.03	Balance

It is important to mention that the insignificant content of nickel (~ 0.1%) in the steels HNS and HNS-Mo was derived from the raw materials, it was not added.

2.3. Ultrasonic Shot Peening

The HNS and HNS-Mo nickel-free high nitrogen grade of stainless steels were Ultrasonic shot peened (USPed). The device used for the USP is shown in **Fig. 2.1**. It is a Stress Voyager (SONATS) system that comprises of an auditory assembly with a piezoelectric transducer, booster and sonotrode. The acoustic body generates mechanical vibration and transfers it to the hard shots of 100C6 grade steel to put them in random motion.

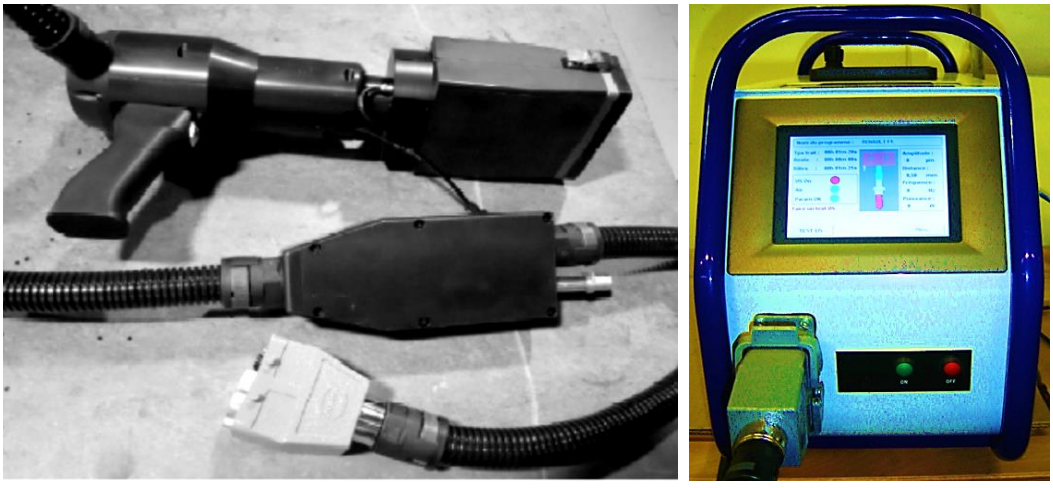

Fig. 2.1. The peening head (left) and the central unit (right) of the ultrasonic shot peening device.

Ultrasonic waves of 20 kHz frequency are emitted by the piezoelectric transducer and amplified. The amplitude of vibration remains constant at 80 µm throughout the process of USP. The resonated flying shots impact the specimens' surface in random directions at a very high speed over a short time. Shot size and duration of USP were varied, keeping the other variables constant, in the present study. Shots of 2 mm and 3 mm diameter were used for USP for different durations and the samples are designated in **Table 2.2**.

Table 2.2. Designations of the differently USPed samples of the nickel-free nitrogen stabilized austenitic stainless steels.

Shot diameter (mm)	USP duration (min)	Sample designation
2	0.5	USP 2-0.5
	1	USP 2-1
	2	USP 2-2
3	0.5	USP 3-0.5
	1	USP 3-1
	2	USP 3-2
	3	USP 3-3
	6	USP 3-6
	10	USP 3-10
	14	USP 3-14
	18	USP 3-18

The non-treated samples are designated Un-USP. These USPed samples were used for the microstructural characterization, hardness, electrochemical corrosion, biocompatibility, low cycle fatigue, high cycle fatigue and corrosion fatigue tests.

2.4. Microstructural Characterization

The microstructures of the various steels were examined under optical microscope, SEM and TEM. The phase identification was carried out by the XRD.

2.4.1. Optical Microscopy

The samples were mechanically polished for microstructural characterization, using different grades of emery paper. The final polishing was done on velvet cloth, mounted on a rotating wheel, using alumina powder suspension in water as an abrasive. The polished samples were etched with aqua regia and examined for their microstructure under an optical microscope. According to the ASTM standards, the microstructure was analyzed for phases, precipitation, and cleanliness (inclusion rating). ASTM E-407-07 (2015) standard was followed to analyze precipitation at the grain boundary, and electrolyte of 10% oxalic acid solution was used for HNS and HNS-Mo, whereas 10% ammonium persulphate solution was used for 316L. ASTM E-562 (2011) and ASTM E-45 method A was followed for the volume fraction measurement of delta ferrite and non-metallic inclusion content, respectively.

2.4.2. Scanning Electron Microscopy

The surface morphology of the Un-USP and USPed samples was examined using SEM (Model: Zeiss EVO18), operated at 20 kV. In order to examine the effect of USP on the microstructure, the USPed samples were sectioned, normal to the USPed surface, polished and analyzed under the SEM.

Fracture characteristics of the fatigue-tested samples were examined using SEM. Small pieces of fatigue-tested samples of ~ 4 mm length were sectioned transversely from the fracture end. These samples were ultrasonically cleaned in acetone for 5 min and their fracture surfaces were examined to trace the sites of crack initiation. Also, the crack

propagation regions were examined for striations, if any. Longitudinal sections of these samples were examined for the size and number density of fatigue cracks.

2.4.3. Transmission Electron Microscopy

The USPed samples were examined by TEM (Model: TECNAI 20 G^2) operated at 200 kV. TEM foils were prepared from sections of ~ 600 μm thick, partitioned parallel to the USPed surface, by a slow-speed precision cutter. Those were reduced by mechanical polishing to a thickness of ~ 40 μm from the side, opposite to the USPed surface. Discs of 3 mm diameter were punched and electropolished using a twin jet electropolisher (Struers-Tenupol-5) in a solution of 5% perchloric acid ($HClO_4$) and 95% alcohol (CH_3OH) by volume, cooled to - 33 °C, at a voltage of 22 V.

2.4.4. X-Ray Diffraction

X-ray diffraction of the different stainless steels samples in the non-treated (Un-USP) condition was carried out by a Rigaku X-ray diffractometer with Cu-Kα radiation in 2θ range of 20° to 110°. Analysis of the phases in the USPed samples was done using X'pert PANalytical diffractometer at the acceleration voltage and current intensity of 40 kV and 40 mA, respectively, with Cobalt radiation of wavelength 1.79021 A°. The data of diffraction lines were recorded by the 'Step scanning' method in 2θ range from 30° to 130° with a step size of 0.03° and 0.3 s time per step. The microstrain and mean crystallite size were determined from the X-ray peak broadening.

The evaluation of residual stress, induced in the samples subjected to USP, was carried out by $\sin^2\psi$ method with the PANalytical X-Ray diffractometer (Cu-Kα radiation). The variation of residual stress with depth was measured, removing the material layer by layer, using iterative electrolytic polishing.

2.5. Surface Roughness Measurement

The surface profiles of the Un-USP and USPed samples were determined using the Surftest-Mitutoyo SV-2100 over the gauge section of the samples as per the ISO 4288:1996 standard.

2.6. Mechanical Testing

2.6.1. Hardness Testing

The Vickers hardness was measured at a 5 kg load. Microhardness of the USPed samples was measured, on their longitudinal section (perpendicular to the USPed surface), along the diameter, from the USPed surface towards interior, at 0.1 kgf load with a dwell time of 10 sec, using Leco microhardness tester (Model: LM 248 AT).

2.6.2. Tensile Testing

The geometry of tensile test samples was as per the ASTM A370. The gauge diameter and gauge length were 6.25 mm and 25 mm, respectively. Testing under tensile loading was conducted using a 100 kN (Instron 5982) universal testing machine, at the strain rate of 5×10^{-4}/sec. An extensometer was mounted in gauge section of the sample for measuring the strain.

2.6.3. Low Cycle Fatigue Testing

Low cycle fatigue (LCF) tests were performed under total strain control mode at room temperature (RT), using an MTS^{TM} Landmark servo-hydraulic test system (Model 370.10) with a capacity of 100 kN, as shown in **Fig. 2.2**. An extensometer of 10 mm gauge length (Model: MTS 632.53F) was used for controlling the strain. The plastic, elastic and total strains with associated stress were recorded by controller software (Flex test 40) during testing.

Blanks of 110 mm length, and width of 16 mm, were sectioned from the solutionized plates and cylindrical LCF samples were machined as per the geometry shown in **Fig. 2.3**. These samples include a gauge section of 5.5 mm diameter and 15 mm length, 25 mm shoulder radii and gripping threaded ends of 12 mm diameter and 30 mm length.

Fig. 2.2. MTS^{TM} Landmark servo-hydraulic fatigue test system (Model 370.10).

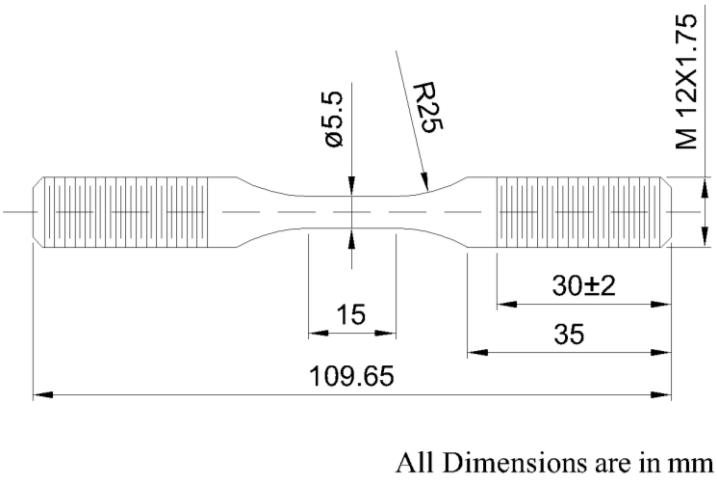

Fig. 2.3. Geometry of the LCF fatigue test sample.

The gauge section of the samples was polished with emery papers of 1/0 to 4/0 grades and finally with alumina lapping powder suspension. The USP was performed in the

gauge section of the polished samples using hard steel shots of 3 mm, at a frequency of 20 kHz and a vibrational amplitude of 80 μm, using Stress Voyager (SONATS). The samples were kept in a horizontal position and rotated continuously along the axis, at 6 rpm, during the USP for 3, 6, 10, 14 and 18 min, to ensure uniformity of USP over the surface.

LCF tests were conducted at various total strain amplitudes ($\pm\Delta\varepsilon_t/2$) of ± 0.40 %, ± 0.50 %, ± 0.60 % and ± 0.80 %, at 0.25 Hz constant frequency, using a triangular waveform under fully reversed loading ($R\varepsilon = -1$). The test matrix of the LCF tests is displayed in **Table 2.3**.

Table 2.3. Test matrix of low cycle fatigue tests.

Stainless steels	Treatment condition	Total strain amplitude (%)	Frequency (Hz)
316L	Un-USP	± 0.40, ± 0.50, ± 0.60 and ± 0.80	0.25
HNS	Un-USP	± 0.40, ± 0.50, ± 0.60 and ± 0.80	
	USP 3-3		
	USP 3-6		
	USP 3-10		
	USP 3-14		
	USP 3-18	± 0.40	

2.6.4. High Cycle Fatigue and Corrosion Fatigue Testing

High cycle fatigue (HCF) tests were conducted in stress control mode (pull-push type) at different stress levels, at a stress ratio (R) of 0.1. Stress ratio is the ratio of minimum stress to maximum stress during cyclic loading. The specimens were prepared according to ASTM E 466 standard and geometry of the sample used for HCF testing is shown in **Fig. 2.4**.

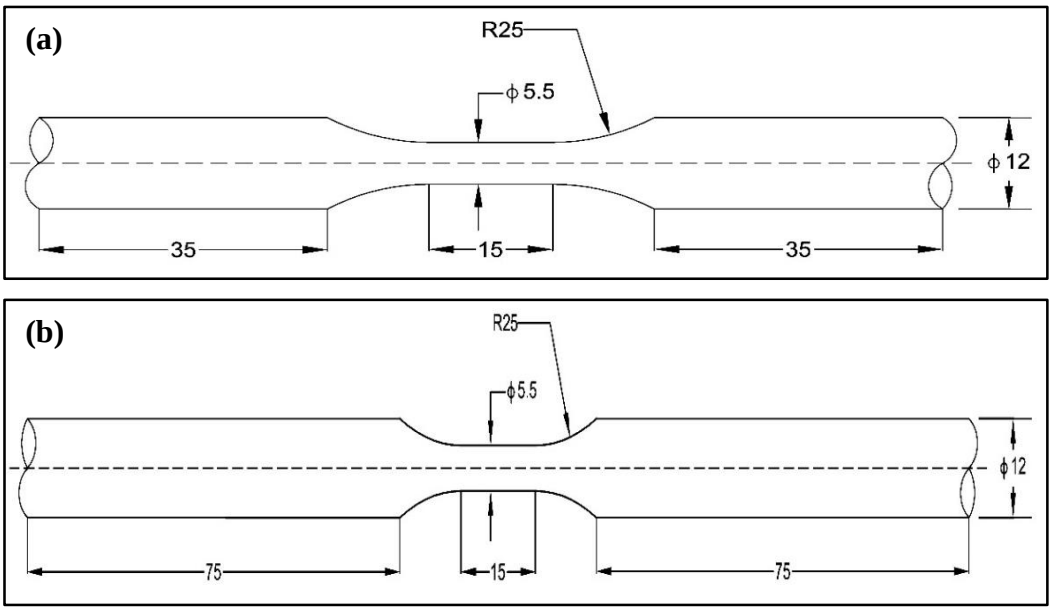

Fig. 2.4. Sample geometry for stress-controlled high cycle fatigue tests in (a) air and (b) simulated body fluid (corrosion fatigue).

Servo-hydraulic test system of MTS (model 370.10) of 100 kN capacity was used for fatigue testing with a sinusoidal waveform. Stress-controlled cyclic tests were conducted at a frequency of 30 Hz in air. Fatigue tests in corrosive medium were conducted at 5 Hz frequency in simulated body fluid (SBF: Ringer solution). The corrosion fatigue test set-up is shown in **Fig. 2.5.**

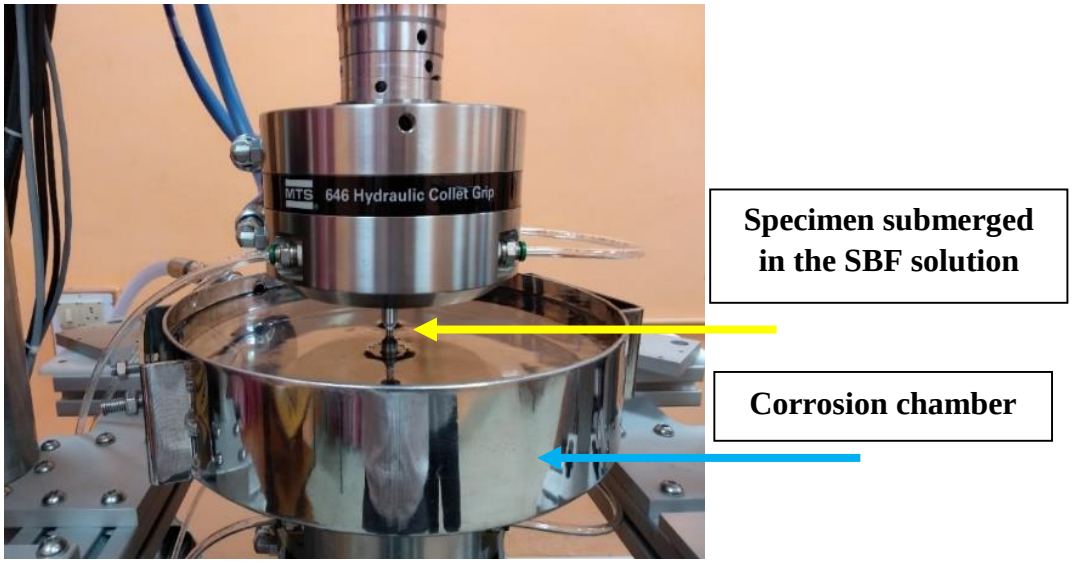

Fig. 2.5. Corrosion fatigue test set-up.

In the corrosion fatigue chamber, SBF solution was continuously circulated using a submersible pump attached with a sink tank filled with SBF fluid. The gauge section of the sample was submerged in the simulated body fluid (SBF) during the testing. Tests were performed till failure or up to the endurance limit. Endurance limit was determined by measuring the stress level corresponding to 10^7 cycles. Tests were stopped after 10^7 cycles. The HCF tests were also conducted following the USP for 3 minutes with 3 mm diameter shots (details are given in section 2.6.3). The USP 3-3 samples were tested at different stress values in the air and SBF.

2.7. Corrosion Testing

Corrosion tests were performed for evaluating pitting and intergranular corrosion (IGC) resistance according to the ASTM A 262 practice A and E, ASTM G-48 11 method A and D. Corrosion resistance is strongly related to Cr, Mo and N levels present in the alloy. Corrosion resistance of the nickel-free grade of stainless steels was studied according to the ASTM A-262 practice A, using 10% oxalic acid electrolyte, whereas 10% ammonium persulphate electrolyte was used for 316L. The microstructure was also examined after 15 h of boiling in copper-copper sulphate, 16% sulphuric acid solution according to ASTM 262 practice E. 316L was sensitized at 675 ºC for 1 h whereas there was no need of sensitization of nickel-free steels. The pitting resistance was evaluated according to ASTM G 48-11 Method-A in 6% $FeCl_3$ solution. ASTM G 48-11 Method-D standard was followed for the crevice corrosion resistance and 6% $FeCl_3$-1% HCl solution was used for testing.

Electrochemical corrosion of stainless steels in the Un-USP and various USPed conditions was studied by potentiodynamic polarisation test. Specimens of 11.5 mm diameter and 4.5 mm thickness were machined and mechanically polished. The flat surface of one side was subjected to USP according to the details in the **Section 2.3**. The

circumferential surface and flat surface of the other side of the samples were covered with lacquer before the test. The corrosive medium used for the testing was Ringer's solution. It was according to the "Cold spring harbour protocols (2008)". An amount of 7.2 gm of NaCl, 0.17 gm of $CaCl_2$ and 0.37 gm of KCl was dissolved in 1 liter of distilled water to prepare the solution.

A 45 minutes stabilization was given before the start of the tests. The initial voltage was -500 mV vs open circuit potential (OCP). The scan rate of 2 mV/s was used during the testing. The tests were done over a biologic corrosion station (model: VSP) using a three-electrode set-up comprising saturated calomel electrode (SCE) as reference electrode and platinum as a counter electrode.

2.8. Biocompatibility Testing

Both *in vivo* as well as *in vitro* tests were conducted for biocompatibility evaluation of the nickel free austenitic stainless steels, following the ISO 10993-5, 2009 and industry standards. It includes cytotoxicity, cell culture and adhesion, and animal study. HNS-Mo was also subjected to a series of biocompatibility tests to evaluate potential of this steel for biomedical applications.

2.8.1. Cell Culture

Specimens of 10 mm diameter and 2 mm thickness were used for the cell culture study. The samples were mirror polished and rinsed with acetone for 15 min to remove contamination, if any. MG-63 human bone osteosarcoma cells were used for the study. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (10,000 U/mL)/streptomycin (10 mg/mL) (PS)) was used to maintain the cells at 37 °C, in a humidified 5% CO_2 incubator (Galaxy® 170 S, Eppendorf, Germany). MG-63 cells, with a number density of 1×10^5 cells/sample were seeded on the surface of the sterile steel samples and incubated at 37 °C in a 5% CO_2

incubator. After 1, 3 and 5 days of incubation, cells cultured on steel samples were fixed in 4% paraformaldehyde (HiMedia) at RT for 20 min, followed by a brief phosphate buffer saline (PBS) wash. After that, 0.5% Triton X-100 (HiMedia) in 1% BSA/PBS was used to permeabilize the cells for 10 min at RT followed by blocking with 1% BSA (HiMedia) in PBS for 30 min at RT and PBS wash. Subsequently, the cells were incubated with rhodamine-conjugated phalloidin (1:1000 in 1% BSA/PBS, AAT-Bioquest) for 1 h at RT to stain the actin cytoskeleton filaments. After washing, 1 μg/mL DAPI (4,6-diamidino-2-phenylindole, HiMedia) solution in 1% BSA/PBS was added and kept in the dark for 45 min at RT to visualize the nucleus. Then a final wash was given to the samples before imaging under a fluorescence microscope.

2.8.2. Cell Proliferation

Disc type samples of 4 mm diameter and 2 mm thickness were prepared, mechanically polished and ultrasonically cleaned using acetone as stated above. They were used for cell proliferation study using MG-63 human bone osteosarcoma cells. The proliferation of cells was determined by MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide, HiMedia) reduction assay. This assay presents a simple colorimetric method for determining comparative cell viability using a standard microplate absorbance reader. The principle behind this standardized metabolism-based screening procedure is the reduction of MTT (slightly yellow) to formazan crystals (purple). The increase in metabolic activity of the cell population increases the formation of formazan crystal, thereby increasing the absorbance value, which is directly proportional to the metabolically active cells. The MTT assay was performed in a 96-well plate as per the standard procedure. The samples were sterilized by washing them in 70% ethanol overnight inside a biosafety cabinet and air-dried for further use. Cells cultured in the wells not having any test samples were taken as a positive control, whereas the blank

medium was considered the negative control for the experiments. MG-63 cells were seeded on each sample at a number density of 1×10^4 cells/well and maintained in a CO_2 incubator for 1, 3 and 5 days. After incubation, the culture medium was removed from each well and 100 µL of MTT (5 mg/mL in PBS) solution was added. The formazan crystals formed after 4 h of incubation with MTT were solubilized using 100 µL dimethyl sulfoxide (DMSO, HiMedia) solution for 15 min. The optical absorbance was measured at 570 nm on a multimode reader (Synergy H1 hybrid, Biotek, USA). The entire experiments were performed in triplicates. Cell proliferation in percentage was calculated as follows:

$$\% \text{ cell proliferation } = [\text{A (sample)}/\text{A (control)}] \times 100,$$

Where, A is the absorbance at 570 nm. The results have been presented as mean with standard deviations (SD) of the triplicate measurements. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests was performed to find the statistical difference between the different groups of samples.

2.8.3. *In vivo* Animal Study of HNS

Ethics statement: Institutional animal ethics committee approved the experimental protocol (reference number: 542/GO/ReBi//S/02/CPCSEA dated 26.5.2017).

Eight adult rabbits with weights ranging from 1.5 kg to 1.7 kg were utilized. The rabbits had been kept into two groups and anesthetized using a combination of ketamine (60 mg/kg) and xylazin (6 mg/kg) administered by intramuscular injection. The various surgical steps are shown in **Fig. 2.6.** The hair over the anterior aspect of a knee joint and the skin was disinfected with betadine. An incision was made over the anterior aspect of the knee and the patella was retracted laterally. Femur fracture was created in distal 1/3 and in a retrograde fashion, the fracture was fixed with HNS wire. The wound was closed in layers.

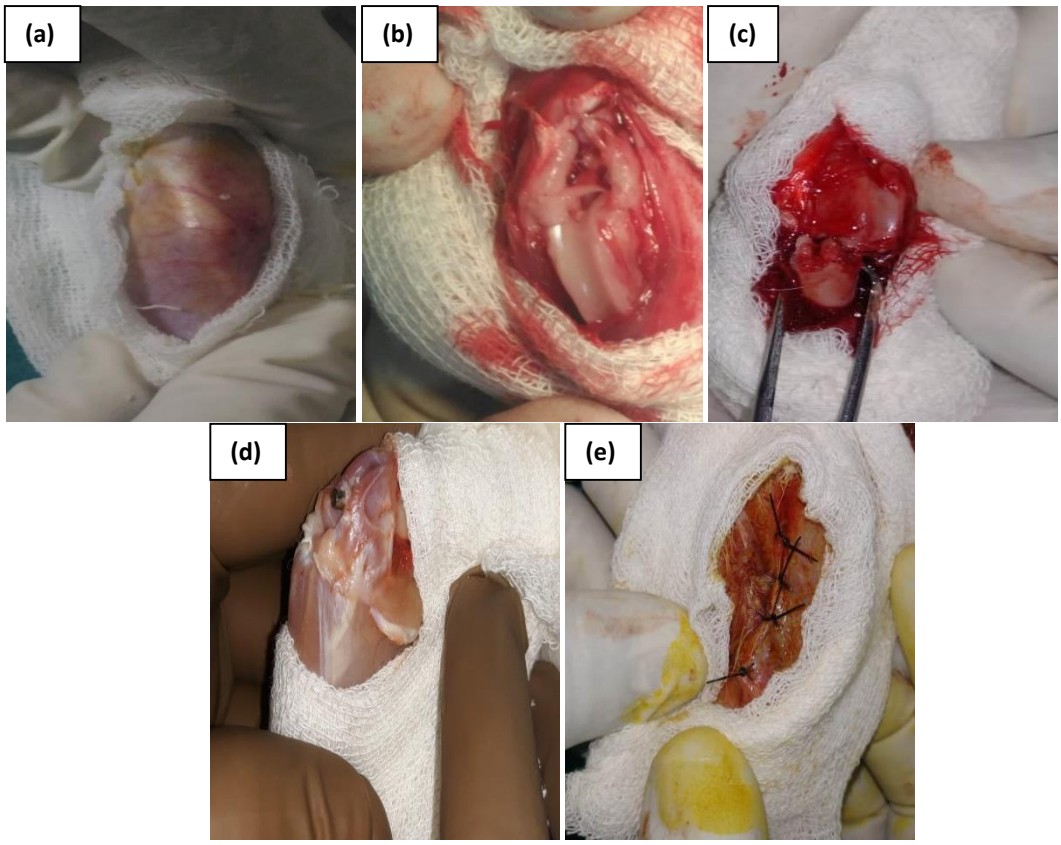

Fig. 2.6. Various surgical steps: (a) Step 1: Part preparation, (b) Step 2: Anterior midline incision over knee, patella retracted laterally and exposure of knee joint, (c) Step 3: Fracture created in distal femoral shaft, (d) Step 4: Fracture fixed retrogradely with HNS wire, and (e) Step 5: Wound closed in layers.

Three doses of gentamicin (10 mg) were administered to prevent post-operative infection. Radiographs were taken at 1st post-operation day in every case. Both the groups were evaluated for any delayed hypersensitivity and irritation during the period of study. At the 3rd week, the first group (group 1) was sacrificed, and at the 6th week, the second group (group 2) was sacrificed and the fracture sites were examined grossly and radiologically for the signs of union at 3rd week and 6th week in each group. The femur was harvested in each rabbit and the fracture sites were examined histologically in both groups. A soft-tissue biopsy (soft-tissue adjacent to the fracture site) was sent for both groups.

In addition to cell culture and proliferation study with MG-63 cells, HNS-Mo was also subjected to a series of biocompatibility tests to further evaluate it for potential use in biomedical applications. Extensive biocompatibility tests were conducted by Shri Chitra Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

2.8.4. Cell Adhesion

In vitro cell adhesion study was performed using L-929 mammalian cells. L-929 is an established and well-characterized cell that has demonstrated reproducible results. Test specimens were sterilized at 121°C for 20 minutes before start of the test. MEM supplemented with FBS was the culture medium used for this study. L-929 cells were trypsinized, seeded on the test materials and control glass cover slip at density of 1×10^4 cells/cm^2 and incubated for 48 h at 37°C under a humidified atmosphere containing 5% CO_2. After 48 h, cell-seeded test materials and glass cover slips were fixed in 2.5% glutaraldehyde and processed by dehydrating using graded alcohol. Samples were then subjected to critical point drying, followed by gold coating. Samples and control slip were examined under SEM.

2.8.5. *In vitro* Cytotoxicity

An *in vitro* cytotoxicity test using the direct contact method was performed as per the ISO 10993-5, 2009. Test specimens were sterilized by steam at 121°C for 20 minutes before start of the test. MEM supplemented with FBS was the culture medium used for this study. The culture medium from the L-929 monolayer was replaced with a fresh medium. Test samples, negative controls (Ultra-high molecular weight polyethylene-UHMWPE) and positive controls (Stabilised PVC disc) in triplicate were placed on the cells. After incubation at 37±1 °C for 24 to 26 h, cell monolayer was examined microscopically for the response around the test samples. The reactivity was graded as 0,

1, 2, 3 and 4, based on the zone of lysis, vacuolization, detachment and membrane detachment, according to **Table 2.4**.

Table 2.4. Grading criteria for reactivity of cells.

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending specimen size up to 1 cm
4	Severe	Zone extending farther than 1 cm beyond specimen

2.8.6. *In vitro* Hemocompatibility

ISO standard 10993-4:2002 (E): selection of tests for interaction of materials with blood was followed for the hemocompatibility testing. The percentage hemolysis, blood counts (Leukocyte counts) and platelet counts in blood were determined as per WPTRU015. The free hemoglobin liberated into the plasma after exposure to samples was measured as per WPTRU022 and the percentage hemolysis was calculated using the formula (free HB/Total Hb/1000) X 100. The leukocyte and platelets were measured in initial and 30 min blood samples using a hematology analyzer (Sysmex-K 4500) as per WPTRU015.

2.8.7. Irritation and Skin Sensitization

This study was designed to determine the irritation potential of the physiological saline (PS) and cotton seed oil (CSO) extracts of HNS-Mo, intended to be used as an orthopedic implant. This study was conducted to meet the requirements of the ISO 10933-10: 2010 (E), Biological evaluation of medical devices-part 10: Test for irritation and skin

sensitization test, Clause 6.4: Animal Intracutaneous (Intradermal) reactivity test and USP41/NF36:2018 and in accordance with the OECD principles of GLP.

In this study there were 3 rabbits (New Zealand white) for each material. Healthy adult animals not less than 2 kg (2.4 to 3.3 kg) and not previously used were used for the test. The PS and CSO extracts of the HNS-Mo was aseptically injected into 5 sites (0.2 ml/site) on the upper left-hand side and upper right-hand side of 3 rabbits. The PS (control) alone and CSO (control) alone were injected into 5 sites on the same rabbits' lower left-hand side and lower right-hand side. All injected sites were observed at 24, 48 and 72h after injection for the evidence of any tissue reactions like erythema or oedema. The grading of erythema and oedema of all the animals' test and control sites was recorded at 24, 48 and 72h as per ISO 10993-10: 2010 (E).

2.8.8. Guinea Pig Maximization Test of Physiological Saline Extract of HNS-Mo

This study was designed for determination of the skin sensitization potential in Guinea pigs by maximization test of the physiological saline extracts of HNS-Mo, intended to be used as orthopedic fracture/implant. This study was conducted as per ISO 10993-10: 2010 (E): Biological evaluation of medical devices-part 10: Test for irritation and skin sensitization test, Clause 7.5: Guinea pig maximization test (GPMT) and in accordance with the OECD principles of GLP. According to the ISO 10993-10: 2010 (E): clause 7.5 recommends the usage of guinea pigs for skin sensitization studies by topical application and also guinea pigs are reported to be a suitable model for pre-clinical safety evaluation. The PS extracts of HNS-Mo and control (PS alone) was intradermally injected at three sites per animal. After seven days of intradermal injection, the test (sample) and control extracts were topically applied to intrascapular region of each guinea pig by saturating a patch of absorbent gauge (8 cm^2) with the test/control extracts. After 48h, the dressing and patches were removed and the sites were treated with 10% sodium lauryl sulfate 24h

prior to topical application. A challenge test was carried out after fourteen days of topical application on all the animals. The patches and dressings were removed after 24h. The appearance of challenge skin sites of test and control animals were observed at 24, 48 and 72h after removal of dressings and patches. The skin reactions for erythema and oedema were scored and recorded the numerical grading as per ISO 10993-10: 2010 (E).

2.8.9. Acute Systematic Toxicity

These tests were conducted to meet the requirement of the ISO 10993-11: 2017 (E), Annex A.7 & A.8 Test for systematic toxicity: Acute systematic toxicity test: Acute intraperitoneal & Acute intravenous applications and USP 41/NF 36: 2018, systematic injection test. These tests were designed for evaluation of the systematic response of mice following intraperitoneal and intravenous injection of CSO and PS extracts of HNS-Mo, respectively, intended to be used as orthopedic implant.

2.8.10. Implantation of HNS-Mo

Implantation test was performed according to the ISO 10993-6: 2016 (E): Biological evaluation of medical devices - Part 6: Test for local effects after implantation: Annex A: Test method for implantation in subcutaneous tissue and in accordance with OECD principles of GLP. Implants were disc shaped with 10 mm diameter and 2 mm thickness. They had been prepared by machining and polishing. The study was conducted in 6 rabbits, 3 rabbits each for 1 and 4 weeks. At the end of each observation period, animals were sacrificed and collected the implants with surrounding tissues for histopathological analysis.