Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 10, 2014

Original Article

DEVELOPMENT AND EVALUATION OF FREEZING RESISTANT INTRAVENOUS FLUID

PRIYANKA CHHABRA[1], SURBHI SARAF[1], RASHID ALI[1], HARISH RAWAT[1], GAURAV MITTAL[1], ASEEM BHATNAGAR[1], SITANSHU SHEKHAR LAHIRI[2], AMIT KUMAR[1*]

[1]Division of Nuclear Medicine, Institute of Nuclear Medicine and Allied Sciences, Defence R&D Organization, Brig. S K Mazumdar Marg, Delhi 110054, India, [2]Amity Institute of Biotechnology, Amity University Campus, Noida 201303 (U. P.)
Email: amityagi@gmail.com

Received: 05 Sep 2014 Revised and Accepted: 03 Oct 2014

ABSTRACT

Objectives: Hemorrhagic or hypovolemic shocks accounts for a large portion of civilian and military trauma deaths due to life-threatening blood loss which requires intravenous fluid infusion to prevent essential deficiencies of fluids. However, at low temperature (-15^{0}C) fluid bottles freeze out and can not be used in emergency. In view of that, objective of the present work is to develop a freezing resistant intravenous formulation (FRIV) and its *in vivo* safety and efficacy evaluation.

Methods: FRIV formulations were developed using standardized ringer lactate (RL) formulation protocol, in which varying concentrations of ethanol and glycerol were added to induce desired physiochemical properties. Efficacy of FRIV was evaluated in terms of survival percentage of hemorrhagic animal models (Swiss albino strain mice). Acute toxicity studies were carried out through an infusion at dose levels (0, 20 and 40 ml/Kg b. wt.).

Results: *In vitro* data showed that optimized FRIV (**F-10**) takes more time (360 ± 21 min) for freezing and less time in thawing (50 ± 4.50 min) in comparison to control which takes (110 ± 15 min) in freezing and (80 ± 7.25 min) in thawing. Formulations were found to be stable and sterile up to six months. *In vivo* efficacy data showed ≥ 75% survival in animals infused with FRIV as compared to control group in hemorrhagic animal models and no treatment related toxic effects of optimized formulation in terms of hematological, serum biochemistry and histopathological analysis.

Conclusion: Pre-clinical safety and efficacy data of the present study indicated that developed FRIV formulation could be used for fluid recovery during the hemorrhagic shocks conditions in the combat scenario.

Keywords: Freezing Resistant Intravenous Fluid, Infusion, Pre-clinical, Serum biochemistry, Histopathology.

INTRODUCTION

Hemorrhagic or hypovolemic shocks accounts for a large portion of civilian and military trauma deaths, results due to life-threatening blood loss or decrease in blood pressure [1-3]. This also leads to tissue ischemia, shock, insufficient oxygen delivery for cellular metabolism [4]. Hemorrhage is responsible for more than 40% of trauma mortality. Up to 56% out of them occurs during the pre-hospital period [5]. Mortality is linked directly to severe blood loss or indirectly due to multiple organ failure. Specifically, loss of pulmonary function, renal, hepatic, and gastrointestinal is common after hemorrhagic shock [6]. Insufficient fluid resuscitation as well as catecholamines stabilize the cardiovascular function, uncontrolled hemorrhagic shock occurs which is also life-threatening [7]. Although resuscitation of shock induced alteration in morbidity and mortality involves an appreciation for the quantity of fluid loss that might be required, however it is difficult to measure the amount of blood loss in most situations [8-11]. To overcome this problem instant administration of intravenous (IV) fluids therapy plays a vital role.

Intravenous fluid infusion in critical injuries aims to prevent essential deficiencies of fluids, to overcome hypovolemia, replace lost electrolytes to correct dehydration. It is also used as a means of treating various diseases, blood transfusion and act as a vehicle for the excretion of waste products and redundant metabolites [12]. Intravenous route is the fastest way to deliver fluids and medications throughout the body as compared with other routes of administration and has been widely accepted therapy as a mean of parenteral administration. More than 80% of hospitalized patients receive IV fluid infusion at some point during their admission [12, 13]. The intracellular fluid volume in the body system is a function of the health of the cells and therefore it can only be indirectly affected by fluid infusion [11]. Body electrolytes and extracellular fluids are tightly regulated by neural, humoral and renal mechanism to maintain osmolarity of extracellular fluid and normal extra cellular volume. About two third of total body water is intracellular fluid which is distributed into intracellular, interstitial, and intravascular compartments [14]. Each of the compartments has a fixed volume and electrolyte composition that must be considered in the development of a resuscitation strategy.

Three main types of intravenous fluid that are used to restore and maintain body systems during shock resuscitation includes, blood and blood products, colloidal fluids, and crystalloid solutions. Crystalloids are true aqueous solutions of mineral salts and are often associated with physical characteristics which affect the balance of these fluids in two of the body fluid compartments: intravascular and interstitial making crystalloids to pass readily through biological membranes [15, 16]. Crystalloids used for IV infusion should be isotonic so that they do not cause a significant shift of water between the blood vessels and cells [2, 17]. Intravenous fluids are sterile aqueous solutions or emulsions having water as the continuous phase. They are free from pyrogens or microbial contamination, usually made isotonic with blood and do not contain any added antimicrobial preservatives that can be observed on visual inspection with the naked eye. The intravenous fluid should have the purpose specific formulation, balanced physiochemical composition to dissolve solutes called electrolytes (charged particles such as sodium, potassium, and chloride).

At high altitude (above 10,000 feet) due to sub-zero temperature intravenous fluids freeze out and cannot be used in trauma / emergency scenario due to the longer time (hrs) taken in thawing or defreezing. In case of hemorrhagic shocks,/ injuries immediate administration of fluids is necessary for the quick management of electrolytes concentration in the body fluid for maintenance of homeostasis. First 60 min following a traumatic injury has been recognized as vital to save lives of the patient, which depends on the quick administration of the IV fluids. Apart from more thawing time, sub-zero temperature also causes denaturation or degradation of the number of essential components of the injectables making them

unusable. In view of that, objective of the present study is development and characterization of a freeze resistant intravenous (FRIV) formulation for high altitude. The developed FRIV will be able to replace the fluid/ blood loss from the body with reduced thawing time in addition to survival efficacy.

MATERIALS AND METHODS

Chemical and Reagents

Sodium chloride, potassium chloride, calcium chloride, lactic acid and analytical grade (AR) reagents were purchased from Merck Ltd. India. Ringer Lactate was procured from Denis Chem Lab Ltd., Ahmedabad, India. Glycerol was purchased from SD Fine Chemical Ltd, Mumbai, India. All other chemicals were of AR grade and used as obtained.

Experimental Animals

Male Swiss albino strain mice, weighing 28 ± 2g from the animal house facility of the Institute were used for the study. All animal experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC). Experimental animals were housed in polypropylene cages in groups of six mice per cage and kept in a room maintained at 25 ± 2^{0}C with a 12h light/dark cycle, and were acclimatized for a week before conducting an experiment. They were given free access to standard laboratory animal feed (Golden Feed Laboratory. Delhi, India) and water ad libitum.

Methods

Preparation of freezing resistant intravenous (FRIV) formulations

Different FRIV fluid formulations based on Ringer lactate were prepared using the standard protocol and varying concentration of ethanol and glycerol was also added. Briefly, the solvent used for preparation of FRIV fluids formulations was water for injection. After addition of different constituents of ringer lactate (Calcium Chloride, Potassium Chloride, Sodium Chloride, and Sodium Lactate) in the given concentration as per British Pharmacopeia 2007, ethanol (0-4%), and glycerol (0-20%) were also added and mixed thoroughly using mechanical stirrer at 100 rpm for 30 min at 25 ^{0}C [18]. The formulations thus prepared were evaluated for clarity against alternate light and black backgrounds. After aseptic preparation the fluids were filtered through 0.22 µm membrane filter (Acrodisc®, Pall Corporation, USA) and thereafter transferred to the pre-sterilized glass containers.

In-vitro characterization

Evaluation of Freezing Resistant Characteristics

Fourteen (14) different formulations were prepared and evaluated for their freezing and thawing properties. Formulations were filled in plastic bags used for IV fluid storage and kept at sub-zero temperature (-20 ± 0.1 ^{0}C) in refrigerating circulating bath (RW-1025G, JEIO TECH, Korea), to observe freezing time. Complete frozen bottles were taken out and kept at room temperature (25^{0}C) to observe thawing time.

Physiochemical Analysis

Freezing and Thawing Stability

After six repetitive cycles of freezing and thawing, formulations were evaluated for changes in their pH, color, and aggregation if any, to confirm their stability (British Pharmacopeia, 2007). Briefly; 10 milliliters (ml) of the solution into a 20 ml colorless ampoule was filled and sealed, and used as the sample. Separately, 10 ml of the solution was put into a 20 ml colourless ampoule and sealed which was wrapped in aluminum foil to protect completely from light, and used as the control. The sample and control were exposed to the light source for an appropriate number of hours. Samples were then analyzed for physical appearance, pH, and color.

Determination of pH

The pH of developed formulations was measured using a pH meter (Microprocessor pH system, Punjab, India) for analysis of acidic or basic nature of the fluids. pH of the formulations was also evaluated after repetitive freezing and thawing to establish stability of the formulation.

Microscopic observation

The slides of optimized fluid formulation were prepared and observed under the microscope (Olympus BX 60) for any physiochemical change or contamination in the formulation at different time intervals (1, 3, and 6 months) so as to confirm the stability of developed fluid formulation.

In-vivo evaluation of FRIV formulation

Efficacy evaluation

In vivo efficacy evaluation studies of developed FRIV fluid formulation was carried out on hemorrhagic animal models. Hemorrhagic shock was induced withdrawing the blood (1 ml) from retro-orbital plexus of the animals. Twenty-four Swiss albino strain male mice were selected and randomly divided into 3 groups, 8 animals in each. Animal groups were designated as group I, group II, and group III. The animals of group I, was kept as control and no treatment was given while animals of group II and III were treated with intravenous infusion of Ringer Lactate and developed FRIV formulation respectively (equal to the volume to the total blood withdrawn) instantly after blood collection using a calibrated portable infusion pump (Pilot E, A2 India 01, Fresenius vial SA). A 27G X19 mm Top Winged Infusion Set (Meditop Corporation (M) SDN BHD Malaysia) was inserted into tail vein for infusion of fluids. Thereafter, clinical sign and symptoms of toxicity and survival studies were observed in all the animals [19].

Acute toxicity study

Eighteen (18) Swiss albino strain male mice weighing 28 ± 2g were selected and randomly divided into three groups of six animals in each. The animals groups were designated as group I, group II, and group III. The animals of group I served as control and received no treatment. Group II and III animals received a single dose of optimized FRIV fluid (20 and 40 ml/kg b. wt.) respectively using a calibrated portable infusion.

A 27GX19 mm, Top Winged Infusion was inserted into tail vein for infusion of FRIV fluid. Animals were observed prior to infusion, throughout the infusion process, and again approximately 1h after the infusion process. Untreated control mice were weighed and returned to their cages they were observed before and after other mice were injected. Animals were observed daily after dosing up to 14 days for any clinical sign of toxicity and mortality [**20**]. At 14th day of IV infusion experimental animals were sacrificed and dissected for further evaluation.

Observations

Gross pathology

The animals were observed for any changes in skin, fur, eyes, mucous membranes, autonomic, central nervous systems, somatomotor activity and behavior pattern. Attention was also directed toward observations of tremors, convulsions, salivation, diarrhoea, lethargy, and sleep if any.

Body weight, food and water intake

All animals were observed at least twice a day and their body weights, feed, and water intake were recorded daily throughout the experiment.

Hematological analysis

The animals were fasted overnight prior to necropsy and blood collection. Blood was collected by cardiac puncture in vials containing EDTA for immediate analysis of hematological parameters. Standard hematological parameters like red blood cells (RBCs), white blood cells (WBCs), erythrocyte sedimentation rate (ESR), P/C ratio, hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH) and platelets (PLT) counts were analysed using hematological automatic analyzer (Roche Integra, 400 Plus, Diagnostic Systems).

Serum biochemistry analysis

Blood samples for biochemical investigations were collected in plain tubes and centrifuged at 4000 rpm at 4°C for 10 min to obtain the serum. Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase activities (SGPT), alkaline phosphatase (ALP), protein, albumin, globulin, A/G ratio, urea, uric acid, and creatinine levels were estimated using the reagent kit of Randox Laboratory Ltd, UK in an automated analyzer (DREL 3000 HACH).

Organ/ body weight ratio analysis

After detailed gross necropsy examination, vital organs (heart, kidneys, liver, lungs, and spleen) were collected carefully for microscopic examine. Further their weights were analysed in relation to total body weight.

Histopathological analysis

The tissue samples of different vital organs viz; heart, kidney, liver, lung, and spleen were collected from all the animals and preserved in 10% buffered neutral formalin. They were adequately sliced wherever necessary. After a minimum of 24h fixation, they were sampled and processed by conventional methods [**21**], paraffin blocks were made and 5µm size paraffin sections were stained with hematoxylin and eosin [**22**]. The sections were deparaffinized using xylene and ethanol. The slides were washed with phosphate buffer saline (PBS) and permeabilized with permeabilization solution (0.1M citrate, 0.1% Triton X-100). Tissue histology was evaluated and microscopic changes were analyzed under a light microscope and compared with control.

Statistical analysis

Numerical data from experiments are presented as Mean ± SD. Differences between groups were analyzed following one way ANOVA using Dunnett's test and minimum criterion for statistical significance was set at $p < 0.05$ for all comparisons.

RESULTS

Preparation of FRIV fluid formulations

Fourteen (14) different types of FRIV formulations were developed as shown in Table 1. Physical properties of the developed formulations were also observed by naked eye any change in transparency and texture of the formulation was observed with change in the concentration of ethanol and glycerol.

Table 1: Concentrations of different constituents present in FRIV fluid formulations prepared.

Formulation No.	Different constituents in FRIF fluid formulation			
	Ringer Lactate	Ethanol	Glycerol	Physical Appearance
F-1	Normal Saline	-	-	Clear, Transparent
F-2	+	1%	-	Clear, Transparent
F-3	+	-	5%	Clear, Transparent
F-4	+	2%	-	Clear, Transparent
F-5	+	4%	-	Clear, Transparent
F-6	+	-	10%	Clear, Transparent
F-7	+	-	15%	Clear, Transparent
F-8	+	-	20%	Transparent with phase separation
F-9	+	2%	10%	Clear, Transparent
F-10	**+**	**2%**	**15%**	**Clear, Transparent**
F-11	+	2%	20%	Transparent with phase separation
F-12	+	4%	10%	Transparent with phase separation
F-13	+	4%	15%	Transparent with phase separation
F-14	+	4%	20%	Transparent with phase separation

Table 2: Time taken in complete freezing and thawing by different FRIV fluid formulations.

Formulation No.	Initiation of freezing Time (min)	Completion of freezing Time (min)	Thawing Time (min)
F-1	50 ± 4.50	110 ± 15.00	80 ± 7.25
F-2	60 ± 3.64	150 ± 11.50	60 ± 6.40
F-3	70 ± 5.24	160 ± 14.60	55 ± 6.25
F-4	90 ± 6.40	180 ± 16.20	45 ± 5.54
F-5	120 ± 10.50	210 ± 18.75	35 ± 5.20
F-6	210 ± 14.25	315 ± 23.10	70 ± 7.90
F-7	270 ± 20.60	375 ± 25.45	50 ± 4.94
F-8	300 ± 21.85	405 ± 28.00	45 ± 4.52
F-9	210 ± 13.50	290 ± 22.70	60 ± 5.04
***F-10**	**300 ± 19.75**	**360 ± 21.00**	**50 ± 4.50**
F-11	265 ± 20.25	350 ± 21.30	45 ± 4.74
F-12	225 ± 14.30	300 ± 19.65	60 ± 5.94
F-13	300 ± 20.90	405 ± 26.40	40 ± 4.34
F-14	345 ± 22.48	465 ± 28.85	35 ± 3.85

* Optimized Freezing Resistant Intravenous Formulation

***In-vitro* characterization**

Evaluation of Freezing Resistant Characteristics

Freezing and thawing time of developed FRIV fluid formulations at sub-zero temperature (-20^{0}C ±0.1^{0}C) are shown in **Table 2**. Freezing and thawing cycle was repeated six times and depression in the freezing point was observed in all the developed formulations. Freezing time increases from ~110 min to ~465 min after addition of 4% ethanol and 20% glycerol.

Increase in the freezing time and decrease in the thawing time was observed uniformly by an increase in the concentration of ethanol and glycerol.

Physical Analysis

Stability

After six repetitive freezing/ thawing cycles of developed formulations no significant change in physiochemical properties

(precipitation, pH and color) of the formulations were observed in comparison to control IV fluid.

Evaluation of pH

Repetitive freezing/ thawing of developed formulations has not showed any effect on the pH of developed FRIV formulations and found to be between 6.5 ± 0.3.

Microscopic observation

No microbial contamination was observed up to six months. The microscopic evaluation of developed formulation was carried out at 0, 1, 3, and 6 month time interval.

***In-vivo* efficacy evaluation**

The survival of animals of different groups has been shown in Fig. 1. All animals of group I were found dead within 24 hours of blood withdrawn, ~ 25% of animal survival rate was observed in the animals of group II, which was infused with normal ringer lactate intravenous fluid. On the other hand, group III animals infused with developed FRIV formulation showed more than 75% survival rate.

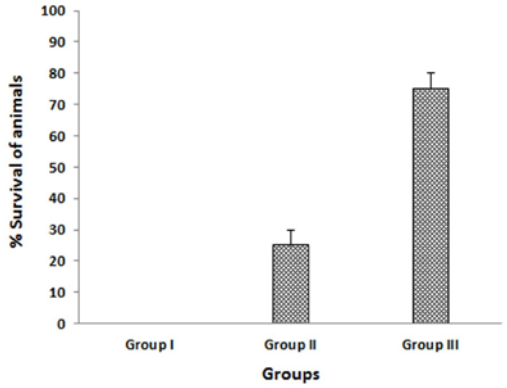

Fig. 1: Efficacy evaluation of FRIV fluid formulation intravenous infusion on hemorrhagic shocked animals. Values are expressed as mean ± S. D; (n=8).

Acute toxicity studies

Gross pathology

No mortality and morbidity or any signs of behavioural changes or toxicity were observed up to 14-days period after intravenous infusion of dose levels of 20 and 40 ml/kg body weight. Morphological characteristics (fur, skin, eyes, and nose) appeared normal. No tremors, convulsion, salivation, diarrhea, lethargy or unusual behaviours such as self-mutilation, walking backward and so forth were observed; gait and posture, reactivity to handling or sensory stimuli, grip strength were all normal.

Food, water intake and body weight analysis

No significant changes were observed in weight of treated groups as compared to control (Fig. 2). No significant difference was observed in test group of experimental animals in comparison to the control group of animals in terms of nutritional status/ feeding habits. Body weights of treated animals increased and there were no changes as compared to control.

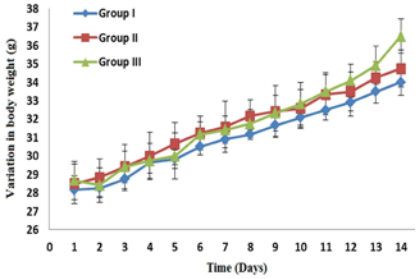

Fig. 2: Body weight variations in experimental animals treated with FRIV fluid formulation infusion during acute toxicity study. Values are expressed as mean ± S. D; (n=6).

Hematological analysis

As shown in Table 3, no significant changes were observed in hematological parameters in all the treated groups as compared to control group animals.

Table 3: Hematalogical evaluation of experimental animals treated with different dose of FRIV fluid formulation.

Hematological Parameters	Group I	Group II	Group III
	Average ± SD	Average ± SD	Average ± SD
Hb (gm/dl)	12.17 ± 1.38	11.067 ± 1.53	12.36 ± 1.42
WBC (10^3/µL)	10.84 ± 1.88	9.96 ± 1.94	10.78 ± 1.96
ESR(mm/1sthr)	16.00 ± 1.46	19.66 ± 1.52	12.66 ± 1.49
P/C	1.43 ± 0.058	1.653 ± 0.045	1.43 ± 0.208
MCV (fl)	41.41 ± 2.58	42.30 ± 2.62	41.71 ± 2.618
MCH (pg)	30.11 ± 1.12	30.37 ± 1.16	30.35 ± 1.32
MCHC (g/dl)	33.13 ± 2.431	33.43 ± 2.516	34.15 ± 2.551
RBC (10^6/µL)	9.45 ± 1.235	9.63 ± 1.353	8.92 ± 1.372
HCT (%)	36.00 ± 1.400	35.33 ± 1.528	36.06 ± 1.462

*Group I: Control; Group II: 20 ml/Kg b. wt., and Group III: 40 ml/kg b. wt. infusion of developed FRIV formulation. Values are expressed as mean ± S. D; (n=6) and p > 0.05; group II and III versus group III.

Table 4: Serum biochemical parameters in blood samples of experimental animals treated with different dose of FRIV fluid formulation.

Serum biochemistry parameters	Group		
	Group I	Group II	Group III
SGOT/AST (IU/L)	88.33 ± 7.53	92.30 ± 8.50	94.20 ± 8.75
SGPT/ALT (IU/L)	57.00 ± 4.65	59.67 ± 5.59	60.67 ± 5.06
ALP (IU/L)	72.00 ± 3.96	69.67 ± 4.25	76.00 ± 4.61
Protein (g/dl)	7.13 ± 1.15	7.43 ± 1.32	7.90 ± 1.36
Albumin (g%)	3.37 ± 0.65	3.55 ± 0.62	3.76 ± 0.73
Globulin (g%)	2.40 ± 0.32	2.52 ± 0.35	2.70 ± 0.34
A/G	1.52 ± 0.16	1.49 ± 0.15	1.56 ± 0.16
Urea (mg/dl)	31.00 ± 3.42	25.00 ± 3.34	29.00 ± 3.66
Uric acid (mg/dl)	8.00 ± 1.20	7.64 ± 1.28	7.38 ± 1.18
Creatinine (mg/dl)	0.52 ± 0.040	0.56 ± 0.042	0.62± 0.046

*Group I: Control; Group II: 20 ml/Kg b. wt., and Group III: 40 ml/kg b. wt. Infusion of developed FRIV formulation. Values are expressed as mean ± S. D; (n=6) and p > 0.05; group II and III versus group III.

Serum biochemistry analysis

Serum biochemistry parameters are shown in **Table 4.** There were no significant changes in treated animals as compared to control group animals.

Organ/ body weights ratio analysis

No statistically significant differences in organ/ body weights ratio of vital organs (heart, kidney, liver, lung, and spleen) of experimental animals as compared to control group were observed as gave in **Figure 3**.

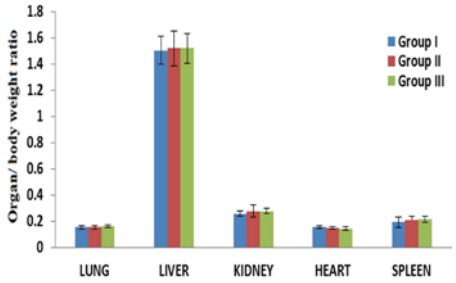

Fig. 3: Organ/ body weight ratio determination of vital organs of experimental animals treated with FRIV fluid formulation. Values are expressed as Mean ± S. D; (n=6).

Histopathalogical analysis

At necropsy no abnormalities were detected in pathological examinations of the tissues during microscopic examination of vital organs in comparative histology of tissues of control and test animals. As shown in Figure 4, Infusion of FRIV formulation did not affect the histology of vital organs viz., heart, kidney, and liver lungs, and spleen.

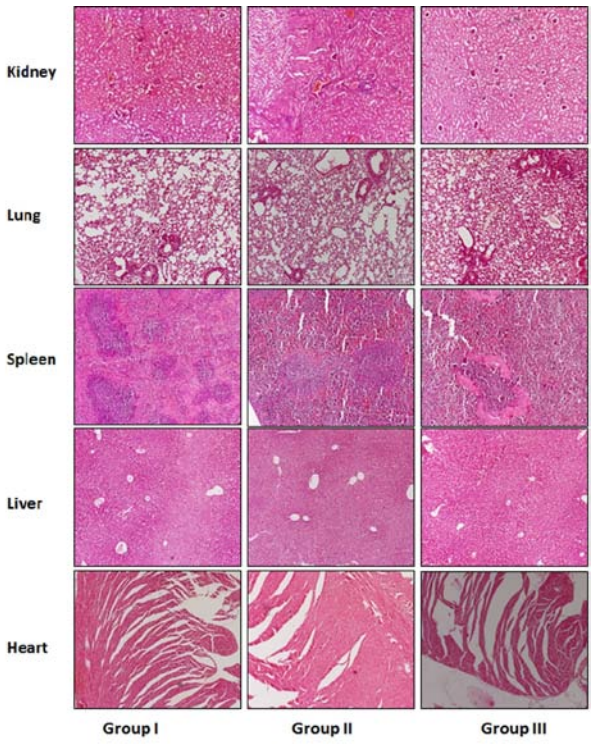

Fig. 4: Histological micrographs of different vital organs of experimental animals treated with FRIV fluid formulation (Magnification: 20X).

DISCUSSION

Hemorrhagic shock remains one of the leading causes of death in battlefield [4]. It is critical to provide medical aid with real time monitoring of soldiers in traumatic injuries. The excessive loss of fluid leads to the dysfunction of immune cells, resulting in tissue damage and finally organ failure [23, 24]. Intravenous fluids are pharmaceutical products that are administered intravenously to recover fluid loss. At high altitude, due to the extreme low temperature the intravenous fluid gets freeze and is not used in case of emergency.

We have formulated and optimize freezing resistant intravenous (FRIV) fluid which remains in liquid form even at sub-zero (-20°C) temperature. Freezing resistant property in developed intravenous fluids was induced by adding variable concentration of ethanol (0-4%) and glycerol (0-20%). It was observed that, freezing resistant property increases with increase in the concentration of the ethanol and glycerol. It was due to the hindrance in the hydrogen bonding developed after addition of crystalloids and increases with increase in concentration of the same. Physical observation of the developed formulations showed phase separation after addition of 20% glycerol, while the formulation having 0-15% glycerol showed uniformity in the texture and no phase separation was observed. On the other hand addition of ethanol up to 4% showed no effect on the texture of the formulation. Addition of ethanol (0-4%), and glycerol (0-20%) in normal ringer lactate also prevents cryo-degradation of the formulations which resists to freezing at subzero temperature [25]. As shown in table 2, formulation containing 4% ethanol with 15% (F-13) and 20% (F-14) glycerol has the highest freezing resistant property and takes maximum time to freeze out and minimum for thawing. As reported earlier intravenous formulations containing more than 2% ethanol and 15% glycerol showed toxicity [26, 27]. In view of that, formulation **F-10** (containing 2% ethanol, and 15% glycerol) was considered to be the best and chosen for further safety and efficacy studies. In addition to depression in freezing point, developed formulation also provides crystalloid solution and additional energy by means of glycerol. Stability of the formulation was evaluated by microscopic examination and confirmed as no aggregation and no variation in pH was found. Infusion of large quantities of blood can increase mortality or result in serious complications, such as disseminated intravascular coagulation (DIC), acute respiratory distress syndrome (ARDS), or systemic inflammatory response syndrome (SIRS) to the exposing patients [28, 29].

Efficacy evaluation of developed FRIV formulation was carried out by induction of hemorrhagic shocks in experimental animal models (Swiss albino strain mice). As shown in Figure 1, developed FRIV fluid formulation was found to be more effective in terms of survival rate (75%) of animals as compared to ringer lactate infused animals (25%), while no survival was observed in case of control (group I) animals. A large volume of fluids occasionally must be administered repeatedly by the intravenous route to test the safety and tolerability studies of a product. Therefore it is important for pathologists, toxicologists, and investigators to be aware of all mortalities, clinical signs, time of onset, duration, and reversibility of toxicity produced by intravenous infusions of large volume of formulations. Other properties of the solution or suspension, such as pH, irritancy, pharmacologic effects, and systemic toxicity, may also affect the maximal tolerated volume and rate of infusion of a particular fluid.

Safety studies of the developed formulation were carried by means of the toxicological evaluation using standard techniques like pathological studies, hematological analysis, serum biochemistry etc. [30-34]. No sign of local injury and inflammatory response was observed at site of injection in the treated groups of animals. As shown in Table 3, hemogram was estimated and results showed no deleterious effect on blood cell count, hemoglobin and other related parameters. Liver is the vital organ of paramount importance involved in the maintenance of metabolic function and detoxification of drugs. The fluid infused eliminates through renal excretion. Thus it was mandatory to estimate its effects on kidney and liver functions.

Transaminases (AST and ALT), ALP, creatinine and uric acid are generally used as an indicator for liver and kidney toxicity [35, 36]. No significant change was found in serum levels of AST, ALT, and ALP enzymes post intravenous infusion of formulation showing no detrimental effect on liver and kidney as shown in Table 4. Similarly, no significant change in the organic body weight of the FRIV treated animals were observed in comparison to the control group animals as shown in Figure 3. The safety of formulation on specific organs was further confirmed by histopathological assessment. Histopathological examination of selected vital organs (heart, lung, liver, kidney, and spleen) from both treated and control animals showed normal architecture, suggesting that no microscopic changes and morphological disturbances were caused due to an infusion of fluid at all dose levels. The fluid volumes used in current study were taken large as small animals require higher doses per kg body weight than larger animals due to their higher metabolic rate [37]. However, we did not examine the potential adverse effects of long-term repeated intravenous infusion of large fluid volumes or possible changes in many clinical laboratory parameters. The infusion rates used in this study were considerably below the estimated glomerular filtration rate of mice, suggesting that infusions of large volumes at higher rates may be possible.

Limitations

The developed freezing resistant formulation presented in this study is in the pre-clinical stage and need to be further evaluation for its safety and efficacy. The developed formulation can be used in emergency conditions if found safe in further clinical studies. The long term stability and sterility of the formulation is also required to assess for its long term storage so that it can be used in case of emergency.

CONCLUSION

Our results confirmed that developed and optimized formulation has the freezing resistant characteristic and it takes more time to freeze out and less time in thawing in comparison to standard ringer lactate fluid. Physiochemical characterization confirmed stability and sterility of the developed intravenous formulation. Enhanced survival rate (75%) of the small experimental animals induced with hemorrhagic shock confirmed the efficacy of FRIV formulation. An acute toxicity study of developed formulation indicates no adverse clinical effects in mice when administered intravenously at volumes up to 40 ml/kg and at rates up to 4 ml/h. In conclusion, our data suggest that developed FRIV formulation is efficient, safe and indicates no clinically relevant alterations of any of physiological, biochemical and histopathological parameters. Developed FRIV formulation can use as lifesaving and possessing widely clinical application and worth for wide use in emergency at high altitude.

CONFLICTS OF INTERESTS

Authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENT

Authors are thankful to Defence Research and Development Organization, Ministry of Defence, Government of India for providing the funds to carry out this work.

REFERENCES

1. Dutton RP. Resuscitative strategies to maintain homeostasis during damage control surgery. Br J Surg. 2012;99(1):21-8.
2. Moore FA, McKinley BA, Moore EE. The next generation in shock resuscitation. Lancet 2004;363:1988-96.
3. Hak DJ, Smith WR, Suzuki T. Management of hemorrhage in life-threatening pelvic fracture. J Am Acad Orthop Surg 2009;17(7):447-57.
4. Gutierrez G, Reines HD, Wulf-Gutierrez ME. Clinical review: hemorrhagic shock. Crit Care 2004;8(5):373-81.
5. Letson HL, Dobson GP. Unexpected 100% survival following 60% blood loss using small-volume 7.5% NaCl with adenocaine and Mg++ in the rat model of extreme hemorrhagic shock. Shock 2011;36(6):586-94.
6. Rönn T, Lendemans S, de Groot H, Petrat F. A new model of severe hemorrhagic shock in rats. Comp Med 2011;61(5):419-26.
7. Wenzel V, Raab H, Dunser MW. Arginine vasopressin: a promising rescue drug in the treatment of uncontrolled haemorrhagic shock. Best Pract Res Clin Anaesthesiol 2008;22(2):299-16.
8. Committee on Trauma, Shock. Advanced Trauma Life Support, Student Course Manual, 6th ed. Chicago: American College of Surgeons; 1997. p. 87-107.
9. Jordan KS. Fluid resuscitation in acutely injured patients. J Intraven Nurs 2000;23(2):81-7.
10. Martel MJ, MacKinnon KJ, Arsenault MY. Arsenault, Hemorrhagic shock. J Obstet Gynaecol Can 2002;24(6):504-20.
11. Shires GT, Browder LK, Steljes TP, Williams SJ, Browder TD, Barber AE. The effect of shock resuscitation fluids on apoptosis. Am J Surg 2005;189:85-91.
12. Waitt C, Waitt C, Pirmohamed M. Intravenous therapy. PGD Med J 2004;80:1-6.
13. Tjon JA, Ansani NT. Transdermal nitroglycerin for the prevention of intravenous infusion failure due to phlebitis and extravasation. Ann Pharmacother 2000;34:1188-92.
14. Schrier RW. The science behind hyponatremia and its clinical manifestations. Pharmacotherapy 2011;31(5):9S-17S.
15. Cook LS. IV fluid resuscitation. J Infus Nurs 2003;26(5):296-303.
16. Tremblay LN, Rizoli SB, Brenneman FD. Advances in fluid resuscitation of hemorrhagic shock. Can J Surg 2001;44(3):172-9.
17. Kaplan LJ, Philbin N, Arnaud F, Rice J, Dong F, Freilich D. Resuscitation from hemorrhagic shock: fluid selection and infusion strategy drives unmeasured ion genesis. J Trauma 2006;61(1):90-8.
18. Flomenbaum NE, Goldfrank LR, Hoffman RS, Howland MA, Lewin NA, Nelson LS. Goldfrank's Toxicologic Emergencies, 8th ed. McGraw-Hill Professional; 2006.
19. Moore EE, Johnson JL, Cheng AM, Masuno T, Banerjee A. Insights from studies of blood substitutes in trauma. Shock 2005;24(3):197-205.
20. Olson H, Betton G, Robinson D. Concordance of the toxicity of pharmaceuticals in humans and animals. Regul Toxicol Pharmacol 2000;32(1):56-7.
21. Slaoui M, Fiette L. Histopathology procedures: from tissue sampling to histopathological evaluation. Methods Mol Biol 2011;691:69-82.
22. Kiernan JA. Histological and Histochemical Methods. Theory and Practice. 4th ed. Bloxham,UK: Scion; 2009. p. 12-73.
23. Hierholzer C, Billiar TR. Molecular mechanisms in the early phase of hemorrhagic shock. Langenbecks Arch Surg 2001;386(4):302-08.
24. Rhee P, Koustova E, Alam HB. Searching for the optimal resuscitation method: recommendations for the initial fluid resuscitation of combat casualties. J Trauma 2003;54(5):S52-S4.
25. Kumana CR, Chan GTC, Yu YL. Investigation of intravascular haemolysis during treatment of acute stroke with intravenous glycerol. Br J Clin Pharm 1994;29:347-53.
26. Nau R, Prins FJ, Kolenda H, Prange HW. Temporary reversal of serum to cerebrospinal fluid glycerol concentration gradient after intravenous infusion of glycerol. Eur J Clin Pharmacol 1992;42(2):181-5.
27. Braun LJ, Tyagi A, Perkins S. Development of a freeze-stable formulation for vaccines containing aluminum salt adjuvants. Vaccine 2009;27:72-9.
28. Linker CA, McPhee SJ, Papadakis MA. (Eds.), Current Medical Diagnosis & Treatment. New York: Lange Medical Boods/ McGraw-Hill. 45th ed; 2006. p. 481-535.
29. Thompson RCC. Physiologic 0.9% saline in the fluid resuscitation of trauma. J R Army Med Corps 2005;151(3):146-51.
30. Baker AJ, Park E, Hare G, Liu, Mazer D. Effects of resuscitation fluid on neurological physiology after cerebral trauma and hemorrhage. J Trauma 2008;64(2):348-57.
31. Kiraley LN, Jerome A, Differding. Resuscitation with normal saline (ns) vs. lactated ringer's (lr). modulates hypercoagulability and leads to increased blood loss in an

uncontrolled hemorrhagic shock swine model. J Trauma 2006;61:57-65.

32. Koustova E, Stanton K, Guschin V, Alam AB, Stegalkina S, Rhee PM. Effects of lactated Ringer's solutions on human leukocytes. J Trauma 2002;52:872-8.
33. Todd SR, Malinoski D, Muller PJ, Schreiber MA. Lactated ringer's is superior to normal saline in resuscitation of uncontrolled hemorrhagic shock. J Trauma 2007;62:636-9.
34. Waters JH, Gottlieb A, Schoenwald. Normal saline versus lactated ringer's solution for intraoperative fluid management in patients undergoing abdominal aortic aneurysm repair: an outcome study. Anesth Analg 2001;93:817-22.
35. Hayes AW. Principles and Methods of Toxicology. 5th ed. Informa Health care, New York; 2007.
36. Raza M, Al-Shabanah OA, El-Hadiyah TM. Effect of prolonged vigabatrin treatment on hematological and biochemical parameters in plasma, liver and kidney of swiss albino mice. Sci Pharm 2002;70:135-46.
37. Olsson J, Hahn RG. Glycine toxicity after high-dose I. V. infusion of 1.5% glycine in the mouse. Br J Anaesth 1999;82(2):250-4.