

# Anti-piroplasmic Activity of Novobiocin as Heat Shock Protein 90 Inhibitor Against in-vitro Cultured Theileria Equi and Babesia Caballi Parasites

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## Research

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## Abstract

**Background:** *Theileria equi* and *Babesia caballi* are the causative agents for equine piroplasmosis (EP). Currently, imidocarb dipropionate (ID) is the only available drug for treating clinical form of EP. Serious side effects and uncompleted clearance of infection is major drawback of ID. Heat-shock proteins (HSP) play a vital role in the life cycle of these haemoprotezoa by way of preventing alteration in protein conformation. These HSPs are activated during transfer of EP sporozoites from tick vector (poikilotherm) to natural host (homeotherm) and helped it for survival. In this present study we have targeted the heat shock protein 90 pathway of *T. equi* and *B. caballi* by its inhibitor drug - novobiocin.

**Methods:** Dose-dependent efficacy of novobiocin drug was observed on growth of *T. equi* and *B. caballi* in *in-vitro* culture. Cell cytotoxicity on host peripheral mononuclear cells (PBMCs) was also checked with different concentration of novobiocin. It was also checked for its haemolytic activity on equine erythrocyte (RBCs) by standard technique. *In-vivo* organ toxicity of novobiocin was also assessed in mice model with identified methods.

**Results:** IC<sub>50</sub> (50 % Inhibitory concentration) value of novobiocin against *T. equi* and *B. caballi* was 165 µM and 84.85 µM, respectively. Novobiocin significantly arrested the *in-vitro* growth of *T. equi* and *B. caballi* parasites at respective 100 µM and 200 µM drug concentration. *In-vitro* treated parasites become dead with distorted nuclear material and showed no further viability. The drug was found safe on the equine PBMCs and RBCs cell line even at 1000 µM concentration and CC<sub>50</sub> (50 %, cytotoxicity concentration) values were 11.628 mM and 261.97 mM. A very high specific selective index (SSI) was also observed 70.47 and 1587 for respective equine PBMCs and RBCs. Organ specific biochemical markers and histopathological examination indicated no adverse effect of the drug at a dose rate of 50 mg/kg of body weight in mice model.

**Conclusions:** The results clearly indicating the growth inhibitory efficacy of novobiocin against *T. equi* and *B. caballi* parasites and its safety on host cell lines with very high SI. Hence, it can be inferred that *Theileria/Babesia* Hsp-90 family are the potential drug targets worthy of further investigation.

## Highlights

1. *In-vitro* *equi* and *B. caballi* growth inhibitory efficacy of a Hsp-90 inhibitor drug novobiocin was investigated.
2. Novobiocin significantly arrested the *in-vitro* growth of *equi* and *B. caballi* parasites with respective IC<sub>50</sub> value of 165 µM and 84.85 µM.
3. The drug was found safe on the horse's PBMCs and erythrocytes cell line with very high SI.
4. Organ toxicity of novobiocin was also accessed in mice model and no adverse effect of the drug was observed at a dose rate of 50 mg/kg body weight.

*Theileria equi* and *Babesia caballi* are haemoprotozoa responsible for causing equine piroplasmosis. The disease is primarily transmitted by tick of the genus *Dermacentor*, *Hyalomma* and *Rhipicephalus* [19]. Prevalence of equine piroplasmosis have been reported from many countries and usually coincide with distribution of tick-vectors [14, 20, 25, 30]. International movement of piroplasm affected equids have been restricted as per trade guidelines of Office International des epizooties (OIE) [13, 15]. The currently available drugs are not suitable to completely clear the *T. equi* or *B. caballi* parasite from latently infected animals [2, 12, 28].

Most of the haemoprotozoan have to cope with repeated host body temperature fluctuation after transmission of sporozoite from arthropod vector. Protozoa specific heat shock proteins 90 (Hsp-90) get activated during such temperature change and plays a vital role in the life-cycle of parasite within host [1, 22]. In spite of fact that Hsp-90 has a critical role in its multiplication inside the equine RBCs, not much efforts have been exerted in pursuing researches against this drug target [6]. In this study we targeted the heat shock protein pathway of *T. equi* and *B. caballi* by its inhibitor novobiocin drug. Novobiocin is an antibiotic and inhibitor of chaperone-Hsp-90 [17]. Novobiocin derivates have shown a promising result as anticancer agents by inhibiting the function of this chaperone [3]. Novobiocin also has *in-vitro* growth inhibition efficacy against *Plasmodium falciparum* and inhibited 80% ATPase activity of the parasite at 30  $\mu$ M concentration [24]. Keeping in view anti-bacterial, anti-cancerous and anti-plasmodial potential of novobiocin drug, this present study was planned for investigating its anti-*T. equi/B. caballi* activity and *in-vitro/in-vivo* cytotoxicity or organ toxicity.

## Methods

### The parasites and in-vitro cultivation

*Theileria equi* and *Babesia caballi* parasites (Indian isolates) were cultured in horse red blood cells (RBCs) through continuous microaerophilic stationary-phase (MASP) culture system. The culture medium M 199 and RPMI 1640, (Sigma-Aldrich, India) were used for MASP culture of *T. equi* and *B. caballi*, respectively. These mediums were supplemented with 40% defibrinated equine serum, antibiotic solution (containing 60 IU/ml penicillin and 60 mg/ml streptomycin) and 200  $\mu$ M hypoxanthine solution. *Theileria equi* and *B. caballi* MASP cultures were maintained at a temperature of 37 °C with micro-aerophilic atmosphere of 5% CO<sub>2</sub>, 3% O<sub>2</sub>, and 95% N.

### In-vitro growth inhibition assay

*Theileria equi* and *B. caballi* parasitized RBCs were obtained from primary MASP cultures at 10% and 6% parasitaemia respectively. These parasitized RBC's (*T. equi* and *B. caballi*) were adjusted to 1% parasitemia by diluting with uninfected RBCs obtained from a healthy horse and used for *in-vitro* evaluation of drug. The parasite growth inhibition assay was performed in 48 well culture plates. Fifty microliter of *T. equi* or *B. caballi* parasite infected RBCs (at 1% parasitaemia) were dispensed per well (in triplicate) together with 500  $\mu$ l of the culture complete medium containing the indicated drug

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concentrations. Novobiocin drug (cat no.: N1628-1G; Sigma–Aldrich, India) was tested at 1  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 200  $\mu$ M concentrations against *T. equi* and 10  $\mu$ M, 20  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 200  $\mu$ M against *B. caballi* parasites. Cultures without novobiocin drug molecule and cultures containing only DMSO (0.005% and 0.5%) were also prepared and initiated in MASP system for control experiments. ID drug was diluted to 0.5  $\mu$ g/ml, 1.0  $\mu$ g/ml and 10.0  $\mu$ g/ml concentration and kept as positive drug control experiment against *T. equi* and *B. caballi* parasites in MASP *in-vitro culture* system. These *in-vitro* cultures with or without drug molecules concentrations were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>, 3% O<sub>2</sub>, and 95% N, for a period of 96 hours (h). The overlaid culture medium was replaced daily with fresh medium containing indicated drug molecule concentration. IC<sub>50</sub> value was calculated by standard curve fitting technique. [2].

### In-vitro viability test

After 96 h of *in-vitro* treatment with novobiocin, 20  $\mu$ L of drug-treated/un-treated parasitized RBCs were collected (at different concentration) and transferred to a fresh culture plate (48 wells) containing 30  $\mu$ L of parasite-free fresh equine RBCs in 500  $\mu$ l of growth medium (without any drug molecule). The overlaid growth medium was replaced after every 24 h for the next 72 h, and parasite recrudescence was determined by examining its stained blood smears [2].

### In-vitro cytotoxicity assay on equine PBMCs

Cytotoxicity of each concentration of drug molecules was analysed on peripheral blood mononuclear cells (PBMCs) by resazurin-based cell viability assay [8]. Briefly, freshly collected equine PBMCs were suspended in 1 ml complete growth medium consisting of RPMI-1640 supplemented with 2 mM L-glutamine, 60  $\mu$ g/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% foetal bovine serum (Sigma Aldrich, India). Enriched PBMCs suspension with complete growth media was adjusted to a final concentration of 3  $\times$  10<sup>7</sup> cells/100  $\mu$ L and distributed to each well (100  $\mu$ L volume) in 96 well culture plate. Simultaneously, phytohaemagglutinin-A (PHA, at the concentration of 10  $\mu$ g/ml) in 50  $\mu$ L volume was also added to each of these well. The culture plate was incubated at 37 °C having 5% CO<sub>2</sub> in air for 48 h. After 48 h, PBMCs in cultured wells were treated with 100  $\mu$ l volume of the different respective concentration of novobiocin drug molecule – 1  $\mu$ M, 5  $\mu$ M, 10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 1000  $\mu$ M, 2000  $\mu$ M. The 96 well culture plate was again incubated (as above) for another 24 h, followed by addition of 25  $\mu$ L of resazurin dye (150  $\mu$ g/ml) and culture plate was kept in an incubator for another 4 h. The change of dye colour was monitored by measuring optical density (OD) at 570 nm and 650 nm. The effective OD value for each well was calculated by deducting OD<sub>570</sub> value from its respective OD<sub>650</sub> value. The IC<sub>50</sub> of each drug molecule on PBMCs was calculated from a regression equation based on the effective OD value, as mentioned above. Effect of drug molecules on PBMCs in terms of per cent viable cell population was determined as below:

$$\text{PBMCs viability}(\%) = \frac{\text{OD of test sample} - \text{OD of positive control}}{\text{OD of negative control} - \text{OD of positive control}} \times 100$$

$$\% \text{Cytotoxicity} = \frac{\text{ODofnegativecontrol} - \text{ODoftestsample}}{\text{ODofnegativecontrol}} \times 100$$

### In-vitro drug haemolytic assay

*In-vitro* drug haemolytic assay was performed as per standard protocol [23]. Freshly collected 5 ml equine whole blood was centrifuged at 1200 *g* for 10 min and final pellet was washed three times with 1X PBS (Phosphate buffer saline) at 1200 *g* for 5 min after discarding the supernatant. Twenty microliters of RBCs suspension were added to each well of 96 well culture plate. Further, 180  $\mu\text{L}$  of each of novobiocin concentration (1  $\mu\text{M}$ , 5  $\mu\text{M}$ , 10  $\mu\text{M}$ , 25  $\mu\text{M}$ , 50  $\mu\text{M}$ , 100  $\mu\text{M}$ , 1000  $\mu\text{M}$ , 2000  $\mu\text{M}$ ) prepared in solubilising buffer (10% dimethylformamide in PBS) was also added to each well. RBCs suspension (20  $\mu\text{L}$ ) was added to 180  $\mu\text{l}$  of distilled water or solubilising buffer and taken as positive or negative control analysis. The 96 well plate was incubated at 37°C for 90 min. Contents of each well after incubation were transferred into 2 ml micro-centrifuge tube, followed by centrifugation at 3000 *g* for 5 minutes. Supernatants were transferred to new 96 well plates and OD was measured at 543 nm in UV spectrophotometer. Percentage of haemolysis was determined as below:

$$\text{Percentage hemolysis} = \frac{\text{ODofdifferentdrugconcentration} - \text{ODofnegativecontrol}}{\text{ODofpositivecontrol} - \text{ODofnegativecontrol}}$$

## Specific Selectivity Index (ssi)

In the present study, the degree of selectivity of novobiocin drug molecule against *T. equi* or *B. caballi* in comparison to mammalian cells (equine PBMCs or RBCs) at respective IC<sub>50</sub> concentration was analysed as per standard method [16] using the below mentioned formula:

$$\text{SpecificSelectivityIndex(SSI)} = \frac{\text{IC50ofdrugmoleculeonhorsePBMCs}}{\text{IC50ofdrugmoleculeonprotozoanparasite}}$$

### In-vivo organ toxicity of novobiocin in mice

The organ toxicity of novobiocin was analysed in six groups of mice ( $n=6$ , in each group). Each five group was administered (by intraperitoneal route) the drug at respective dose rate of 5, 10, 20, 50, 100 mg/kg body weight, whereas sixth group was kept as no-drug control and administered PBS only. Mice in each group were observed for 14 days. Blood from these group of mice were collected at 0 h, 24 h and 72 h interval from retro-orbital sinus under general anaesthesia. Serum was isolated from these mice and processed for biochemical analysis with-respect-to changes in organ specific biochemical markers. Mice were sacrificed on day 14 post-drug treatment and biopsy sample from vital organs were collected (liver, lung, heart, kidney and spleen) and processed for histopathological examination as per standard protocol.

## Statistical analysis

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Anti-piroplasmic activity of novobiocin drug molecules against *T. equi* and *B. caballi* was computed by Two Way ANOVA test followed by Bonferroni Post-hoc test ( $p < 0.05$ ). The P values  $< 0.05$  were considered as statistically significant differences between the novobiocin treated wells and control wells. Correlation between novobiocin concentration and its cytotoxicity and haemolytic activity was accessed by using Graphpad prism version 4.0 software (San Diego California, USA).

## Results

### In-vitro growth inhibitory efficacy of novobiocin against *T. equi* and *B. caballi*

*In-vitro* growth inhibitory effects of different concentrations of novobiocin against *T. equi* and *B. caballi* were analysed by Bonferroni Post-hoc test (Two Way ANOVA) in-between treated and control groups (Fig. 1A and 1C). *Theileria equi* *in-vitro* growth inhibition observation at 24 h and 48 h exhibited significant difference from its respective control well ( $p < 0.05$ ) at higher concentrations of novobiocin only (100  $\mu$ M and 200  $\mu$ M). While, at 72 h and 96 h of *in-vitro* drug treatment with novobiocin concentrations of 20  $\mu$ M and more revealed significant difference from respective control well ( $p < 0.05$ ) (Fig. 1A). *Babesia caballi* *in-vitro* novobiocin drug trial studies at 24 h, 48 h, 72 h and 96 h, showed significant growth inhibition at a concentration of 100  $\mu$ M and 200  $\mu$ M from its respective control well ( $p < 0.05$ ) (Fig. 1C).

*Theileria equi* and *B. caballi* parasites were observed dead at 100  $\mu$ M and 200  $\mu$ M concentration of novobiocin on 96 h of *in-vitro* drug treatment, whereas drug concentration from 1  $\mu$ M to 50  $\mu$ M (*T. equi*) and 10  $\mu$ M to 50  $\mu$ M (*B. caballi*) were incapable in inhibiting the growth of these parasites in viability studies (Fig. 1A and 1C). Computed IC<sub>50</sub> values of novobiocin against *T. equi* and *B. caballi* growth inhibition were 165  $\mu$ M and 84.85  $\mu$ M, respectively.

### Morphological alterations in *T. equi* and *B. caballi* parasites after *in-vitro* drug treatment

Stained blood smears prepared from *in-vitro* drug treated/control wells over a period of 96 h were examined for morphological changes in parasite (*T. equi*/*B. caballi*) at different concentrations of novobiocin (Fig. 1B and 1D). *T. equi* or *B. caballi* parasite with conspicuous outline cytoplasm membrane and nuclear material were indicative of live dividing parasites while, dead parasites usually were pyknotic with condense/distorted nuclear material. *T. equi* and *B. caballi* parasites were dead, showing dot-shaped/distorted nuclear material, at 100  $\mu$ M and 200  $\mu$ M concentration of novobiocin at 96 h of *in-vitro* treatment. While, in control culture parasites were live, healthy and dividing throughout the 96 h of *in-vitro* period (Fig. 1B and 1D).

### In-vitro cytotoxicity of novobiocin on PBMCs

Various concentrations of novobiocin (ranging from 1  $\mu$ M to 1000  $\mu$ M) tested for cytotoxicity on equine PBMCs and less than 10% cytotoxicity was observed at highest concentration of novobiocin (1000  $\mu$ M) (Fig. 2A). The extrapolated cytotoxic concentration (EC<sub>50</sub>) by regression analysis was 11628  $\mu$ M. The

specific selective index (SSI) of novobiocin in this *in-vitro* study was 70.47, indicative of its safety on mammalian cell lines.

### In-vitro haemolytic activity of novobiocin on equine erythrocytes

Haemolytic activity of novobiocin on equine RBCs was also assessed. Different concentrations of novobiocin (from 1  $\mu$ M to 2000  $\mu$ M) were analysed and 0.01% to 0.44% haemolysis was observed (Fig. 2B). CC<sub>50</sub> of novobiocin on horse erythrocyte was analysed by regression analysis and found to be 261973  $\mu$ M. A very high SSI value (1587) was observed for horse erythrocyte which indicated its safety.

### In-vivo toxicity of novobiocin in mice model

Organ toxicity of novobiocin was tested in different mice groups and biochemical parameters were analysed. A significant ( $p < 0.05$ ) rise in SGOT and SGPT was observed in group I mice (dosage @ 100 mg/kg body weight) at 24 h interval of inoculation of novobiocin drug, which thereafter decreased to insignificant level (Fig. 3C and 3D). An insignificant difference ( $p > 0.05$ ) in kidney biochemical markers like creatinine, blood urea nitrogen (Fig. 3G and 3H) and other parameters like bilirubin and total protein (Fig. 3E and 3F) was observed at different time interval after inoculation of novobiocin in different group of mice. Tissue samples collected from different organs from these mice groups (I to VI) were subjected to histopathological examination. Hepatocytes in group I mice showed minimal diffuse cytoplasmic rarefaction and vacuolation as compared control group (VI) mice (Fig. 3A and 3B). It is indicative of liver damage in this group of mice. No adverse histopathological changes were observed in any organ of mice of other groups. As such, 50 mg/kg dose rate of novobiocin may be considered as 'No Observed Adverse Effect Level' (NOAEL) concentration.

## Discussion

Heat shock protein (Hsp) are multiunit chaperon complexes and help in proper folding of the target protein. Heat shock protein-90 (Hsp-90) is the most extensively studied and largest protein of this family. Hsp-90 is obligatory for maintaining integrity of number of client proteins involved in cellular and signal transduction processes [10, 27]. Hsp-90 has four structural domains – N-terminal, charged linker region, middle domain and C-terminal [26]. N-terminal is the ATP-binding domain of the Hsp-90 and interacts with most of its inhibitors – geldanamycin [26].

Novobiocin is an amino-coumarin class of antibiotic and exhibits potent activity against Gram-positive bacteria. Novobiocin possess Hsp-90 C-terminal nucleotide-binding site activity and induced degradation of Hsp-90 dependant client protein ErbB2, mutant p53 in a concentration-dependent manner in cancer cell lines [18]. In this study we examined *in-vitro* growth inhibitory efficacy of novobiocin against *T. equi* and *B. caballi* parasites. Novobiocin significantly arrested the *in-vitro* growth of *T. equi* and *B. caballi* parasites leading to their death at respective 100  $\mu$ M and 200  $\mu$ M drug concentration. Novobiocin greatly reduced *Plasmodium falciparum* parasitaemia during first cycle of growth (trophozoite to schizont) in *in-vitro*

inhibiting its ATPase activity. Novobiocin IC<sub>50</sub> value against *T. equi* and *B. caballi* was 165 µM and 84.85 µM in the present study. While other author [24] observed IC<sub>50</sub> value of 280 µM and 210 µM against respective FCC<sub>1</sub> (a chloroquine-susceptible) VNS (Viet Nam Smith, a chloroquine-resistant) strains of *P. falciparum*. The IC<sub>50</sub> observed in our experiments against *T. equi* and *B. caballi* is approximately 1.48 to 2.88 times low as observed against *P. falciparum*, indicating its effectiveness. Further, novobiocin *in-vitro* treatment also altered morphology of *T. equi* and *B. caballi* parasites. After 96 h of *in-vitro* treatment (at 100 µM and 200 µM concentration) these parasites become dead with distorted nuclear material (Fig. 1B and 1D). *Theileria annulata* (bovine) has four isoforms of Hsp-90 and respective genes were aligned with orthologues from other closely related apicomplexan parasites [11]. Phylogenetic tree derived from this alignment indicated close relationship between the isoform within the Apicomplexa. Further, sequence alignment analysis of Hsp-90-like proteins across *Theileria*, *Babesia*, and *Plasmodium* species indicated conservation of C-terminal sequences motif. The inhibitory efficacy of novobiocin against *T. equi* and *B. caballi* in the present study may be attributed due to its anti-Hsp-90 properties as evidence in other Apicomplexa parasites.

Novobiocin drug was found safe on the horse PBMCs as well as erythrocytes and < 10% cytotoxicity was observed on PBMCs at highest concentration of 1000 µM or 2000 µM. The CC<sub>50</sub> value was 11.628 mM and 261.97 mM and respective SSI was 70.47 and 1587 for horse PBMCs and erythrocytes. Another group of researchers [7] tested novobiocin for *in-vitro* inhibition of Kaposi's sarcoma-associated herpesvirus (KSHV). A marked inhibition of KSHV was reported with SSI of 31.62 (and CC<sub>50</sub> of 871 µM), indicating its safety on lymphoma cell line (BCBL-1). In our experiment novobiocin was observed as safe drug on horse PBMCs and erythrocytes with very high CC<sub>50</sub> and SSI values.

Organ toxicity of novobiocin was also assessed in mice model at different dosage. Organ specific biochemical markers and histopathological examination indicated no adverse effect of the drug at a dose rate of 50 mg/kg of body weight. Therapeutically, novobiocin was used primarily for infections due to penicillin-resistant *Staphylococcus aureus* or against pneumococcal pneumonia [4, 5]. Oral dosage of novobiocin (@ 1–2 g/day) was well absorbed and therapeutically useful concentration (18.8 µg/ml following 0.5 g dose) was readily achieved in the bloodstream [29]. Novobiocin has been observed to be safe in non-infection clinical studies at high dosage (3–9 g/day, orally administration) while high plasma concentration was achieved (150 µM sustained for 24 h @ 5.5 g dosage) and no serious toxicities were observed [9, 21]. Our study generated novobiocin organ toxicity data in mice and indicated its *in-vivo* safety.

It is crucial to continually look for target specific novel anti-Theilerial/Babecidal compounds. In this study, we tested novobiocin drug, a Hsp-90 inhibitor and results suggested its anti-*T. equi*/*B. caballi* potential. The drug has low IC<sub>50</sub> value for *T. equi* and *B. caballi* and very high SSI for horse PBMCs and erythrocytes. These results clearly indicated *in-vitro* *T. equi* and *B. caballi* growth inhibitory efficacy of novobiocin and its safety on host cell lines with very high SSI.

# Conclusions

*In-vitro* *T. equi* and *B. caballi* growth inhibitory efficacy of a Hsp-90 inhibitor drug novobiocin was investigated. Novobiocin significantly arrested the *in-vitro* growth of *T. equi* and *B. caballi* parasites with respective IC<sub>50</sub> value of 165 µM and 84.85 µM. The drug was found safe on the host PBMCs and erythrocytic cell line with very high SSI. Organ toxicity of novobiocin was also assessed in mice model and no adverse effect of the drug was observed at a dose rate of 50 mg/kg body weight. It can be inferred that *Theileria/Babesia* Hsp-90 family are the potential drug targets worthy of further investigation.

## Abbreviations

**EP:** Equine Piroplasmosis; **ID:** Imidocarb dipropionate; **Hsp:** Heat shock proteins; **Hsp-90:** Heat shock protein-90; **CC<sub>50</sub>:** 50 % cytotoxicity concentration; **IC<sub>50</sub>:** 50 % Inhibitory concentration; **PBMCs:** Peripheral mononuclear cells; **RBCs:** Red blood cells; **SSI:** Specific Selective index; **OIE:** Office International des epizooties; **MASP:** Microaerophilic stationary-phase; **h:** Hours; **PHA:** Phytohaemagglutinin-A; **OD:** Optical density; **KSHV:** Kaposi's sarcoma-associated herpesvirus; **PBS:** Phosphate buffer saline; **NOAEL:** No Observed Adverse Effect Level .

## Declarations

### Ethics approval and consent to participate

All animal protocols were approved by the Institute Animal Ethic Committee (IAEC) of ICAR-National Research Center on Equines, Hisar (meeting proceeding vide order no. NRCE/CPSCEA/2018-18 dated 18. 09. 2017). The study was performed in accordance with good animal practices guidelines issued by *Committee for the Purpose of Control And Supervision of Experiments on Animals (CPSCEA), India*.

### Consent for Publication

All author read the whole manuscript and gave their consent for publication.

### Availability of data and materials

All supporting data of presenting manuscript was included within this article and its additional files. The data generated during present study is available from corresponding author for further request.

### Competing interests

The authors declare that they have no conflict of interest.

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## Authors' contributions

Authors' contributions, AS and CM performed the experiments. AG performance of *in-vitro* cytotoxicity and hemolytic trials. RK designed and conducted *in-vivo* organ toxicity trial. SHR performed organ histopathology. SK conceptualized, designed and supervised the whole study. Performed statistical calculations and prepared the graphs. Also drafted the final version of manuscript. All authors read and approved the final manuscript.

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## Figures

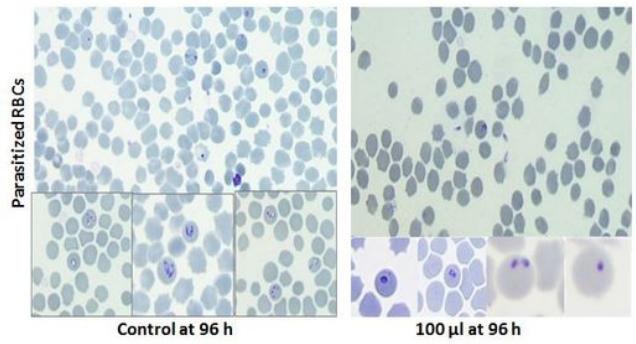
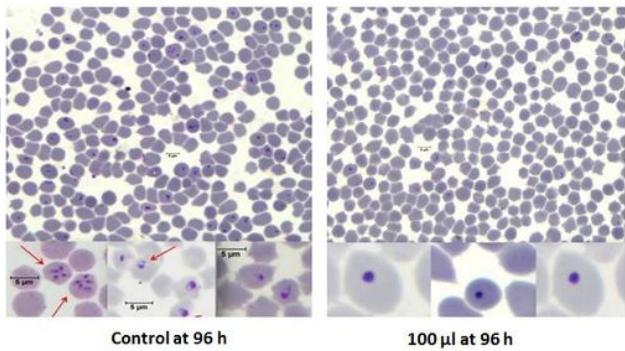
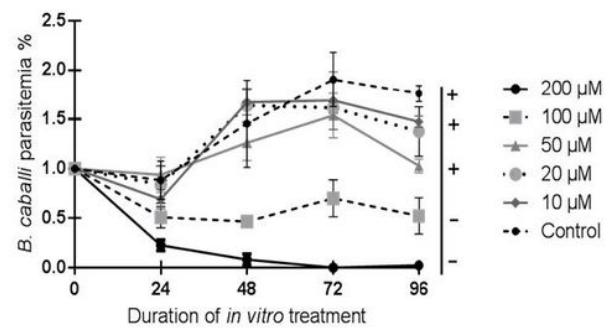
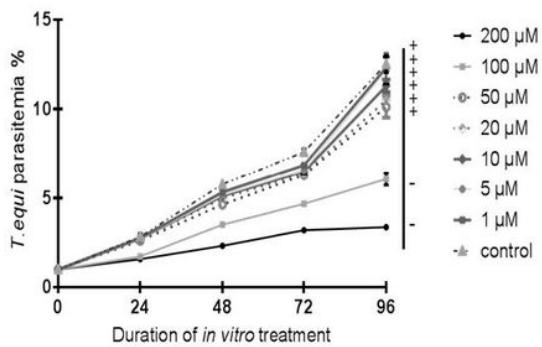
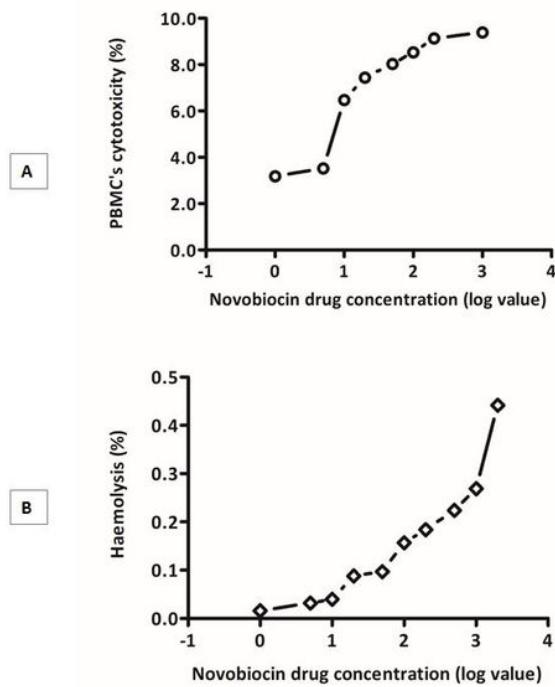


Fig. 1, Suthar et al, 2020

## Figure 1

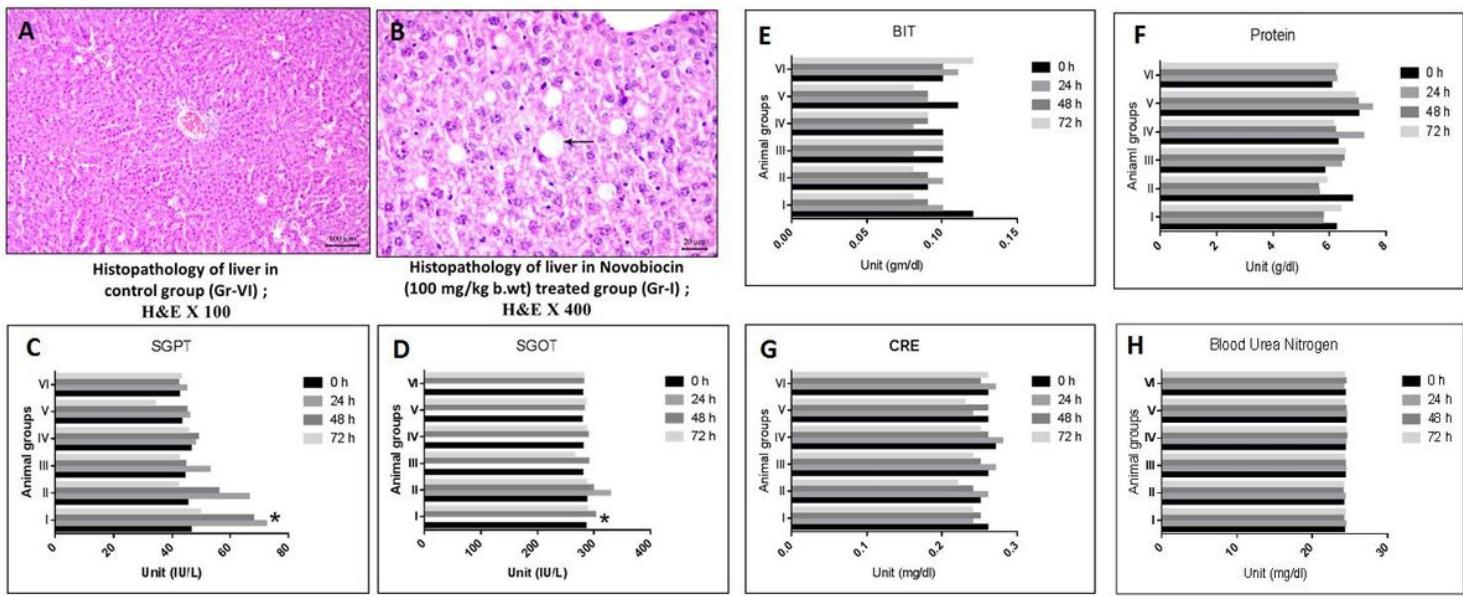
Linear growth curves of *Theileria equi* (1 A) and *Babesia caballi* (1 C) parasitaemia after in-vitro treatment with different concentrations of novobiocin. Each value in the individual graph represent percent parasitaemia (mean  $\pm$  SD) observed at each respective concentration of the novobiocin. '+' '-' numeral on right hand side of the graph represented viability status of the *T. equi* or *B. caballi* parasite after 96 h of in-vitro treatment trial. Parasite viability has been indicated as +: live or -: dead. Morphological changes observed in *T. equi* (B) or *B. caballi* (D) parasites after in-vitro treatment with novobiocin have been depicted in microphotographs. Control culture showed tetrad or pyriform shaped *T. equi* or *B. caballi* merozoites, whereas the novobiocin treated parasites were degenerated or with condensed nucleus and appeared pyknotic. Giemsa X 1000. Bars, 5  $\mu$ m.



**Fig. 2, Suthar et al, 2020**

## Figure 2

Effect of different concentrations of novobiocin on the viability of equine peripheral blood mononuclear cells (PBMCs) shown as cytotoxicity percentage in the graph (A). Effect of novobiocin drug was also observed on equine erythrocytes and represented as haemolysis percentage in the graph (B). Different concentration of novobiocin and percent cytotoxicity/heamolysis were shown after log transformation.



**Fig. 3, Suthar et al, 2020**

### Figure 3

Effect of different dosage of novobiocin drug on vital organs observed in groups of mice model. Different mice groups ( $n = 6$  mice/group) administered novobiocin by intraperitoneal route – Gr I: 100 mg/kg body weight (b. wt); Gr II: 50 mg/kg b. wt; Gr III: 20 mg/kg b. wt; Gr IV: 10 mg/kg b. wt; Gr V: 5 mg/kg b. wt; Gr VI: PBS only, control group. A significant ( $p < 0.05$ ) rise in SGOT and SGPT was observed in group I mice at 24 h interval (C and D) whereas, an insignificant difference ( $p > 0.05$ ) in kidney biochemical markers (creatinine; G, blood urea nitrogen; H) and other parameters (bilirubin; E and total protein; F) was observed at different time interval after inoculation of novobiocin in different groups of mice. Histopathological examination revealed that hepatocytes in group I mice have minimal diffuse cytoplasmic rarefaction and vacuolation as compared control group mice (VI) (A and B). It is indicative of liver damage in group I mice. No adverse histopathological changes were observed in any organ of mice of other groups. H & E, 100 X and 400 X.