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Original Research Article

Safety and efficacy evaluation of plain OTC-HCL in acute and subacute toxicity tests and *Brucella abortus* infected mice

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ABSTRACT

Aims and Objectives: The study aimed to evaluate the safety and *in vivo* efficacy of plain Oxytetracycline hydrochloride (OTC-HCL) in mice experimentally infected with *Brucella abortus (B. abortus)* 544 strain.
Materials and Methods: The safety of plain OTC-HCL was assessed through acute and subacute toxicity testing. The LD_{50} value was determined, and hematologic, biochemical, and histopathological analyses were performed. For efficacy evaluation, mice were intraperitoneally injected with 3 × 10^6 CFU of *B. abortus* 544 in 0.5 ml. Following infection, the mice received 25 mg/kg of plain OTC-HCL intravenously either daily or on alternate days for 21 days, starting from day 14 post-infection. Efficacy was assessed by measuring the logarithmic decrease in *B. abortus* counts in the liver, lymph nodes, and spleen on days 1, 7, and 14 after treatment.
Results: Plain OTC-HCL has an LD_{50} of greater than 250 mg/kg. In both acute and subacute toxicity evaluations, the hematologic and biochemical data of the drug were within the normal range. Histopathology studies revealed no significant changes in the safety studies. In the subacute toxicity study, the drug was administered to mice received plain OTC-HCl @ 25 mg/kg intravenously for 21 days. Efficacy was measured by the decrease of *B. abortus* counts in the liver, lymph nodes, and spleen.
Conclusion: Plain OTC-HCL was found to be safe in both acute and subacute toxicity tests and effectively reduced the infection load of *B. abortus* in mice. Our findings and novelties are**:** 1) LD_{50} is more than 250 mg/kg for plain OTC-HCL by intravenously as a single dose. 2) Histopathology indicates that there is no organ toxicity for plain OTC-HCL in safety studies. 3) Plain OTC-HCL was found to be effective in reducing the infection load in *Brucella*-infected mice.

1. Introduction

Clinical control of brucellosis, a zoonotic intracellular bacterial illness, remains a significant challenge. This disease is primarily transmitted to humans through the consumption of unpasteurized milk from infected animals. One of the key difficulties in managing brucellosis is that conventional antibiotics only partially target intracellular *Brucella*, which can evade the immune system by residing and reproducing within host cells. Additionally, *Brucella* has the capability to induce abortions in animals and may also proliferate extracellularly, contributing further to the complexity of the disease. Oxytetracycline (OTC) is a broad-spectrum antibiotic with bacteriostatic activity against a wide range of gram-positive and gram-negative

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bacteria, including certain anaerobes found in livestock and poultry. The introduction of OTC, particularly long-acting formulations, either alone or in combination with streptomycin, has proven effective in alleviating symptoms of brucellosis and reducing *Brucella* shedding in infected cows during parturition.[1]

Oxytetracycline hydrochloride (OTC-HCl) is commonly used for systemic therapy as well as local therapy for gastric or intestinal infections of bacteria. Rana et.al. 2011 reported that the additional functions of OTC-HCl include anti-inflammatory, antioxidant and immunosuppressive activity. It has recently been considered as therapy of choice for skin infections, perioral dermatitis, papulopustular acne and rosacea.[2] Oxytetracycline has been found to have strong iron chelating properties.[3] Conventional oral formulations of OTC-HCl lead to gastrointestinal irritative effects such as stomach upset, epigastric burning, nausea and vomiting due to the high solubility of this drug in the gastric fluid.[4] OTC is widely distributed in tissues like the heart, kidney, lungs, muscles, and body fluids like pleural fluid, bronchial secretions, sputum, bile, saliva, urine, synovial fluid, ascitic fluid, ocular aqueous humor, and vitreous humor. The advantages of intravenous administration of OTC-HCL are that it promises full and instantaneous drug bioavailability and results in speedy therapeutic blood levels. In *brucella* infectious, it is especially important where quick and efficient treatment is required. Also, the benefit of intravenous delivery is that exact dosing and control over the drug's pharmacokinetics are possible.

This is primarily due to two major factors: the high cost of treatment, which necessitates large doses of drugs over extended periods, and the inability to completely eradicate *Brucella* from infected animals, leading to lifelong carriers. A significant challenge to antibiotic therapy is the intracellular sequestration of the bacteria in lymph nodes, mammary glands, and reproductive organs, where they are protected from the immune response and antimicrobial agents.[5] Given these complexities and challenges, the present study was undertaken to assess the safety of plain OTC-HCL through acute and subacute toxicity tests, as well as to evaluate its *in vivo* efficacy in mice.

2. Materials and Methods

2.1. Ethical permission

All animal procedures followed CPCSEA rules and were sanctioned by the Institutional Animal Ethics Committee (IAEC) under resolution no. 10/2022 dated June 2022, as well as prior approval from the Institutional Biosafety Committee (IBSC).

2.2. Sample collection

A total of 72 blood samples were collected for hematological and biochemical analysis from the mouse's retro-orbital plexus using capillary tubes. The liver, kidneys, heart, spleen, and lungs were also collected for histopathological investigation..

2.3. Acute toxicity test

Following sanction from the Institutional Animal Ethics Committee (IAEC), twenty-four adult Swiss albino mice weighing 20-25 grams and of either gender were sourced from the Central laboratory animal house facility at Mumbai Veterinary College for both acute and subacute toxicity studies. As per OECD guideline No. 420, the acute toxicity of plain and nanoparticulate oxytetracycline was conducted. These mice were housed in an individually ventilated cage (IVC) at a temperature of 21°C and a relative humidity of 65-68 percent, with a balanced 12-hour light and 12-hour dark cycle. The mice were randomly selected, weighed, and labeled to enable individual identification. To ensure acclimatization, the mice were individually housed in IVC under similar management and feeding conditions for seven days before commencing the toxicity studies at the laboratory animal inoculation facility of the Department of Microbiology, Mumbai Veterinary College. Following the acclimatization period, six mice for each group were administered intravenously, receiving a dosage of 250 mg/kg of plain oxytetracycline as a single dose, along with control groups.

They were individually monitored for the initial 30 minutes, followed by subsequent observations at 30-minute intervals for the next 4 hours, and then at regular intervals for the subsequent 24 hours. Conducted daily checks over the following 14 days to assess mortality and detect toxicity symptoms, including tremors, convulsions, salivation, diarrhea, lethargy, sleep, etc. Recorded the body weight of the mice on days 0, 7, and 14. On day 14, the mice were humanitarianly sacrificed. On day 14, capillary tubes were used to take blood samples from each mouse's retro-orbital plexus. The samples were then processed for hematological and biochemical tests. A thorough postmortem examination was conducted, recording detailed gross lesions. Gathered and preserved tissue samples from the liver, kidneys, heart, spleen, and lungs were in 10% formalin for subsequent histopathological studies.

2.4. Subacute toxicity test

Following approval from the Institutional Animal Ethics Committee (IAEC), Swiss albino mice weighing 20-25 grams and of either gender were sourced from the Central laboratory animal house facility at Mumbai Veterinary College for acute and subacute toxicity studies. The subacute toxicity assessment of plain and nanoparticulate oxytetracycline was conducted by OECD guideline No. 407. These mice were housed in a well-ventilated environment maintained at a temperature of 21°C and a relative humidity

of 65-68 percent, with a balanced 12-hour light and 12-hour dark cycle. The mice were randomly selected, weighed, and labeled to enable individual identification. To ensure familiarization, the mice were individually housed in ventilated cages under similar management and feeding conditions for seven days before commencing the toxicity studies at the laboratory animal inoculation facility of the Department of Microbiology, Mumbai Veterinary College. Following OECD 407 criteria, a sub-acute toxicity study (a 28-day repeated toxicity study) was conducted.

The subacute intravenous toxicity of plain oxytetracycline drugs was performed using repeated doses of 125 mg/kg, 25 mg/kg, 12.5 mg/kg of plain oxytetracycline in mice for 28 days. The mice were individually checked for tremors, convulsions, salivation, diarrhea, lethargy, sleep, etc. The animals were checked daily and for the next 28 days for mortality and signs of toxicity. The individual body weight of mice was recorded on days 0, 7, 14, 21 and 28 of the experiment. The mice were humanely sacrificed on day 29. On day 29, capillary tubes were used to draw blood samples from each mouse's retro-orbital plexus. The blood samples were then processed for hematological and biochemical tests. A comprehensive postmortem investigation was carried out, documenting specific gross lesions. For ensuing histopathology investigations, tissue samples from the liver, kidneys, heart, spleen, and lungs were collected and kept in 10% formalin.

2.5. In vivo efficacy of plain oxytetracycline in the clearance of Brucella infection in mice

2.5.1. Inoculation of mice with the B. abortus 544 bacterial strains

B. abortus 544 bacterial strain was used for the experiment. *Brucella* was grown on *Brucella* agar media for two to three days at 37ºC in ambient air to reach the stage of exponential growth. A few pure colonies of *B. abortus* from the BAM plate were transferred to 0.85% normal saline solution, and the turbidity of the bacterial suspension was adjusted against the background of the Wickerham card using the McFarland standard. All mice were inoculated intraperitoneally with 0.5 ml of saline containing 3 x 10^6 cfu's of *B. abortus* inoculum. The infected mice were closely monitored for up to 14 days after infection.

2.5.2. Treatment of infected mice with OTC-HCl antibiotics

The infected mice were kept under close observation for up to 14 days post-infection. On the 15th day post-infection, the mice were randomly divided into 2 groups, each group consisting of 6 mice, and were treated intravenously with plain and nanoparticulate oxytetracycline along with controls, as shown in Table 1.

Both the mice from the treatment and control groups were sacrificed under anesthesia on 1, 7, and 14 days after the last dose of treatment.

2.5.3. Estimation of a total viable count of B. abortus from the spleen, liver and lymph nodes of treated mice

Detailed postmortem examination was performed for each mouse after the treatment. The organs including spleen, liver and lymph nodes were collected aseptically and processed further for detection of *B. abortus* by total viable count (TVC). TVC was obtained by spreading 100 μl of each dilution of the spleen, liver, and lymph node homogenates throughout the surface of BAM plates with an L-shaped spreader. Plates were incubated at 37°C for up to 9-10 days in CO_2 incubator and were checked for the presence of colonies on a regular basis before being categorized as sterile. The colonies on each plate were then counted and documented for each dilution. Every plate's colony count was noted except for those with too many colonies to enumerate.

Viable plate count of the bacteria was calculated by the following formula:

1. Viable count (CFU/ml) = CFU/V × DF

Where,
CFU: Number of colonies on the plates
V: Volume plated
DF: Dilution factor

3. Results

3.1. Acute toxicity study

3.1.1. Body weight

The acute intravenous toxicity of plain oxytetracycline drugs was performed using a single dose of 250 mg/kg of plain oxytetracycline in mice. During 14 days of acute toxicity studies, no mortality in mice, no weight loss, and no behavioral disorders were recorded in both plain and nanoparticulate oxytetracycline. The difference in weight gain between the mice treated with plain and nanoparticulate oxytetracycline was found to be statistically insignificant(p<0.05). Observations were made at least twice daily for 14 days of study. Intravenous LD_{50} of plain oxytetracycline is greater than 250 mg/kg in mice.

3.1.2. Hematological parameters

Plain OTC-HCL showed a non-significant difference with the control group in parameters such as white blood cell (WBC), platelet count (PLT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), eosinophil (E%), and monocyte (M%). Significant differences were observed in hemoglobin (HB), red blood cell (RBC), packed cell volume (PCV), mean corpuscular hemoglobin

Table 1: Treatment groups of mice used in efficacy studies

S. No.	Treatment	Dose	Number of Mice	Frequency of injection	Duration (days)
1.	OTC-HCL	25 mg/kg	6	Daily	21
2.	Vehicle control	DNS 5%	6	Daily	21

concentration (MCHC), neutrophils (N%), and lymphocytes (L%).

3.1.3. Biochemical parameters

Plain OTC-HCL showed non-significant differences in Aspartate Aminotransferase (AST), alanine transaminase (ALT), Alkaline phosphatase (ALP), albumin (ALB), Total protein (TP), globulin (GLB), blood urea nitrogen (BUN), and creatinine (Creat) when compared with control.

3.2. Histopathological study

After necropsy, liver, lungs, heart, kidney, and spleen samples were fixed in 10% buffered formalin and stored at room temperature for histopathological examination. All histological sections were normal, with no significant difference and without any cell degeneration, congestion, necrosis, etc. In the case of the heart, the myocardium was normal. Unremarkable renal glomeruli and tubules were observed in all groups. The kidney architecture was normal. In the case of the liver, the hepatocytes and portal tracts were found to be normal in all groups, and no inflammation was observed. The spleen was also not affected. Normal red and white pulp were observed in the spleen. There were no significant changes observed in the lungs.

3.3. Subacute toxicity test

Table 2: Details of groups for subacute toxicity

Group	Treatment	Dose
1	Plain OTC-HCL (E)	125 mg/kg
2	Plain OTC-HCL (F)	25 mg/kg
3	Plain OTC-HCL (G)	12.5 mg/kg
4	Vehicle control (H)	

3.4. Body weight

Body weight changes are a sensitive indicator of an animal's overall health.[6] The Subacute intravenous toxicity of plain oxytetracycline drugs was performed using repeated doses of 125 mg/kg, 25 mg/kg, 12.5 mg/kg of plain oxytetracycline in mice for 28 days. A total of 24 mice of both sexes were divided into four groups. The first three groups had plain OTCHCL, and the fourth group had vehicle control (5% DNS). No behavioral disorders were recorded during the 28 days of observation. Observations were made at least twice daily for 28 days of study. Body weight reflects the general health status of animals. In the present study, there was an average increment in body weight. No significant changes like tremors, convulsions, salivation, diarrhea, or coma were observed in mice.

3.4.1. Hematological parameters

The mice treated with repeated doses of plain oxytetracycline for 28 days showed statistically significant by using one-way ANOVA in parameters such as Hb, RBC, PCV, and E% in groups 1, 2, and 3, as compared with vehicle control group 4. At the same time, remaining parameters like WBC, PLT, MCV, MCH, MCHC, N%, L%, and M% showed non-significant differences ($p < 0.05$) by using one-way ANOVA in the levels of hematological parameters.

3.4.2. Biochemical parameters

3.4.2.1. Plain OTC-HCl. The mice were treated with repeated doses of plain oxytetracycline for 28 days showed non-significant changes ($p > 0.05$) by using one-way ANOVA in ALT, Alb, TP, GLB, BUN, and CREAT in groups 1, 2, and 3, as compared with vehicle control group 4. Significant changes were recorded in AST and ALP.

3.5. Histopathological study

After necropsy, liver, lungs, heart, kidney and spleen samples were fixed in 10% buffered formalin and stored at room temperature for histopathological examination. All histological sections were normal, with no significant difference and without any cell degeneration, congestion, necrosis, etc. In the case of the heart, the myocardium was normal. Unremarkable renal glomeruli and tubules were observed in all groups. The kidney architecture was normal. In the case of the liver, the hepatocytes and portal tracts were found to be normal in all groups, and no inflammation was observed. The spleen was also not affected. Normal red and white pulp was observed in the spleen. There was no significant change observed in the lungs

4. Discussion

In the present study, we observed the intravenous LD_{50} plain oxytetracycline its greater than 250 mg/kg in mice. The toxicity studies may provide initial information on the mode of toxic action of an agent and help in deciding the dose of novel compounds in animal studies.[6] In screening new formulations for pharmacological activity, evaluating the

toxic characteristics of formulations is usually a preliminary step. In the present study, we observed non-significant differences in the body weight of mice in all the groups, and no mortality was recorded. During acute toxicity of injectable oxytetracycline in rats at doses of 300 mg/kg and 100 mg/kg of body weight, no mortality was reported.[7] Weight differences could be influenced by hormonal changes, such as growth hormone and somatostatin levels, as well as changes in neurotransmitters that impact food consumption.[8] With regular feed and water consumption, mice in all groups showed steady growth in body weight comparable to the control group. There was no significant alteration in average body growth, and there was no toxic effect of plain oxytetracycline on body weight. Similarly, (Bacharach et al., 1959) investigated the toxicity studies of ten antibodies in mice and reported 154 mg/kg body weight as an LD_{50} dose of oxytetracycline drugs by intravenous route. Griffin et al. (1979) reported that in feedlot heifers a dose of 33 mg/kg when administrated by intravenous route for three days cause nephrotoxicity but not death. Whereas, reported that oxytetracycline causes a moderate toxic effect at doses as high as 2,000 mg/kg intraperitoneally in rats.[9]

Group D (plain OTC-HCL) showed a non-significant difference with control group A in parameters such as white blood cell (WBC), platelet count (PLT), Mean corpuscular volume (MCV), Mean Corpuscular hemoglobin (MCH), eosinophil (E %), and monocyte (M%). Significant differences were observed in hemoglobin (HB), red blood cell (RBC), packed cell volume (PCV), Mean corpuscular hemoglobin concentration (MCHC), neutrophils (N%), and lymphocytes (L%). Group D showed an increase in PLT as compared with Control Group A. Reduction of PLT may be the ability of nanoparticles to specifically bind anti-platelet autoantibodies.[10] Destruction of RBCs and shrinkage in RBCs might be the reason for the decline in cell count in group D. The hematopoietic system is very susceptible to harmful substances and plays a crucial role in both normal and diseased conditions. The blood profile provides crucial information about the body's response to stress and damage. As a result, toxic substances are first introduced to blood cells.[8]

In plain OTC-treated mice, statistical significance was observed in parameters such as Hb, RBC, PCV and E% in groups 1, 2, and 3, as compared with excipient control group 4. The HB were decreased in group 1 (high dose) and 2 (Medium dose) of plain oxytetracycline as compared to Vehicle control group H. Even though the mean value of HB shows decreased trends, they are in the normal range of 11.1 to 14.8 g/dl.[11,12] Meanwhile, the low-dose group 3 shows a minor increase in the mean value of HB 15.33 g/dl as compared with vehicle control group 4. The normal range of RBC is 5.2 -10.4 x 10^6/cu mm and E is 0-80 per cent.[11] Hence, even though other parameters such as RBC, PCV and E show significant differences within the groups, they are under the normal range, indicating that the drug doses do not reveal any subacute toxicity in mice.

Serum biochemistry was evaluated to identify the possible alterations in renal and hepatic functions affected by plain oxytetracycline. ALT, AST, and ALP are good indicators of normal health of the liver also TP, ALB, and GLB are responsible for hepatocellular and secretory functions of the liver. Since blood AST, ALT, ALP, LDH, and bilirubin levels are cytoplasmic in nature and are discharged into the circulation following cellular damage, they are the most sensitive markers used in the diagnosis of liver impairment.[13] ALT, AST, and ALP are good indicators of normal liver health. TP, ALB, and GLB are responsible for hepatocellular and secretory functions of the liver. ALT is a liver-specific enzyme that is released into the bloodstream during cell damage or injury. Benrahou et al. (2022) stated that AST is an enzyme found primarily in red blood cells, the heart, skeletal muscle, and kidneys. In the present study, significant differences were recorded in AST and ALP in mice that were treated with a repeated dose of plain oxytetracycline. Santana et al., 2020 reported that the normal range of AST is 121.6-163 Iu/L, ALP 147-162 Iu/L, TP 5.14-5.31 g/dl, Alb 1.84-2.77 g/dl and GLB 2.6-3.34 g/dl. Oxytetracycline may have produced liver injury, leading to a substantial rise in AST activity in the treated groups.[14] Significant changes in AST indicate hepatic damage due to repeated administration of plain OTC-HCL. To examine renal function, blood urea, creatinine, and BUN levels were measured.[15] In the present study, there were significant differences recorded in AST and ALP in mice that were treated with repeated doses of plain oxytetracycline, but their values are within the normal range, hence indicative that the drug did not cause renal damage on repeated administration of the drug and found to be safe for a kidney. Histopathological studies were carried out to understand the effect plain oxytetracycline on the vital organs. Similar observation were reported by Silva et al.(2014) and Pokharkar *et al*.(2009).[15,16]

5. Conclusion

The histology of all organs of all mice was normal, and no significant changes were seen. Even though the elevated biochemical markers indicate liver and renal damage, histologically, all organs of all mice in all four groups were normal without any significant changes being reported, hence suggesting that plain oxytetracycline drug did not cause any toxic effect in mice during the 14 days of acute toxicity study period and in subacute toxicity study.

6. Source of Funding

None.

7. Conflict of Interest

None.

8. Acknowledgment

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