MATERIAL AND METHOD
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The present study was conducted in the animal research laboratory of M.L.B. Medical College, Jhansi. The study was designed to evaluate the role of antibiotic impregnated acrylic bone cement in prevention and treatment of bone infection.

Experiments were conducted on 150 Albino rats (114 male and 6 female). All the animals were fed on a standard animal feed and the weight of animals ranged between 250 to 400 grams. At a time ten animals were operated.

COMPOSITE PLAN OF STUDY:

The study was done, dividing all the experimental animals in following three groups of 50 each (48 male and 2 female).

CONTROL GROUP:

Metaphyseal end of right tibia was drilled in the animals of this group, to make a hole of 2 mm in diameter. This hole was filled with 0.01 ml of bacterial suspension (10^7 staphylococcus aureus in 1 ml of normal saline) and was plugged with plain bone cement bead. In this way pyogenic bone infection was produced experimentally.
FIRST TEST GROUP:

In this group of animals, tibia was drilled, filled with the same concentration and amount of the similar organism as in the control group of animals. After inoculation of bacterial suspension tibial hole was plugged with bone cement bead having antibiotic mixed with it. (one gram of benzyl penicillin in 40 grams of bone cement powder). Inoculated organisms were sensitive to this antibiotic, thereby, evaluating preventive aspect of antibiotic impregnated bone cement, in cases of bone infection.

SECOND TEST GROUP:

Bone infection was produced experimentally in tibia of animals of this group. The technique was same as in control group. On 14th day, plain bone cement bead of tibial hole was replaced after another operation with antibiotic impregnated bone cement bead, which was prepared in the same manner as in second group of animals.

On the 14th day the animals of control and first test groups were sacrificed. The inoculated legs of these animals were radiographed (in antero-posterior and lateral views) and then examined microscopically, bacteriologically and histopathologically, to find out evidences of bone infection. The animals
of second test group were anaesthetised and examined radiologically. They were then examined macroscopically, before undergoing a second operation, to replace the bone cement bead, while, at the same time bacterial, the remaining part of macroscopic and microscopic examination (separating a very small bone piece adjacent to tibial hole) was done, in the same manner, as in other groups.

On 26th day these animals were also sacrificed and examined on the same parameters as in other groups. The difference in number of infected bone on 26th day than on 14th day showed therapeutic effects of antibiotic impregnated bone cement bead in infected bone.

DETAILS OF STUDY:

PREPARATION OF BACTERIAL SUSPENSION

ORGANISM:

Staphylococcus aureus, being the commonest causative organism in human bone infection, was chosen for our study. We used NCTC 6571 strains of staphylococcus aureus. (Supplied from K.E.M. Hospital,
Bombay). All the instructions of laboratory were followed.

**REACTIVATION OF OMNASIN:**

Organisms were supplied in lyophilised form and were reactivated by inoculating one colony of bacteria in nutrient broth. This broth was incubated at 37°C for 24 hours.

**SUSCULTURE**

After 24 hours of incubation, one platinum loop full fluid from broth was taken and sub cultured in blood agar culture plate for next 24 hours at the same temperature (37°C).

**PREPARATION OF STOCK SUSPENSION:**

Single colony of bacteria was taken out from culture plate and diluted in 5 ml of sterile normal saline. Number of bacteria were $10^6$ in each ml of suspension prepared in this manner (based on instructions of laboratory). One ml of this suspension was further diluted upto 10 ml, using the same sterile saline. In this way, stock bacterial suspension was prepared for experiments. One ml of stock suspension was having $10^7$ staphylococi. Fresh suspension was prepared for experiments every time. We also confirmed the following characteristics of these bacteria.
(i) Beta haemolysis.
(ii) Coagulase positive reaction.
(iii) Sensitivity to benzyl penicillin.
(10 units of benzyl penicillin in each disc).

PREPARATION OF BONE CEMENT BEADS

BONE CEMENT:

Simplex P bone cement (Manufactured and supplied by instrument Orthopaedics Ltd, Bombay) was used. It was having total 40 grams of powder content, present in three packets of unequal weights and 20 ml of liquid component. Fresh packs were used for experiments every time.

PREPARATION OF BEADS (PLAIN):

Total powder content of bone cement was poured in an autoclaved stainless steel bowl, all liquid component was added to it and stirred thoroughly with the help of a stainless steel spatula. All aseptic measures were adopted throughout the procedure. When consistency of mixture was changed to rubber like, non-sticky material, it was poured into sterile molds and cement beads were made two mm in diameter (average weight of beads was 20 mg). Beads were allowed to set at room temperature.
PREPARATION OF ANTIBIOTIC IMPREGNATED BONE CEMENT

BEADS:

One gram of benzyl penicillin powder was mixed with total powder content (40 gms) of bone cement, then beads of same size and weight were made, using the same technique as to make the plain bone cement beads. Therefore each such bead was having 0.5 mg of antibiotic in it.

EXPERIMENT:

(a) ANAESTHESIA:

A cotton swab, soaked in ether was placed in a glass jar. The animal was kept in it and jar was then covered with a metallic lid. This metallic cover was having holes to allow free flow of atmospheric air in the jar. Rate were carefully observed while anaesthetised, to avoid any mortality during anaesthesia.

(b) PART PREPARATION AND ANTISEPSIS:

The anaesthetised animal was taken out of the jar and hair around its right leg were cut with scissors (Shaving or application of hair removers may cause local allergic or hypersensitive manifestations). This leg was then thoroughly painted with 2% aqueous solution of sodium several times and draped with autoclaved towels.
(c) **OPERATION**:

Fresh set of autoclaved instruments were used every time for operation and other aseptic measures were also undertaken. Antero medial vertical incision was given in right leg of rat in its upper 1/4th. Tibia of the animal was exposed, retracting soft tissues aside. Periosteum was also incised in the line of skin incision and reflected on either sides. Metaphyseal end of tibia was exposed and a hole of 2 mm diameter was drilled into medullary cavity of exposed bone. Burr of 2 mm diameter was used for making this hole. Release of cortical resistance and coming of dark red blood suggested that medullary cavity had been opened. In this hole 0.01 ml of stock bacterial suspension was insculated with the help of a pipette. In this way one hundred thousand staphylococci were poured in the hole. Flooded suspension over operation field was not applied.

**PLUGGING OF THE HOLE**:

(a) **CONTROL GROUP**:

After insculnation of bacterial suspension, tibial hole in this group was plugged with one plain bone cement bead.

(b) **FIRST TEST GROUP**:

Tibial hole after instillation of bacterial suspension was plugged, with one antibiotic impregnated bone cement bead.
BACTERIAL INOCULATION IN THE HOLE OF RAT'S TIBIA
Implantation of bone cement beads in the hole of rat's tibia.
(c) **SECOND TEST GROUP**

In animals of this group bacterial suspension was poured in their tibial hole, which was then plugged with plain bone cement head. On 14th day, this plugged hole was re-explored by the same operative technique and after that plain bone cement head was replaced with antibiotic impregnated bone cement head.

**CLOSURE**

Operative wound over leg of rat was closed using autoclaved, fine cotton thread (2 to 4 sutures were given). Stitches were sealed with a thin film of healer spray.

**RECOVERY AFTER ANAESTHESIA**

Operated animals were replaced to their respective cages and observed carefully till recovery after anaesthesia.

**POST OPERATIVE CARE**

Animals were kept in similar environmental conditions after operation. They were watched carefully in their post operative days till the date of sacrifice then.

**RADIOLOGICAL EXAMINATION**

On 14th day of operation all the animals were radiographed. Animals of control and first test group were sacrificed and placed over a marked and
loaded radiographic cassette, while animals of second test group were anaesthetised and placed in the same manner. Intact hind limbs were positioned and fixed to the cassette. Remaining part of x-ray film was blocked with the help of lead blocks over already marked area over the cassette. Antero-posterior and lateral radiographs of animals limbs were taken. Following radiological factors were used.

(a) s a : 10
(b) k v : 35
(c) Distance between leg and x-ray tube = 30 inches.
(d) Grid was not used.

Standard radiographic and dark room techniques were used.

In second test group of animals x-ray of their operated limbs were again taken on 20th day, when these were sacrificed. All factors and technique used was the same.

MACROSCOPIC EXAMINATION

Macroscopic examination was done after sacrificing the animals of control and first test group on 14th day. But in second test group, animals were anaesthetised on 10th day and findings were noted at the time of second operation of these animals. On 20th day animals of this group were sacrificed and again examined.
macrocopically alike the animals of other groups.

Following features were noted on macroscopic examination of animals:

(A) SOFT TISSUE CHANGES

(a) EXTERNAL FINDINGS :

(i) Condition of scar.
(ii) Swelling of operated leg.
(iii) Consistency of swollen leg.
(iv) Presence of cystic swelling on the operated leg.

(b) FINDINGS AFTER INCISION :

(i) Discharge after incision.
(ii) Oedema of muscular tissues.
(iii) Granulation tissue adjacent to hole.
(iv) Fibrosis in muscles.
(v) Thickening of periosteum.
(vi) Adherent periosteum.
(vii) Presence of sub periosteal abscess.

(B) CHANGES IN FOWL AND HOG CHEEK PLUG

(i) Thickening of bone.
(ii) Roughening of bone.
(iii) Destruation of bone.
(iv) Consistency of bone.
(v) Whether bone was brittle or not.
(vi) Sequestration.
(vii) Formation of involucrum.

(viii) Condition of cement bead in hole.
   a. Loose or b. Tight.

(ix) Condition of cement bead itself
   (after its removal from the hole).
   a. Any change in shape size or consistency.
   b. Whether surface of cement was smooth or rough.
   c. Any change in weight of cement bead.

(x) Presence of pus inside the hole, after removal of bone-cement plug.

BACTERIOLOGICAL EXAMINATION:

Culture of the following material was studied:

(a) Specimen from discharge of sinus (if sinus was seen in animal).

(b) Skin of animals was incised and any discharge if seen was drained and examined.

(c) Pus collection beneath the periosteum was also drained and examined microbiologically.

(d) Cement plug was taken out and examined bacteriologically.

(e) A small bone piece adjacent to tibial hole was also taken out and cultured.

Above mentioned material or specimen was taken and incubated in nutrient broth at 37°C for 24 hours. Next day broth was examined for development
of turbidity in it. At the same time one platinum loop full suspension from broth was taken and subcultured in blood agar culture plate. Plates were again incubated at 37°C for next 24 hours. Growth on culture plate was examined. If contaminants were also grown in these plates, they were also identified. All these procedures were done with all possible aseptic precautions.

IDENTIFICATION:

Green organisms on culture plates were distinguished on the basis of following features.

(i) Colony characteristics.

(ii) Beta hemolysis.

(iii) Their coagulase activity.

(iv) Microscopic examination: A smear from grown organisms on culture plate was made over a clean glass slide. This smear was fixed and stained by Gram's staining with standard techniques, thereafter, organisms were recognized by the microscopic examination of this smear.

HISTOPATHOLOGICAL EXAMINATION:

(A) REMOVAL OF SPECIMEN:

The animals of control and first test groups were sacrificed on 14th day and a small bone piece (about 0.5 cm in size) was taken adjacent to the tibial hole and
preserved for histopathological examination. In second
test group of animals specimen was removed on 14th day
at the time of second operation in them, for replacing
the bone cement bead. All precautions were taken to
avoid much change in size or shape of the tibial hole,
while removing bone piece. On 28th day, animals of this
group were also sacrificed and specimen was removed
similarly as in other groups. All aseptic precautions
were taken during removal of specimen for microscopic
examination.

**PRESERVATION :**

Specimen was preserved in 10% formaline
solution, after its removal.

**DECALCIFICATION :**

Decalcification of preserved specimen was
done by keeping it in 5% aqueous nitric acid solution
for 24 hours.

**WASHING :**

Washing to remove the excess of nitric
acid was done by keeping the decalcified specimen under
running tap water for 12 hours.

(b) **PROCESSING :**

Decalciﬁed specimen was passed in an
automatic tissue processor. It was passed through the
following chemicals for the duration shown against the name of the chemical.

<table>
<thead>
<tr>
<th>S.No. Stage</th>
<th>Chemical</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) DEHYDRATION</td>
<td>(a) 70% Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td>(b) 90% Ethanol I</td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td>(c) 90% Ethanol II</td>
<td>2 hours</td>
</tr>
<tr>
<td></td>
<td>(d) 100% Ethanol I</td>
<td>1 hour</td>
</tr>
<tr>
<td></td>
<td>(e) 100% Ethanol II</td>
<td>2 hours</td>
</tr>
<tr>
<td></td>
<td>(f) 100% Ethanol III</td>
<td>2 hours</td>
</tr>
<tr>
<td>(2) CLEARING</td>
<td>(a) Chloroform I</td>
<td>3 hours</td>
</tr>
<tr>
<td></td>
<td>(b) Chloroform II</td>
<td>4 hours</td>
</tr>
<tr>
<td></td>
<td>(c) Chloroform III</td>
<td>4 hours</td>
</tr>
</tbody>
</table>

**Wax Impregnation:**

(a) Paraffin I 1 hour
(b) Paraffin II 1 hour
(c) Paraffin III 2 hours

(E) EMBRIOLOG:

A square shaped space approximately 1.5 cm x 1.5 cm was made by placing two 'L' plates over a glass slide. Molten wax was poured in this space and processed tissue was put in it, wax was then allowed to solidify, central depression, which was made in the wax block at the time of solidification, was filled with more wax. Solid mould was taken out removing 'L' plates and then trimmed.
(F) **SECTION CUTTING**

Sections were made from tissue, embedded in wax block. Microtome was used to prepare the tissue sections. Average thickness of sections was 5 micron.

(C) **STAINING**

(i) Wax-removal: Section was placed in Xylol for 1 to 2 minutes, to dissolve the wax.

(ii) Hydration: Section was taken out of Xylol and transferred to 100% and 90% alcohol and left for 1 minute in each respectively.

(iii) Staining: Hydrated section from 90% alcohol was removed and kept in hematoxylin stain for 10 minutes. Excess of stain was removed by keeping the slide in acid alcohol. It was then washed until section showed blue colour.

(iv) Counter staining: Stained section was dehydrated again, by passing it through 90% and 100% alcohol respectively for 10-15 seconds in each, and then to absolute alcohol II for 30 seconds.

(v) Clearing: Dehydrated section was transferred to Xylol and left in it till became clear.
(vi) Mounting: Section was taken cut of xylol and placed in middle part of a cleaned glass slide, excess of xylol was removed by wiping around the section, one drop of Canada balsam was placed over the section and cover slip was applied.

**MICROSCOPIC EXAMINATION:**

The prepared slide was examined under a microscope to find out the following features of bone infection.

(i) Congestion of sinusoids.

(ii) Increase in number or size of sinusoids.

(iii) Presence of inflammatory exudates.

(iv) Cellular infiltration, including type of infiltrated cells.

(v) Empty lacunar spaces.

(vi) Loss of trabecular striations.

(vii) Bone necrosis.

(viii) Engorgement of arterioles or venules in Haversian canals.

(ix) Raw osteonal surface of bone.

(x) Hemorrhages around the vessels.

(xi) Shrinkage of fat cells in bone marrow.

(xii) Fibroblast proliferation.

(xiii) Periosteal congestion.