Original Article

# Inhibition of *Escherichia coli* Induced Bacterial Meningitis by Ansamycin Loaded Polymeric Nanoparticles

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## ABSTRACT

**Background:** Bacterial meningitis is a serious medical disorder that should be treated as soon as possible. **Aim:** The effect of ansamycin as a nanoformulation on *Escherichia coli*-induced meningitis was investigated. **Materials and Methods:** By injecting *Escherichia coli* directly into the brains of Swiss Albino mice, an experimental meningitis model was established. **Results:** As expected, TNF-α, IL-6, and IL-10 secretion was stimulated, following neutrophil infiltration. Additionally, the BBB's vasopermeability was raised. Ansamcyin nanoparticles were delivered to assess their therapeutic potential in preventing bacterial meningitis caused by *Escherichia coli.* Ansamycin nanoparticle-treated mice produced less tumour necrosis factor alpha and interleukins when brain tissue was stained, compared to nontreated mice. *E. coli*-induced increases in the permeability of the blood-brain barrier and infiltration of inflammatory neutrophils were also prevented. **Conclusion:** These findings suggest that ansamycin nanoparticles could be used to treat bacterial meningitis. It has been shown that ansamycin nanoparaticles can prevent the mice from *E. coli*-induced mortality, therefore enhancing their outcome.

**Keywords:** *Escherichia coli*, Bacterial meningitis, Ansamycin, polymeric nanoparticles, Improved outcomes.

## INTRODUCTION

As a life-threatening infection, bacterial meningitis must be treated immediately. *Streptococcus pneumoniae, Nesseria eningitidis*, and *Haemophilus influenzae* are perhaps the most prevalent pathogens that cause bacterial meningitis in humans. Annually, it is anticipated that more than 1.2 million instances of bacterial meningitis would develop in different parts of the world. The prevalence and overall mortality rates for bacterial meningitis vary depending on the area, the nation, the microorganism, and the patient's age. It is possible for bacterial meningitis to be fatal if not treated, and one out of every five victims might suffer irreversible consequences such as hearing loss, cognitive issues, or the loss of an arm or leg.[1]

An infection of the central nervous system (CNS) by bacteria known as meningococcal meningitis is known as bacterial meningitis. Anti-inflammatory cytokines and prostaglandins are produced by the bacteria or their breakdown products. This increases the permeability of blood-brain barrier (BBB) in the CNS. This causes neutrophils to migrate across the endothelium and plasma proteins to flow into the brain, causing additional damage.[2] Bacterial meningitis can be exacerbated by the release of proinflammatory cytokines, as has been discovered. Patients with bacterial meningitis and experimental animals with elevated levels of the cytokines TNF-α and interleukin-1b (IL-1b) had their brain fluid tested.[3-4]

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The great majority of small molecule drugs are unable to pass the BBB. There are just 5% of the medicines in the comprehensive medical chemistry data source that are active in the CNS. In general, CNS active medications are only used to treat conditions such as anxiety, schizophrenia, and also sleeplessness. The CNS active drug has a typical molecular mass of 357 Da, which is considered to be average. 12% of medicines were found to be active in the central nervous system, while just 1% of all medications for non-affective illnesses investigated were found to be active in the central nervous system.[4-5]

Ansamycin, an antibiotic, is used to prevent bacterial meningitis. Oral ansamycin has an 85% bioavailability, but only 4% reaches the brain, limiting its therapeutic potential. Thus, developing a novel ansamycin formulation that can reach the targeted brain area quickly is critical.

The reasons for this assumption are mostly tied to the possibility of nanoparticles multifunctionalization, as well as their potential to transport pharmaceuticals that are BBB-impermeant. Polymeric nanoparticles are composed of a polymer matrix and fragments smaller than one nanometer.[6-8] The presence of nanofillers alters the polymer's mechanical, thermal, and rheological properties, affecting its ability to cross the blood-brain barrier and deliver the drug.[9-11] Polymeric nanoparticles can perform well in a variety of important drug delivery applications if they are correctly engineered.[12-13] Otherwise, the same would undoubtedly encounter a variety of complications relating to certain formulation components, which would necessitate that they be dealt with in a much less substantial manner.[9,14]

*Escherichia coli* was injected directly into the brains of Swiss albino mice in this investigation to establish a model of experimental meningitis. Following the stimulation of TNF-α and IL-1 production, neutrophil infiltration was seen. As a result, BBB vasopermeability has also been improved. In an *E. coli* meningitis model, researchers looked into the possibility that polymeric nanoparticles loaded with ansamycin might reduce the development of meningitis. Its medicinal potential for the treatment of bacterial meningitis is being investigated.

## MATERIALS AND METHODS

### Materials

Ansamycin, PLGA, Poloxamer, DMSO of analytical grade and water for the experiment were purchased were purchased from Sigma Aldrich, China. Swiss Albino mice procured from SPF Biotechnology Co., Ltd., China. The protocol was reviewed and further approved by the Animal Ethics Committee of Jinan Central Hospital, Jinan City, Shandong Province, 250013, China.

### Formulation of nanoparticle

Ansamycin loaded polymeric nanoparticles were prepared using emulsion/solvent diffusion method.[15] There were two phases: an organic one, made of PLGA and ansamycin dissolving in 10ml of DMSO, and an aqueous one, made of poloxamer dissolving in 100 ml water. Drop by drop, at a rate of 1 ml/min, 10 ml of organic phase was added to 10 ml of aqueous phase. Ansamycin PLGA nanoparticles were suspended in a colloidal suspension and stirred for three hours at 300 rpm at 300°C to dissipate the DMSO and return the suspension to the aqueous phase. The encapsulated ansamycin pellets were prepared by centrifuging the colloidal nano solution at 12,000 rpm for about 30 min at 40°C to yield out the nano-precipitation. Any remaining drug on the nanoparticle's surface was flushed away with deionized water twice. Re-distribution of nanoparticle pellets was carried out by using water. The characterization parameters that includes Particle size, Zeta potential, Surface morphology, and drug release *in vitro* of the prepared nanoparticles were examined.[15]

### Animals and Treatments

The animals were isolated for three weeks to ensure that they were stable before being used. The mice were given a conventional pelleted feed and free access to water. The rats were split into four groups, each with six rodents. Normal saline was given to the control group ($n$=6) (0.9% NaCl). Normal saline was given to the meningitis-induced group ($n$=6) (0.9% NaCl). For 7 days, the meningitis caused + treated group ($n$=6) received an intraperitoneal (i.p.) injection of 300 mg/kg body weight ansamycin once every 24 hr, either before or after intracerebral injection of *E. coli*. For 7 days, the meningitis-induced group and the treated group received an intraperitoneal (i.p.) injection of an equivalent amount of ansamycin nanoparticle once per 24 hr, either before or after intracerebral injection of *E. coli*. For various biochemical and enzyme studies, a CSF sample (0.02 to 0.08ml) was taken from the animals by lumber puncture.

### Induction of Bacterial meningitis

*E. coli* ATCC 10536 was cultivated for 12 hr in Luria-Bertani (LB) broth (1% NaCl, 1% tryptone, 0.5% yeast extract), then subcultured for another 3 hr in fresh media. A spectrophotometer with an optical density of 1 equal to 108 CFU/ml at 600 nm was used to determine the *E. coli* concentration.[16] Animals were given a 20 µl volume of $5 \times 10^5$ *E. coli* cells diluted in saline straight

into the temporal area to induce meningitis. For a total of 7 days, the animals were watched every 12 hr.

### Survival Study

The survival rate was determined in the meningitis-induced group, the ansamycin-treated group, and the ansamycin nanoparticle-treated group. Meningitis supposed to be a life-threatening condition and the study animals being much prone to the disease condition, a 7-day survival analysis was performed at 12-hr intervals. In addition, blood pressure and heart rate were measured. Blood pressure and heart rate were measured using the tail pressure method.

### Enzyme Assays

Superoxide dismutase (SOD) activity,[17] Catalase activity,[18] glutathione reductase (GR) activity,[19] adenylate kinase (AK) activity[20] were determined. The xanthine oxidase (XO) assay was carried out largely in accordance with the method described by Roussos.[21] The extent of lipid peroxidation in terms of malondialdehyde (MDA) formed was used to assess free radical mediated damage.[22]

### Biochemical Analysis

Aside from survival analysis, CSF specimens have been examined to determine preferable markers of organ damage, if there were any, in order to explore the therapeutic potential in mice. Interleukin IL-6, IL-10, and tumour necrosis factor TNF-α were measured as inflammatory mediators. For further analysis, a CSF stock solution was prepared and plated on trypsin blood plates. The diluent has been introduced to the discs, which were then placed in a 37°C incubator for 24 hr.[22] For each sample, colonies were counted separately. The bacterial load is calculated by multiplying the mean value of each sample's colony count by the dilution ratio and then by a constant of 20. Platelets (PLTs), were supposed to be the primary clotting factor and the platelets were estimated using a haematology analyzer. For the biochemical analysis, CSF samples were collected at 0, 8, 16, 24, and 48 hr after meningitis induction. To investigate the biochemical parameters, a biochemical analyzer was introduced. Alanine aminotransferase (ALT), aspartate transaminase (AST), total protein (TP), globulin, and albumin levels were measured to investigate hepatic functional changes. Urea nitrogen (UN) and creatinine levels were measured to investigate renal functional changes. Glucose level was also determined. The thromboplastin time, prothrombin time, and international normalised ratio, as well as the coagulation factor, were all calculated. Serum lactate, another important parameter for evaluating menigitis physiological functions, was also measured. The latate levels in blood were determined using a lactic acid kit. Lactate dehydrogenase converts lactate to pyruvate and hydrogen peroxide, which then interacts with the colorimetric probe to form a red dye whose intensity is measured using a spectrophotometer at 530 nm. The activity of the tissue myeloperoxidase (MPO) is used to measure neutrophil sequestration in inflamed tissues[23] 48 hr following the induction of meningitis, the liver was isolated. Haematoxylin and eosin stained and histologically investigated afterwards the liver tissues were preserved by formaldehyde.

### Statistical Analysis

A comparison of statistically significant groups was made of the control group, meningitis induced group, the ansamycin treatment group, and ansamycin nanoparticle treated group. The statistical significance was checked by two-way ANOVA with $p < 0.05$ using the GraphPad Prism V. 7.0. All the data is shown to be a mean ± standard deviation.

## RESULTS

According to the toxicity profile, Ansamycin nanoparticles did not cause any death or behavioral abnormalities or toxicity in mice at doses up to 3500 mg/kg body weight. For 28 days, ansamycin at a dose of 100 mg/kg/day was administered to mice with no side effects, and the same dose of ansamycin nanoparticles is also safe.

Exposure to ansamycin nanoparticles improves the outcome of bacterial meningitis in terms of both mortality and immunomodulation. Mice given ansamycin nanoparticles following meningitis induction had considerably better outcomes and lower mortality, with an overall 7-day survival rate of 83.3% compared to mice not given ansamycin nanoparticles (Figure 1). The meningitis mouse showed 100% mortality by day two (diseased control).

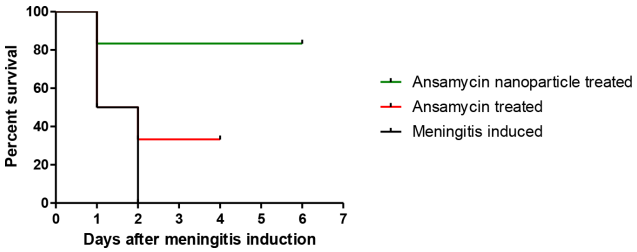


**Figure 1: Kaplan-meier curve showing the percentage survival. Survival analysis observed for seven days among the meningitis induced mice and infected mice treated with ansamycin and its nanoformulation (*n*=6).**

**Table 1: Enzyme parameters in cerebral spinal fluid (CSF) of meningitis induced and treated mice.**

| Sl. No | Parameter | Control | Meningitis induced | Ansamycin treated | Ansamycin nanoparticle treated |
|---|---|---|---|---|---|
| 1 | SOD (mMole/min/ml) | 424.41±1.76 | 234.82±1.65 | 302.71±1.54 | 403.2±1.23 |
| 2 | Catalase (mMole/min/ml) | 118.34±0.27 | 60.85±1.01 | 75.74±0.89 | 96.25±1.21 |
| 3 | GR (mMole/min/ml) | 0.233±0.011 | 0.178±0.001 | 0.185±0.004 | 0.280±0.008 |
| 4 | MDA (nmole/min) | 5.26±0.07 | 17.38±0.87 | 12.27±0.76 | 6.58±0.12 |
| 5 | XO (unit/min/ml) | 0.201±0.082 | 1.23±0.26 | 1.0±0.15 | 0.934.05±0.011 |
| 6 | AK (mMole/min/ml) | 138.10±17.31 | 220.52±28.76 | 200.41±27.65 | 184.05±8.76 |

Value are expressed as Mean ± SD. Value represents the changes in the enzyme activities in the control, meningitis induced, ansamycin treated and ansamycin nanoparticle treated groups, where SOD (superoxide dismutase); GR (Glutathione reductase); MDA (Malondialdehyde); XO (xanthine oxidase) and AK (adenylate kinase).

Hypothermia was observed in animals both before and after meningitis induction, with the body temperature rising in infected animals and falling in ansamycin nanoparticle-treated animals. Similarly, decreases in HR and SBP occurred in infected animals, but were reversed by ansamycin nanoparticle treatment. The values in the ansamycin-treated group did not change significantly. When comparing the meningitis-induced group to the control group, it was discovered that the activities of SOD, MDA, XO, AK, and catalase were considerably greater in the meningitis-induced group. After ansamycin nanoparticle treatment, the activities were restored to normal levels. When comparing the meningitis-induced group to the control group, it was discovered that GR was substantially lower in the former. The ansamycin nanoparticle-treated group's activity increased and then returned to normal. Table 1 shows that the group treated with ansamycin reversed back a little but not significantly more than the group treated with ansamycin nanoparticles.

The bacterial load in CSF after meningitis induction was measured at 0, 8, 16, 24, and 48 hr. The bacterial load, as shown in Figure 2, increased significantly after 8 hr and remained high for 48 hr. The same thing did not happen in the ansamycin nanoparticle-treated group, but there was a lower count at the start. The meningitis-induced group had a higher bacterial load than the ansamyicn nanoparticle-treated group.

Inflammatory mediators like IL-6, IL-10, and TNF-α were all measured in this study. At 8 hr after meningitis induction, levels of IL-6 (Figure 3A), IL-10 (Figure 3B), and TNF-α (Figure 3C) increased significantly, with a subsequent decrease. Inflammatory mediators did not alter significantly between the control and ansamycin nanoparticle treatment groups.

Both ALT (Figure 4A) and AST (Figure 4B) increased steadily from 0 to 16 hr following meningitis induction in rats that did not receive therapy, and these levels were observed to be sustained throughout the course of the

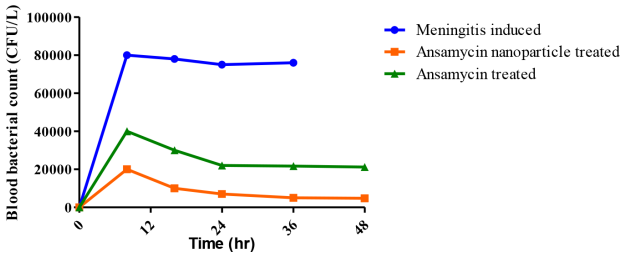


**Figure 2: Bacteria load observed among the control group, meningitis induced mice, infected mice treated with ansamcyin and infected mice treated with ansamycin nanoparticles (*n*=6).**

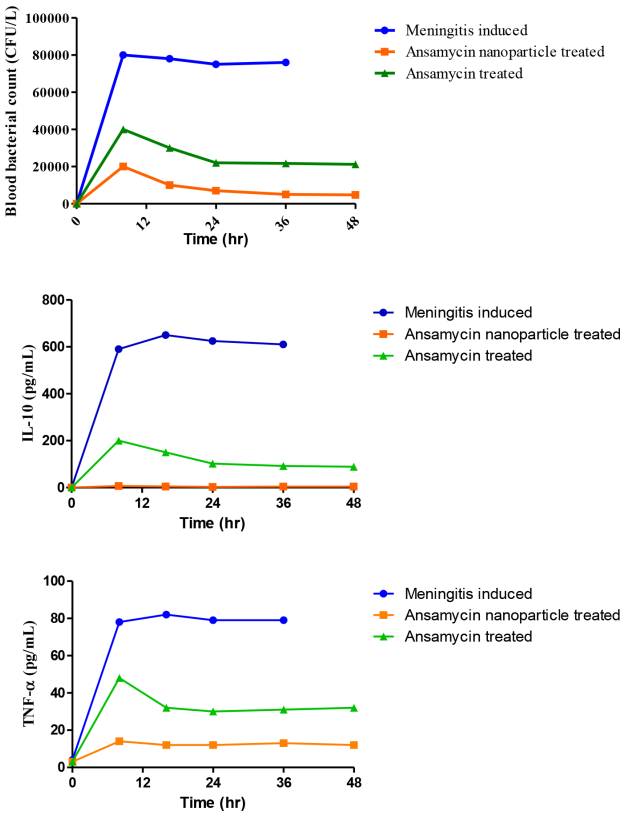


**Figure 3: The inflammatory parameters 3(a) IL-6; 3(b) IL-10; 3(c) TNF-α observed among the control group, meningitis induced mice, infected mice treated with ansamcyin and infected mice treated with ansamycin nanoparticles (*n*=6) (IL, interleukin; TNF, tumor necrosis factor).**

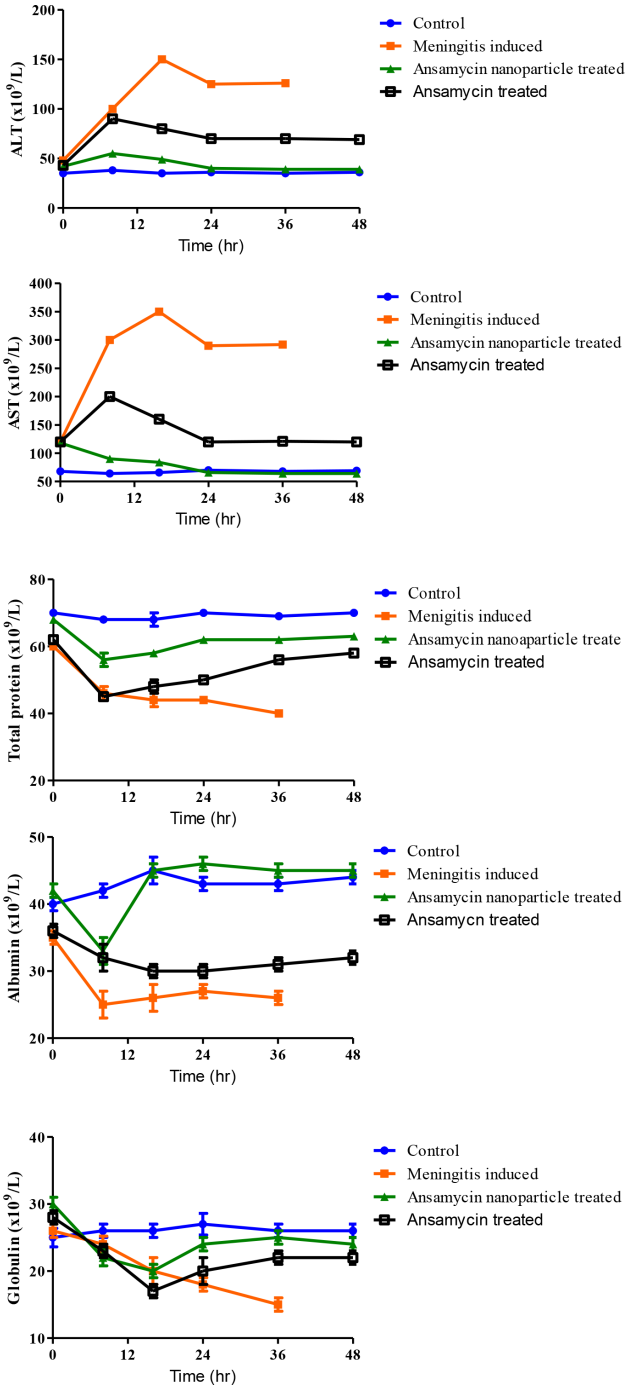


**Figure 4: Hepatic function 4(a) ALT, Alanaine Transaminase; 4(b) AST, Aspartate transaminase; 4(c) Total protein; 4(d) Globulin; 4(e) Albumin observed among the control group, meningitis induced mice, infected mice treated with ansamcyin and infected mice treated with ansamycin nanoparticles (*n*=6).**

trial. As illustrated in Figure 4C and Figure 4D, total protein and globulin levels dropped from 0 to 48 hr. The albumin level stayed lesser during the 48-hr observation period, as seen in Figure 4E.

In animals that were not treated with ansamycin nanoparticles, urea nitrogen (Figure 5A) and creatinine

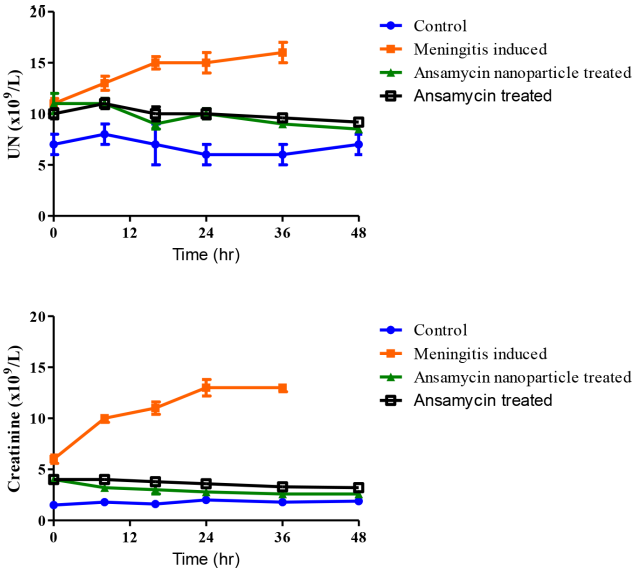


**Figure 5: Renal function 5(a) BUN, Blood urea nitrogen; 5(b) Creatinine observed among the control group, meningitis induced mice, infected mice treated with ansamcyin and infected mice treated with ansamycin nanoparticles (*n*=6).**

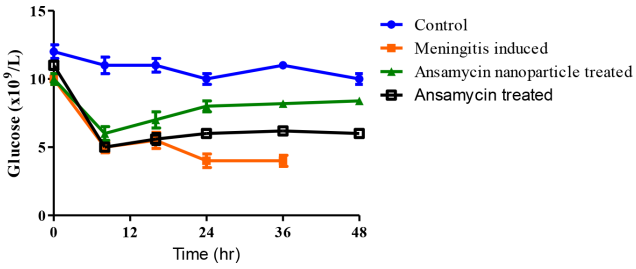


**Figure 6: Blood glucose level observed among the control group, meningitis induced mice, infected mice treated with ansamcyin and infected mice treated with ansamycin nanoparticles (*n*=6).**

(Figure 5B) levels gradually increased from 0 to 16 hr after meningitis induction. Both creatinine and urea nitrogen well reversed within 48 hr upon the treatment. There were no significant differences in hepatic and renal functions between the control and ansamycin nanoparticle treated groups.

CSF glucose levels decreased significantly 8 hr after meningitis induction and remained stable throughout the experiment (Figure 6). As seen in Figure 7, hypoxia-induced metabolic alterations raised CSF lactate levels. However, levels reduced at 8 hr, but did not change at 16 or 48 hr. The control and ansamycin nanoparticle treated groups had similar CSF lactate levels.

Histology of the brains of meningitis-infected patients demonstrates liver necrosis, inflammatory cell infiltration, and hepatocyte disintegration. The central vein was abnormally narrowed (Figure 8). Despite this, the ansamycin nanoparticle-treated group showed no or minor histological changes (Figure 9).

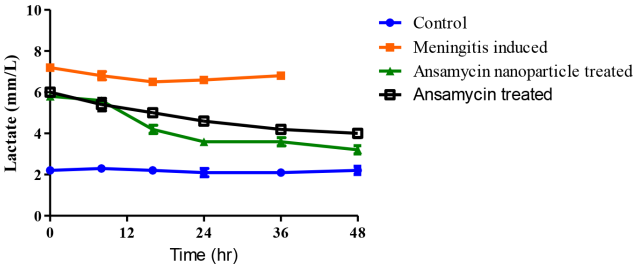


**Figure 7: CSF lactate observed among the control group, meningitis induced mice, infected mice treated with ansamcyin and infected mice treated with ansamycin nanoparticles (*n*=6).**

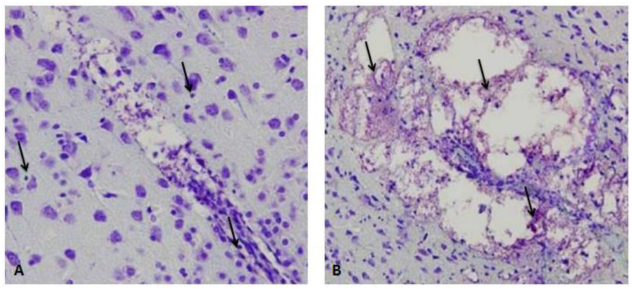


**Figure 8: Histological examination of brain tissues of mice induced with meningitis. Hematoxylin and eosin (HE) and Nissl staining were performed to visualize cortical necrosis, vasculitis and abscess formation. Nissl staining of the cortex of mice with bacterial meningitis, showing (A) extensive inflammations, perivascular lymphocytes and perivascular lymphocytic cuffing, (B) perivascular necrosis (100× magnification).**

The CSF MPO activity of the meningitis-induced group was found to be 3.5 times higher than that of the ansamycin nanopaarticle-treated group (Figure 10).

## DISCUSSION

In order to prevent things (especially water-soluble compounds or bigger molecules from the bloodstream) from passing through the BBB, a layer of endothelial cells is present.[24] Adsorptive endocytosis and receptor-mediated endocytosis are the two most common methods by which big water-soluble molecules can pass through the BBB.[24] As a result of adsorption endocytosis, polymeric nanoparticles produced in our study may enter the brain tissue via CSF diffusion.

Meningitis being a bacterial infection, the bacteria have an higher incidence to rapidly enter the systemic circulation and begin to multiply. When bacteria circulate and multiply in the bloodstream, symptoms appear much faster. As a result, it's vital to keep a close eye on the bacterial load in meningitis infections because it's linked to a high fatality rate. The greater the bacterial load, the greater the mortality. In the study here, bacterial load

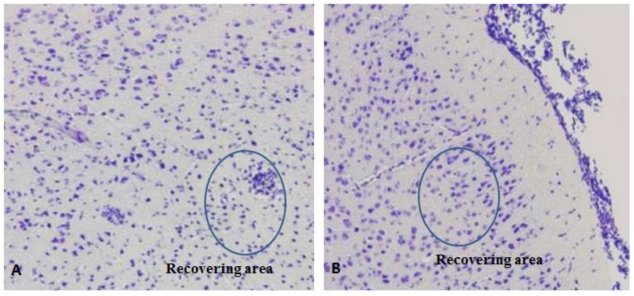


**Figure 9: Histological examination of brain tissues of mice treated with ansamycin nanoparticles. Nissl staining of the cortex of meningitis mice treated with ansamycin nanoparticles, showing near normal neurons and distribution of Nissl granules. Also no Kupffer cell proliferation and necrosis. Inflammation recovering (100× magnification).**

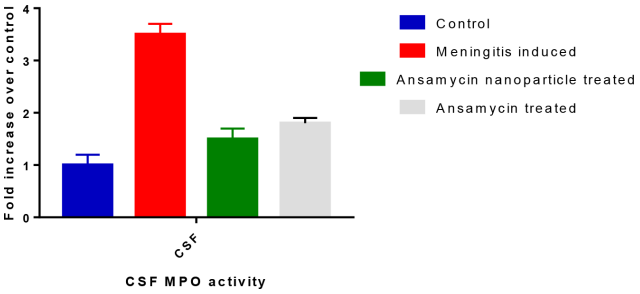


**Figure 10: Quantification of neutrophil in CSF by measuring the MPO activity.**

in CSF after meningitis induction upon treatment with ansamycin nanoparticles got reversed.

Evidence suggests that bacteria in the blood stream proliferate and trigger an inflammatory reaction, and that inflammatory markers serve a key role in the development of meningitis. Macrophages, neutrophils, and platelets are activated by cellular mediators such as lipopolysaccharide (LPS), lipoteichoic acid (LPA), peptidoglycan, super antigens, endotoxin, and humoral mediators, which produce cytokines and other mediators. They can, in fact, trigger inflammatory responses. These mediators activate B and T cell proliferation by acting as a foreign body in the biological system. Upon the treatment of meningitis with ansamycin nanoparticles, inflammatory mediators were well maintained within the range.

The stimulation of humoral and cell mediated inflammatory mediators has a major impact on clinical features in hepatic and renal function, as previously discussed. To learn about the biological alterations in hepatic and renal efficiency in a meningitis mice model, biochemical research was undertaken where it was observed the increased levels of ALT and AST in meningitis condition was well reversed with the treatment of ansamycin nanoparticles.

The glomerular filtration rate is the determinant of changes in creatinine and urea nitrogen levels in relation to renal function. Increased levels of creatinine and urea nitrogen were seen as a result of renal failure. The increased levels of creatinine and urea nitrogen very well reversed upon the treatment of ansamycin nanoparticles.

Glycolysis produces lactate. As a marker of cell hypoxia in meningitis, lactate rate is an important predictor of death.[25] Lactate is a naturally occurring chemical created by cells when food is turned to energy. Lactates in the cells circulate to the liver as needed. The Cori cycle converts lactate to pyruvate, then to glucose. Lactate can be used as an energy source by all tissues since it is easily regenerated into pyruvate. In the body, lactate is quickly converted to pyruvate. Because tissue oxygenation is diminished in meningitis, pyruvate cannot be metabolised as fast, causing intracellular levels to grow, eventually leading to elevated lactate levels. The decreased levels of CSF lactate in the meningitis induced animals were very well reversed by ansamycin nanoparticle treatment.

However, the cell-death process called apoptosis reduced neutrophil numbers in the bloodstream, but at a lower rate than previously thought. This can be assessed by assessing the MPO activity in tissues injured by neutrophil sequestration. Neutrophils and macrophages contain a haemoprotein called MPO that helps to produce haemoglobin in the bloodstream. MPO, a catalytic enzyme, transforms chloride and hydrogen peroxide into hypochlorite. Active neutrophils secrete it during an inflammatory reaction. Activated neutrophils. Measurements of CSF neutrophil activity were made as part of this investigation in order to quantify the number of neutrophils where the CSF MPO activity of the meningitis-induced group was found to be 3.5 times higher than that of the ansamycin nanoparticle-treated group.

In conclusion, nanodelivery of ansamycin improves outcome in an experimental meningitis caused animal model while also lowering surrogate marker levels. Ansamycin was administered in both its pure form and as a nanoformulation. Out of the various assays performed, the group treated with ansamycin reversed back a little but not significantly more than the group treated with ansamycin nanoparticles. This adds to the evidence that ansamycin, when administered and so is, does not cross the BBB to deliver the medicament to the target side, requiring the use of a nanocarrier, and the developed nanocarrier to deliver ansamycin across the BBB has been tested successfully.

## CONCLUSION

To summarise, the data obtained shows that ansamycin nanoparticles have a beneficial effect on survival in a meningitis-induced model, with enhanced outcomes. First study to report a survival benefit associated with all molecular mechanisms, including inflammatory mediators, clotting factors, hepatic and renal features, neutrophil evaluation, and pathological changes, when ansamycin nanoparticles were administered in a meningitis mouse model induced by intracerebral injection of a 20 µl of $5\times 10^5$ *E. coli* cells in saline. The findings should be implemented clinically, and the authors intend to do so in the near future. Clinicians should experiment with ansamycin nanoparticles as a therapeutic option for bacterial meningitis.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**CNS:** Central Nervous System; **BBB:** Blood-Brain Barrier; **IL:** Interleukin; **TNF:** Tumour necrosis factor; **PLGA:** poly(lactic-co-glycolic acid); **DMSO:** Dimethyl sulfoxide; **NaCl:** Sodium chloride; **Ip** Intraperitoneal; **E. coli:** *Escherichia coli*; **LB:** Luria- Bertani broth; **CFU:** Colony Forming Unit; **SOD:** Superoxide dismutase; **GR:** Glutathione reductase; **AK** Adenylate Kinase, **XO:** Xanthine Oxidase; **MDA:** Malondialdehyde; **CSF** Cerebrospinal fluid; **PLT:** Platelets; **ALT:** Alanine aminotransferase; **AST:** Aspartate transaminase; **TP:** Total protein; **UN:** Urea nitrogen; **MPO:** Myeloperoxidase; **ANOVA:** Analysis of variance; **LPS:** Lipopolysaccharide; **LPA:** lipoteichoic acid.

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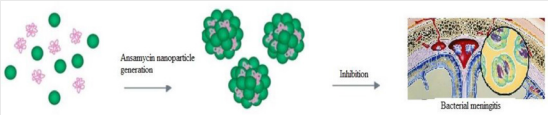
## PICTORIAL ABSTRACT

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## SUMMARY


It is imperative that patients suffering from bacterial meningitis receive prompt medical attention. Ansamycin as a nanoformulation was tested for its effect on *Escherichia coli*-induced meningitis. An experimental meningitis model was created using Swiss Albino mice injected with *Escherichia coli* directly into their brains. Following neutrophil infiltration, TNF-α, IL-6, and IL-10 production was increased. Increased BBB vaso permeability was also observed. *Escherichia coli* meningitis was prevented by administering ansamycin nanoparticles to test their therapeutic potential. When mice treated with ansamycin nanoparticles was compared to that from mice not treated, the treated mice produced less tumour necrosis factor and interleukins. Ansamycin nanoparticles may be useful in the treatment of bacterial meningitis, according to these findings. Ansamycin nanoparticles have been proven to protect mice against *E. coli*-induced death, improving their prognosis.


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