**In Vitro and In Vivo Synergism between Tetracycline and the Cardiovascular Agent Oxyfedrine HCl against Common Bacterial Strains**

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The cardiovascular drug oxyfedrine HCl revealed noteworthy *in vitro* antibacterial action against 501 strains of Gram positive and Gram negative bacteria. It also offered significant protection to mice challenged with a mouse-virulent bacterial strain. Prompted by such results, the present study was carried out to ascertain whether this drug could augment the efficiency of an antibiotic when used in combination with it. For this purpose, ten bacterial strains were selected, which were sensitive to oxyfedrine as well as to six antibiotics, like benzyl penicillin, chloramphenicol, ciprofloxacin, erythromycin, streptomycin and tetracycline. Distinct and statistically significant ($p<0.01$) synergism was observed between oxyfedrine and tetracycline by disc diffusion tests, compared with their individual effects. The fractional inhibitory concentration (FIC) index of this combination, evaluated by checkerboard analysis, was 0.37, which confirmed synergism between the pair. This synergistic drug duo was further dispensed to infected mice. The results of the mouse-protection tests advocated that the combination was significantly synergistic ($p<0.0001$), according to Student’s *t* test. Hence, the capacity of extended antibiotic therapy in several microbial diseases may be improved with the help of this synergistic drug pair, and the study might throw light on newer directions to contest drug-resistant bacterial infections.

**Key words** synergism; oxyfedrine; tetracycline; antimicrobial activity; non-antibiotic

Ever since their launch, antibiotics have been one of man’s most imperative weapons in combating bacterial infections. At present, there are effectual antibiotics to heal almost all major infectious diseases. However, over the past few decades, these health benefits have come under threat, not only because many of them generate toxic reactions, but also due to the surfacing of antibiotic-resistant bacteria. The escalating levels of drug-resistance render it indispensable to explore newer drugs with lesser degrees of resistance. Moderate to powerful antibacterial action, both *in vitro* and *in vivo*, has been exhibited by a number of agents belonging to different pharmacological groups, such as the antihistaminics bromodiphenhydramine and diphenhydramine, methdilazine, trimeprazine, the antipsychotics promazine, chlorpromazine, fluphenazine, trifluoperazine, trifluromazine, the antihypertensives methyl-DOPA, dobutamine, anilopidine, the antispasmodic dicyclomine and local anesthetic procaine. Such compounds, having antimicrobial action in addition to their predesignated pharmacological properties, have been collectively termed as the ‘Non-antibiotics’. Many of these non-antibiotic agents have further shown remarkable synergistic action with several antibiotics and other non-antibiotics. The antianginal and coronary vasodilator oxyfedrine HCl (Oxy) was previously reported to be a non-antibiotic agent that demonstrated significant antimicrobial action. This drug could inhibit bacteria in the following order: *Staphylococcus aureus*, vibrios, *Bacillus* spp., *Shigella* spp., *Salmonella* spp., *Escherichia coli*, *Klebsiella* spp. and pseudomonads. Oxy could offer statistically significant protection to mice challenged with a virulent bacterium. The present study was conducted to demonstrate the synergism between Oxy and an antibiotic tetracycline (Tc).

### MATERIALS AND METHODS

**Bacteria** The bacterial strains were identified and preserved in freeze-dried condition.

**Drugs** All the agents were obtained as pure dry powder from their respective manufacturers in India and stored at 4 °C.

**Media** The liquid media used in the study were peptone water [PW; 1.0% bacteriological peptone (Oxoid) plus 0.5% NaCl (Analar)], Nutrient broth (NB, Oxoid) and Mueller-Hinton broth (MHB, Oxoid). The solid media were peptone agar (PA), nutrient agar (NA) and Mueller-Hinton agar (MHA), which were prepared by solidifying PW, NB and MHB, respectively with the help of 1.5% agar (Oxoid No.3).

**Inoculum** All the bacterial strains were grown overnight (24 h) in PA/NA/MHA at 37 °C and harvested during the stationary growth phase. From these cultures, the organisms were directly suspended in 5 ml sterile distilled water. The turbidity of each suspension was adjusted to match with a 0.5 McFarland standard with the help of a spectrophotometer (Chemito UV 2600 Double Beam UV-Vis Spectrophotometer) at 625 nm, which corresponded to $2.4 \times 10^8$ cfu/ml. The suspension was further diluted 1:100 with sterile distilled water.

**Determination of Minimum Inhibitory Concentration (MIC) of Drugs** Agar dilution method was employed to determine the MIC of antimicrobial agents with respect to different test bacteria. The MIC of an agent was taken to be its lowest concentration in which there was no visible growth or only a faint haze.

**In Vitro Synergism Tests between Oxyfedrine and Antibiotics** Disc diffusion technique, as described by National Committee for Clinical Laboratory Standards (NCCLS), was used to determine the combined effects of Oxy and an antibiotic. Sterile filter paper discs ($7.25$ mm, Whatman No.1) were prepared according to Cruickshank. Each disc...
contained 5 \( \mu g \) of an antibiotic or 200 \( \mu g \) of Oxy. The selected bacterial strains were grown in liquid media for 18 h. Each such culture was flooded on appropriate solid media in triplicate. The plates were dried at 37 °C for 45 min. The drug-discs were placed on the flooded plates at appropriate positions, and the plates were incubated at 37 °C for 18 h. The zones of inhibition produced by each drug were measured in 3 different directions around its disc and mean diameter was recorded. At first, the individual inhibitory effects of Oxy and the antibiotic were determined. The data obtained were used for determination of their combined effects; the drug-discs were placed on the flooded agar plates in such a manner that the inhibitory circles would just touch each other tangentially. Finally, the diameters of inhibition zones produced due to individual and mutual effects of two drugs were recorded on the same plate. The mutual influence/interference encountered when two drugs were used in combination was assessed as follows: (i) indifference, when both the zones of inhibition remained unaffected; (ii) antagonism, when the zones of inhibition receded and assumed kidney shape; (iii) synergism, in which there was enlargement of zones. The statistical evaluation of the increase in surface area (\( \pi r^2 \)) of a zone, if any, due to a combination of effects was done by the \( \chi^2 \) test for its level of significance.

**Checkerboard Assessment** The degree of synergism between Oxy and Tc was confirmed by the checkerboard method in microtiter trays with MHB with respect to *Sh. dysenteriae* NCTC 519/66. The trays were prepared with a 96-channel dispenser and stored at −70 °C until use. The MIC of Tc with respect to the test organism was 2 \( \mu g/ml \), and that of Oxy was 50 \( \mu g/ml \). The concentrations tested for the antibiotic were 0, 0.15, 0.3, 0.6, 1.25, 2.5 and 5 \( \mu g \), and those for Oxy were 0, 6.25, 12.5, 25, 50, 100 and 200 \( \mu g \) (using two-fold dilutions). Also included was a row of tubes without each drug. The checkerboard was arranged in the following manner: in the first row, all the tubes contained 200 \( \mu g \) of Oxy and either of 0, 0.15, 0.3, 0.6, 1.25, 2.5 and 5 \( \mu g \) of Tc in a final volume of 1 ml of MHB. Similarly, in the second row, all the tubes contained 100 \( \mu g \) of Oxy and either of the above concentrations of Tc in a final volume of 1 ml of MHB. An exactly similar pattern was followed for all the rows of tubes (Fig. 2B). In the last or seventh row, the tubes had only different concentrations of Tc but not Oxy. Thus, each tube in the checkerboard was a unique combination of the two drugs.

In each well, an inoculum of 0.5 McFarland’s standard was applied using multipoint inoculator. The trays were incubated aerobically at 37 °C for 24 h. For each run, standard control strains were included.

The presence or absence of growth was noted by visual observation. Results were taken by drawing a line connecting the MIC for each row of tubes on the isobologram.

**End Point Determination** The MIC was read as the lowest concentration of an agent showing no visible growth. MICs were determined for each agent individually and in combination.

**Analysis of Data** The fractional inhibitory concentration (FIC) index\(^{23}\) was determined by the formula: FIC index = FIC\(_A\) + FIC\(_B\) = \([A]/MIC\(_A\) + [B]/MIC\(_B\)\), where \([A]\) is the concentration of drug A, MIC\(_A\) is its MIC and FIC\(_A\) is the FIC of drug A for the organism, while \([B]\), MIC\(_B\), and FIC\(_B\) are defined in the same fashion for drug B. The FIC index thus obtained was interpreted\(^{24}\) as follows: <0.5, synergy; 0.5 to 0.75, partial synergy; 0.76 to 1.0, additive effect; >1.0 to 4.0, indifference; and >4.0, antagonism. Finally, the varying rates of synergy between two agents were determined by performing Chi-square analysis.

**Animal Experiments** In vivo tests were carried out on Swiss albino male mice (each weighing 20 g), abiding by the ethical guidelines. The test bacterial strain was *Salmonella typhimurium* NCTC 74, as it was naturally virulent to mice. The virulence of the strain was enhanced after repeated passage through mice. The median lethal dose (MLD/LD\(_{50}\)) of the passaged strain was determined by injecting graded challenges in batches of mice and recording the mortality up to 100 h. Freeze-drying and reconstitution did not affect the MLD. The MLD of the passaged strain, corresponding to 0.95 \times 10^7 colony forming units (cfu)/mouse, suspended in 0.5 ml NB, served as the challenge dose.\(^{17}\) Standardizing its optical density at 640 nm in a Klett-Summerson colorimeter ensured reproducibility of the challenge dose.

A total of 20 mice were divided into 4 groups, having 5 animals per group. On the basis of standard pharmacological data\(^{17,25}\) Oxy was intraperitoneally administered at a dose of 1.5 \( \mu g/g \) of a mouse, while Tc was injected at 3 \( \mu g/g \) body weight. Each animal in Group 1 was injected 30 \( \mu g \) of Oxy, that in Group 2 received 60 \( \mu g \) of Tc, Group 3 was given a combination of Oxy and Tc (30 \( \mu g \) of Oxy plus 60 \( \mu g \) of Tc), and all the animals in Group 4 received 0.1 ml sterile saline in place of the drugs. After 3 h, the challenge dose of *S. typhimurium* NCTC 74 was injected i.p. into each mouse. After 18 h of administration of the challenge, all the mice were autopsied. Heart blood (0.2—0.4 ml) was aseptically collected from each mouse for the subsequent determination of viable counts. Livers and spleens of the animals were removed, homogenized and cfu counts were determined from the samples under aseptic conditions.

**RESULTS AND DISCUSSION**

**MIC of Drugs Used for the Study** Table 1 shows the inhibitory spectra of the drugs used. The MICs of benzyl penicillin (Pc), chloramphenicol (Cm), ciprofloxacin (Cf), streptomycin (Sm) and tetracycline (Tc) with respect to all the test bacteria ranged from 2—25 \( \mu g/ml \), while the MIC of Oxyfedrine HCl was 0.5—4.0, indifference; and >4.0, antagonism. Finally, the varying rates of synergy between two agents were determined by performing Chi-square analysis.

**Effects of in Vitro Combination of Antibiotics with Oxyfedrine HCl** Initially, when disc diffusion tests were performed with Oxy and each of the antibiotics above, synergism was revealed between Oxy-Pc, Oxy-Em and Oxy-Tc. Indifference was observed between Cm and Oxy, while the combinations of Oxy with both Cf and Sm proved to be antagonistic. Most marked synergism was noted between Oxy and Tc (Table 2).

**Synergism between Tetracycline and Oxyfedrine HCl** Subsequently, extensive disc diffusion tests were carried out between Oxy and Tc with respect to 10 bacterial strains. When the drug discs were placed individually on a culture lawn of *S. aureus* NCTC 8530, the diameters of the zones of inhibition were 21.0 mm and 23.5 mm for Tc and Oxy,
respectively. These increased to 22.2 mm and 24.8 mm, respectively, when the discs were placed for determining the combined activity between the drugs. The increase in surface area due to the combination was 11.75% for Tc and 11.37% for Oxy (Table 2).

Tc (5 μg) and Oxy (200 μg) discs when placed separately on plates having Shigella dysenteriae 7 NCTC 519/66, the diameters of the zones of inhibition were 15.0 mm and 21.1 mm, respectively, which increased to 22.0 mm and 25.2 mm, respectively, when the discs were placed for the elucidation of combined effect. The increase in surface area due to the combination was 115.11% for Tc and 42.63% for Oxy (Table 2; Fig. 1). These two drugs in combination showed synergistic activity for the remaining bacteria tested as well (Table 2). The percent increase in surface area with respect to all the test bacteria was found to be statistically significant.

**FIC Index by Checkerboard Technique** The MIC of Oxy with respect to Shigella dysenteriae 7 NCTC 519/66 was 50 μg/ml, while that of Tc was 1.25 μg/ml. In combination, the MIC values were 12.5 μg/ml and 0.15 μg/ml for Oxy and Tc, respectively. The FIC index was determined to be 0.37 for the Oxy-Tc combination, and the same was depicted on isobologram where the synergistic antibacterial effect of the combination was shown by a concave curve (Fig. 2). The FIC index therefore proved significant synergism of the pair.

**In Vivo Synergism** In animal experiments, it was seen that a combination of Oxy and Tc could reduce the number of viable bacteria in heart blood, liver and spleen of the treated animals (Group 3), compared to the control mice, 18 h after the challenge (Table 3).

The statistical analysis of the data by Students’'t' test...
showed that \( p < 0.001 \) for Groups 1 and 2, and \( p < 0.0001 \) for Group 3 versus control, thereby revealing that the results were significant (Table 3).

While deciding on a suitable therapy for a particular microbial infection, the foremost criterion that a clinician takes into account is the MIC of an antimicrobial agent. In many instances, two drugs have been simultaneously used against a microbe, after obtaining the susceptibility profiles of the agents. However, due to the escalating frequency of multidrug resistant organisms, very often only one agent (or one class of agents) remains to which the pathogen is susceptible. In such cases, monotherapy may be preferred, but such treatment might be sub-optimal, if not be a failure altogether. Therefore, it is obvious that the advantage of combination therapy (preferably synergism) is obtained only when the concerned organism is susceptible to both agents.

In the present study, promotion of antimicrobial activity of the antibiotic tetracycline has been achieved when used in combination with the non-antibiotic oxyfedrine HCl, both against Gram positive and Gram negative bacteria. Quantitative estimation using percent increase in surface area of the individual zones of inhibition compared with those produced by the combination showed a statistically significant enhancement (Table 2). As a final confirmation of the in vitro results, the checkerboard titration was performed (Fig. 2).

In vivo studies additionally pointed toward the synergistic activity of these two drugs. It was noted that the in vitro MIC of oxyfedrine is greater than that of tetracycline, but the amount of oxyfedrine required to protect an animal is much less than of tetracycline (Table 3). Since both these drugs have been used satisfactorily for a long time in clinical medicine, they are in a basic compliance with human requirements.

Earlier studies have established synergism between tetracycline and a non-antibiotic promazine. When these two drugs were used in combination, there was a marked enhancement of the inhibitory capacity of each drug, both against Gram positive and Gram negative bacteria, on the basis of disc diffusion tests. Such in vitro synergistic action was further confirmed by in vivo experiments. Demonstration of antibacterial activity in oxyfedrine and its subsequent synergism with an antibiotic indicate that like sulphonamides, nalidixic acid, nitrofurantoins and other chemotherapeutics, this non-antibiotic may display an extensive spectrum of antimicrobial activity independently and in appropriate combinations. Oxyfedrine shows structural resemblance to the tricyclic phenothiazines, the most potent class of non-antibiotics, in having one benzene ring attached to another that may be considered as an incomplete tricyclic ring. In this study, it can be seen that the rates of synergy for this drug and tetracycline against the test organisms are comparable. Even though the mechanism of action of most non-antibiotics still remains to be ascertained, it could be due to multiple factors interfering with cellular biosynthesis. The same factors could reduce the MIC of two drugs in combination, even below their break-point concentration, thereby making the pair synergistic. With quite low FIC index as determined in most synergism tests, it was observed that the actual concentration of each drug in the test pair was much lower than that required in individual tests, implying that a suitable synergistic combination in case of most non-antibiotics would allow a reduction in the dosage of both the drugs, thus overcoming the problem of unrealistic break-point concentration of the drug(s) for prolonged use.

![A](image1.png)

**Fig. 2.** FIC Index of Tc-Oxy Combination by Checkerboard Technique with Respect to *Shigella dysenteriae* 7 NCTC 519/66

(A), Isobologram plotted from the result of checkerboard test. (B), Arrangement of microtiter trays in checkerboard.

<table>
<thead>
<tr>
<th>Time of sampling</th>
<th>Group (5 mice per group)</th>
<th>Drug/mouse</th>
<th>Cfu/ml counts in</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Heart blood</td>
</tr>
<tr>
<td>18 h</td>
<td>1</td>
<td>Oxyfedrine HCl (30 μg)</td>
<td>2.0×10⁶ to 8.9×10⁶</td>
</tr>
<tr>
<td>18 h</td>
<td>2</td>
<td>Tetracycline (60 μg)</td>
<td>6.7×10⁶ to 4.0×10⁶</td>
</tr>
<tr>
<td>18 h</td>
<td>3</td>
<td>Oxyfedrine HCl (30 μg)</td>
<td>3.0×10⁶ to 4.9×10⁶</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+Tetracycline (60 μg)</td>
<td></td>
</tr>
<tr>
<td>18 h</td>
<td>4</td>
<td>Control 0.1 ml saline</td>
<td>5.2×10⁶ to 1.5×10⁸</td>
</tr>
</tbody>
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Viable counts significant; \( p < 0.001 \) in Groups 1 and 2 and \( p < 0.0001 \) in Group 3 versus control.
Even if their concentration cannot be brought down to as low as that of the existing antibiotics or chemotherapeutics, shorter duration of treatment may be achieved, and that too within considerable toxic effects. The rewards would be in terms of eradication of drug-infections due to resistance plasmids.29) Such synergistic combination of drugs, as observed between oxyfedrine and tetracycline in this case, may serve as a prospective device in selection of appropriate antibiotic therapy that is likely to contribute in the ongoing crusade against microbial drug-resistance. 

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REFERENCES