Title: Estrogen as a safe therapeutic adjunct in reducing the inflammatory storm in Trauma haemorrhagic shock patients.

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Short Title: Estrogen as a safe therapeutic adjunct

Key words: Trauma haemorrhagic shock (THS), sepsis, inflammation, multiple organ failure, estrogen

List of Abbreviations:

THS: Trauma haemorrhagic shock

ED: Emergency Department

ATLS: Advance trauma life support

ICU: Intensive care unit

MODS: Multiple organ dysfunction syndrome

MOF: Multiple organ failure

SOFA: Sequential organ failure assessment

ISS: Injury severity score

SI: Shock index

GCS: Glasgow coma scale

APACHE: Acute physiology and chronic health evaluation

AIS: Abbreviated injury scale

CU: Compression ultrasonography

PBMCs: Peripheral blood mononuclear cells

ELISA: Enzyme linked immunosorbent assay

FCS: Foetal calf serum

Summary:

Trauma is a major cause of morbidity and mortality throughout the world. Alarmingly the mortality rate, owing to multiple causes with or without sepsis, is now reported to cross the value of 50%. The preliminary study was conducted in humans to investigate the 1) safety of estrogen therapy following trauma haemorrhage 2) Does estrogen reduced the inflammatory storms caused due to trauma 3) Does estrogen affects the survival of THS patients and prevent the advancement of sepsis associated problems. 40, THS patients and 20 healthy controls were recruited. THS patients were divided into experimental group and placebo controls based on the estrogen administration in the ED. Serum level of cytokines and immune cells were measured at different time points on days 0, 3, 7 and 14 in both groups of THS patients. Patients receiving intravenous estrogen beside standard of care as per ATLS guidelines did not develop any major or minor adverse events and showed favourable clinical outcomes during their course of stay in the ED and ICU. The levels of T regulatory cells, monocytes, and systemic cytokines were significantly reduced in THS patients who received estrogen. Again, THS patients who received estrogen recovered early, do not have side effects and showed balanced inflammatory response.

In conclusion, this preliminary study showed that intravenous estrogen therapy is safe and overcame the problem of inflammatory insults caused due to trauma haemorrhagic shock. It may protect from sepsis associated complications among THS patients.

1. INTRODUCTION:

Trauma haemorrhagic shock (THS) remains a significant public health problem throughout the world and is the fourth leading cause of death in person younger than 40 years(1). The higher rate of mortality in THS patients are usually associated with development of sepsis, septic shock, and the development of the multiple organ dysfunction syndrome (MODS) (2,3). Despite the advancement in pre-hospital care, early balanced resuscitation in the Emergency Department (ED) and damage control surgery mortality rate among THS has yet not been improved (4). The outcome of patients following trauma haemorrhage is not determined only by severity of injuries, but also influence by altered immune cells functions, hormonal status, and secreted cytokines(5,6). The inflammatory response is essential to host defence against infections, but inflammatory disparity caused by imbalance production of inflammatory and anti-inflammatory cytokines may lead to MODS and subsequently poor clinical outcomes (7,8).The management of trauma patients who develop sepsis associated problems in the ICU are challenging for the physician. Previous findings from our laboratory and others showed that THS patients who developed sepsis had elevated serum levels of IL-6 and IL-10 (9). The serum levels of IL-6 and IL-10 were significantly elevated in non-survivors as compared to survivors and subsequently showed positive correlation with Sequential Organ Failure Assessment score (SOFA score). We have also reported that T cell anergy, depressed Th1 cytokines production and skewing toward increased Th2 type immune reactivity in post-traumatic sepsis patients may lead to poor outcome among THS patients(5,9).

Several clinical and experimental studies demonstrated that gender dimorphism may determine the immune response following trauma haemorrhage which may differentially affects the release of Th1 and Th2 cytokines and subsequently determine the occurrence of sepsis and clinical outcome (10,11). In this respect, the cell mediated immune response has been shown to be depressed in the animal models following trauma haemorrhage (11,12). The sex hormones

estrogen and androgen play a pivotal roles in regulating the host immune response against haemorrhage in animal models (11,13). Estrogen administration restored adversely affected immune alterations in the animal models of THS. Therefore, estrogens may be use as a therapeutic adjunct for the treatment of THS by preventing sepsis associated problems and may subsequently reduce the mortality. In this respect, previous studies of animal models of haemorrhage showed that male gender as an independent risk factor for sepsis associated complications and for the poor clinical outcomes (14,15). Female rat animals with THS had lower chance to develop sepsis complications and showed favourable outcomes when compared to male (13). Recent finding of *Mars et al.,* 2016 also reported a significantly higher incidence of bacteraemia in traumatized males as compared to females(12). *Al-Tarrah et al.,* 2018 have shown a significantly higher survival rate in women (74%) compared to men (31%) following the onset of sepsis(16). Estrogen therapy is being commonly used to treat the haemorrhage occur due to massive dysfunctional uterine bleeding, however clinical and therapeutic applications of this therapy among THS patients in the humans have yet not studied. Based on these encouraging results from animal studies, we hypothesised that early administration of Estrogen in male THS patients may restore the inflammatory storm caused due to trauma, reduced the mortality, and also prevent from sepsis associated problems at the later stages in the ICU.

This preliminary study was conducted to investigate the 1) safety of estrogen therapy to THS patients 2) Does estrogen therapy reduce the inflammatory storm caused following trauma haemorrhage 3) Does estrogen affects the survival of THS patients and prevent the advancement of sepsis associated problems in the ICU.

2. MATERIALS and METHODS:

2.1 Patients Characteristics:

This pilot study was conducted as per the "Ethical Guidelines" issued by Indian Council of Medical Research (ICMR), New Delhi for conducting the biomedical research work on Human subjects and GCP guideline issued by Department of Ethical Committee, ICMR, Government of India, New Delhi. The preliminary study was approved by Human ethics committee, AIIMS, New Delhi (India) with Ref. No: NP-303/2010 & OP-16/11.04, 2011) and Director General of Health Science, Office of Drugs Controller General, India, New Delhi with Ref. No: 12-223/10-DC.

Male THS patients hospitalized in the ED at Trauma Centre, AIIMS, New Delhi were recruited. Eligibility criteria for enrolment in this study includes the haemorrhagic shock patients of male sex of age 16-60 years with systolic blood pressure (SBP) (≤90 mm Hg), presenting within 8 hours of injuries. Patients were recruited after taking proper consent from the patient or their relative. THS patients, who have neurogenic shock, traumatic brain injury (TBI), septic shock and already resuscitated with fluids (colloids or crystalloids), anti-inflammatory drugs or corticosteroids and blood or blood components before reporting to the ED were excluded.

2.2 Patients resuscitation and Samples Collection: All patients were managed in the ED according to the standard protocol of American College of Surgeons Advanced Trauma Life Support (ATLS). THS patients were divided into two groups based on estrogen therapy in the ED. Estrogen was administered intravenously (25 mg) at a single time point on day 0 in the ED. A double blind randomisation was performed to conduct the preliminary study. THS patients who received the intravenous estrogen (25 mg) with initial treatment 2 litre ringer lactate +fluid/blood resuscitation as per ATLS protocol are called experimental group and others who received 2 litre ringer lactate only with fluid/blood resuscitation as per ATLS protocol are called Control group. Blood samples were collected twice on day 0 in the ED. A total of 2ml blood

sample was collected in the EDTA vial at the time of arrival to measure the base line parameters from both groups of patients. Once patients were stablished in the ED, further 8 ml blood samples were collected within eight hours of injuries from them in the ED. The blood samples collected after drug infusion was designated as Day 0. The individual patients information (demographic and clinical parameters) were collected in the ED from all patients as per the Annexure I and II **(Supplementary Tables 1 and 2)**. The demographic and clinical parameters collected from every patients in the annexure sheets were used to correlate the all possible sequence of events of recovery and death up to one year of injuries. The details of inclusion criteria, exclusion criteria and outcome analysis of THS patients are shown in Consort Flow chart **(Fig.1).**

The severity of injuries of patients were assessed by Shock Index (SI) and Injury severity score (ISS) in ED and the extent of organ dysfunction was assessed by SOFA score (SOFA; range, 0–24) and Acute Physiology and Chronic Health Evaluation II (APACHE II; range, 0-71) later in the ICU.

2.3 Adverse Events Reporting:

We have performed hypersensitivity test to see the primary side effects of estrogen at the time of admission in the ED (by injecting the small amount of intravenous estrogen in a circulated area of patient arm and waited for 20 minutes to see any sign of hypersensitivity) in all patients of experimental group. All major, minor thrombotic events and non-thrombotic adverse events were noted and reported to the drug monitoring committee at AIIMS, New Delhi. Wells score and Compression Ultrasonography (CU) was done to asses deep vein thrombosis at different time points on days 0, 3, 7, and 14.

2.4 Collection of First and Second Blood Samples: On arrival 2 ml blood sample was collected in EDTA vial by vein puncture from every patients to investigate the base line levels

of analytes in the ED. The second blood sample (8 ml) was taken after infusion of 2L (Litre) resuscitation fluid with estrogen (Experimental group) and without estrogen (Control group) within eight hours of injuries in the ED. Further, sequential blood samples were collected on Days 3, 7 and 14. The blood samples collected after infusion of estrogen from experimental group and without estrogen from controls groups were divided as follows; 4 ml blood samples collected in plain vial to estimate the serum cytokines and hormones, and remaining 4 ml samples was collected in EDTA vial to isolate the Peripheral Blood Mononuclear Cells (PBMCs) to performed the immunophenotyping of monocytes, Treg cells, NK cells and B reg cells.

As per study protocol blood sample were collected consecutively on days 3^{rd}, 7^{th}, and 14^{th} from every patient. Patients discharged before completion of 14^{th} day after injuries were excluded from final analysis. Furthermore, if patient died in this time intervals we recorded the details with intent to find the plausible immunological and clinical reasons of death.

2.5 Severity Scores and Outcome:

The severity scores of patients in the ED were measured by ISS score, Glasgow Coma Scale (GCS) and Shock index (SI), whereas SOFA and APACHE scores were noted in the ICU(17)(18). ISS score is the sum of the square of Abbreviated Injury Scale (AIS) scores for each body regions including head and neck, face, chest, abdomen and extremities and external. The GCS score is used to measure the extent of neurological injury of patients. The extent of organ failure of patients were measured by SOFA and APACHE II scores in the ICU. SOFA score reflects the respiratory, hepatic, cardiovascular, coagulation, renal and neurological systems while, APACHE II is applied within 24 hours of ICU admission with worst value recorded for each component part of physiology variable.

2.6 PBMCs isolation: PBMCs were isolated from 4 ml of venous blood samples by density gradient centrifugation over Ficoll–Hypaque (Sigma-Aldrich). Blood sample was mixed with RPMI-1640 media in an equal ratio and poured slowly on Histopaque followed by centrifuged at 400 g for 30 minutes. The resultant buffy layer was collected from the interface after centrifugation and washed twice with 1X-PBS. Finally. PBMCs were pelleted down and suspended in complete media having 10% foetal calf serum (FCS) made in RPMI-1640 having antibiotics (50ug/ml gentamycin and streptomycin).

2.7 Cell Viability and Counting: The viability of isolated PBMCs were measured by trypan blue exclusion dye. The viability of cells were assessed under microscopic observation by their shiny appearance. PBMCs was incubated with an equal volume of dye for 2-3 minutes and loaded on haemocytometer for counting in the WBC chamber. The average number of cells was expressed in per ml.

2.8 Immunophenotyping: Immunophenotyping of T regulatory cells, B regulatory cells, Monocytes and NK cells were performed by multicolour Flow cytometry. PBMCs ($1X10^6$ cells) were stained with various cocktails of anti-human antibodies and incubated for 30 minutes at 4^0C. After that cells were centrifuged and washed with 250 µL of 1x PBS for 2 times at 2000 rpm at 4^0C for 5 minutes. Finally, cells were suspended in 100 µL of staining buffer and samples were acquired on Flow cytometry. For the staining of transcription factor FoxP3, cells were permealised and fixed with Perm Fixed buffer as per recommended standard protocol of BD Bioscience, USA.

Anti-human CD4-FITC, anti-human CD25-PE and anti-human FoxP3-APC were used for the immunophenotyping of Treg cells and anti-human CD5-APC, anti CD19-FITC and anti-human IL-10-PE were used to analysed the B regulatory cells. The percentage of total

monocytes were performed by using anti-human monoclonal antibodies, $CD14^{+}$FITC, $CD16^{+}$ PE and $CD11c^{+}$ APC and NK Cells by $CD3^{-}$APC, $CD56^{+}$FITC and $CD16^{+}$PE.

2.9 Estimation of serum cytokines by ELISA:

Serum levels of TGF-β, TNF-α, IFN-γ, IL-6, IL-10, IL-12, MCP-1, and MIP-1 were evaluated at different time points on days 0, 3, 7 and 14 in both groups (Control and Experimental) of THS patients as per the standard ELISA e-bioscience kits protocol.

2.10 Estimation of serum levels of estrogen and androgen:

The basic principle of estimation of estrogen and androgen are based on sandwich ELISA. Serum level of Estrogen and Androgen were evaluated in the serum samples collected on day 0 by ELISA as per given instructions by manufacturers. The serum levels of Estrogen was measured by using the ELISA kit of ALPACO, USA (Cat No-11ESTHU-E01) having sensitivity 10 pg/ml. The 96 well coated anti-human rabbit monoclonal antibodies microwell plate was provided by manufacturers. As per given instruction, 50 µl/well samples, standard and blanks were added in the duplicate wells and incubated for 1 hours at room temperature. After that plate was washed thrice with 300 µl of wash buffer and centrifuge at 2500 rpm for 5 minutes at 4^{0}C. The enzyme labelled secondary antibodies (100 µl/well) were added and incubated for 1 hour at room temperature. Further, washing were performed 5 times and 100 µl/well substrate (TMB) was added and incubated for 30 minutes at room temperature. After that 2N H_2SO_4 solution (50µl/well) was added and plate was read at 450 nm.

Similar to estrogen, the serum levels of androgen was measured by using the ELISA kit of ALPACO, USA (Cat No-11DHTHU-E01) having sensitivity 6 pg/ml.

2.11 Statistical Analysis:

The statistical analysis were performed by using Graph Pad Prism version 5.0 for Windows (Graph Pad Software, San Diego, CA, USA) and SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA).The categorical variables and continuous variables were expressed as percentage and mean (SD) / median (range), respectively. The difference among the groups were assessed by using t-test / Mann-Whitney *U*-test. Analysis of variance (ANOA) was used to compare the samples collected over time. Karl Pearson coefficient was used for correlation among continuous variables.

3. RESULTS:

3.1 Clinical and Demographic Variables:

A total of 44, THS patients were enrolled in present study. Out of 44 patients, four patients were excluded from the analysis as they did not fulfilled the inclusion criteria. In the experimental group, a total of (n=20) patients, mean age (31±10.8 years) and a total of (n=20) patients, mean age (36.7±11.5 years) were included in control group **(Table 1)**. The clinical and demographic details recorded to both groups of THS patients in the ED at the arrival are shown in **Table 1**. The baseline data's of heart rate, SPO2 in percentage (%), Respiratory rate, Blood transfusion (number of units), and Fresh frozen plasma (number of units) were found to be comparable among both groups **(Table 1)**. The clinical details such as mode of injuries, types of injury, drug allergy, X-ray reports and response of patients to fluid resuscitation are shown in **Table 2**. Based on response to fluid resuscitation in the ED, THS patients were divided into responder, non-responder and transit responders **(Table 2)**. As compared to controls (20.83%), patients of experimental group (5.26%) have comparatively lower percentage of non-responders. The arterial blood gases (ABG) of both groups of THS patients were observed in the ED are shown in **(Table 3A)**. The PH, Po2, PCo2, $HCO3^-$, and base deficit was found to be comparable ($p>0.05$) among both groups of THS patients, However, Lactate was found to be significantly

($p<0.05$) two folds higher in experimental group as compared to control. We have also measured the serum levels of metabolic waste (urea, creatinine), Na^+, K^+ and blood haemoglobin among both groups of THS patients **(Table 3B)**. These values were found to be comparable in both groups **(Table 3B)**. The severity score used for the evaluation of THS patients are shown in **Table 4**. THS patients who received estrogen (Experimental Group) had significantly ($p<0.05$) lower SOFA score as compared to control **(Table 4).** As compared to control (30%), Experimental group (10%) have significantly lower mortality rate and subsequently showed favourable outcomes within one year of observation period **(Table 4)**. Our study also showed, THS patients who received estrogen have comparatively lower chances of developing sepsis associated complications in the ICU **(Table 4)**.

Further, we followed the patients on different time intervals (day 1, day 3, day 7, day 14 and day 28) and see the occurrence of any minor or major thrombotic events due to estrogen therapy. We have not observed any thrombotic events in patients who received estrogen therapy, therefore we could not performed D-dimer test.

3.2 Expression of different subsets of white blood cells:

As compared to control, patients of experimental group have significantly ($p<0.05$) lower percentage of Treg cells on days 7 and 14. However, percentage of Treg cells was found to be comparable on days 0 and 3 among both groups **(Fig. 2A)**. As like Treg cells, B regulatory cells (Breg) works as a negative regulator of the immune system, prevent potentially damaging autoimmune and protective immune responses that lead to uncontrolled inflammation(19). The percentage of Breg cells was found to be comparable ($p>0.05$) among both groups **(Fig. 2B)**. Patients received estrogen have significantly ($p<0.05$) lower percentage of monocytes on days 3, 7 and 14 **(Fig. 2C)**. However, percentage of monocytes observed comparable among both groups on Day O.

NK cells are important for host innate and adaptive immune response to fight against obligate intracellular pathogens, their role is very critical in disease conditions (20). The current data showed that levels of NK cells are comparable among both groups **(Fig.2D)**.

3.3 Analysis of cytokines:

The serum levels of TGF-β, IFN-γ, IL-12, MCP-1, and MIP-1 were found to be comparable ($p>0.05$) among both groups of THS patients are shown in supplementary Table **(Table 3S)**. The serum levels of TNF-α, IL-6, and IL-10 observed significantly ($p<0.05$) lower in experimental group when compared with controls **(Table 5)**. The serum levels of TNF-α was reported about two folds lower on day 0, 76.8(20.6-132.8) pg/ml and day 3, 84.6(22.3-148.2) pg/ml in experimental group as compared to controls. However, levels of TNF-α observed comparable ($p>0.05$) on days 7 and 14 **(Table 5)**. Similar to TNF-α, serum levels of IL-6 observed significantly lower in experimental group when compared with controls on days 0 and 3. The level of IL-6 found to be 195.6 (54.2-267.8) pg/ml vs. 132.7(38.5-201.8) pg/ml on day 0 and 216.7 (62.5-307) pg/ml vs. 101.6 (56.2-187.5) pg/ml days 3 among control and experimental groups, respectively. The serum level of anti-inflammatory cytokine IL-10 was also reported to be significantly ($p<0.05$) lower in experimental group when compared with controls on days 0 and 3 **(Table 5)**.

3.4 Serum levels of Estrogen and Androgen: The serum levels of estrogen and androgen were measured twice on Day 0, at the time of arrival in the ED (Basel line sample) and after 2 Litre of Fluids resuscitation with estrogen (Experimental Group) and without estrogen (control Group). The serum levels of estrogen and androgen were observed comparable among both groups at the time of arrival (Table 6). After 2 L of fluid resuscitation, levels of estrogen was found to be about 10 folds (295.24±32.7 pg/ml) higher in experimental group when compared with control (29.68±2.8 pg/ml). While level of androgen was found to be comparable in both groups (Table 6).

3.5 Correlation of serum cytokines with severity of sepsis: The correlation of severity scores of patients (SOFA and APACHE II) with serum levels of cytokines TNF-α, IL-6 and IL-10 were measured on days 7 and 14. We were not observed any correlation of cytokines with SOFA score and APACHE II score.

4. DISCUSSION:

The finding of present preliminary study on humans following trauma haemorrhage shows that estrogen therapy is safe, did not show side effects, and significantly reduced the systemic levels of serum cytokines. Our results indicate that administration of intravenous estrogen in humans following trauma haemorrhage have several beneficial effects. THS patients recovered early, protected from sepsis associated problems, and subsequently showed favourable clinical outcome. Our results showed that estrogen therapy was associated with a significant reduction in levels of T regulatory cells, monocytes, and cytokines TNF-α, IL-6 and IL-10. To the best of our knowledge, this is the first human trial that showed estrogen application after trauma haemorrhage resulted in reduction of TNF-α, IL-6 and IL-10 levels. The role of TNF-α and IL-6 as a crucial mediators of pathological process of sepsis leading to MOF have been confirmed by several studies (2,5). Furthermore, as a key inflammatory cytokine, TNF-α stimulates the release of diverse mediators which themselves might directly or indirectly cause immune depression and MOF(21). The previous finding from our laboratory and others confirmed the elevated levels of serum cytokines IL-6 and IL-10 during post-traumatic sepsis that caused defective cell mediated immunity that may leading to sepsis associated problems(22,23). The anti-inflammatory cytokine IL-10 has previously been recognised as an important immunosuppressant of cell mediated immunity in animals following trauma haemorrhage (24). These mechanisms might be possible explanations for the observed beneficial effects of estrogen therapy on sepsis associated problems and MOF after trauma haemorrhage. Therefore, it might be assumed that estrogen exerts its beneficial effects on MOF and sepsis by decreasing

levels of TNF-α, IL-6 and IL-10. Our present findings conducted in humans are supported by studies done in animal models of haemorrhage. The finding of *Trentzsch et al*., 2014 in male mice of haemorrhage showed that administration of estrogen restore the inflammatory storm caused due to haemorrhage and also protect the mice from sepsis associated complications (25). *Knoferl et al*., 2002 already demonstrated that female sex hormones play a critical role in maintaining immune response after trauma haemorrhage by suppressing the inflammatory cytokines and preventing from sepsis (26). Furthermore, study of *Zeckey et. al*., 2011 confirmed that administration of Finasteride, specific inhibitor of androgen in male mice following trauma haemorrhage increased the levels of estradiol concentration, reduced the sepsis occurrence and subsequently improved the survival (27). In contrast to this, few studies reported the elevated levels of IL-10 in female mice following trauma haemorrhage, with higher levels of estrogen and estradiol in septic patients particularly non-survivors (11,28). However, none of the studies confirmed why female mice following THS have elevated levels of IL-10 and septic patients have elevated levels of estradiol. We assume pre-existing disease conditions, genetic heterogeneity of patients, varying nutritional status, and due to aberrant hormonal levels can contribute differential response to estrogen.

The level of T regulatory cells and monocytes were reduced and showed similar trends at different time points on days 3, 7 and 14 in THS patients who received estrogen when compared with placebo control group. Monocytes is a crucial component of innate immunity that activates the adaptive immune response (29). The depressed function of monocytes and altered secretion of cytokines has been reported by several studies in male mice and humans following trauma haemorrhage(30). Consistent with previous findings in animal models, our results indicate that administration of estrogen following THS increased the resistance of humans to infections and also protected from sepsis associated problems. THS patients who received estrogen have significantly lower SOFA score and have comparatively higher survival rates than controls.

Therefore, we assume estrogen restored functioning of monocytes by modulating cytokine secretions and preventing host from infections and sepsis associated problems.

The primary function of Treg cells was originally defined to prevent against autoimmune diseases by maintaining self-tolerance(19). In male animals, the increased level of Treg cells following THS and its association with depressed immune response has been well established. The previous work published from our laboratory also confirmed the elevated levels of Treg cells in THS patients are associated with immune suppression (9). The present finding is supported by work of *Kovacs et al.*, 2002 that administration of estrogen in male mice following THS reduced the levels of Treg cells and also protect the animals from sepsis(10).

Although, our study confirmed the reduced levels of Treg cells, monocytes, and serum cytokines in humans following trauma haemorrhage. But multivariate logistic regression analysis could not confirm estrogen as an independent factor that determine the immunological changes occurred in THS patients. Therefore, our results could not allow us to conclude whether observed affects are directly associated to estrogen or they are mediate via indirect mechanism. Further clinical trials in more patients are required to conduct the safety and efficacy profiles of intravenous estrogen in humans following trauma haemorrhage. Drug dose, its frequency needs attention in large randomized control trials. Nonetheless, additional studies are required to conduct in more patients to elucidate how estrogen therapy is helpful in reducing cytokine storm, improving the functions of monocytes and other immune cells, and increasing the survival rate following trauma haemorrhage.

4.1 Limitations of study:

This study has some limitations. Firstly, it is a single-centre study and has been done in small sample size, thus restricting generalizability. However, the finding of present study observed

THS patients who received estrogen showed favourable outcomes and had lower level of monocytes and T regulatory cells.

In addition, all the scoring system used to measure the disease severity and MOF has some limitations (18) (17).

5. CONCLUSION:

In conclusion, the present study showed that administration of intravenous estrogen in trauma haemorrhagic shock is safe and without any major or minor adverse events. It restored the inflammatory insult and also overcome the problem of inflammation. Estrogen group showed trend towards favourable clinical outcomes and had lower level of monocytes and T regulatory cells.

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Author contributions:

Dr. D.L.Gupta designed the study, analysed the data, and prepare the figures and tables of the manuscript and carried out the experiment with Shrestha. Dr. Kapil and Dr. Subhod help in recruiting the patients from the ICU and providing the clinical details of the patients and samples. Dr. Sagar drafted the manuscript and suggested the outline of the manuscript. Dr.DN Rao did the editing of manuscript and suggested necessary correction's. Dr.Bhoi brought the grant from the ICMR to carry out the research work and take the approval of human ethical clearance from the ICMR, Drug control of India and from the Institute.

Declaration of interest: The authors have no conflicts of interest.

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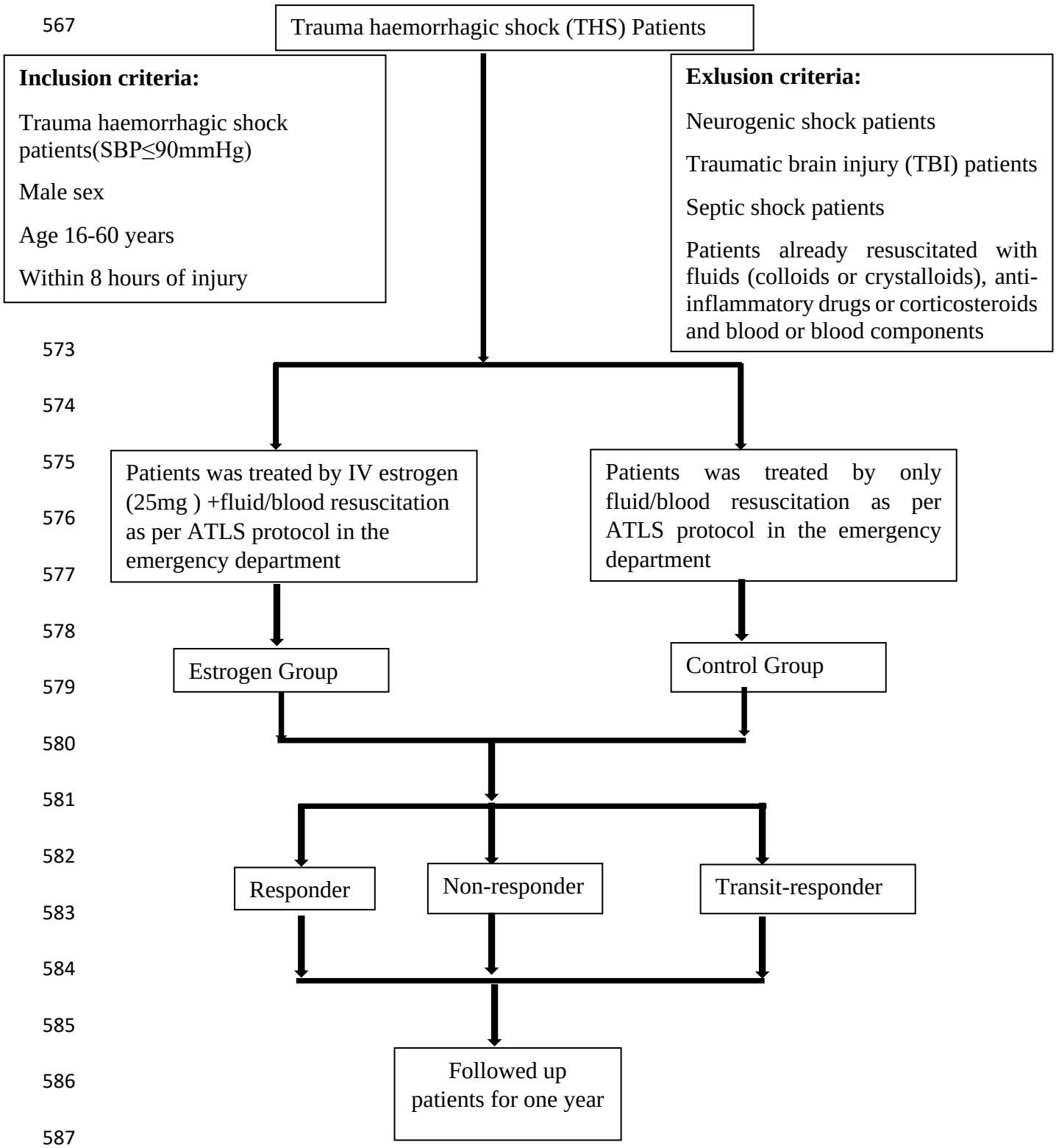
Figure Legend:

Fig. 1: The details about the inclusion criteria, exclusion criteria and outcomes analysis of the THS patients

Fig.2: Shows the percentage of Treg($CD4^+CD25^+FoxP3^+$), Breg ($CD5^+$ $CD19^+$ $IL\text{-}10^+$)cells, monocyes ($CD14^+$, $CD16^+$ and $CD11c^+$) cells and NK cells($CD3^-$ $CD56^+$ $CD16^+$) in placebo group of trauma patients(control) and estrogen treated trauma patients(Estrogen) on days 0, 3,7 and 14.

Figure 1: Consort flow chart showing the inclusion criteria, exclusion criteria and outcome analysis of THS patients



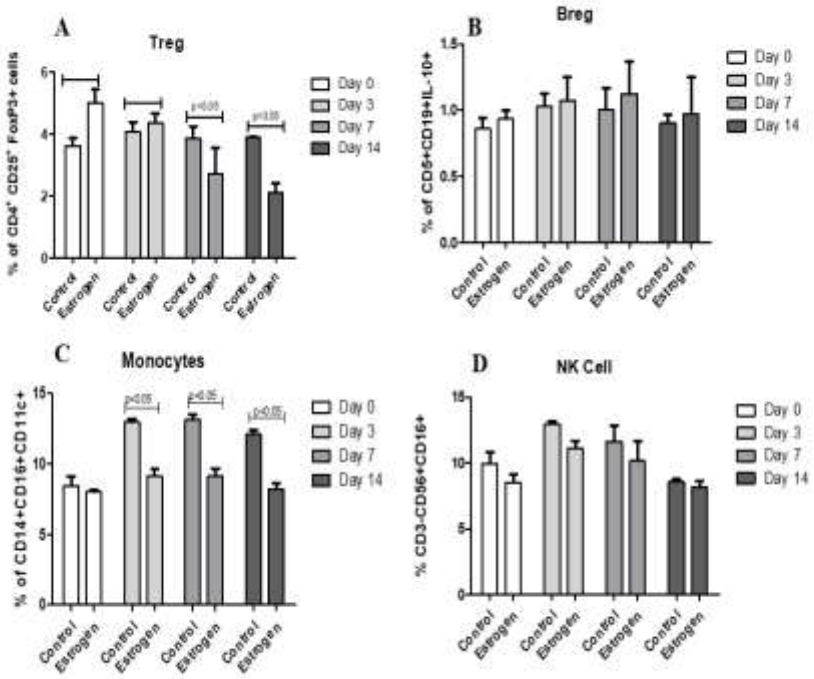


Figure 2: Shows the percentage of Treg(CD4+CD25+FoxP3+), Breg ($CD5^+$ $CD19^+$ $IL\text{-}10^+$)cells, monocytes (CD14+, CD16+ and CD11c+) cells and NK cells($CD3^-$ $CD56^+$ $CD16^+$) in both groups of THS patients on days 0, 3,7 and 14.

Table 1: Clinical and demographic details of control and experimental group:

Parameters	**Control Group n=20** (Mean ± SD)	**Experimental Group n=20** **(**Mean ±SD)	p- Value
Age (yrs)	36.7±11.5	31.0±10.8	0.07
SBP (mmHg) at arrival	81.4±11.2	77.4±9.1	0.23
SBP (After 2 liter fluid /blood transfusion)	99.9±13.8	101.0±30.9	0.19
Heart rate	107.4±23.4	108±23.2	0.94
SPO2 in percentage (%)	100.5±4.1	98.6±2.3	0.09
Respiratory rate	19.8±4.8	21±5.2	0.38
Blood transfusion (no.of units)	4.6±1.8	4.8±1.7	0.19
FFP (no.of unit)	4.6±1.9	4.1±1.8	0.49

Abbreviations:

SBP- Systolic blood pressure

SPO2-Oxygen saturation

FFP- Fresh frozen plasma

Table 2: Clinical Details of control and experimental group:

Clinical details	Control Group (n=20)	Experimental Group (n=20)
MOI (Mode of injury)	RTA =62.5%, Fall= 16.67%, Assault=4.17%, Other=16.67%	RTA = 41.18%, Fall= 17.65%, Assault=41.18%, Other=0
Type of Injury	Penetrating = 4 Blunt= 16	Penetrating = 5 Blunt=15
Any drug allergy	None	None
Fast	Positive= 37.5% Negative=62.5%	Positive= 48.72% Negative=51.28%
Chest X-ray	Hemothorax=4 Pneumothorax= 2, Rib fracture= 2	Hemothorax=0 Pneumothorax= 3, Rib fracture= 4
Pelvis X ray	fracture present =3	fracture present =0
Long bone Fracture	fracture=25%	fracture present =25.64%
Alcohol	Present= 9.09% absent=90.9%	Absent=100%
Response to fluid	Responder= 62.5% Non responder= 20.83% Transit responder=16.67%	Responder= 73.68% Non responder= 5.26 % Transit responder=21.05%

Table 3 (A): ABG details of control and experimental group:

ABG details	Control Group(n=20) Mean±SD	Experimental Group (n=20) Mean±SD	p-Value
PH	7.1±1.1	7.2±1.0	0.52
Po2	116.9±61.9	165.2±71.0	0.03
Pco2	40.2±10.6	37.8±6.7	0.45
$HCO3^-$	30.6±4.3	31.8±5.5	0.23
Lactate	1.5±0.9	3.6±1.6	0.005
Base deficit	-7.42+-7.36	-7.8+-5.12	0.54

Table 3 (B): Lab details of control and experimental group:

Lab details	Control Group (n=20) Mean±SD	Experimental Group (n=20) Mean±SD	p- Value
Hemoglobin (Hb)	10.9±2.7	11.1±2.4	0.89
Serum Na+	138.2±6.8	138.5±5.9	0.9
Serum K+	4.2±0.6	3.9±0.8	0.21
Blood Urea	23.1±7.9	24.5±7.5	0.58
Serum Creatinine	0.8±0.24	0.7±0.20	0.17

Table 4: Severity scores of control and experimental group:

Severity score	Control group (n=20) (Mean±SD)	Experimental group (n=20) (Mean±SD)	p-Value
Injury severity score (ISS)	33.9±15.5	31.2±10.8	0.58
Glasscow coma score (GCS)	13.1±3.9	12.7±4.2	0.77
Shock Index (SI)	1.27+0.4	1.38+0.5	0.41
APPACHE (ICU score)	11.2±4.9	9.3±3.6	0.155
Sequential organ failure assessment score (SOFA) (ICU score)	4.6±2.0	3.2±1.0	0.013
OUTCOME	Survivors= 14(70%) Non-survivors=6(30%)	Survivors= 18(90%) Non-survivors=2(10%)	0.04

Table 5: Serum levels of cytokines at different time points in THS patients who received estrogen (Experimental Group) and didn't received estrogen (Control group).

	Day 0	Day 3	Day 7	Day 14
(Pg/ml)	TNF-α			
Control	154.7 (52.6-181.4)	167.5 (35.6-205)	90.8 (16.7-128.5)	50.5 (8.4-86.6)
Experimental	76.8 (20.6-132.8)	84.6 (22.3-148.2)	88.2 (30.5-138.7)	46.4 (10.2-67.2)
p-value	0.005	0.005	0.06	0.07
	IL-6			
Control	195.6 (54.2-267.8)	216.7 (62.5-307)	135.7 (45.2-216.8)	105 (37.6-156.8)
Experimental	132.7 (38.5-201.8)	101.6 (56.2-187.5)	98.5 (23.7-156.3)	97.2 (18.6-168.5)
p-value	0.005	0.005	0.02	0.04
	IL-10			
Control	92.4 (32.5-126.7)	156.7 (45-201.6)	189.8 (56.7-298.5)	192.6 (68.4-278)
Experimental	94.6 (15.5-128.5)	92.2 (22.5-167.8)	67.8 (12.5-104)	60.4 (14.6-102.5)
p-value	0.08	0.005	0.005	0.005

Table: 6 Serum levels of estradiol and testosterone in experimental group and control.

		Control Group (HS) n=20 (Mean ± SD)	**Experimental Group (HSE) n=20** **(**Mean ±SD)	**p-value**
At the time of arrival in the ED (Base line data)	Estradiol(pg/ml)	31.79±3.5	30.56±2.7	0.221
	Testosterone(ng/dl)	756±21	759.7±13.6	0.512
After 2L of Fluid resuscitation in the ED	Estradiol(pg/ml)	29.68±2.8	295.24±32.7	0.0001
	Testosterone(ng/dl)	702±18.70	710.8±11.4	0.080