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Ayesha Humayun

Faculty of Veterinary and
Animal Sciences, Pir Mehr Ali
Shah, Arid Agriculture
University, Rawalpindi,
Pakistan

Ghulam Muhammad

Faculty of Veterinary Science,
University of Agriculture,
Faisalabad, Pakistan

Zafar Iqbal

Faculty of Veterinary Science,
University of Agriculture,
Faisalabad, Pakistan

Muhammad Arif Zafar

Faculty of Veterinary and
Animal Sciences, Pir Mehr Ali
Shah, Arid Agriculture
University, Rawalpindi,
Pakistan

Asad Manzoor

Faculty of Veterinary Science,
University of Agriculture,
Faisalabad, Pakistan

Misbah Ijaz

Faculty of Veterinary Science,
University of Agriculture,
Faisalabad, Pakistan

Correspondence**Ayesha Humayun**

Faculty of Veterinary and
Animal Sciences, Pir Mehr Ali
Shah, Arid Agriculture
University, Rawalpindi,
Pakistan

Evaluation of therapeutic efficacy of deferroxamine as an adjunct to rational therapy alone or in combination with vitamin C in induced endotoxemia in dogs

**Ayesha Humayun, Ghulam Muhammad, Zafar Iqbal, Muhammad Arif
Zafar, Asad Manzoor and Misbah Ijaz**

Abstract

The present study was conducted to evaluate the therapeutic efficacy of deferroxamine (DFX) as an adjunct to rational therapy alone or in combination with vitamin C in induced endotoxemia in a dog model in 2013 at Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad. A total of 18 adult, healthy dogs of either sex were randomly selected and divided into three equal groups viz. A, B and C. All above parameters were recorded at baseline, during septic shock, 3, 6, 12 and 24 hrs after treatment. Survival index of one week was also recorded. Group A and group B showed better survival rate of 83% than group C (66 %). The body temperature was elevated during shock in all groups but both A and B group attained the baseline value. As also was the case with the other clinical parameters. The haemoglobin concentration, RBCs count, neutrophils and lymphocytes were at lower level during shock in all groups and attained the baseline values 24-hour post treatment. Animals of group A (treated with DFX along with antibiotic and vitamin C) as well as group B (dosed with DFX & antibiotics showed better results than group C which received DFX without antibiotics and vitamin C. It was concluded that the DFX along with antibiotics can combat the septic shock and resulted in early and better recovery.

Keywords: Endotoxaemia, Dehydration, Septic shock, Induction, Deferroxamine

1. Introduction

Sepsis is a biological phenomenon of unconstrained, unregulated and self-sufficing intravascular inflammation that occurs due to imbalance between pro inflammatory and anti-inflammatory response ^[1]. This clinical condition is usually associated with gram negative bacteria, although gram positive organisms are also sometimes held responsible for the induction of sepsis which mostly includes staphylococci and streptococci ^[2]. Gram negative bacteria contain lipopolysaccharide (LPS) in their outer membrane consisting of lipid and polysaccharide that is the primary mediator of inflammatory process ^[3]. Pathophysiology of the sepsis starts with detachment of LPS from bacterial cell wall. These LPS then bind to the LPS binding protein (LBP) to form a complex in the plasma ^[4]. Then these LPS binds with CD14 receptors present on the surface of host macrophages ^[5]. After binding of CD14 receptors, a signal transduction starts towards mammalian toll-like receptor 4 (TLR- 4) ^[6]. In innate immune defense mechanism TLR-4 are very important, identifying conserved patterns on pathogens ^[7].

Sepsis is associated with severe oxidative stress due to overproduction of free oxygen radicals ^[8]. Reactive oxygen species (ROS) produced by the inflammatory cells kill the pathogenic microorganisms in normal condition but excessive formation of ROS leads to cell damage ^[9]. Sepsis is also mediated with cascades of inflammatory responses due to the production of cytokines and nuclear factor kappa B (NF-kB). Systemic inflammatory response syndrome (SIRS) starts with the infectious or noninfectious agents and ensues in inflammation, thrombosis and hypotension ^[10]. There are certain events in SIRS which leads to septic shock ^[11].

Normally, highly reactive chemical species like reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an important role in mitochondrial respiration, prostaglandin production and host defence ^[12]. However, for cellular homeostasis, a balance is maintained

between the formation of these reactive entities and their removal by scavenger activity of endogenous antioxidants^[13]. Progression of sepsis is linked with severe oxidative stress due to imbalance in this delicate equilibrium of ROS and antioxidant^[8]. In sepsis, there are several potential sources of ROS, including activation of xanthine oxidase due to ischaemia and mitochondrial respiratory electron transport chain^[13]. ROS have some pro-inflammatory effects like endothelial damage: neutrophil recruitment and cytokines release thus contribute in worsening the outcome of sepsis^[14]. In addition to this, the oxidative stress due to overwhelming release of free radicals during sepsis is also responsible for the development of septic shock^[8].

Fluid therapy reportedly improves the outcomes of sepsis due to early volume replacement and decreased blood viscosity^[15]. Fluid therapy with normal saline solution is very helpful in septic shock^[16].

In sepsis, vitamin C causes a decrease in the expression of iNOS^[12]. Vitamin C reacts with the hydroxyl and superoxide radicals that inhibit the bacterial replication and protects the endothelial cells from injury^[17]. It also prevents the endothelial dysfunction without changing the response of vascular smooth muscles caused by LPS^[18]. To combat severe oxidative stress, ascorbate is a powerful antioxidant vitamin and it acts as an enzyme cofactor and reductant^[19].

It is obvious from literature that inclusion of antioxidant should be a corner stone of therapy of sepsis. However, the use of DFX as an adjunct therapy to rational treatment of sepsis is not in vogue. It has not been evaluated in a canine induced sepsis model. So the present study was conducted to evaluate the therapeutic efficacy of DFX in septic dogs and to compare the therapeutic efficacy of DFX as an adjunct to rational therapy and with addition of vitamin C in rational therapy of sepsis.

2. Materials and Methods

The present study was conducted to evaluate the therapeutic efficacy of deferoxamine (DFX) as an adjunct to rational therapy alone or in combination with vitamin C in induced endotoxemia in a dog model in 2013 at Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad.

2.1 Experimental Animals

A total of 18 adult, healthy Mongrel dogs of either sex were selected and divided into three groups comprising of six animals in each group. All animals were kept indoors in separate kennels. During acclimatization period of one week, health status of all the animals was checked through clinical and laboratory examinations. For ethical considerations, animals were managed, handled and utilized in experiment as per guidelines of Animal Committee, University of Agriculture, Faisalabad.

2.2 Induction of Septic Shock

Intra-abdominal sepsis was induced through caecal ligation and perforation. For this purpose, laparotomy of each dog was performed for exteriorization of caecum. Aseptically surgical site was prepared. Animals were anaesthetized with the injection of ketamine hydrochloride (Ketamax) @ 13-30 mg/kg B.Wt. An incision of 5-7 cm was given on caudal midline for exteriorization of caecum. After exteriorization the caecum was ligated at base of ilio caecal junction, after stripping of iliac contents into it. After ligation, 4mm punctures were made at five different sites. Abdominal muscles and skin

was closed systematically and animals were observed for septic shock. Induction of septic shock was observed through clinical signs.

2.3 Intravenous Cannulation

All the animals of group A, B, and C experienced the maintenance of intravenous infusion lines for the administration of resuscitation fluid, vitamin C and other treatments respectively. For this purpose, the cephalic vein was cannulated. Cannulated site was prepared aseptically. Following skin preparation, the cephalic vein was cannulated properly and secured and respective treatment was given through cannula to the respective groups.

2.4 Experimental Design

A total of 18 adult, healthy dogs of either sex were randomly selected and divided into three equal groups viz. A, B and C through random number table^[20]. Treatment protocol A comprised of vitamin C @500 mg/Kg b.w, enrofloxacin @5 mg/kg b.w, ketoprofen @0.2 mg/kg b.wt, DFX @20 mg/kg b.w and normal saline @90 ml/kg b.wt. Animals of group B received enrofloxacin @5 mg/kg, DFX @20 mg/kg, ketoprofen @0.2 mg/kg b.w and normal saline @90 ml/kg. Group C was treated with DFX @ 20 mg/kg b.w, ketoprofen @0.2 mg/kg b.w and normal saline 90 ml/kg b.w after the establishment of septic shock and treatment was repeated after 24 hrs.

2.5 Measurement and Analysis of Samples

After septic shock induction followed by treatment protocol following parameters were taken to check the effectiveness of treatment regime.

2.6 Clinical Parameters^[21]

The body temperature was measured with the help of mercuric thermometer. First of all the dogs were properly restrained and the thermometer was shaken down just before inserting into the rectum. I inserted the thermometer approximately 3 to 4 inches into the rectum and it should be touching the wall of rectum. Then placed it for 2 minutes and after that it removed. Reading of thermometer were taken. Pulse rate was taken by placing the fingers on the femoral artery and counted for 15 seconds and it was multiplied by 4 to obtained the average pulse rate per minute. Respiration rate was taken by placing a wet finger over the nares for 15 seconds or by observing the motion of the flank region and it was multiplied by 4 to get the respiration rate per minute. All the animals were evaluated for a week for their survival period.

2.7 Haematological parameters^[22]

2.7.1 White blood cell count (WBCs)

Took blood sample having anticoagulant (EDTA) in diluting pipette upto 0.5 mark. I added leukocyte diluting fluid (N/10 HCl at the concentration of 1 ml in 100 ml of distilled water) by slight suction to 11 mark. Then before filling the counting chamber discarded 2-3 drops of fluid. WBCs were calculated by following formula^[22].

$$\text{WBC /microliter} = \frac{\text{Cell counted} \times 10 \times 20}{4}$$

2.7.2 Red blood cells count (RBCs)

Blood samples were collected and anticoagulant was added into them. Moved the anticoagulant added blood sample to the

thoma erythrocytes diluting pipette upto 0.5 mark. Erythrocytes were added in diluting fluid (0.85 mg of NaCl in 100 ml of distilled water) upto mark 101 by suction. Shaked the pipette slowly and leave it for 3 minutes. Then first 3 drops of fluid were discarded before touching the counting chamber. Then under 10x magnification the RBCs located at central 9 large squares were counted. The total number of RBCs were counted by formula ^[22].

Erythrocytes /microliter = cell counted x 5 x 200

2.7.3 Haemoglobin concentration (Hb concentration)

Cyanmethemoglobin method was used for determining the haemoglobin concentration in the blood sample ^[22]. For this purpose, mix the 20 µl of blood of each dog in drabkin solution. Color was produced when blood was mix into the drabkin solution and spectrophotometer give its precise idea about the absorbance at 540 nm. Haemoglobin concentration with the help of following formula:

Haemoglobin concentration = sample absorbance x standard concentration/ standard absorbance

2.8 Statistical Analysis

Data thus generated was analysed with repeated measure analysis technique and difference in means was compared by least significance test (LSD) at 5% level of significance ^[23].

3. Results

3.1 Clinical Parameters

3.1.1 Survival Rate: At the start of study period each treatment group comprised of 6 dogs. After the provision of allocated treatments to each group there was a different survival rate i.e mortality rate was higher in group C while it was low in group A and B. Group A and group B showed greater number of revived dogs while group C showed 66% mortality. The percent survival rate that was observed 7 days post shock in groups A, B and C were 100, 100 and 66 percent, respectively.

3.1.2 Body temperature (BT): In all the groups body temperature (BT) was normal at baseline. Before the induction of septic shock, a non-significant difference was observed in all the groups. During septic shock an increase in the BT was recorded. A quick drop in the BT was noted after providing the treatment to the groups. All the groups representing non-significant difference that sustained up to t=24h ($p>0.05$) and at the end of the study period baseline

values were almost attained. BT of the dogs of group A was elevated as compared to base line ($p<0.05$) at t=0 after the induction of septic shock. After the provision of treatment, the gradual decrease in the trend was noted till the end of the study period statistically different at t=6 and t=24 h ($p>0.05$). BT of group B was at higher levels from baseline at t=0 h during shock and after the treatment it showed decreasing trend ($p>0.05$) throughout study period and similar trend was observed in group C but it could not attain baseline values till 24 h post treatment than previous study interval (Table 1).

3.1.3 Respiration rate (RR): At baseline a non-significant ($p>0.05$) difference was observed between all the groups. A significant ($p<0.05$) increase was noted after the septic shock induction in all the groups. The allocated treatment was given to the particular group and a decreasing trend was observed in RR. The group A and B were non-significantly ($p>0.05$) lower than group C at all study intervals except t=12 hrs post treatment. While group A was significantly ($p<0.05$) lower than group B at t=3 hr, 6 hr, 12 hr and 24 hr post treatment. All the groups successfully achieved the baseline values at the end of study period. In group A after the septic shock induction an increase trend was observed in the animals, which was significantly ($p>0.05$) different. After the provision of particular treatments, a reducing trend was presented during the course of the study period being statistically significant ($p<0.05$) at each study interval. The group B and C also showed the same trend. (Table 1)

3.1.3 Pulse rate (PR): A significant ($p<0.05$) increase was observed in all groups during septic shock, but after the administration of treatment a decreasing trend was observed. Group B showed a better recovery in terms of PR as compared to other groups, being non-significant ($p<0.05$) at 3 hr, 6 hr, 12 hr and 24 hr. While group A was significantly ($p<0.05$) lower from group C at t=3 hr post treatments. The PR of the animals of all groups generally increased after the induction of septic shock. After the provision of particular treatments, group A showed a decrease in PR during the course of the study period being non-significant ($p>0.05$) at each study interval while in group B showed a decrease, trend being significant ($p<0.05$) at baseline and during sepsis. In group C an increased trend was observed being significant ($p<0.05$) at t=12 hr and 24 hr. (Table 1).

Table 1: Mean comparison of (Means \pm SE) of three groups viz infusion with desferal, vitamin C, NS, ketoprofen and enrofloxacin during shock while in group B, desferal, NS, ketoprofen and enrofloxacin during shock, group C comprises of desferal, NS and ketoprofen given during shock.

Time	Body temperature values F°	Respiration rate breath/min	Pulse rate beat/min
Baseline	102.02 \pm 0.07F	20.78 \pm 0.37E	101.61 \pm 0.78D
During Sepsis	104.93 \pm 0.10A	47.06 \pm 0.57A	126.89 \pm 3.83A
3 hr	104.39 \pm 0.12B	40.56 \pm 0.55B	118.68 \pm 4.01B
6 hr	103.94 \pm 0.14C	34.44 \pm 0.92C	116.10 \pm 3.95B
12 hr	103.32 \pm 0.13D	28.11 \pm 0.94D	108.81 \pm 2.29C
24 hr	102.57 \pm 0.13E	23.28 \pm 0.96E	106.01 \pm 1.36C

*Means sharing similar letter in a row or in a column are statistically non-significant ($P>0.05$). Capital letters represent overall mean.

3.2 Hematological Parameters

3.2.1 Red blood cells count (RBCs): The red blood cells (RBCs) count decreased in all the experimental groups after septic shock induction. After the treatments were given, group A shown non-significant ($p>0.05$) increasing trend at t=6 hr, 12 hr and 24 hr. Group B also showed the same trend. In group C a significant ($p<0.05$) difference was observed at

baseline. At each study intervals group, A and B showed non-significant ($p>0.05$) increase in RBCs count after the allocated treatments were given to the groups. Group A and B successfully achieved the baseline values successfully. Group C also shown non-significant ($p>0.05$) increasing trend but could not attain the baseline values. (Fig. 1)

3.2.2 White blood cells count (WBCs): After the septic shock induction, the WBCs count was decreased in all the three groups. When the treatments were given to the experimental groups the non-significant ($p>0.05$) increase was observed at each study interval. After the allotted treatments were given, group A showed non-significant ($p>0.05$) difference during sepsis and 3 hr. The group B showed non-significant ($p>0.05$) difference at baseline, during sepsis and 24 hr. In group C there was a non-significant ($p>0.05$) difference at baseline. Group A and B attained the baseline values successfully but group C could not have achieved the values (Fig. 2)

3.2.3 Haemoglobin concentration (Hb): After the septic shock induction, the Hb concentration was decreased in all the three groups. When the treatments were given to the experimental groups a non-significant ($p>0.05$) increase was observed till 24 hr. After the provision of allotted treatments group, A showed increased Hb concentration being non-significant ($p>0.05$) at each study intervals and successfully attained the baseline values. The group B showed similar trend. In group C there was a non-significant ($p>0.05$) difference but could not attain the baseline values (Fig. 3).

3.2.4 Neutrophil cell count: The neutrophil cells count of all the groups was decreased after the septic shock induction. After the allocated treatments were given to each group the non-significant ($p>0.05$) increasing trend of neutrophils was observed till 24 hr. After the provision of allocated treatments group, A showed increased neutrophils cell count that was non-significant ($p>0.05$) at $t=12$ hr and 24 hr. The group B showed significant ($p<0.05$) increased trend at each study interval. In group C a non-significant ($p>0.05$) difference at $t=3$ hr and 6 hr was observed. Group A and B successfully achieved the baseline values (Fig. 4).

3.2.5 Lymphocyte cell count: The lymphocyte cells count of all the groups was decreased after the septic shock induction. After the allocated treatments were given to each group the non-significant ($p>0.05$) increasing trend of neutrophils was observed till 24 hr. After the provision of allotted treatments group, A showed increased trend being non-significant ($p>0.05$) at $t=3$ hr and $t=6$ hr. The group B showed non-significant ($p>0.05$) difference during sepsis and $t=3$ hr. Group A and B successfully attained the baseline values. Regarding group C a significant ($p<0.05$) difference was observed at baseline (Fig. 5)

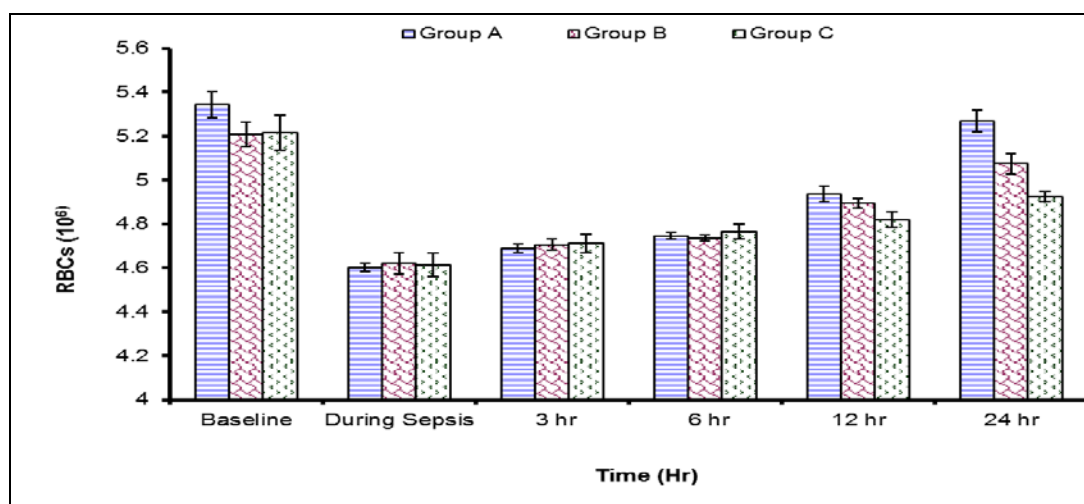


Fig 1: Red Blood Cells (10^6) in dogs ($n=6$ animals/group) with induced endotoxaemia in three groups viz infusion with desferal, vitamin C, NS, ketoprofen and enrofloxacin during shock while in group B, desferal, NS, ketoprofen and enrofloxacin during shock, group C comprises of desferal, NS and ketoprofen given during shock.* non-significant ($p>0.05$) increase was observed till 24 hr. Means (\pm SE) sharing similar letters within a group are statistically non-significant.

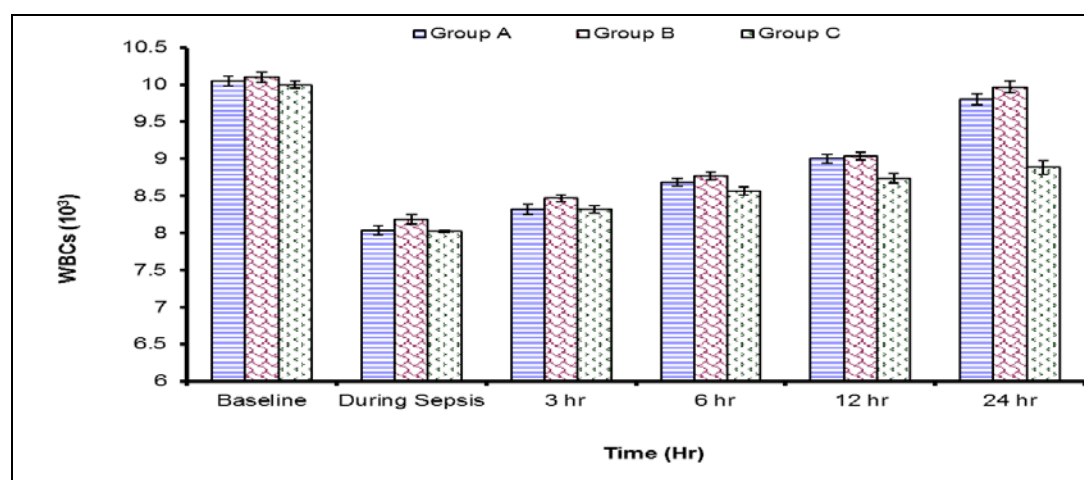


Fig 2: White blood cells (10^3) in dogs ($n=6$ animals/group) with induced endotoxaemia in three groups viz infusion with desferal, vitamin C, NS, ketoprofen and enrofloxacin during shock while in group B, desferal, NS, ketoprofen and enrofloxacin during shock, group C comprises of desferal, NS and ketoprofen given during shock.* non-significant ($p>0.05$) increase was observed till 24 hr. Means (\pm SE) sharing similar letters within a group are statistically non-significant.

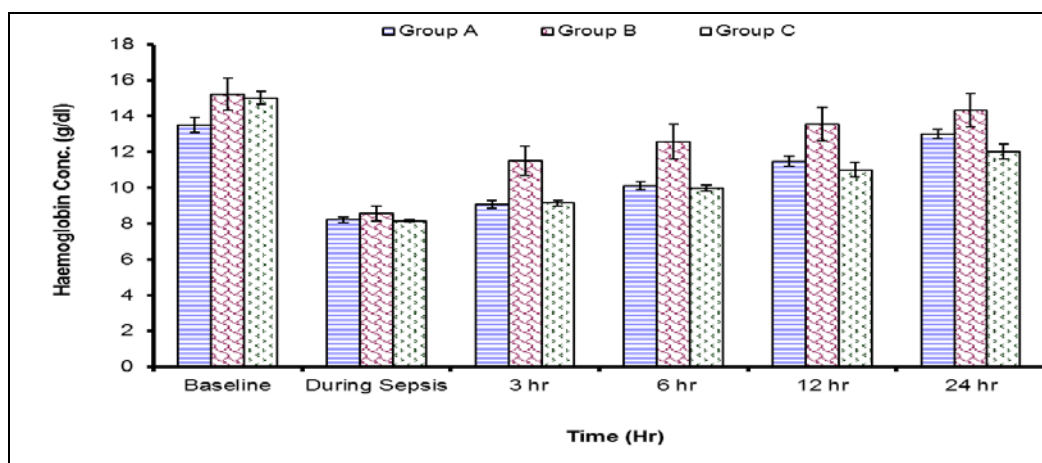


Fig 3: Haemoglobin concentration (g/dl) in dogs ($n=6$ animals/group) with induced endotoxaemia in three groups viz infusion with desferal, vitamin C, NS, ketoprofen and enrofloxacin during shock while in group B, desferal, NS, ketoprofen and enrofloxacin during shock, group C comprises of desferal, NS and ketoprofen given during shock. * non-significant ($p>0.05$) increase was observed till 24 hr. Means (\pm SE) sharing similar letters within a group are statistically non-significant.

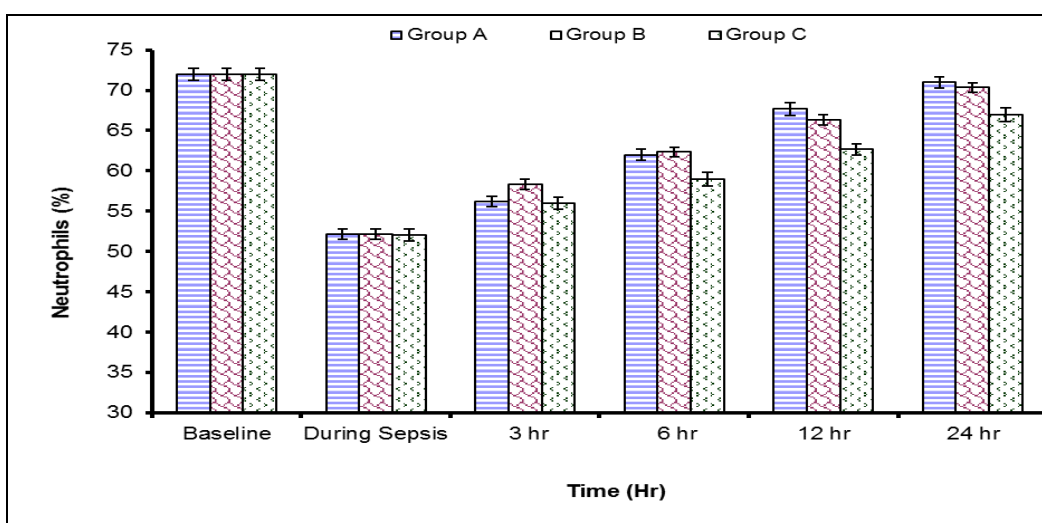


Fig 4: Neutrophils (%) in dogs ($n=6$ animals/group) with induced endotoxaemia in three groups viz infusion with desferal, vitamin C, NS, ketoprofen and enrofloxacin during shock while in group B, desferal, NS, ketoprofen and enrofloxacin during shock, group C comprises of desferal, NS and ketoprofen given during shock. * non-significant ($p>0.05$) increase was observed till 24 hr. Means (\pm SE) sharing similar letters within a group are statistically non-significant.

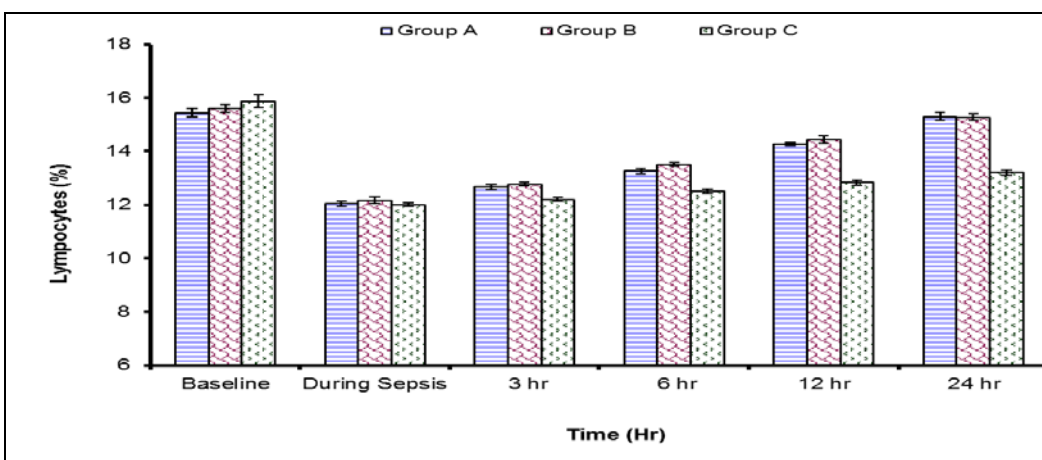


Fig 5: Lymphocytes (%) in dogs ($n=6$ animals/group) with induced endotoxaemia in three groups viz infusion with desferal, vitamin C, NS, ketoprofen and enrofloxacin during shock while in group B, desferal, NS, ketoprofen and enrofloxacin during shock, group C comprises of desferal, NS and ketoprofen given during shock. * significant difference ($p>0.05$) was observed till 24 hr. Means (\pm SE) sharing similar letters within a group are statistically non-significant.

4. Discussion

Sepsis is serious and complex syndrome that is characterized by serious inflammation due to infection. The severity of the sepsis leads to the origin of different condition like severe sepsis and septic shock. In general view the bacterial lipopolysaccharide (LPS) is activator of various pro and anti-immune responses and stimulates the monocytes and macrophages to release a variety of inflammatory cytokines. However, excessive production of inflammatory mediators in response to bacterial infections is known as septic shock [24].

Sepsis comprises series of abnormalities due to the involvement of broad array of humoral interleukins, TNF- α , arachidonic acid, free oxygen radicals, proteases and cellular like lymphocytes, monocytes, and neutrophils factors interact with each other that may cause to increase vascular permeability, up-regulate expression of adhesion molecules, and direct inflammatory cells that may lead to generalized body insult in the form of septic shock [25]. Sepsis is mediated with alteration of clinical, hemodynamic as well as thermoregulatory parameters such as fluctuation in body temperature usually lower than 36°C or greater than 38°C, increased heart rate greater than 90 beats per minute, arterial pressure of carbon dioxide less than 32 mmHg [26]. White blood cell count less than 4000 cells/mm or greater than 12,000 cells/mm with the contribution of more than 10% immature neutrophils [27].

Rational therapy of the sepsis is based on the use of broad spectrum antibiotics like aminoglycosides beta lactams, imipenem and fluoroquinolones and effective and early therapy should be ensured because mortality rate goes up to 35% for every hour delay in treatment [28]. Fluid therapy has been shown to improve the outcome in sepsis due to recovering from hypovolemic condition due to the volume replacement [29]. Normal saline improves the perfusion of microvasculature by increasing the driving pressure and decreasing blood viscosity [30].

As stated earlier sepsis is associated with the depletion of endogenous antioxidants therefore, supplementation of exogenous antioxidants has a key role in restoring antioxidant capacity [14]. It reacts with both superoxide and hydroxyl radicals which inhibits the bacterial replication and prevents the hydrogen peroxide injury to endothelial cells [17]. Intravenous (IV) infusion of DFX reduced brain edema and attenuates the lipid peroxidation, and blocks the IL-6 production, and conferred renoprotection [31]. It was observed that the body temperature was elevated during septic shock, and after treatment of respective therapy a decrease in body temperature was observed in all the groups.

In present study the level of Hb was reduced during septic shock due to the production of immature and less number of blood cells (RBCs, WBCs and platelets) and after the administration of treatments the level was achieved towards baseline in both groups A and B as compared to group C. These findings are in agreement with the findings of (Tyler *et al.*, 1994) [32] that correlate the hemoglobin level directly with the number of cells like RBCs, WBCs and platelets.

In septic shock the number of RBCs becomes at low level that is indicative of hypovolemic and shock condition. In septic shock Hb level becomes low due to circulating precursor's cells with decreased Hb binding capacity.

It has been demonstrated in this study that treatment with an antibiotic in combination with DFX improves survival rate these findings are in agreement with the findings of (Yoo *et al.*, 1999) [33]. The study has also demonstrated the beneficial effect of DFX as a supplement to antibiotics, in the survival

rate of peritonitis in group A and B: these findings are also in agreement with the findings of (Bullen *et al.*, 2000) [34]. DFX in combination with antibiotics have a significant effect on survival rates when compared with the survival rate of dogs treated with DFX alone. However, treatment with a combination of DFX with antibiotics resulted in a survival rate significantly higher are in agreement with the findings of (Soybir *et al.*, 2002) [35].

The result of this study is that the use of DFX along with antibiotics along with rational therapy is very beneficial in the septicemic dogs and gives better results in the terms of clinical, biochemical and hemodynamic parameters associated with an increase in survival rate.

5. Conclusion

Keeping in view of the results of this study it is concluded that the therapeutic efficacy of deferoxamine as an adjunct to rational therapy along with antibiotics is very useful in combating the endotoxemia and has better recovery rate in terms of haemodynamic, haematological and serum biochemical profile.

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