Original Article

Safety Assessment of Intravenous Injection of Rat Embryonic Proteome Extract for *in-vivo* Regenerative Therapies

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Abstract

Tissue differentiation and organogenesis are exclusively embryonic-phase activities, and these two activities are strictly under the control of the embryonic proteome, the developing embryo is a prime source of molecules for regenerative therapy. Many *ex-vivo* rudimentary organoid culture studies confirmed the inherent biological function of embryonic factors. Before using embryonic proteins for in vivo therapies, their safety should be confirmed first. Hence, we aimed for an *in vivo* study to inject rat embryonic proteome extract (EPE) through the intravenous route and investigate the impact on immunological, biochemical, and haematological parameters in the adult rats. **Methods**: In this study, we isolated rat embryonic proteins from the 14th, 16th, and 19th embryonic days by homogenization of embryos and isolated protein extracts through ultra-centrifugation. Six pairs of rats have been taken; six are allotted for control, and six are for the embryonic extract injection. The isolated embryonic protein extract was injected intravenously into the treatment group of rats and the normal saline into the control group. After the injections, blood samples are collected from both the treated and control groups to analyse immunological markers Il6, CRP, biochemical parameters creatinine, urea, sugar, proteins, albumin, globulin, and haematological parameters total leucocyte count, neutrophils, and lymphocyte percentage. **Result:** After the EPE injection, biochemical parameters, immunological markers, and haematological parameters were analysed in both control and treated groups. All the above mentioned parameters are within normal limits. Statistical analyses were done using the T-test for unequal variance (p 0.05). We observed no significant difference between the control and treated groups, so in vivo injections of embryonic protein extract is safe for the use of *in vivo* regenerative therapies.

Key words: Embryonic Proteome Extract (EPE); Immunological markers; Biochemical parameters; Haematological parameters; In-Vivo Regenerative Therapy.

Introduction

Regenerative medical research has stumbled into a new direction in the use of embryonic protein extracts (EPE). The dynamics of tissue differentiation, organogenesis, and growth are strictly operated through specific embryonic factors [1]. During the development of an embryo, tissue differentiation and organogenesis are manifested with embryonic differential expressions of the transcriptome [2] and proteome [3]. Proteomic analysis of a study has shown that 977 embryonic proteins are unique among 288 adult proteins. This determines that embryonic cell transcriptomes [4] differ from adult cell transcriptomes [5]. Thus, the embryo is a reservoir of different proteins that might be involved and can be used for regeneration and growth of damaged organs in the adult stage. Recent in vitro studies have confirmed the regenerative function of embryonic proteins [6]

One of the prominent studies is the transformation of adult somatic cells into induced pluripotent stem cells by using the Yamanaka factors Oct3/4, Sox2, Klf4, and c-Myc [7]. All these factors are exclusively expressed on the 14th day of the rat embryo. One more in vitro study has confirmed that early nephrogenesis is due to epigenetic factors like Cbx1, Cbx3, Cbx5, and Trim28 isolated from developing kidneys [8]. Cell cultures, while supplemented with these factors, will be differentiated into ureteric buds and elongated into renal vesicles, forming comma- or S-shaped bodies. In another study, proteins from vesicles of amniotic fluid stem cells are injected through the intra-ventricular route; these injected cells are homed in the damaged kidney and exert a reno-protective function [9]. All these studies have paved the way for embryonic molecules' application in the regeneration of organs.

Application of embryonic molecules is a recent advancement over stem cell therapy, as stem therapy has crucial limitations tumorgenicity,[10] heterogeneity[11] and immunogenicity[12]. Tumorgenicity and heterogeneity are intact cellular functions, so in this study we have deployed homogenization of embryonic stem cell to decellurized [13], so that tumorgenicity and heterogeneity can be completely resolved. Present study also confirmed that embryonic protein extract (EPE) does not activate immune cells, so it is safe for In vivo therapies. Total experimental work flow is elucidated in (Fig.1). There are four major advantages to the application of embryonic proteins: non-tumorgenicity, non-heterogeneity, non-immunogenicity, and regenerative potency [14]. Very few studies and scant literature were observed regarding the determination of embryonic stem cell proteins compatibility during the development and repair of tissues and organs. The compatibility of embryonic

protein extract injection was confirmed by biochemical, haematological, and immunological manifestations [15].

Materials and Methods

Selection of Rats

One pair of Sprague Dawley rats were provided with unrestricted access to food and water while being kept on a 12:12 h light cycle. All the animal were housed in isothermic environment as per animal ethical committee guidelines. To ensure biological replication, six pregnant rats were examined based on the cytology of vaginal smear microscopy and visible vaginal plug formation [16]. This first set, consisting of one female and one male, was allowed for mating to produce the first filial generation of six female and five male rats. Again, these six female and five male rats were arranged for mating to generate their second filial generation. In second filial generation 3 females are arranged for mating, these F2 pregnant rats are scarified for isolation of embryonic proteins in 14th 16th and 19th day embryonic days respectively [17] and in remaining F2 generation six males and six female rats allocated for this study, 6 are for EPE injection and 6 are for control group.

Isolation and quantification of embryonic proteins

A total of 30 embryos were isolated on 14th, 16th and 19th embryonic days from 3 pregnant rats. A single embryo from each day was taken, washed six times with 10x PBS buffer and homogenized using an ultrasonicator in EDTA 0.85 mg/ml to inhibit the proteolytic enzyme activity [18]. The EPE used for *in-vivo* injections, and the entire process of extraction is performed under cold chain at 4^0c. Remaining un-homogenized embryos are stored at -80^0 c for future use. The isolated EPE were quantified on nanodrop spectrophotometer [19]. Protein concentrations of 14th, 16th and 19th embryonic days are 5.21 mg/mL, 12.19 mg/mL, and 19th 24.1 mg/mL respectively.

Polyaccralamaid Gel Electrophoresis of EPE

The 10% polyacrylamide denaturing gel was used for the EPE protein separation process. In the gel in the first well, 5 µL of the biorad reference standard was used, and then EPE samples from days 14, 16, and 19 were added to the following wells and duplicate gel run was performed. After separation, gels were stained with Coomassie brilliant blue staining. The gel was distained and scanned for further analysis, as shown in the (Fig .2).

Injection of Embryonic Extract through Intravenous Route

Numerous studies have confirmed the biological functions of specific embryonic factors for *in vivo* usage in regenerative therapies [20]. In the present study, the embryonic protein extract were injected through intravenously [21] for *in vivo* safety evaluation. In this study, 12 adult rats with an age of 13 weeks are divided into two groups, i.e., the control group with 6 rats and the treatment group of 6 rats for EPE injection. Embryonic extract was prepared from 14th, 16th and 19th days embryos and all the treatment group rats were injected with EPE of 120 μL volume , with a concentration of 724 μg/120 μL, 1548 μg/120 μL, and 2532 μg/120 μL respectively. After 48 hours of intravenous injection, blood sample was collected from both control and treated groups and tested for biochemical, haematological and immunological parameters [22].

Tested Parameters

After intravenous injection of EPE to the treated group rats, blood samples were tested for serum creatinine, blood urea, blood sugar, serum proteins, albumin, globulin, CRP, IL6, total leucocyte count, neutrophils%, and lymphocytes% were compared with control group rats. There was no significant difference between the injected group and the control group following intravenous injection of 14th, 16th and 19th day of EPE.

Histological Examination of tissues After EPE injection

Histological examination procedures were followed as described by Adeyemi and Akanji [23]. Organs are collected after euthanization of a single treated rat as per Committee for Control and Supervision of Experiments on Animals (CCSEA), India guidelines. The brain, heart, kidney, large intestine, liver, small intestine, spleen and stomach samples were collected and fixed in 10% (v/ v) formaldehyde, the tissue samples were dehydrated through ascending grades of ethanol (70%, 90%, and 95%, v/v), then cleaned in xylene, and

embedded in the paraffin wax (melting point 56^{0}C) and stained with hematoxylin and eosin (H & E). The photomicrographs were captured at 100X using the software Presto Image Folio package (Fig .6)

RESULTS

Biochemical Parameters

After intravenous injection of 14th 16th and 19th day of EPE, the serum biochemical profile were estimated [24] with fully automated biochemical analyser Erba EM 200 and compared the biochemical profile with control group values and all parameters are with in normal range.

Serum creatinine, urea, sugar, proteins, albumin, and globulin were estimated. Biochemical parameters employed for post-injection serum biochemical analysis are creatinine, urea, blood sugar, serum total proteins, albumin, and globulin. Serum samples consumed for these tests are in the following sequence from creatinine to globulin, respectively: Creatinine 12 uL, urea 2 uL, sugar 2 uL, proteins 20 uL, albumin 2 uL. A total of 38 uL serum is utilized for biochemical analysis in each group. Graphical data presented in (Fig-3)

Table:1 The Mean values of (mean ± SE) of serum biochemical profile in control and treatment rats after 48 hours of intravenous injection of EPE.

S.NO	PARAMETERS	ROUTE	CONTROL	14th Day	16th Day	19th Day
				48h	48h	48h
1	**CREATININE**	**IV**	0.62±0.0.25	0.48±0.038	0.53±0.02	0.57±0.021
2	**BLOOD UREA**	**IV**	17.93±0.0.52	16.2±0.037	15.7±0.29	15.9±0.24
3	**BLOOD GLUCOSE**	**IV**	102.0± 4.8	77.0± 5.5	81.5± 3.7	75.8±3.3
4	**SERUM PROTEINS**	**IV**	6.5±0.13	6.8±0.09	7.1±0.06	6.9±0.104
5	**ALBUMIN**	**IV**	3.9±0.08	4.1±0.05	4.3±0.04	4.2±0.06
6	**GLOBULIN**	**IV**	2.6±0.06	2.8±0.04	2.9±0.03	2.8±0.04

Haematological Parameters

250 µL of blood was collected in anti-coagulated tubes and was used to assess the haematological parameters [25], the total leucocyte count, per centage of neutrophils and lymphocytes are analysed on Cell counter Erba H 360, the graphical data presented in (Fig-4)

Table -2: Mean and standard error values of haematological parameters after 48 hours of intravenous injection of 14th 16th and 19th day of EPE to rats under control and treatment groups (n=6)

S.No	Parameters	Control	14th Day	16th Day	19th Day
			48h	**48h**	**48h**
1	**TLC**	9,890±206.5	7,300±206.55	7,067±396.4	7,340±320.17
2	**Neutrophils %**	20±1.06	24±1.13	22±1.68	24±2.3
3	**Lymphocytes %**	77±1.18	69±1.97	70±1.68	72±2.02

Immunological Markers

Vertebrate Immune system is more advanced and highly protected with immune system [26], if any foreign protein enters into the body, immune system responds instantaneously by synthesizing CRP from liver and IL6 by macrophages. 100 µL of blood sample was used for estimation of CRP and IL-6 using Elab science ELISA kit. Interleukin 6 is important pro-inflammatory marker produced macrophages [27], it plays a crucial role production of acute phase proteins, enhances the production of Neutrophils as well as B lymphocytes. In this study the estimated CRP and IL-6 are in normal range in both control and treatment groups. Graphical data presented in (Fig-5)

Table -3: Mean and standard error values of CRP and IL6 at two different intervals 48 Hours of Intravenous injection of 14th 16th and 19th day embryonic extract to rats under control and treatment groups (n=6)

S.No	Parameters	Control	14th Day	16th Day	19th Day
			48h	**48h**	**48h**
1	**CRP pg/mL**	198.5±11.5	156.48±3.58	157.5±2.68	164.07±3.4
2	**IL-6 pg/mL**	19.5±0.8	17.1±0.38	16.78±0.41	16.67±0.28

Histological Examination of tissues After EPE injection

After EPE intravenous injection, results of the histopathological examination of rat brain, heart, kidney, liver, small intestine and large intestine of the treated rat and compared with normal organs of rat, showed no signs of teratomas or tumours in the tissue sections in above organs and cellular architecture in both treated and control groups are normal (Fig – 6). The data confirms that, EPE can be used for *in-vivo* for regenerative therapy in adult stage.

Statistical Analysis and Quantification

Bar overlap diagrams were generated using Origin software. The p-value was calculated by t-Test: Two-Sample Assuming Unequal Variances using Microsoft Excel indicated. All experiments (except the ones involving the animal models) were done at least with duplicates for each condition within the experiments. The data are represented as mean ± standard error, p-Value is less than 0.05.

Discussion

Application of embryonic molecules was started in the production of induced pluripotent cells, which are isolated from 14th-day mouse embryos by Shinya Yamanaka (21) (Oct3/4, Sox2, Klf4, c-Myc) [28]. Based on this, we have designed an *in vivo* study to isolate EPE that contain various tissue differentiation, organogenesis, and growth factors to analyse their immunological, biochemical, and haematological acceptance after intravenous injection into adult rats. The most recent study by Devid Sincliar confirms that Yamanaka factors from exclusive embryonic sources have reversed cellular aging [29].

Embryonic proteins inherent functions are tissue differentiation and organogenesis. Many *in-vitro* studies have also proven the biological function of embryonic factors, but direct *in-vivo* application of these factors needs to confirm their safety; they should be non-tumorigenic, non-heterogenic, and non-immunogenic. For this, we have isolated embryos and homogenized embryos to decellularize, so that to avoid tumorigenicity and heterogeneity complications, this is confirmed by histological examination of different organs.

In this work, we confirmed the safety of embryonic proteins by testing immunological, biochemical, and haematological markers after 48 hours of EPE injection. We injected embryonic extracts of different embryonic days, i.e., 14th, 16th, and 19th day embryos, which were isolated and homogenized embryonic stem cells in EDTA solution to prevent proteolysis of embryonic proteins. This homogenized embryonic protein extract was injected into healthy rats, and all rats didn't manifest any biochemical, immune, or haematological fluctuations.

Strengths and limitations

Direct injection of embryonic factors is possible with our study, as immunological and haematological parameters are within normal limits. We need to extend our study for proteomic analysis of embryonic protein extracts, confirmation of embryonic proteins in organ function restoration. Future applications in regenerative therapy, there is no need for scarification of embryos, as bulk industrial production can be feasible using biotechnological tools, and it should be carried out on a large group of experimental animals. Simultaneously, we are continuing this study, in which we injected embryonic protein extract through ultrasound-guided intra-renal injection, with this it is confirmed the safety of intra-organ

injection. After this injection, renal parameters came back to normal in the drug-induced chronic kidney disease (CKD) model. A proteomic study and a histopathology study of recovered kidneys are under analysis.

Conclusion:

Our study has ratified the safety of embryonic proteins by direct intravenous injection. Our study laid the foundation for future embryonic molecular regenerative therapy and also provided alternatives for stem cell-based therapy complications like heterogeneity, immunogenicity, and tumorigenicity, as embryonic proteins are non-immunogenic, non-tumorigenic, and non-heterogenic. With this study molecular based regenerative therapy replaces Stem cell-based Regenerative therapy.

Conflicts of interest:

Authors declared that they have no conflicts of interest.

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Ethical committee approval

Entire study was approved by Institutional Animal Ethical Committee (IAEC) of Jeeva Life Science with the approval number: CPCSA/IAEC/JLS/13/08/20/04.

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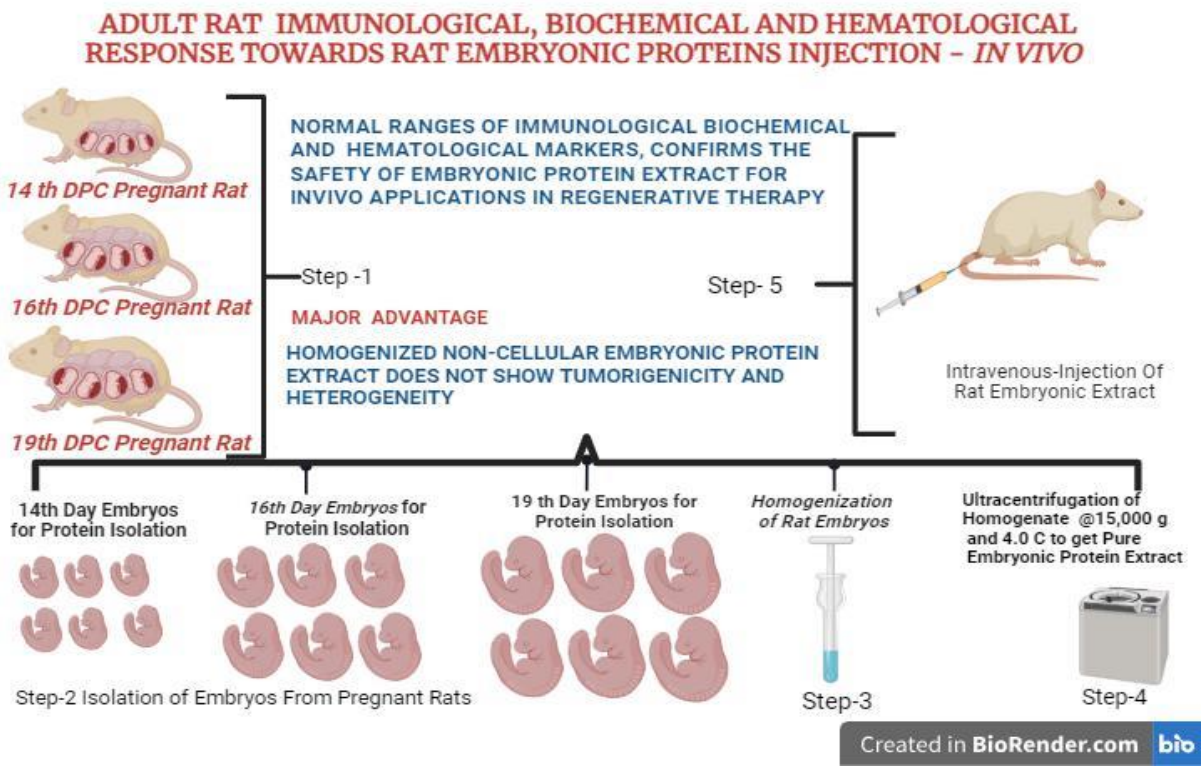

Fig. 1 Total experimental Procedure presented in figure 1 in 5 different steps. **Step 1** includes selection of animals and caged for mating, conformation of mating through vaginal swab microscopy and vaginal plug formation. **Step 2** Scarification of 3 pregnant rats, on three different embryonic days 14th, 16th and 19th to isolate embryos. **Step 3** Homogenization of embryos of 14th, 16th and 19th embryonic days separately in EDTA 0.85 mg/ml. **Step 4** Protein extraction, after homogenization of 14th, 16th and 19th homogenates were, centrifuged out at 13,000× g and 4 °C for 30min. The supernatant was recentrifuged at 13,000× g and 4 °C for an additional 30min to get maximal purity. The pellet was discarded, and the final samples were was stored at −80 °C until use. **Step 5** Embryonic extract of different was given through intravenous injection (Image created using Biorender creator)

b

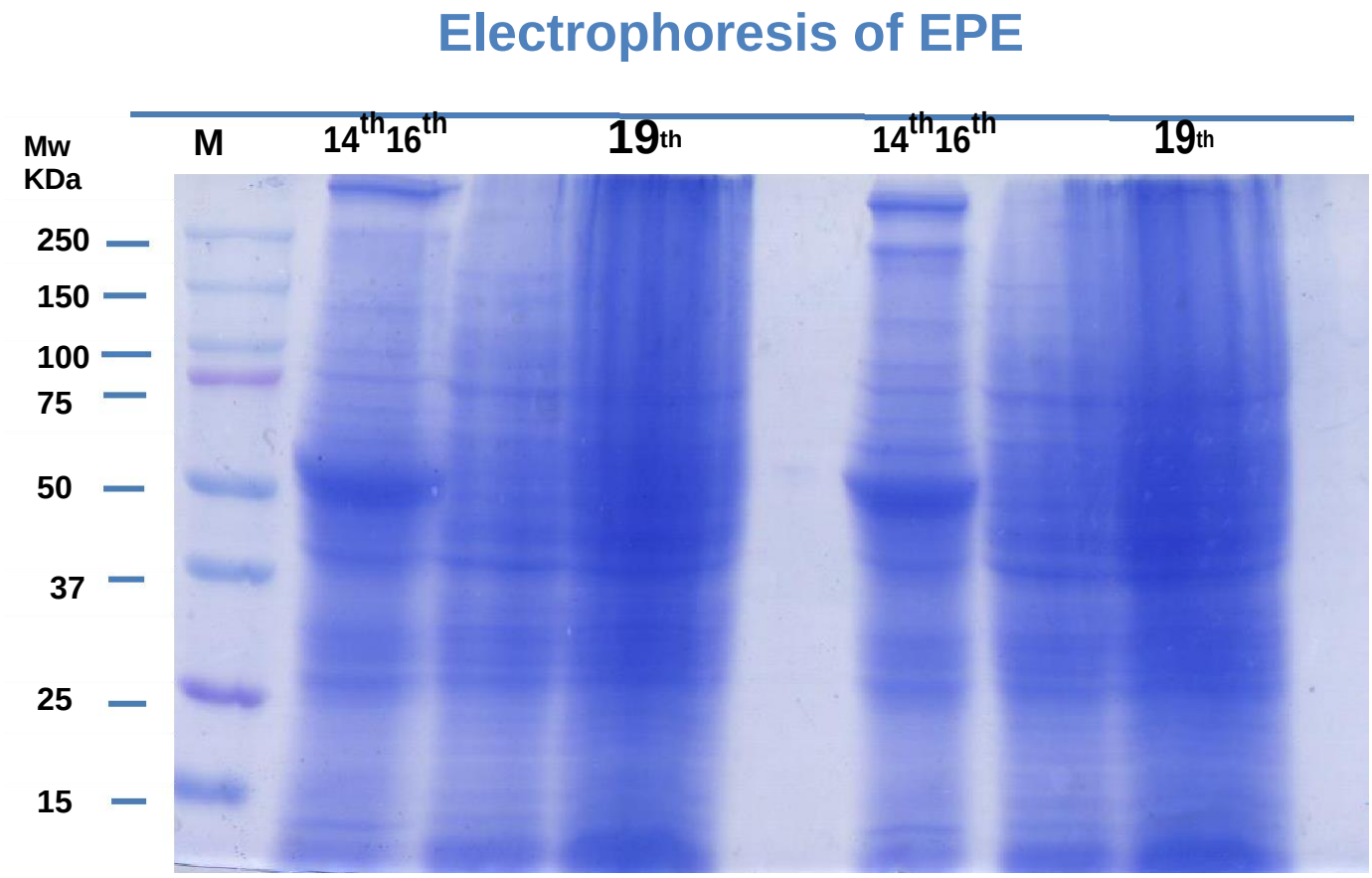

Fig: 2 Proteins in the homogenized embryonic protein extract were separated on a 10% polyacrylamide gel. 1st well loaded with Biorad reference standard; 2nd, 3rd, and 4th are loaded with 14th, 16th, and 19th EPE; the same EPE samples were in duplicate.

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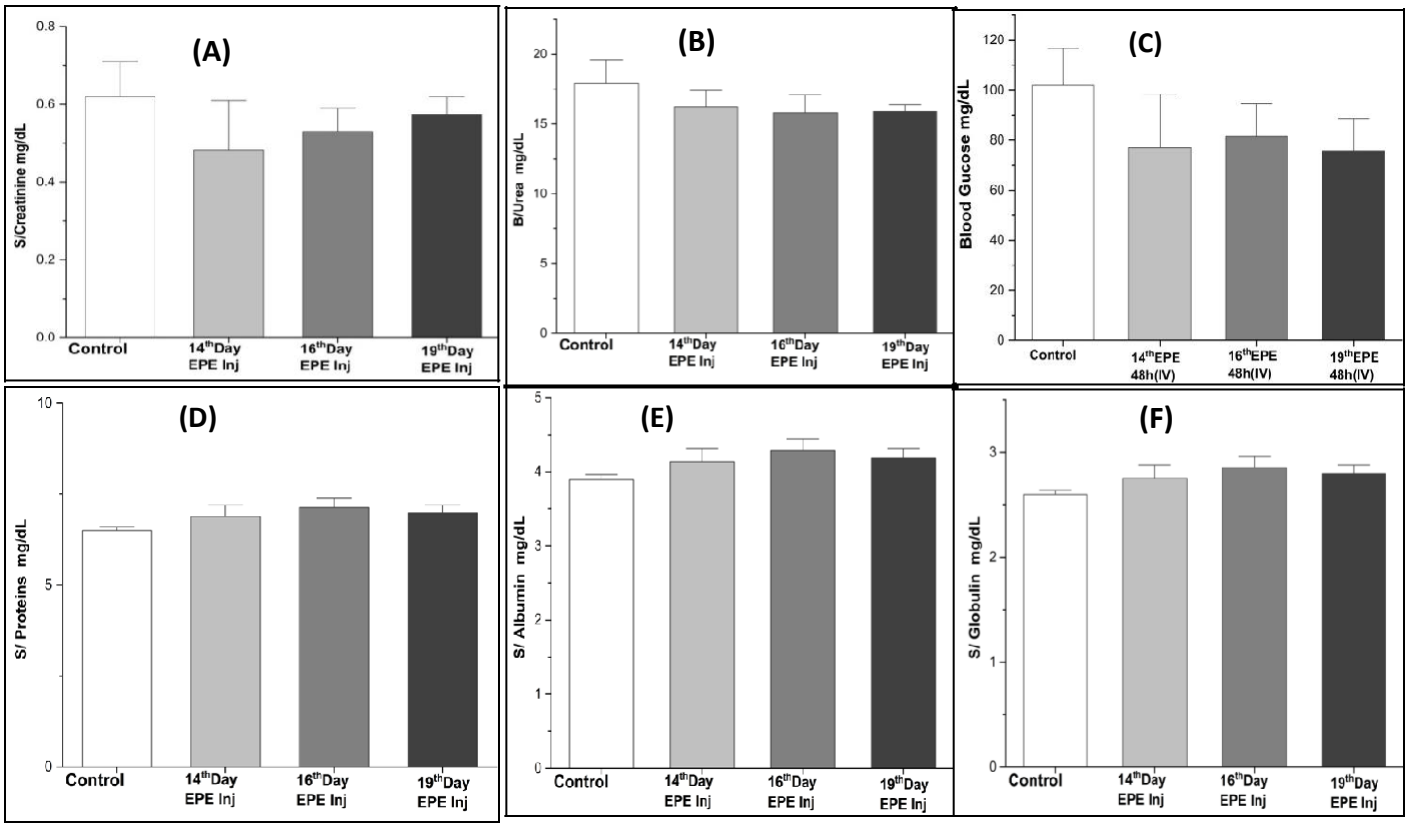


Figure : 3 After 48 Hours Intravenous injection of embryonic extract, following biochemical parameters A) S/creatinine, B) B/urea, C)S/Proteins, D) S/Albumin, E) S/Globulin are tested and values are expressed as Standard error of the mean(mean ± SEM) table 1. Values are also plotted in bar graphs with standard error in each column includes control group, embryonic days 14th, 16th and 19th day embryonic extract injection. t-Test: Two-Sample Assuming Unequal Variances was also performed as mean ± SEM and P value for all parameters are significantly less then ($p < 0.005$).

ical Markers

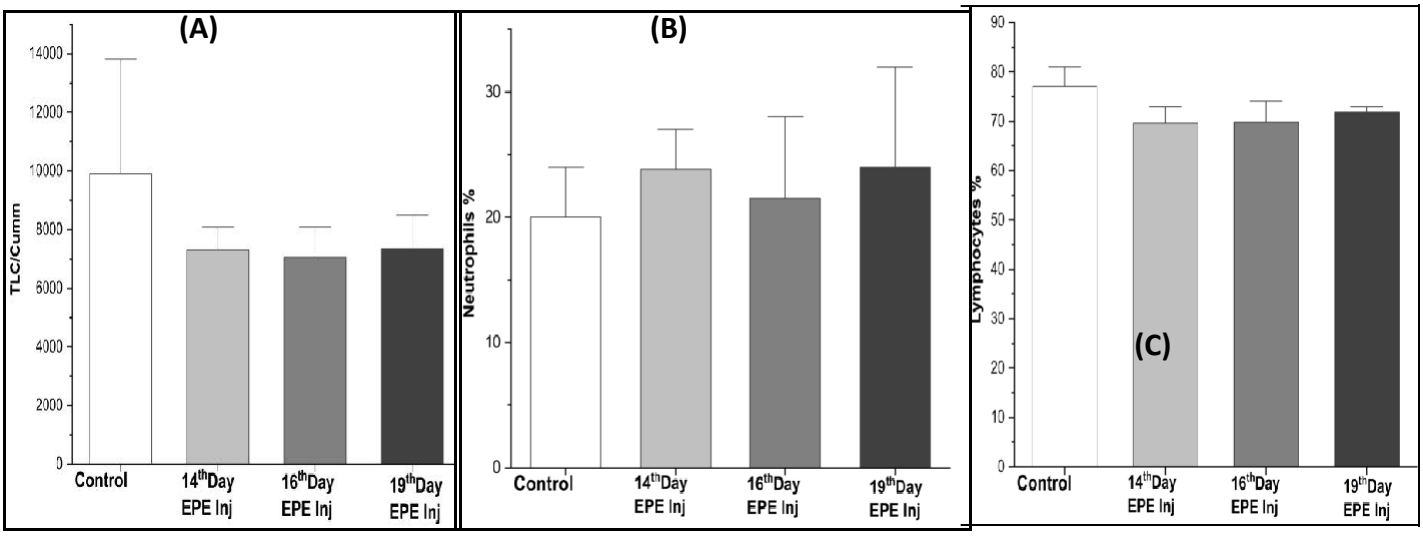


Figure: 4 After 48 Hours Intravenous injection of embryonic extract, following haematology parameters (Total count, Neutrophils and Lymphocytes %) were performed and values are expressed as Standard error of the mean (mean ± SEM) table 2. Values are also plotted in bar graphs with standard error in each column includes control group, embryonic days 14th, 16th and 19th day embryonic extract injection. t-Test: Two-Sample Assuming Unequal Variances was also performed as mean ± SEM and P value for all parameters are less then ($p < 0.05$).

Inflammatory Markers:

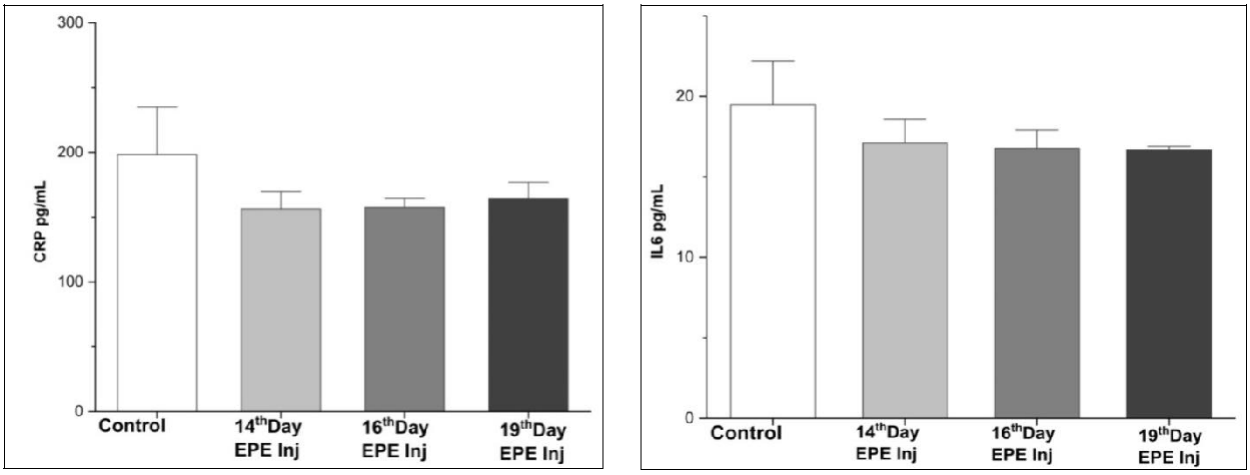


Figure: 5 After 48 Hours Intravenous injection of embryonic extract, following immunological parameters (CRP and IL6) were performed and values are expressed as Standard error of the mean (mean ± SEM) table 2. Values are also plotted in bar graphs with standard error in each column includes control group, embryonic days 14th, 16th and 19th day embryonic extract injection. t-Test: Two-Sample Assuming Unequal Variances was also performed as mean ± SEM and P value for all parameters less then ($p < 0.05$).

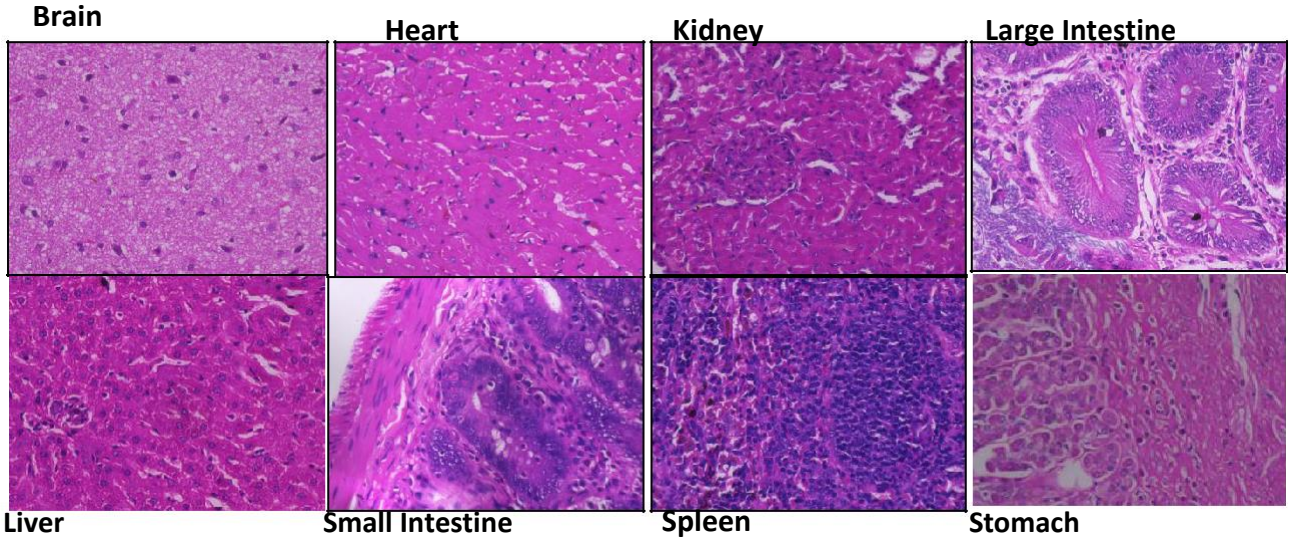


Figure: 6 Histopathology study of tissues Following EPE Intravenous injections, histopathology examination different tissues of treated rat was studied by staining with hematoxylin and eosin A) Brain, B) Heart, C) Kidney D) Liver, E) Small intestine and F) Large intestine, G) Spleen and H) stomach are with normal histoarchitecture without tarotamas.

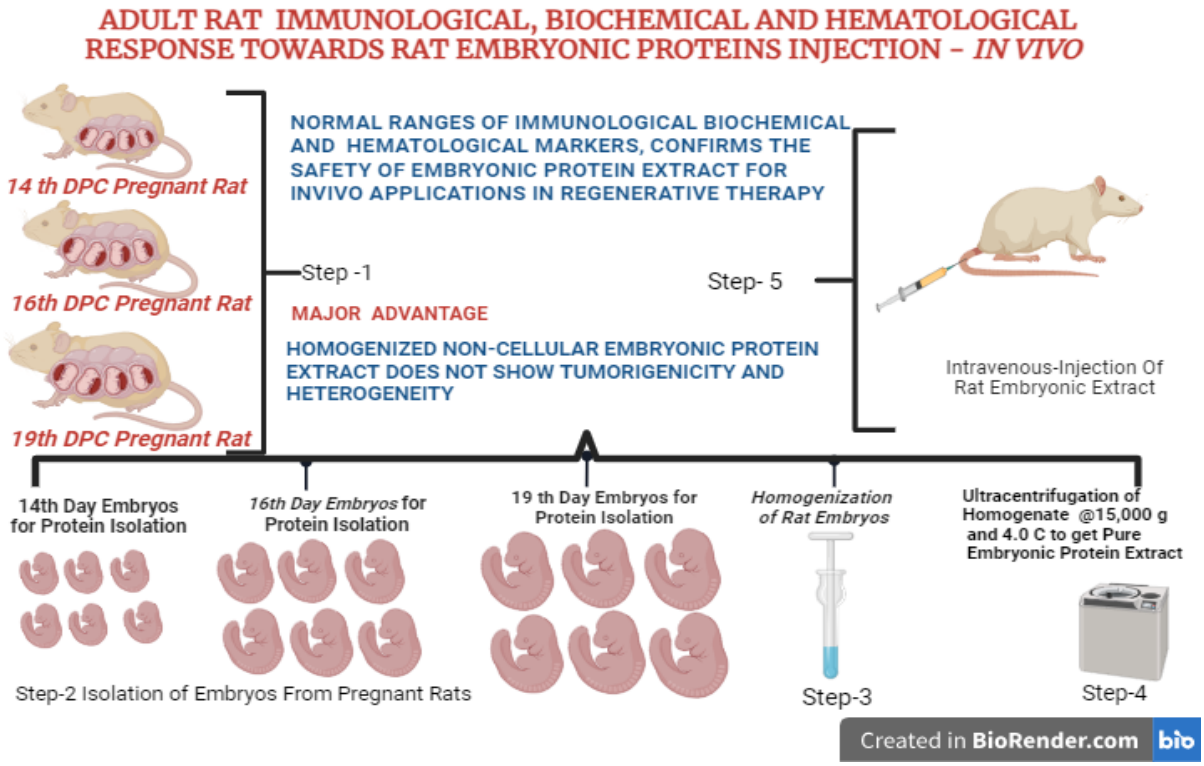


Fig. 1 Total experimental Procedure presented in figure 1 in 5 different steps. **Step 1** includes selection of animals and caged for mating, conformation of mating through vaginal swab microscopy and vaginal plug formation. **Step 2** Scarification of 3 pregnant rats, on three different embryonic days 14th, 16th and 19th to isolate embryos. **Step 3** Homogenization of embryos of 14th, 16th and 19th embryonic days separately in EDTA 0.85 mg/ml. **Step 4** Protein extraction, after homogenization of 14th, 16th and 19th homogenates were, centrifuged out at 13,000× g and 4 °C for 30min. The supernatant was recentrifuged at 13,000× g and 4 °C for an additional 30min to get maximal purity. The pellet was discarded, and the final samples were was stored at −80 °C until use. **Step 5** Embryonic extract of different was given through intravenous injection (Image created using Biorender creator)

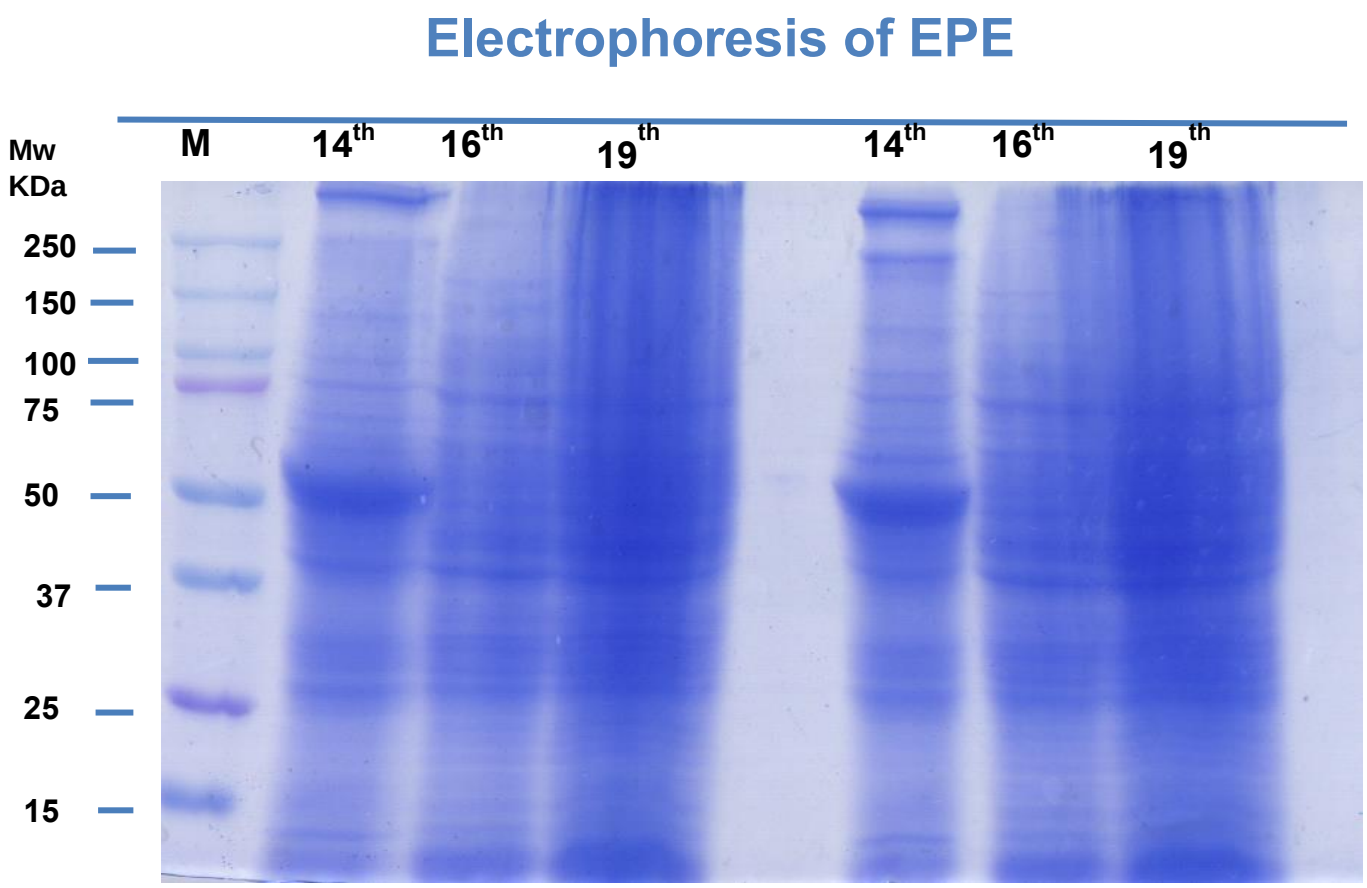


Fig: 2 Proteins in the homogenized embryonic protein extract were separated on a 10% polyacrylamide gel. 1st well loaded with Biorad reference standard; 2nd, 3rd, and 4th are loaded with 14th, 16th, and 19th EPE; the same EPE samples were in duplicate.

BIOCHEMICALMARKERS

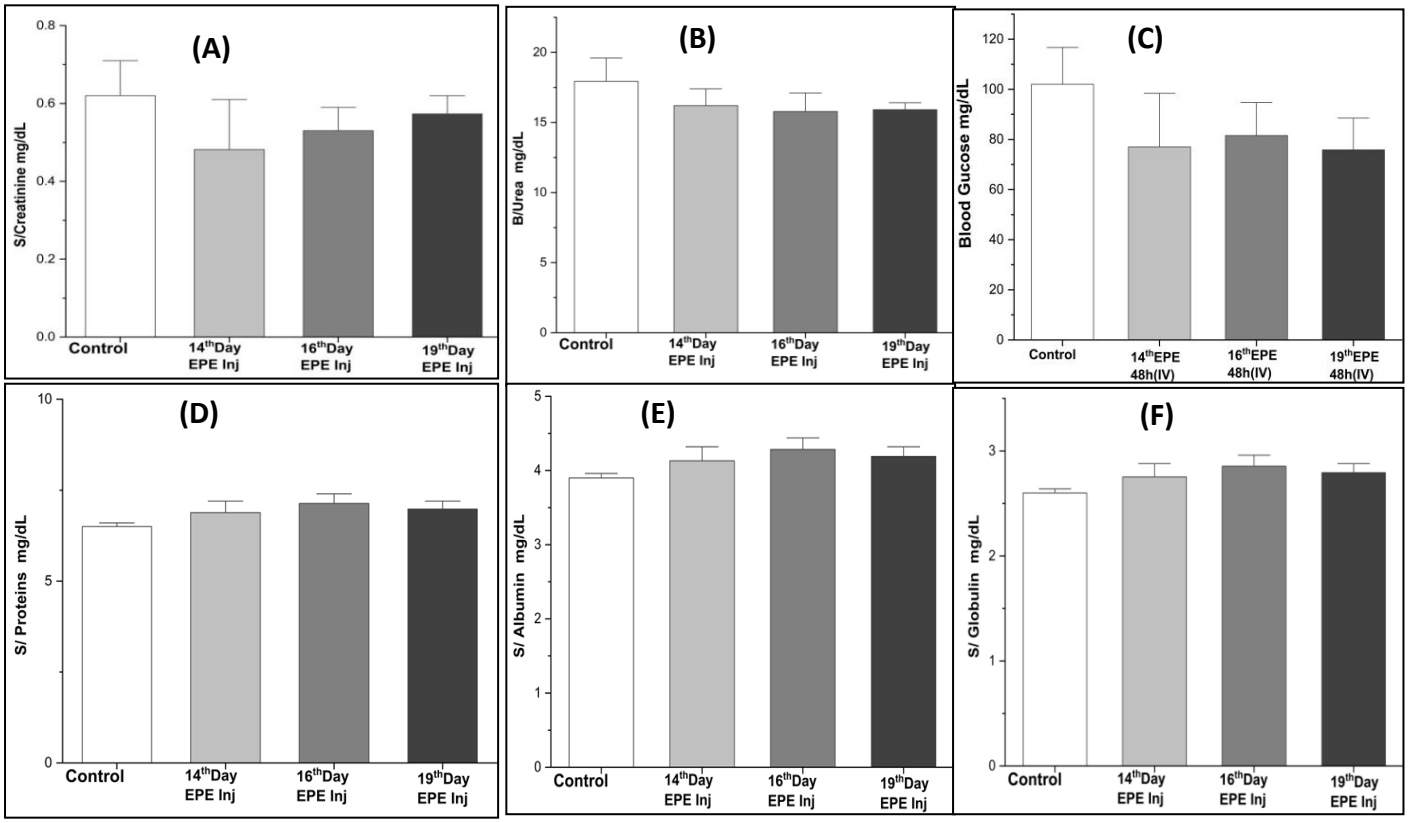


Figure : 3 After 48 Hours Intravenous injection of embryonic extract, following biochemical parameters A) S/creatinine, B) B/urea, C)S/Proteins, D) S/Albumin, E) S/Globulin are tested and values are expressed as Standard error of the mean(mean ± SEM) table 1. Values are also plotted in bar graphs with standard error in each column includes control group, embryonic days 14th, 16th and 19th day embryonic extract injection. t-Test: Two-Sample Assuming Unequal Variances was also performed as mean ± SEM and P value for all parameters are significantly less then ($p < 0.005$).

Hematological Markers

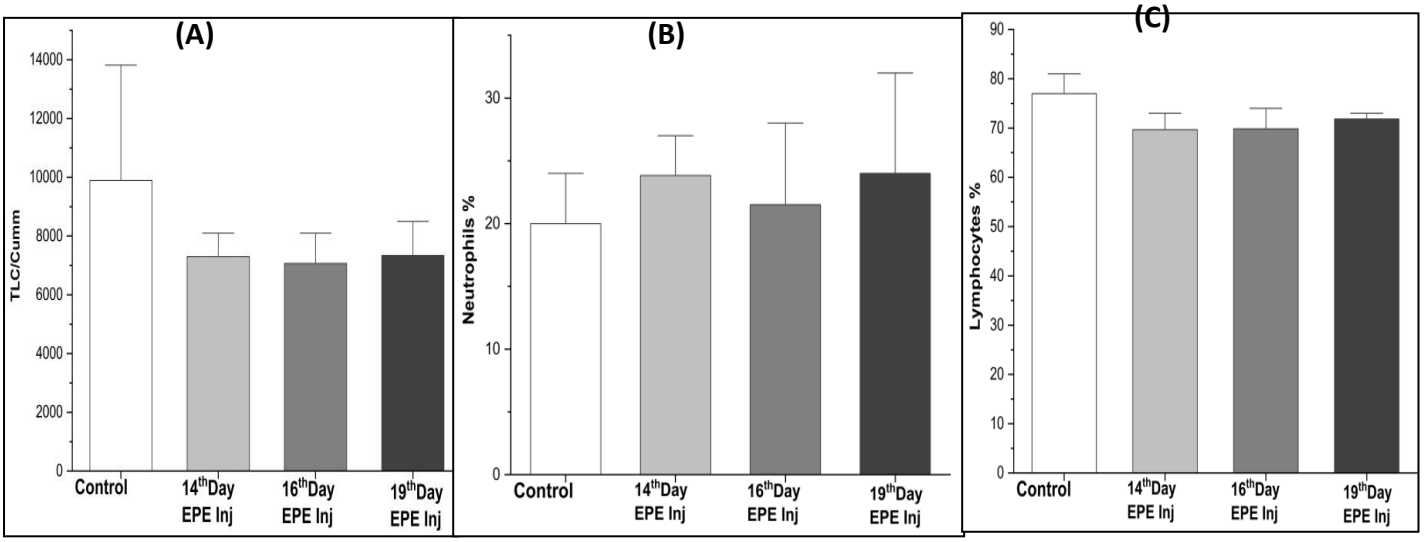


Figure: 4 After 48 Hours Intravenous injection of embryonic extract, following haematology parameters (Total count, Neutrophils and Lymphocytes %) were performed and values are expressed as Standard error of the mean (mean ± SEM) table 2. Values are also plotted in bar graphs with standard error in each column includes control group, embryonic days 14th, 16th and 19th day embryonic extract injection. t-Test: Two-Sample Assuming Unequal Variances was also performed as mean ± SEM and P value for all parameters are less then ($p < 0.05$).

Inflammatory Markers:

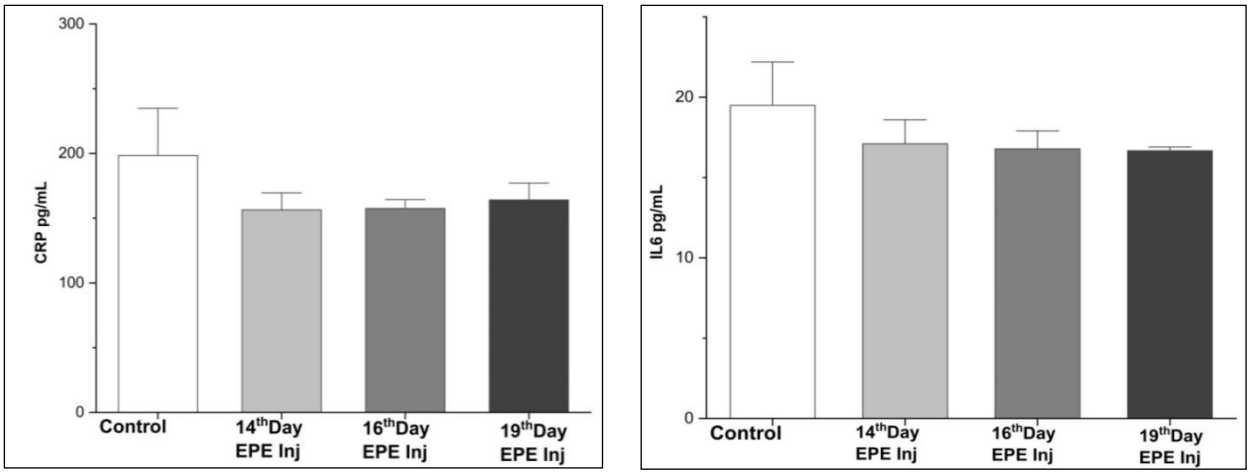
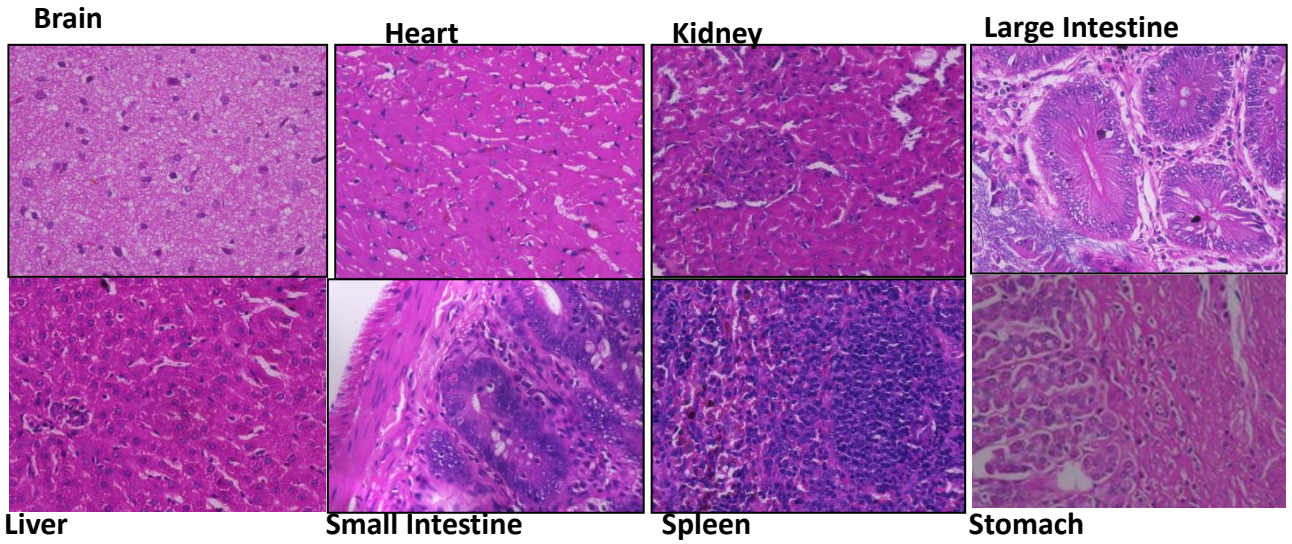


Figure: 5 After 48 Hours Intravenous injection of embryonic extract, following immunological parameters (CRP and IL6) were performed and values are expressed as Standard error of the mean (mean ± SEM) table 2. Values are also plotted in bar graphs with standard error in each column includes control group, embryonic days 14th, 16th and 19th day embryonic extract injection. t-Test: Two-Sample Assuming Unequal Variances was also performed as mean ± SEM and P value for all parameters less then ($p < 0.05$).



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