ANTIBACTERIAL ACTIVITY OF TRIFLUOPERAZINE; *IN VITRO* SUSCEPTIBILITY OF MRSA *STAPHYLOCOCCUS AUREUS*, *PSEUDOMONAS AERUGINOSA* AND *E. COLI*, AND *IN VIVO* EVALUATION AGAINST METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN SURGICAL WOUND INFECTION MODEL

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ABSTRACT

Management of surgical site infections (SSI) becomes very difficult due to Bacterial resistance. Current study was designed to determine *in vitro* susceptibility and minimum inhibitory concentration of trifluoperazine against MRSA, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli*, and determination of *in vivo* antibacterial activity of trifluoperazine in induced surgical site infections by MRSA. *In vitro* antibacterial activity of trifluoperazine was determined by disc diffusion susceptibility testing while determination of MIC by agar dilution technique. *In vivo* antibacterial activity was observed by microbiological assessment of tissue harvested from the experimentally infected wound site and number of colony forming units per gram (cfu/gm) of tissue. The zones of growth inhibition of trifluoperazine were determined against *MRSA, E. coli* and *Pseudomonas aeruginosa.* At a disc potency of 25 µg no zone of inhibition was produced against any isolate. 50 µg produced 13 mm and 16 mm zone of inhibition against *MRSA and Pseudomonas aeruginosa* respectively. Whereas, No zone against E. coli was observed at this concentration but, 12mm, 15mm and 18mm zones of inhibition were produced by 100 *µg,* 150 µg*,* and 200 *µg* per disc concentration of trifluoperazine respectively. The colony forming units/gram observed in trifluoperazine treated wounds were $2.93\pm0.02 \times 10^6$ while in case of normal saline treated wounds the value was $6.68\pm0.07 \times 10^6$. Results revealed the efficacy of trifluoperazine as a potentially therapeutic agent in the treatment of wound infection beds especially against MRSA.

Keywords: Trifluoperazine, *Staphylococcus,* Methicillin, Antibacterial, Wound Infection

INTRODUCTION

Surgical site infection (SSIs) can be defined as infections that develop in the operated area and can be categorized as incisional (superficial and deep) or organ/space (Amenu *et al*., 2011). Establishment of infection at a site is a complex mechanism involving many factors like virulence, coincident remote site infections and operation related risk factors (Olsen *et al.*, 2008; Reichman and Greenberg, 2009). Postoperative surgical site infections are considered major source of illness in surgical patients and can lead to delayed wound healing (Nicholas, 2001). Antimicrobial therapy after appropriate surgical intervention remains the mainstays of treatment for prevention against wound infection and this purpose can be achieved by the use of antibiotic and non-antibiotic antibacterial (Dancer, 2001; Berger, 2002; Rahbar *et al*., 2010). Efficacy of currently available antibiotics is decreasing because of numbers of resistant strains which because infections are increasing (Nawaz *et al*., 2009). Resistance has been commonly found in *Pseudomonas aeruginosa* and Enterobacteriaceae against tetracycline,

penicillin, aminoglycosides and cephalosporin. Beta-lactamase resistance has also increased significantly in Haemophilus, Neisseria, Pseudomonas species and Enterobacteriaceae (Neu, 1984; Okonko *et al*., 2009).

Non antibiotic antibacterial, term coined by Kristiansen in 1990, are medicinal compounds other than antibiotics that are used therapeutically in non-infectious pathology but endowed with anti-microbial activities in addition to their already existing pharmacological actions (Kristiansen, 1990; Rahbar *et al*., 2010). The group of 'non-antibiotics' comprises of three subgroups; (a) non-antibiotics anti-bacterial having direct antimicrobial activity, (b) helper compounds altering the microorganism permeability against a given antibiotic, (c) macrophage modulator regulating the killing activity of microorganism containing macrophages after phagocytosis (Martin *et al*., 2008).

The antimicrobial property of some synthetic and non-chemotherapeutic agents like phenothiazine and methylene blue has been already reported and known since the time of Ehrlich (1854–1915). Paul Ehrlich, the pioneer behind non-antibiotics, studied the antimicrobial activity

of methylene blue and resulted in discovering colorless neuroleptic group Phenothiazine, namely Chlorpromazine. phenothiazine belongs to group of heterocyclic compounds having a three-ring structure comprise of two benzene rings united by a nitrogen and sulpher atom at non-adjacent positions having a growth stopping activity on a wide variety of microbes (Amaral *et al.*, 2004). Many phenothiazine derivatives like chlorpromazine, thioridazine, prochlorperazine, bromodiphenhydramine and psychotherapeutic phenothiazine viz trifluoperazine also possesses antibacterial action against Gram positive and Gram-negative strains including Bacillus, *Staphylococcus aureus*, *Vibrio cholera*, *E. coli*, Shigella and *Salmonella typhimurium* that it is more potent than other phenothiazine (Mazumder *et al.*, 2001). Literature has depicted the studies on the use of trifluoperazine as antibacterial in vitro as well as in vivo through parental route. But the effect of trifluoperazine on important antibiotic resistant bacteria like MRSA and Pseudomonas has not been yet investigated. Consequently, the current study was designed with the following objectives; (1) Determination of *in vitro* susceptibility and minimum inhibitory concentration (MIC) of trifluoperazine against MRSA, *Pseudomonas aeruginosa* and *E. coli*. (2) Determination of *in vivo* antibacterial activity of trifluoperazine in induced surgical site infections by MRSA.

MATERIALS AND METHODS

The current study was compartmentalized into two phases viz. phase I and phase II.

Phase I: During phase I, an *in vitro* antibacterial activity of trifluoperazine (Glaxosmithkline, Pakistan) was determined against Methicillin-resistant *Staphylococcus aureus (MRSA), Pseudomonas aeruginosa* and *E. coli* (20 isolates) by following methods:
A) Determination of antibacterial susceptibility by disc diffusion susceptibility testing.
B) Determination of minimum inhibitory concentration (MIC) by agar dilution technique (Ericsson and Sherris, 1971, Yousaf, 2009).

A) Determination of *in vitro* antibacterial susceptibility by disc diffusion susceptibility testing of non-antibiotic antibacterial

Chemicals, Trifluoperazine, non-antibiotic broad-spectrum bactericide, were procured from GlaxoSmithKline Karachi, Pakistan. Filter paper discs were prepared from Whatman® filter paper (Cole-Parmer, USA) and impregnated with the following potencies: Trifluoperazine (25, 50, 75, 100, 150 and 200 µg). Sulpha/trimethoprim was used as a control drug. Different dilutions of non-antibiotic agents were prepared and kept at -20°C until used. **Bacteria,** Purified isolates of

Methicillin Resistant *S. aureus*, *E. coli* and *P. aeruginosa* were used as test organisms, while *S. aureus* ATCC were used as a control. **Media,** Mueller-Hinton agar medium was used, (NCCLS, 1994b). 2.5% NaCl was added to the medium for MRSA.

Preparation of inoculum for susceptibility test: Inoculum was prepared against each bacterial strain by picking 4-5 pure colonies from an overnight growth by sterile inoculation loop, suspended in normal saline and gentle dilution was done till the turbidity was compare visually to 0.5 McFarland turbidity standard with inoculums density approximately 10^8 cfu/ml. For each organism, a sterile swab was dipped into its standardized cell suspension and inoculated the entire surface of Muller Hinton agar plate by streaking the swab in several directions to ensure a uniform growth. Several plates for each organism (*Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa and E. coli*) were incubated to ensure the growth. After drying the plates for 5-10 minutes (at room temperature) are placed in incubator at 35-37°C for 28 hours. The center of a plain filter paper disc was pierced with a sterile disposable syringe needle and held upright with the disc above. Micro-dispenser was used to discharge non-antibiotic till the disc was thoroughly soaked. The disc was then stamped on the surface of Mueller-Hinton agar medium. Changing the tips of micro-dispenser and needles, the process was repeated. All the discs were kept at the same distance from the edge of the plate and from each other.

Incubation of plates, the inoculated plates were incubated at 35°C for 18 hours. Then, after the inhibition zones were measured using Kirby Bauer ruler. Since, the size of the zones of inhibition in respect of the non-antibiotic do not exist in the available literature; the size of zones of inhibition were interpreted by referring to the zone diameter standards for sulpha/trimethoprim against the reference strain. The actual zones size has not been standardized as in the Kirby-Bauer method.

B) Determination of MICs of the non- antibiotic anti-bacterial agent against *Staphylococcus aureus* (MRSA), *P. aeruginosa* and *E. coli.*
MIC of non-antibiotic agent was determined by agar dilution technique (Ericsson and Sherris, 1971, Yousaf, 2009). In this test, the lowest concentrations of a non-antibiotic agent that inhibited the visible growth of a microorganism were described as the MIC of that agent, regarding a single colony or a faint haze caused by the inoculums.

Phase II: During phase II, *in vivo* antibacterial activity of trifluoperazine was determined.

Experimental animals: Adult, active and clinically healthy rabbits (n=20) of both sex, managed indoor uniformly during research period, were divided into two

equal groups i.e., group A and B. They were housed (cages) and acclimatized for 2-weeks at Laboratory Animal Facility of the Department of Clinical Medicine & Surgery, University of Agriculture, Faisalabad, Pakistan, endowed with 8 to 12 hours light-14-hour dark cycle in a well-ventilated, temperature maintained (~ 25-28°C) room.

Inoculum preparations: Five MRSA colonies were inoculated in 5ml of sterile Mueller-Hinton broth and later, incubated at 35°C for 18 hours. Dilution 1:20 of inoculum was done in Mueller-Hinton broth, followed by incubation at 35°C for 4 hours, and then centrifuged at 75 rpm for the bacterial growth. 4 hours later, the bacterial inoculum concentration was noted and fix to a 0.5-McFarlane standard related to 10^8 organisms, then stored in refrigerator (4°C) before transportation to laboratory for further testing.

Induction of Surgical Site Infection (SSI): Animals were kept off feed for 12 hours before the operation. Before surgical intervention, area was shaved and sanitized thoroughly with antiseptics. Animals were anesthetized with ketamine hydrochloride (Ketarol®, Global Pharmaceuticals, Pakistan) by intramuscular injection at dose level of 13-30 mg/kg body weight. Clean surgical 4cm vertical incisions were created on the flank region. All the wounds were inoculated with 10^8 CFU/ml of MRSA. The incisions were then closed using silk suture material. Infected wounds of Group A were treated with topical application with 2 % of Trifluoperazine at dose level of 0.5ml, applied topically on the wounds.

Evaluation of Antibacterial efficacy: 24 days post-surgery, animals were sacrificed and 1 cm^2 of tissue was harvested from the wound site for microbiological examination. The piece of the tissue was weighed and homogenized for 15-30 seconds. After homogenization, tissue and broth mixture were used for two 1:10 serial dilutions in Mueller-Hinton broth and then placed at 4°C. Each sample (100-µl), having these serial dilutions, was moved to tryptic soy agar plates, and then kept for incubation at 35°C for 24 hours. Counts were noted for numbers from 30 to 300, and then estimated with these dilutions and alternative plates for reproducibility. Then, colony counts were transferred to colony forming units/gram by given formula.

Cfu/gm= Colony counts X 50 X Dilution factor/Specimen weight (g)

Statistical Analysis: Two-way analysis of variance (ANOVA) was used for statistical analysis. Average number of colonies forming unit's ± standard error of the mean was measured to compare the treated sites with control ones. Significance was explained as $p < 0.05$, as estimated in a sign test.

RESULTS

Determination of *in vitro* antibacterial activity of Trifluoperazine by Disc diffusion testing: Trifluoperazine was proved effective with pronounced antibacterial actions against MRSA, *P. aeruginosa* and *E. coli* as shown in Table 1. On a disc potency of 25 µg of trifluoperazine, it didn't inhibit the growth of MRSA and *P. aeruginosa,* while in case *E. coli* 75 µg of trifluoperazine failed to produce desired results. Increasing the disc potency to 50, 75 and 100 µg caused the further increase in the zone of inhibition of MRSA (13, 18, and 26 mm) and *P. aeruginosa* (16, 20 and 24 mm). While on the other hand, discs of standard drug (Sulfamethoxazole + trimethoprim) produced a zone of growth inhibition measuring 20 and 24 mm in size against MRSA and *P. aeruginosa.* In case of *E. coli,* even the potencies of 100, 150 and 200 µg of trifluoperazine failed to produce larger zone of inhibition (12, 15 and 18mm) as compared to the standard disc of Sulfamethoxazole + trimethoprim (26 mm). These results demonstrated noteworthy antibacterial effect of trifluoperazine against MRSA, *P. aeruginosa* and satisfactory response against *E. coli* as shown in Table 1. MIC of trifluoperazine tested against MRSA and *P. auroginosa* proved to be 50 µg/ml, whereas an MIC of 100 µg/ml was recorded against isolate of *E. coli* as shown in Table 2.

Phase II: *In vivo* antibacterial activity was observed by microbiological assessment of tissue harvested from the experimentally infected wound site and number of colony forming units per gram (cfu/gm) of tissue was calculated and significant difference was noticed in bacterial population was noticed among the trifluoperazine treated wounds and control (Figure 1). Quantitative wound cultures indicated significant decrease ($p < 0.05$) in cfu/gm in the group treated with 2% solution of trifluoperazine ($2.93\pm0.02 \times 10^6$) as compared to that of normal saline treated control group ($6.68\pm0.07 \times 10^6$) as shown in Table 3.
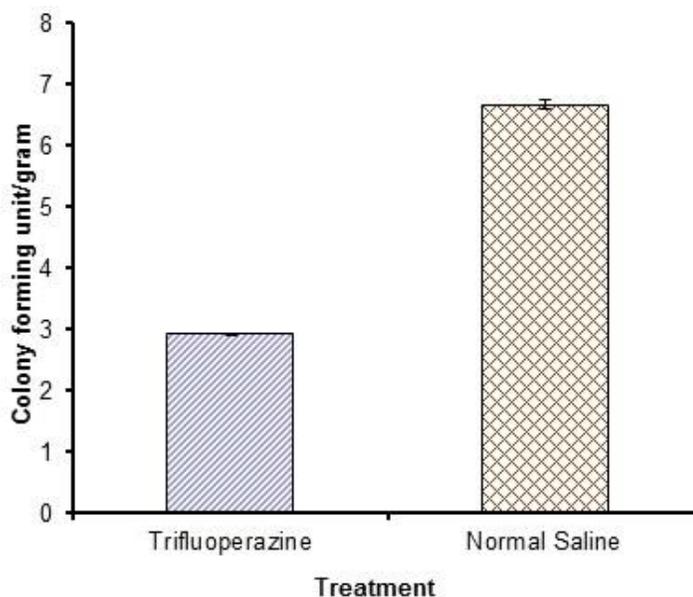
Figure 1. Average quantitative cultures (cfu ± standard error of the mean) of MRSA measured in wounds (*n* = 20) infiltrated with 2% Trifluoperazine compared with controls (Normal saline).

Table 1. Measurement of Zone of Growth Inhibition (mm) of Trifluoperazine against MRSA, *P. aeruginosa* and *E. coli*.

Test Drug	Isolate	Disc Potency	Zone of growth inhibition (mm)
Trifluoperazine	MRSA	25	No zone
		50	13
		75	18
		100	26
Standard	MRSA		20
Trifluoperazine	*P. aeruginosa*	25	No zone
		50	16
		75	20
		100	24
Standard	*P. aeruginosa*		24
Trifluoperazine	*E. coli*	75	No zone
		100	12
		150	15
		200	18
Standard	*E. coli*		26

Table 2. Minimum Inhibitory Concentrations (MICs) of Trifluoperazine determined by Agar dilution method.

Drug	Isolate	MIC (µg/ml)
Trifluoperazine	MRSA	50
	P. auroginosa	50
	E. Coli	100

Table 3. Outcome of 2 days treatment with topical application of trifluoperazine in experimentally induced infection with MRSA.

Group	Drug	Mean Bacterial Count
Treated	Trifluoperazine (2% solution)	$2.93 \pm 0.02 \times 10^6$

Untreated/Control	Normal Saline	$6.68 \pm 0.07 \times 10^6$

DISCUSSION

SSIs in an operated area is a serious issue leading to delayed wound healing and resulting in increased expenses (Amenu *et al.*, 2011; Nicholas, 2001). Antimicrobial therapy after appropriate surgical intervention remains the mainstays of treatment for prevention against wound infection but antimicrobial drug resistance is a serious global health issue compromises the treatment of bacterial, viral, fungal and parasitic infections and effectiveness of the currently available antibiotics is decreasing (Dancer, 2001; Berger, 2002; Nawaz *et al.*, 2009). Phenothiazine are normally used for psychosis therapy but its antibacterial activity is known since the time of Paul Ehrlich (Kristiansen and Amaral, 1997). Its effectiveness as antibacterial agent is well established against gram-positive cocci, Mycobacteria and some gram-negative rods (Amaral *et al.*, 2004). It has been proved that Phenothiazine inhibit efflux pumps responsible for antibiotic resistance in cancer cells and bacteria (Bray *et al.*, 1992; Molnar *et al.*, 1997; Hidika and Naito, 1998). Phenothiazine inhibit the calcium (ca^{2+}) dependent enzyme systems involved in energy generation through ATP hydrolysis by preventing the calcium transport through calcium binding proteins such as calmodulin thus destroy the phagocytized bacteria (Garcia *et al.*, 1995; Amaral *et al.*, 2004). To the best of our knowledge, Phenothiazine such as trifluoperzine have not been yet studied for their antibacterial action *in vitro* against MRSA, *Pseudomonas aeruginosa* and *E. coli* and *in vivo* antibacterial action against MRSA. The present study had been designed to determine *in vitro* susceptibility and Minimum inhibitory concentration (MIC) of trifluoperazine against MRSA, *P. aeruginosa* and *E. coli* along with determination of *in vivo* antibacterial activity of trifluoperazine in induced surgical site infections by MRSA. For this purpose, antibiotic susceptibility was determined by disc diffusion method against selected isolates mentioned previously, MICs against these isolates were determined by using agar dilution method, In the second phase of the trial, *in vivo* efficacy of trifluoperazine was determined against induced MRSA surgical site infection in rabbits.

Regarding zones of growth inhibition against MRSA, trifluoperazine produced 0, 13, 18- and 26-mm zone of growth inhibition at 25, 50, 75 and 100 µg/disc potency respectively. These findings are in line with the studies conducted previously (Hadji- nejad *et al.*, 2010 and Amaral *et al.*, 2004). This antimicrobial activity of trifluoperazine against this MRSA isolate is attributed to property of phenothiazine to inhibit ABC type efflux pumps that account for the antibiotic resistance of the organism. Furthermore, this class results in failure of binding of calcium with different proteins like calmodulin

type proteins, also affecting efflux pumps sensitive to verapamil (Amaral *et al.*, 2004; Kaatz *et al.*, 2003). Whereas, when tested this drug against *P. aeruginosa* isolate, it produced 0, 16, 20 and 24 mm zones of inhibition at similar concentrations as that of previous isolate. Contrary to previous two isolates, the trial drug i.e. trifluoperazine produced no zone of growth inhibition till disc potency of 75µg when applied against *E. coli.* At higher concentrations i.e. 100, 150 and 200 µg/disc, it produced 12, 15- and 18-mm zones of growth inhibition respectively. MICs determined against the said isolates were 50µg/ml for MRSA and *P. aeruginosa,* whereas it was 100 µg/ml for *E. coli.* This MICs obtained for trifluoperazine are in line with the studies conducted previously (Hadji-nejad *et al.*, 2010; Advani *et al.*, 2012).

The colony forming units per gram recorded from trifluoperazine applied wounds ranged up to $2.93 \pm 0.02 \times 10^6$, whereas about $6.68 \pm 0.07 \times 10^6$ colony forming units were calculated in normal saline treated control group. These findings could be correlated with the suggestions of Kistiansen and Amaral, 1997 and Amaral *et al.*, 1992 that Phenothiazine can be used as adjuvants to antibiotics. Moreover, they have also reported that a much lower concentration of Phenothiazine is required for this purpose as compared to their concentration required for *in vivo* antibacterial activity. This study demonstrated that trifluoperzine, psychotherapeutic phenothiazine, could be regarded as a good non-antibiotic antibacterial.

Conclusions: Trifluoperazine is cheap, administered easily, that is previously used as antipsychotic drug. *In vitro* and *in vivo* trials proved it to be a good non-antibiotic antibacterial against MRSA, *P. aeruginosa* and *E. coli.* Its efficacy as a prophylactic agent in case of postoperative wound infection or therapeutic agent in infected wound beds treatment especially against MRSA should be studied in a clinical setting in future along with dose adjustment.

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