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An Experimental Model of Post-Traumatic Osteomyelitis In Rats

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Osteomyelitis is an infection of bone and bone marrow with a propensity for progression, usually caused by pyrogenic bacteria or mycobacteria. It is an acute or chronic inflammatory process of the bone and bone marrow. Findings it was concluded that VRP 1003 has free radical scavenging property which is helpful for the prevention of osteomyeltis infection caused by staphylococcus aureus microorganism and also prevent heptotoxicity in comparison to levofloxacin drug.

Keyword: Beck Depression Inventory, Self-Report, Depression Rating,

1. Introduction

Osteomyelitis (OM) is a bone infection characterized by progressive inflammatory destruction of the bone, bone necrosis and induction of new bone apposition at the site of infection]. Acute bacterial osteomyeltis carried out 15% mortality in the pre antibiotic era because of overwhelming sepsis with metastatic abscesses. During osteomyeltis infection, bacteria induces local bone destruction (osteolysis) and causes an intense inflammatory response, thrombosis of endosteal and periosteal vessels, bone infarcts with subsequent abscess and sequestrum   formation. The pathogenesis of osteomyelitis are very limited, and immune responses in this infection are poorly characterized. In the present study, body weight was   decreased along with increased   body temperature in infected group. High temperature and weight loss of infected group indicate that the development of bone infection occur due to

bacterial infection and loss of bone material during infection. The concentration of TNF-α and IL-6 were elevated in osteomyelitis infected group. IL-6 is likely to have several actions in osteomyelitis. It has an important role in the acute-phase response IL-6 cannot be the only mediator but also have a critical role in altering osteoclast and osteoblast function during bone remodeling[119]. Several studies have been reported that the increased levels of proinflammatory cytokines, such as IL-6 and TNF- α released by the host in osteomyelitis patients. Cytokines such as IL-6, TNF- α may be directly involved in bone desorption and osteoclasts, 'activity regulation' occurring in osteomyelitis. The levels of protein, calcium and sodium were decreased along with increased phosphorus and C- reactive protein level in infected group. Calcium, sodium and phosphorus minerals play a significant role in the repair of bone fractures. Calcium and phosphorus are the

main constituents of bone in the form of calcium phosphate. It provides tensile strength to bone. Due to alteration in calcium and phosphorus levels during bacterial infection, bone become fragile. So calcium and phosphorus level are essential in regulating the elastic stiffness and tensile strength of bone. Protein level was decreased during bacterial infection in bone due to loss of bone minerals. C-reactive protein (CRP) is also acute phase protein that causes inflammation during bacterial infection. Several studies have been reported that the CRP level was increased during osteomyelitis infection. In the present study the levels of urea, creatinine and uric acid were significantly increased in infected group. It means that bacterial infection also altered renal enzymes during osteomyelitis. Various researchers have been reported that Osteomyelitis infection has only rarely been associated with glomerular diseases but on the basis of present findings have concluded that glomerulonephritis diseases occur during chronic osteomyeltis which is caused due to staphylococcus aureus bacteria . Hepatic enzymes (SGPT and SGOT) did not altered during osteomyelitis infection. Similar result was reported by Murdoch in Vertebral Osteomyelitis due to Staphylococcus lugdunensis . Free radical mediated damage may also responsible during bone infection. It has been reported that oxygen-free radicals play a significant role in the formation and activation of osteoclasts. In present study the level of malondialdehyde and myeloperoxidase level were increased in infected group in comparison to both treated groups. various studies have been reported that the levels of MDA and myeloperoxidase were increased during osteomyelitis. Many aspects of the antibiotic treatment of osteomyelitis have not been completely investigated. Antibiotics kill bacteria and prevent their invasive spread, while surgery aims to drain pus, remove necrotic soft and bone tissues and bacterial slime, and restore blood supply. Levofloxacin is commonly referred to as a quinolone drug and is a member of the fluoroquinolone class of antibacterials. VRP 1003 is a fixed dose combination of antibiotic i,e cefepime and amikacin. After treatment of respective drugs for 21 days, all the biochemical parameters were improved in levofloxacin treated as well as VRP 1003 treated groups but inVRP1003 all the parameters reaches nearly equal to control normal value when compared to infected group. When levofloxacin treated group was compared to VRP 1003 treated group, all biochemical parameters along with cytokinine levels and free radical mediated damage (MDA and MPO) levels were improved in VRP 1003 treated group and approx similar to normal value after 21 days treatment. But in case of hepatic enzymes, the level of SGOT and SGPT were significantly increased in levofloxacin treated group. While in case of VRP 1003 treated group, the level of SGOT, SGPT were decreased but did not altered significant. It means that levofloxacin drug causes major side effect in liver during osteomyelitis infection. Several studies have been reported that levofloxacin causes hepatoxicity and tendon damage during osteomylitis treatment . VRP 1003 drug have free radical scavenging properties by using chemical vector mediated technology. Chemical vector mediated technology is used to provides compatibility of cephalosporins and aminoglycosides without interfering with the pharmacokinetic property of drug component and prevents the oxidation of methionine group and thiazolidine and dihyrothiazine present in antibiotics.

2. Materials and Methods
2.1 Antibiotics (Drugs):
All the biochemicals used in the present study were procured from Sigma, St.Louis, MO, USA. Other chemicals purchased locally were of analytical grade. All the antibiotics such as levofloxacin, cefepime plus amikacin (VRP 1003) were obtained from Venus Remedies Ltd. India. The concentration of levofloxacin was 51.6mg/ gm/BW. The concentration and ratio of fixed dose combination of cefepime + amikacin was 25mg/gm/BW.

2.2 Bacteria
The Staphylococcus aureus strain (MTCC no. 737) was used for the preparation of

osteomyelitis induced rat model. Staphylococcus aureus bacterial strain was inoculated on nutrient agar slant were grown in septic culture in nutrient broth at 37 °C for 24 hours. Organisms were harvested and centrifuged at 2348 g for 15 min, washed three times, and suspended in phosphate buffer saline (0.2 M, pH 7.0) to the desired concentration. The Concentration of Staphylococcus aureus was used $1x10^6$CFU/ml for induction of osteomyelitis in the study.In TBS agar plates, a suspension containing approximately 105 colony-forming units (CFU)/ml of S. aureus was used as inoculum.

2.3 Osteomyelitis Model
Osteomyeltis infection was induced to animals according to method of fiza . For the purpose of this investigation, it is necessary to develop a model of osteomyelitis in rats. Total twentyfour rats were anesthetized by intramuscularly administration of 10 mg of ketamine per 100 gm of body weight. The left hind legs were shaved and clean with 70% ethyl alcohol. The proximal part of the tibia exposed anteriorly and a hole was drilled through the cortex into the medullary cavity by using a high speed drill with a 0.6 mm diameter bit. A bacterial strain 100 µl ($1x10^6$ CFU/ml) was injected through the hole and stainless steel implants (4x1x1mm) were inserted in to the medullary cavity. The hole was covered with bone wax to prevent bacterial leakage in to soft tissues. The skin was sutured and the animals were then allowed free movement in their cage for three weeks, before treatment began.

2.4 Animals and Treatments
Total twenty four wistar rats, (weighing 240 to 245 gm) were used in the experiment. They were housed at controlled temperature and humidity in an alternating 12-hr light and dark cycle with free access to food and water. The study was approved by the institutional animal ethical committee (Ref.No,VEN/EC/PRJ/014)drugs were given to Animals intravenously according to their body weight for three weeks treatment. The rats were divided into four groups of six rats containing each group.

Group I : control group treated with normal saline 0.9% w/v

Group II : Infected with S. aureus ($1x10^6$ CFU/ml).

Group III : Infected plus VRP 1003 treated group (25mg/gm/ body weight).

Group IV : Infected plus levofloxacin treated group (51.6mg/gm/ body weight).

Bacterial strain Staphyloccous aureus was injected to all groups except control group. After three weeks induction of infection, treatment was started with respective drugs for three weeks. Body weight and temperature was monitored at every fourth day in infected and treated groups along with control groups upto three weeks. All the animals were scarified on 21th day with a lethal dose of ether. Blood samples from all groups were collected from heart in polypropylene tubes for seperation of blood serum.

2.5 Serum Preparation
2.0 ml Citrate free blood samples were collected and centrifuged at 3500 rpm for 20 minutes. The supernatant was removed vary carefully and stored at 0°C- 4°C at least for 1 hours before estimation of biochemical and enzymatic parameters.

2.6 Measurement of Myeloperoxidase Level
Myeloperoxidase level was determined by O-dianisidine method with slight modificationThe assay mixture consisted of 0.3 ml of sodium phosphate buffer (0.1 M; pH 6.0) 0.3 ml 0.01 M H_2O_2, 0.2ml of 0.02 M O-dianisidine (freshly prepared) in distilled water and added water up to 3.0 ml. The reaction was started by adding 0.025 ml serum. The change in absorbance was recorded at 460 nm wavelength. All measurement was carried out in duplicate. One unit of enzyme activity is defined as that giving an increase in absorbance of 0.001per min.

2.7 Estimation of Reduced Glutathione
The reduced glutahione level was estimated by the method of Hissin and Hiff[114] . In this method

0.25 ml serum was taken and mixed with 3.0 ml of 5 % (w/v) TCA reagent and allowed
to stand for 10 minutes for the precipitation of protein and filtrate out. Take 1.0 ml of filtrate and added to 2.0 ml of 0.3 M phosphate buffer pH 7.4 and 0.5 ml of DTNB (1% w/v aqueous sodium citrate). A blank was run simultaneously using distilled water in place of the filtrate. An appropriate standard solution of 0.075 ml GSH (10 μmol) was also run simultaneously. The pale yellow colored developed and absorbance was recorded immediately at 412 nm wavelength by spectrophotometer.

2.8 Lipid Peroxidation Level
Free radical mediated damage was assessed by the measurement of the extent of lipid peroxidation in the term of malonadialdehyde (MDA) formed. It was determined by thio barbituric reaction. The reaction mixture consisted of 0.25 ml of serum preparation, 0.20 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of (20%, pH 3.5) acetic acid, 1.5 ml of 0.8% thio barbituric acid (TBA) and distilled water to make up the volume to 4.0 ml. The tubes were boiled in water bath at 95^0C for one hour and cooled immediately under running tap water. Added 1.0 ml of water and 5.0 ml of mixture of n-butanol and pyridine (15:1 v/v) and vortexed. The tubes were centrifuged at 3500 rpm for 20 minutes. The upper layer was aspirated out and optical density was measured at 532 nm. The reference standard used was 1,1, 3,3 tetra ethoxy propane.

2.9 Biochemical Parameters
All other biochemical parameters (C-reactive protein, Protein, Calcium, Phosphorus, sodium, hepatic and renal function test) were determined by using a commercially available standard kit (Bayer Diagnostics India Ltd.,Baroda, Gujarat, India).

2.10 Statistical Analysis
The data obtained was analyzed statistically. All values are expressed as mean ± SD. One-way analysis of variance (ANOVA) with student-Newman-Keuls comparison test was used to determine statistical difference between infected vs treated groups and compared with normal control group.

3. Results
3.1 Mortality
Mortality was not seen during experimental study. Swelling symptom was occurred in infected group after inoculation of bacterial strain.

3.2 Body Weight and Temperature
In the present study, the body weight was decreased along with increased temperature (^{0}C) in infected group. After treatment with levofloxacin and VRP 1003 drug for three weeks, the body weight and temperature (^{0}C) were improved in both treated group as compared with infected group. Body weight and temperature (^{0}C) were improved in VRP 1003 treated group in comparison to levofloxacin treated group and reaches nearly to control normal group.

3.3 Effect on Protein and C-Reactive Protein Level
Protein level was decreased along with increased C-reactive protein level in infected group. After treatment from both specified drugs, the level of protein was found to increased along with decreased C-reactive protein levels in both treated group as compared with infected group. When levofloxacin treated group was compared with VRP 1003 treated group, the levels of protein and C-reactive protein were improved but did not alter very significantly in the serum of VRP 1003 treated group.

3.4 Effect on Calcium, Phosphorus and Sodium Levels
Calcium and sodium levels were decreased along with increased Phosphorus level in the serum of infected group. The levels of calcium and sodium were increased significant in levofloxacin treated group as well as in VRP 1003 treated group as compared with infected group after three weeks treatment of respective drugs. But in case of phosphorus, the level was decreased in both treated group. When levofloxacin treated group

was compared with VRP 1003 treated group, the level of calcium was increased but insignificant in VRP 1003 treated group after three weeks treatment of drug. Whereas in case of sodium, the level was increase in VRP 1003 treated group. In case of phosphorus, the level was decreased but did not altered very significant in VRP 1003 treated group after three weeks treatment when compared with levofloxacin treated group.

3.5 Effect On Urea, Uric Acid and Creatinine
These levels were increased in infected group after inoculation of Staphyloccous aureus bacterial strain. When administration of levofloxain and VRP 1003 drugs were administered intravenously in infected group, the levels of urea, uric acid and creatinine were decreased in both treated group as compared to infected group. The levels of urea and creatinine were decreased significantly in VRP 1003 treated group when compared with levofloxacin treated group after treatment of drugs for 21 days. But in case of uric acid, the level was decreased but insignificant in VRP 1003 treated group as compared with levofloxacin treated group.

3.6 Effect on Hepatic Enzymes (SGOT and SGPT)
Serum glutamic-oxaloacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) were not altered in osteomyelitis induced infected group. After administration of levofloxacin drug upto three weeks treatment, these levels were significant increased in the levofloxacin treated group in comparison to infected as well as VRP 1003 treated group. While in case of VRP 1003 treated group, these level were lowered but did not altered significant in comparison to infected group.

3.7 Effect on Reduced Glutathione Level
Reduced glutathione level was found to decreased in infected group. After treatment of both respective drugs for 21 days, the GSH level was found to be significant increased in the VRP 1003 treated group as well as in levofloxacin treated group. On comparison of both treated groups, the level was found to higher in VRP 1003 treated group after end of the treatment.

3.8 Effect on Myeloperoxidase Level
The level of myeloperoxidase was founded higher in infected group. This level was decreased significantly in both treated group after treatment of drug for 21 days. When levofloxacin treated group was compared with VRP 1003 treated group, the myeloperoxidase level was highly reduced in VRP 1003 treated group after end of the treatment.

3.9 Effect on Malondialdehyde Level
The level of malondialdehyde (MDA) was increased in osteomyelitis induced infected group. The level was reduced significantly in levofloxacin treated group as well as in VRP 1003 treated group after administration of both drugs for 21 days treatment. When levofloxacin treated group was compared with VRP 1003 treated group, the level was significantly reduced in VRP 1003 treated group. MDA level is a marker of free radical mediated damage.
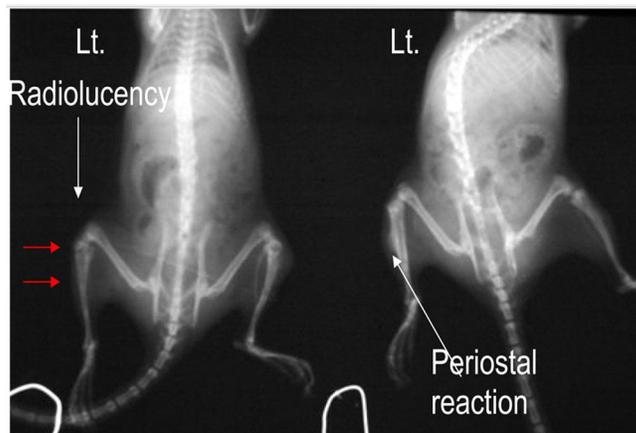

Fig 1: Radiological Examination of Osteomyelitis Induced Rat

3.10 Effect on TNF-α and IL-6
TNF-α and IL-6 were increased in infected group. The levels of TNF-α and IL-6 were significant

decreased in Levofloxacin treated group as well as in VRP 1003 treated group after treatment with respective drugs. When the levels of TNF-α and IL-6 was compared in both treated groups, the levels were found to   lowered in VRP 1003 treated group after 21 days treatment.

4. Observation
From the above radiological examination the left joint bone has swelling and periosteal reaction which indicates the symptoms of osteomyelitis.

Table 1: Status of biochemical parameters and free radical mediate damage (MDA and MPO levels) in   osteomyelitis induce and treated groups

SS.NO	Parameters	Control group	Infected group	Levofloxacin group (L)	VRP1003 Group
1	Protein (mg/ml)	8.75±1.26	7.25 ± 0.25	8.32 ± 0.16	8.48 ± 0.16
2	C reactive protein	5.86±0.26	10.62 ±0.37	5.22 ± 0.04	5.18 ± 0.14
3	Calcium (mg/dL)	7.92±0.15	7.23 ± 0.11	7.53 ± 0.12	7.58 ±0.07
4	Phosphorus (mg/dL)	5.25±1.26	6.28 ± 0.2	4.68 ± 0.16	4.66 ± 0.10
5	Na level (mg/dL)	176.2±3.45	159. 7 ± 1.69	174.54 ± 2.19	177.07 ± 2.04
6	Urea (mg/dL)	48.56±1.26	57.03 ± 1.35	52.54 ± 2.98	49.8 ± 1.15
7	Uric acid (mg/dL)	0.95±0.25	3.18 ± 0.21	2.07 ± 0.25	1.96 ± 0.10
8	Creatinine (mg/dL)	0.26±0.01	0.56 ± 0.02	0.35 ± 0.02	0.29 ± 0.03
9	SGOT level	126.45±21.2	128.33 ± 17.69	198.67 ± 5.59	127.83± 22.15
10	SGPT level	48.5±3.4	48.5 ± 2.5	69.83 ± 4.52	48.17± 4.81
11	Glutathione reduced	0.031±0.002	0.0128 ± 0.0013	0.0253 ± 0.0009	0.0299 ± 0.0015
12	MDA level	0.89±0.102	1.32 ± 0.24	1.10 ± 0.040	0.909 ± 0.106
13	MPO (nmole/min/ml)	3.13±0.55	10.22 ± 0.67	7.46 ± 0.25	3.49 ± 0.44

All values are expressed mean ± SD. Significant data are reported between infected vs levofloxacin and VRP 1003 treated group and control group
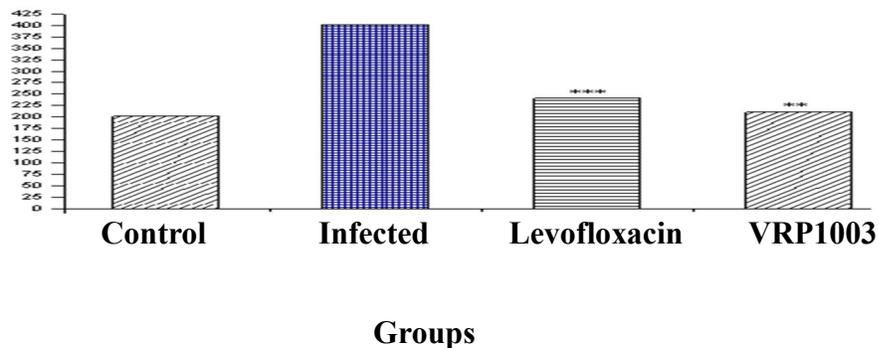

Fig 1: Status of TNF alpha in osteomyelitis induced rat and after treatments

All values are expressed mean±SD. Significant data are reported between infected vs levofloxacin and VRP 1003 treated group along with control group.
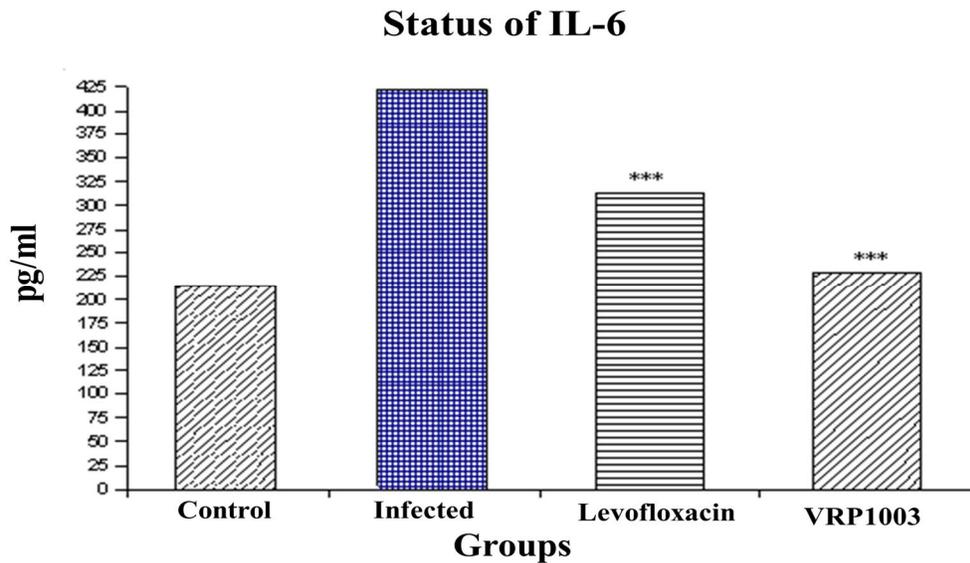
Status of IL-6

Fig 2: Status of IL-6 in osteomyelitis induced rat and after treatments

All values are expressed in mean±SD. Significant data are reported between infected vs levofloxacin and VRP1003 treated group and control normal grup.

Table 2: Status of Body Weight Monitoring In Osteomyelitis Induced and Treated Groups

S.no	Groups	Initial day	4th day	8th day	12th day	16th day	20th day
1.	Infected	250.45±22.03	244.06±19.35	228.37±6.8	212.39±5.98	204.51±5.38	184.76±4.8
2.	Control	251.25±19.85	207.42±4.40	230.29±8.17	240.21±6.49	245.43±3.86	250.24±4.81
3.	Levofloxacin treated	262.06±15.94	190.5±4.3	209.21±9.24	218.84±4.49	228.7±5.94	236.89±5.41
4.	VRP1003 treated	250.45±22.03	205.36±3.21	226.19±7.37	237.18±5.54	243.34±3.75	246.13±2.42

All values are expressed mean ± SD. Significant data are reported between infected vs levofloxacin and VRP 1003 treated group and control group.

Table 3 : Status of body temperature monitoring in osteomyelitis induced and treated Groups

S.no	Groups	Initial day	4th day	8th day	12th day	16th day	20th day
1	Infected	36.6±0.55	37.79±0.46	38.53±0.37	38.95±0.30	38.93±0.30	39.31±0.28
2	Control	38.74±0.68	38.85±0.35	37.96±0.09	38.54±0.46	38.05±0.19	37.91±0.15
3	Levofloxacin treated	39.22±0.20	39.13±0.30	38.28±0.08	38.14±0.52	37.81±0.10	37.28±0.19
4	VRP 1003 treated	39.14±0.08	38.64±0.39	38.17±0.12	38.04±0.52	37.58±0.25	37.14±0.11

All values are expressed mean ± SD. Significant data are reported between infected vs levofloxacin and VRP 1003 treated group and control group.

5. Conclusion

On the basis of present findings it was concluded that VRP 1003 has free radical scavenging property which is helpful for the prevention of osteomyeltis infection caused by staphylococcus aureus microorganism and also prevent heptotoxicity in comparison to levofloxacin drug.

6. References

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