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Preclinical evaluation reveals comparable toxicology and pharmacology of the erythropoietin biosimilar GB*poietin*® and Eprex®

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Abstract

Erythropoietin (EPO) is an essential growth factor for erythropoiesis. We report the results of the preclinical safety evaluation of GB*poietin*®, a recombinant human EPO (rhEPO), through a comparative acute toxicity study with the reference product, Eprex®. The products were administered subcutaneously into Wistar rats for both the single-dose and repeated-dose toxicity studies. Hematological and biochemical parameters were measured for all test subjects before the first dose and the day after the last dose in both studies. Necropsy and histopathology of representative subjects from each group were also performed to find any pathological changes, such as degeneration or cellular necrosis in internal organs such as the kidney, liver, lung, and spleen. Both GB*poietin*® and Eprex® comparative toxicology studies, which were not significantly different ($P > 0.05$), revealed similar pharmacologically driven mechanisms of toxicity. Although hematological parameters stayed within the normal range throughout the study, improved profiles of hemoglobin and hematocrit ($P < 0.05$) confirmed the therapeutic effect of rhEPO in both studies. Moreover, the initial and final values of aspartate aminotransferase, alanine aminotransferase, and blood urea nitrogen were comparable ($P > 0.05$) for both experimental products. The study established that the toxicological profiles of GB*poietin*® and Eprex® were similar and aligned with the known pharmacology of EPO alfa, demonstrating proof of "totality" and "no residual uncertainty."

Keywords: Erythropoietin; Preclinical study; Single-dose toxicity; Repeat-dose toxicity; GB*poietin*®; Drug safety profile

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1. Introduction

The hormone erythropoietin (EPO), a glycoprotein, is essential for the production of red blood cells (RBCs). In adults, EPO is mostly produced in the kidney's peritubular cells and released into the bloodstream.[1] The EPO receptor (EPOR) on bone marrow erythroid progenitors is bound to circulating EPO, initiating a number of signaling pathways that promote the development of mature RBCs.[2] It was originally isolated from

the urine of aplastic anemia patients and characterized as a 34000 Da protein.[3] The expression of this endogenous glycoprotein is controlled by the transcription factor hypoxia-inducible factor.[4] Carbohydrates constitute 40% of the molecule, primarily in the form of sialic acid, which is distributed across three N-linked and one O-linked glycosylation sites. These carbohydrates are necessary for the biological function of EPO.[5] The terminal sialic acid residues prevent rapid hepatic clearance, preserving its activity in the bone marrow. Conversely, the biological activity of EPO *in vivo* is lost when sialic acid is enzymatically removed by neuraminidases.[6] The kidneys are the primary physiological site of EPO synthesis, and its production increases in response to a localized reduction in renal oxygen supply or systemic anemia. EPO interacts with its target cells through EPORs on the cell surface. The receptor undergoes homodimerization upon EPO binding, activating Janus kinase 2 (JAK2) through transphosphorylation. Phosphorylated receptor tyrosines recruit intracellular proteins like STAT5, which, upon activation, enter the nucleus to induce erythroid gene transcription. Phosphatases dephosphorylate JAK2 to downregulate the receptor. EPO can bind to the tissue-protective receptor, specifically EPOR/CD131 heterodimer, to play a critical role in tissue protection and immune regulation in addition to its function in erythropoiesis. These receptors are expressed mostly on erythroid progenitor cells, but they are also present in neuronal, endothelial, multipotent hematopoietic, and embryonic stem cells.[7]

As a therapeutic agent, EPO is made using recombinant DNA technology in mammalian cell cultures that were transfected with the human *EPO* gene.[8] Recombinant DNA technology has enabled the production of recombinant human EPO (rhEPO), allowing its extensive therapeutic application in clinical settings.[9] Non-clinical tests should be conducted following manufacturing changes in a biologic product and for products claimed to be similar to an already approved one.[10,11] A series of *in vitro* receptor binding studies and *in vivo* investigations were required under the guidelines "if there are specific uncertainties or concerns regarding safety."[12] Clinical evidence suggests that rhEPO can be used to treat anemia associated with conditions such as chronic renal insufficiencies, rheumatoid arthritis, premature birth, chemotherapy, transfusions, and hematological diseases.[13] When rhEPO is administered, no allergies have been reported; nevertheless, a small number of patients have had arthralgias and local cutaneous responses.[14] Preclinical trials involving rhEPO are, therefore, important for establishing its safety, and efficacy, and understanding its potential therapeutic applications across various medical conditions.[15]

The purpose of this study is to provide a comprehensive overview of the preclinical studies conducted on rhEPO, with a focus on its safety, efficacy, and potential as a drug product. As a prelude to clinical trials and subsequent regulatory approval, preclinical investigations play a pivotal role in elucidating the pharmacological properties, toxicological profiles, and mechanisms of action of novel therapeutic agents.[16,17] The exploration of rhEPO begins with an examination of the protein's structure and the underlying genetic modifications that render it a recombinant biopharmaceutical.[18] By delving into the molecular intricacies of rhEPO, we aim to establish a foundational understanding of its design and functionality. In addition, this study will highlight the significance of rhEPO in the context of endogenous EPO while emphasizing the potential advantages and limitations of using the recombinant form.

The first-ever alfa epoetin product, Eprex®, is a prescription medication with a track record of safety and effectiveness.[19] Eprex® was manufactured by Johnson & Johnson and it was the first EPO formulation to receive regulatory approval in Europe in 1988. In the early 1990s, physicians outside of the United States adopted the subcutaneous route of administration of EPO for hemodialysis patients due to the socioeconomic benefit for the patients.[20] GB*poietin*® is a biosimilar of Eprex®, which was developed by Globe Biotech Limited and synthesized in genetically engineered Chinese hamster ovary cells. Upstream and downstream process development and validation were done for large-scale production.[1] Step-by-step analytical results confirmed the biosimilarity of GB*poietin*® to Eprex® in terms of molecular characterization.[21]

This study investigated the preclinical safety assessments, encompassing acute and chronic toxicity studies in relevant animal models. Comprehensive evaluations of potential adverse effects, dose-dependent responses, and organ-specific toxicity are crucial in establishing a safety profile for rhEPO, guiding subsequent clinical trial designs, and mitigating risks associated with human administration.[22] This study, designed for the preclinical safety assessment of GB*poietin*®, includes the evaluation of single- and repeated-dose toxicity. With this goal, the product was compared to a commercial homolog (Eprex®) in Wister rats to assess the toxicity of GB*poietin*®.

2. Materials and methods

2.1. Materials

The drug product, GB*poietin*® (code name: GBPD002), was obtained from Globe Biotech Limited (Bangladesh) while the reference product, Eprex®, was purchased from

Janssen (USA). Ethylenediaminetetraacetic acid (EDTA) was purchased from Merck (USA).

2.2. Formulation of GB*poietin*®

The sample was formulated using formulation buffer (L-glycine, sodium chloride, sodium phosphate, Polysorbate 80, water for injection; pH: 6.8 – 7.2) and AKTA Flux S (GE Healthcare, USA). After sterile filtration through a 0.22-micron PES Sartopore 2 filter (Sartorius Stedim, Germany), the pre-formulated bulk sample was transferred to quality control (QC) for testing as per the specification. The sterile, pre-formulated bulk drug substance was then transferred to a fill-finish facility in a 2D single-use bag (Sartorius Stedim, France) for filling and packaging while maintaining a temperature of 4 – 8°C. The sterile 1 mL empty long syringes (Schott, Switzerland) were filled using automatic combo filling and closing machine (Tofflon, China) at 1 mL volume for dose preparation. The pre-filled syringes were packaged (blistered) using the HM-AV Plus blister machine (Hoonga, The Republic of Korea). After blistering completion, the drug product was stored at 4 – 8°C. Finally, the pre-filled syringes were tested for QC as per the specification and transferred to the pre-clinical (animal) study center, maintaining a temperature of 4 – 8°C, for the toxicology study.

2.3. Animal selection

Wistar rats (*Rattus norvegicus*) were used for the single- and repeated-dose safety and toxicity studies. A total of 75 Wistar male and female rats (10 – 15 weeks old) were selected and isolated 5 days before the dosing. After attentive monitoring and conditioning, 36 rats (18 males and 18 females) were subjected to single-dose toxicity analysis and 24 rats (12 males and 12 females) were subjected to repeated-dose toxicity analysis. The temperature of the experimental animal room was 26 ± 2°C and the relative humidity was 60 ± 5%. The room was an HVAC-controlled ISO class 7 room with 70% fresh air intake and full exhaust. The rats were individually housed in a polypropylene cage with proper water and feed and kept under 12 h of the day-night cycle. The target weight of male animals was 185 ± 20 g and female animals were 175 ± 20 g. In the entire experiment, 20% of additional animals (males and females) were used as substitutes for the excluded animals. Healthy young adult animals were used. Females were nulliparous and non-pregnant. At the beginning of the study, each animal was between 10 and 15 weeks old. The weight difference of animals used was minimal, not exceeding ± 20% of the mean weight for each sex. These animals were used to replace any individuals that were excluded during the study periods. The study plan and procedures were approved by the internal ethical review board of Globe Biotech Limited, complying with local ethical regulations. No treatment randomization and blinding methods were used in the study and sample sizes were determined by the resource equation method.

2.4. Single-dose toxicity assay

A total of 36 Wister rats, including both male and female, were used for the study comprising four treatment groups, a placebo group, and a control group – each group consists of three male and three female rats. The four treatment groups were assigned to either normal (500 IU/kg) or toxic dose (1500 IU/kg) to assess the toxicological similarity of GB*poietin*® to Eprex®. Treatment-1 and Treatment-2 were injected with normal and toxic doses of GB*poietin*®, respectively, while Treatment-3 and Treatment-4 were injected with normal and toxic doses of Eprex®, respectively. The placebo group was injected with 100 µL of formulation buffer used for GB*poietin*® and Eprex®. The control group was not given any injection, serving as a negative control of the study. Two days before dosing, whole blood (approximately 200 µL) was collected in 2% EDTA from each rat for the complete blood count (CBC) analysis. Similarly, whole blood was also collected after the last dosing at day 14 for the CBC analysis. Pharmacodynamic (PD) endpoints including RBC count, white blood cell (WBC) count, hemoglobin (HGB) level, platelet (PLT) count, hematocrit (HCT) level, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured for all test subjects before injection and on the last day of the 14-day study using BK-6190-Vet auto hematology analyzer (Biobase, Germany). Similarly, alanine aminotransferase (ALT)/glutamic pyruvic transaminase (GPT), aspartate aminotransferase (AST)/serum glutamic-oxaloacetic transaminase (sGOT), and blood urea nitrogen (BUN) assays were performed on blood serum using a semi-automatic chemistry analyzer (Biobase, Germany) to assess the toxic effect of GB*poietin*® and Eprex® on the liver and kidney. Body temperature and weight were also measured during the whole study period. A necropsy of representative subjects from each treatment, placebo, and control groups was done to check for abnormalities in the kidney, lung, liver, spleen, and heart. During the necropsy, external surfaces, all orifices, cranial cavities, external surfaces of the brain and spinal cord, thoracic, abdominal, and pelvic cavities, cervical tissues, and organs were also examined. Finally, histopathological evaluation was carried out to find any pathological significance like degeneration or cellular necrosis in the rat internal organs such as the kidney, liver, lung, and spleen.

2.5. Repeated-dose toxicity assay

A total number of 24 rats were separated into four different groups consisting of 6 rats (3 males and 3 females) in each group. There were four different treatment groups (1 and 2), one placebo group, and one control group. Each rat of the two treatment groups was subcutaneously (SC) injected with sterile 125 IU/kg, 250 IU/kg, 500 IU/kg, 750 IU/kg, 1000 IU/kg, 1250 IU/kg, and 1500 IU/kg of GB*poietin*® (Group 1) and Eprex® (Group 2) on 7 consecutive days. The placebo and the control groups were similar to those in the single-dose toxicity assay. For CBC analysis, whole blood was collected 2 days before dosing as well as after the last dosing on day 7 using similar protocols described in Section 2.4. PD endpoints including RBC, WBC, HGB, HCT, MCV, MCH, MCHC, and PLT count were measured for all test subjects before the first dose injection and the day after the last dose. ALT, AST, and BUN assays as well as necropsy tests and histopathological evaluations were performed as described in Section 2.4**.** Body temperature and weight were also measured during the whole study period.

2.6. Data evaluation and statistical analysis

The variables used for statistical processing are body weight, body temperature, hematological and biochemical parameters, as well as microscopy findings. Central tendency and dispersion statistics, including mean, standard deviation, maximum, and minimum values, were calculated. The variable FD (difference between the final and initial value) was calculated to assess the treatment effects on hematological and biochemical parameters. The assumptions of normal distribution and homogeneity of variance were verified using the Kolmogorov-Smirnov and Shapiro-Wilk tests, and the Levene test, respectively, before the analysis of body weight and body temperature variables for each evaluation time point. A parametric analysis of variance (ANOVA) or a non-parametric alternative (Kruskall–Wallis test) was used, depending on whether the data followed a normal distribution. Paired comparisons were performed in consecutive intervals, using either the paired t-test or the Wilcoxon test, depending on whether the data followed a normal distribution. The results from the histopathological studies were analyzed using cross-tabulated classification tables and the test for association of independence (Fisher's exact test). The data were processed with Microsoft Excel, 2010, running on the Windows operating system. The mean difference between the test and the comparator product was calculated using linear mixed-effect analysis of variance, along with the corresponding *P*-value. Statistical significance was defined as a $P < 0.05$. A $P > 0.05$ for the PD parameters of the sample formulations was considered similar and non-significant.

3. Results

3.1. Body weight assessment

Body weight increased steadily and significantly during the single-dose toxicity study, as shown in Figure 1A. The differences in body weight for all study groups at different evaluation time points (day 0, 7, and 14) suggested that the body weight gains were significant ($P < 0.05$) for Treatment-2 ($P = 0.01$), Treatment-4 ($P = 0.006$), Control ($P = 0.001$), and Placebo ($P = 0.017$) groups but were not significant in Treatment-1 ($P = 0.09$) and Treatment-3 ($P = 0.07$) groups. On the other hand, during the repeated-dose toxicity study, body weight increased steadily but not significantly, as shown in Figure 1C; the differences in body weight across all study groups at different evaluation time points (day 0, 3, and 7) were not significant ($P > 0.05$). The results remained consistent when the data were analyzed independently per gender or evaluation time points. An increase of body weight for all the groups suggests a normal evolution of body weight which is an indicator of sound health for the animals, substantiating the non-toxicity of the GB*poietin*® under both single- and repeated-dose toxicity studies. The absence of negative effects on body weight gain is favorable for the evaluation of the substance under the study, as a decrease in body weight is one of the primary clinical symptoms of stress or illness in this rat strain. Therefore, the increase in body weight serves as indirect evidence of the non-toxicity of the substance being analyzed.

3.2. Body temperature assessment

During the single-dose toxicity test, the changes in body temperature were not significant ($P > 0.05$) for all four treatment groups (Treatment-1, Treatment-2, Treatment-3, Treatment-4) and Placebo group ($P = 0.009$), while nearly significant in Control group ($P = 0.07$), as shown in Figure 1B. On the other hand, the changes in body temperature were not significant ($P > 0.05$) for all study groups (Treatment-1, Treatment-2, Placebo, and Control) in the repeated-dose toxicity study, as shown in Figure 1D. These results remained consistent when the data were analyzed independently per gender or evaluation time points, indicating normal health for the animals and substantiating the non-toxicity of the GB*poietin*®. In addition, the absence of changes in body temperature outside the normal range serves as an indirect indicator of non-toxicity for the test animals.

3.3. Hematological assessments

When analyzing the results of the hematological tests, differences were detected among treatment groups of both doses of GB*poietin*® and the reference drug, Eprex®.

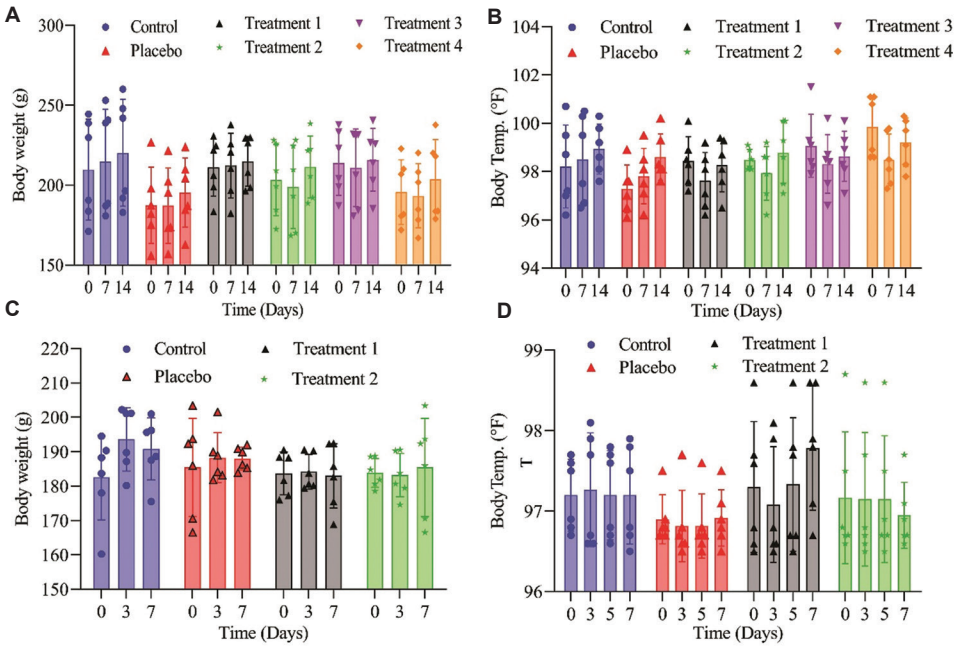


Figure 1. Changes in the body weight and body temperature of the subjects. Body weight (A) and temperature (B) changes in single-dose toxicity studies. Changes in body weight (C) and temperature (D) in repeated-dose toxicity studies. Data are presented as mean ± standard deviation.

3.3.1. Single-dose toxicity study

The change in RBC count was significant ($P < 0.05$) in Treatment-1 ($P = 0.02$), Treatment-3 ($P = 0.02$), and Placebo ($P = 0.00$) groups but not significant ($P > 0.05$) in Treatment-2 ($P = 0.16$), Treatment-4 ($P = 0.14$), and Control groups ($P = 0.43$) as shown in Figure 2A. A significant ($P < 0.05$) change in WBC count in Treatment-1 ($P = 0.05$), Treatment-4 ($P = 0.038$), and Control ($P = 0.001$) groups was observed; however, the changes found in Treatment-2 ($P = 0.80$), Treatment-3 ($P = 0.80$), and Placebo groups ($P = 0.86$) were insignificant ($P > 0.05$), as shown in Figure 2B. The change in HGB level was significant ($P < 0.05$) in Treatment-1 ($P = 0.01$), Treatment-3 ($P = 0.00$), and Placebo ($P = 0.001$) groups but not significant ($P > 0.05$) in Treatment-2 ($P = 0.11$), Treatment-4 ($P = 0.05$), and Control ($P = 0.27$) groups, as shown in Figure 2C. For the PLT count, we did not find any significant ($P < 0.05$) differences in all the groups; Figure 2D. The percentage of HCT was also checked, where the changes in HCT percentage were significant ($P < 0.05$) in Treatment-1 ($P = 0.01$), Treatment-3 ($P = 0.02$), and Placebo groups ($P = 0.00$) but not significant ($P > 0.05$) in Treatment-2 ($P = 0.218$), Control ($P = 0.55$), and Treatment-4 ($P = 0.08$) groups, as shown in Figure 2E. The changes in MCV values were significant ($P < 0.05$) in Treatment-1 ($P = 0.02$), Treatment-4 ($P = 0.01$), and Placebo ($P = 0.02$) groups but not significant ($P > 0.05$) in Treatment-2 ($P = 0.20$), Control ($P = 0.86$), and Treatment-3 ($P = 0.07$) groups, as shown in Figure 2F. The amount of MCH was also measured, and the changes were found to be significant ($P < 0.05$) in Placebo ($P = 0.03$) group but not significant ($P > 0.05$) in Treatment-1 ($P = 0.54$), Treatment-2 ($P = 0.91$), Treatment-3 ($P = 0.37$), Treatment-4 ($P = 0.97$), and Control groups ($P = 0.91$) as shown in Figure 2G. The changes in MCHC count were significant ($P < 0.05$) in Treatment-1 ($P = 0.03$) and Placebo ($P = 0.06$) groups; however, it was not significant ($P > 0.05$) in Treatment-2 ($P = 0.47$), Treatment-3 ($P = 0.28$), Treatment-4 ($P = 0.40$), and Control ($P = 0.90$) groups, where the initial and final values were very close to each other as shown in Figure 2H.

3.3.2. Repeated-dose toxicity study

For the analysis of one-way ANOVA and t-test, the hematology parameters were compared between the measurements on day 0 and day 7 in all the study groups. The RBC count increased in all study groups but Treatment-1 and Treatment-2 groups showed the highest count compared with Control and Placebo groups, as shown in Figure 3A. The differences in RBC count between the two treatment groups were very significant ($P = 0.0002$). We also compared the differences between the groups by doing a t-test, which showed significant differences between Treatment-1 and Control ($P = 0.002$), Treatment-2 and Control ($P = 0.002$), Treatment-1 and Placebo ($P = 0.002$), Treatment-2 and Placebo ($P = 0.002$)

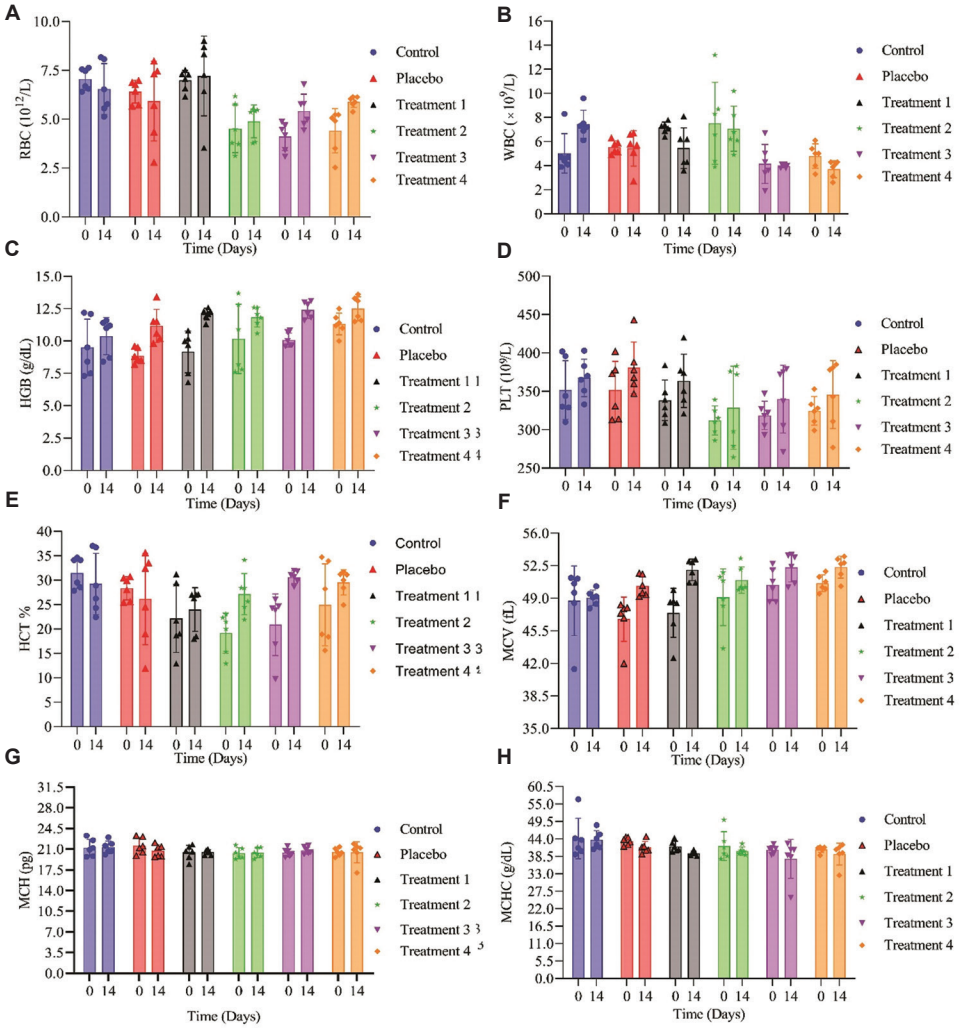


Figure 2. Hematological test findings of single-dose toxicity study. (A) Red blood cell count; (B) White blood cell count; (C) Hemoglobin count; (D) Platelet count; (E) Hematocrit; (F) Mean corpuscular volume; (G) Mean corpuscular hemoglobin; (H) Mean corpuscular hemoglobin concentration. Data are presented as mean ± standard deviation.

but no significant differences between Treatment-1 and Treatment-2 ($P = 0.83$) and Placebo and Control ($P = 0.64$). WBC count decreased within the normal range in all study groups, including Control group, and their results were almost similar, as shown in Figure 3B. However, the differences in WBC count among the groups were not significant ($P = 0.80$). HGB levels increased in all study groups but Treatment-1 and Treatment-2 groups showed the highest levels compared with Control and Placebo groups, as shown in Figure 3C. The differences in HGB levels on day 0 and day 7 among the different study groups were very significant ($P = 0.0004$). HCT also increased in Treatment-1 and Treatment-2 groups but decreased in Control and Placebo groups, as shown in Figure 3E. The differences in HCT percentage among the different study groups were very significant ($P = 0.0001$). We also found significant differences between Treatment-1 and Control ($P = 0.01$), Treatment-2 and Control ($P = 0.000$), Treatment-1 and Placebo ($P = 0.04$), Treatment-2 and Placebo ($P = 0.000$). The MCV increased within the normal range in all study groups, including Control, where Treatment-1 group showed the highest level compared with Treatment-2, Placebo, and Control groups, as shown in Figure 3F. When the differences in HCV count among the groups were analyzed, we did not find any significant ($P = 0.64$) differences. MCH level decreased within the normal range in all study groups except Treatment-2 group where the results were insignificant ($P = 0.46$), as shown in Figure 3G. MCHC decreased in Treatment-2 and Control groups but increased in Treatment-1 and Placebo groups, as shown in Figure 3H. The differences in MCH levels among the groups were not significant ($P = 0.27$).

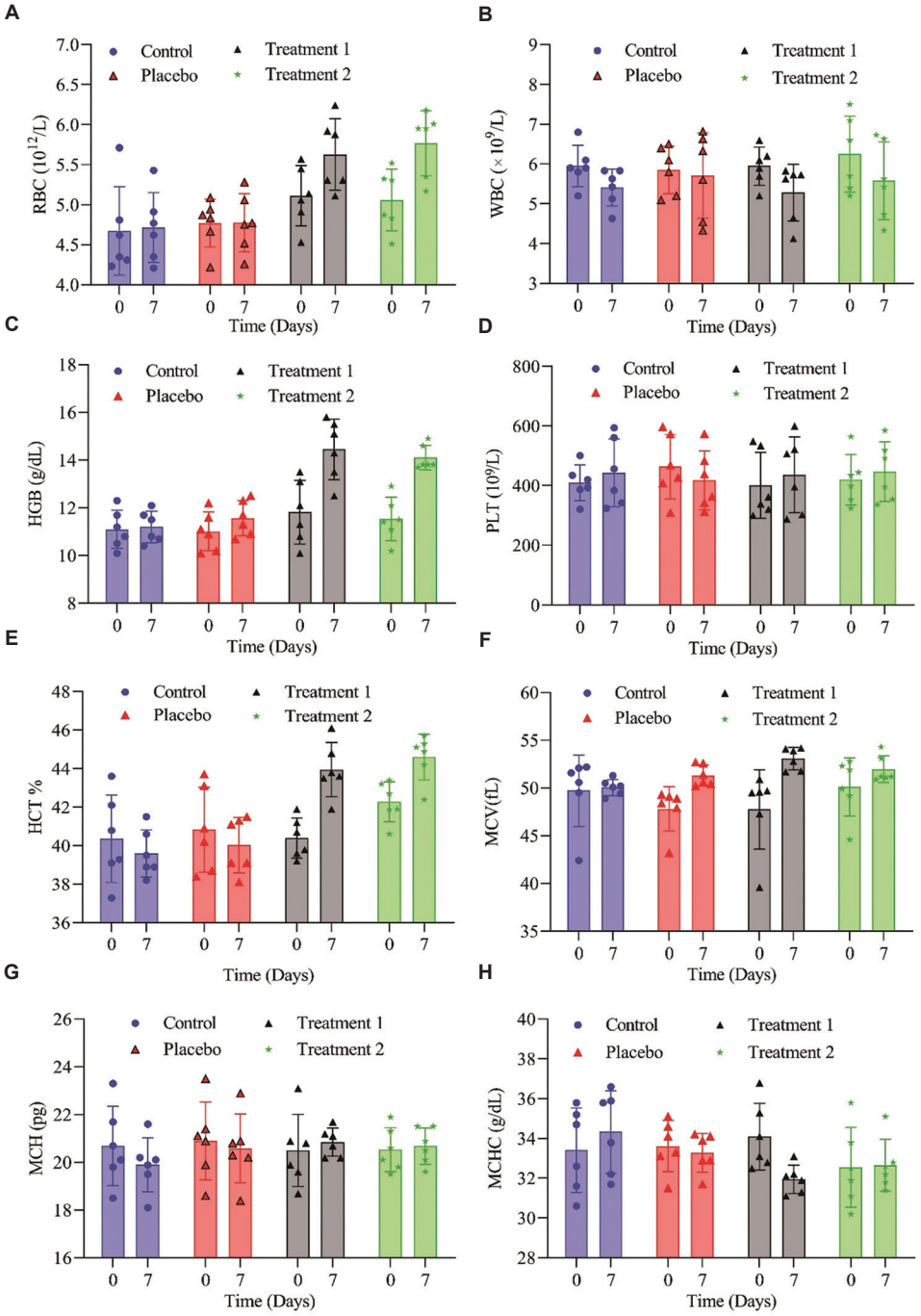


Figure 3. Hematological test findings of repeated-dose toxicity study. (A) Red blood cell count; (B) White blood cell count; (C) Hemoglobin count; (D) Platelet count; (E) Hematocrit; (F) Mean corpuscular volume; (G) Mean corpuscular hemoglobin; (H) Mean corpuscular hemoglobin concentration. Data are presented as mean ± standard deviation.

Finally, PLT count increased within the normal range in all study groups, including Control group, and their results were almost similar, as shown in Figure 3D. However, the differences in PLT count among the groups were significant ($P = 0.44$).

3.4. Biochemical tests

3.4.1. ALT/GPT assay

Increased ALT was observed in all animal groups of the toxicity study. In the single-dose toxicity study, the

Treatment-4 group showed the highest positive value among all the study groups, whereas Treatment-1 showed the lowest value, as shown in Figure 4A. The change in ALT levels was significant ($P < 0.05$) in Treatment-2 ($P = 0.03$), Treatment-4 ($P = 0.00$), Control ($P = 0.01$), and Placebo ($P = 0.06$) groups but not significant ($P > 0.05$) in Treatment-1 ($P = 0.14$) and Treatment-3 ($P = 0.10$) groups. On the other hand, in the repeated-dose toxicity study, ALT levels also increased in all study groups, where Treatment-2 and Control groups showed higher values compared to Treatment-1 and Placebo groups, as shown in Figure 4D. However, the difference in ALT levels between the two treatment groups was not significant ($P < 0.05$).

3.4.2. AST assay

AST level increased in all animal groups under study, where Treatment-4 group showed higher values compared to all other groups in the single-dose toxicity study, as shown in Figure 4B. The change in AST levels was significant ($P < 0.05$) in Treatment-1 ($P = 0.02$), Treatment-2 ($P = 0.02$), Treatment-4 ($P = 0.01$), Control ($P = 0.01$), and Placebo ($P = 0.06$) groups but not significant ($P > 0.05$) in Treatment-3 ($P = 0.28$) group. The AST level increased within the normal range in all study groups, including Control group, and their results were almost similar, as shown in Figure 4E. However, the analysis of AST levels among the groups showed no significant ($P = 0.76$) differences.

3.4.3. BUN assay

BUN level also increased in all groups under toxicity study, except Treatment-3 group in the single-dose toxicity test, as shown in Figure 4C. The change in BUN levels was significant ($P < 0.05$) in Treatment-1 ($P = 0.03$), Treatment-2 ($P = 0.00$), and Treatment-3 ($P = 0.00$) groups. However, it was not significant ($P > 0.05$) in Treatment-4 ($P = 0.80$), Control ($P = 0.31$), and Placebo ($P = 0.12$) groups, in which the initial and final values were very close. The BUN level also increased in Treatment-1, Treatment-2, and slightly in Control groups but decreased in Placebo group in

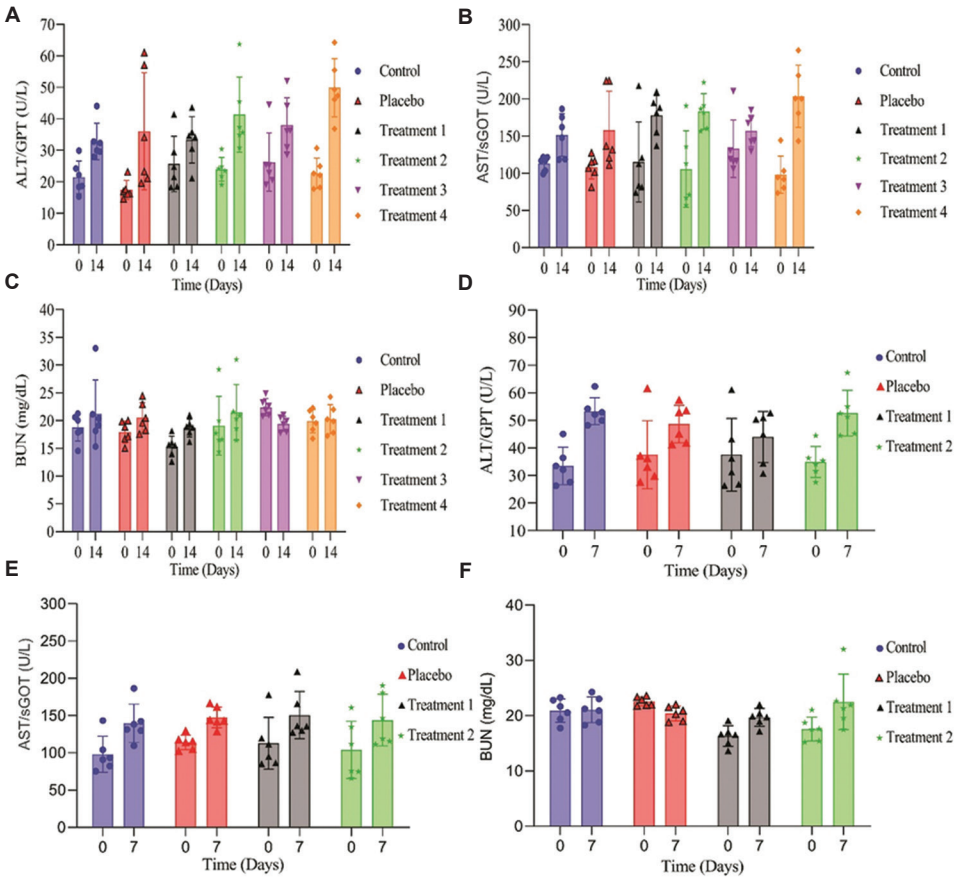


Figure 4. Analysis of biochemical parameters of the study subjects. (A-C) show the findings of alanine aminotransferase (ALT)/glutamic pyruvic transaminase (GPT), aspartate aminotransferase (AST)/serum glutamic-oxaloacetic transaminase (sGOT), and blood urea nitrogen (BUN) assays, respectively, for single-dose toxicity tests. (D-F) show the findings of ALT/GPT, AST/sGOT, and BUN assays, respectively, for repeated-dose toxicity tests. Data are presented as mean ± standard deviation.

the repeated-dose toxicity study, as shown in Figure 4F. However, the differences in BUN levels among the groups were not significant ($P = 0.02$).

3.5. Necropsy findings

Single representative subjects from all Treatment, Placebo, and Control groups were euthanized for necropsy findings. External surfaces, all orifices, cranial cavities, external surfaces of the brain and spinal cord, thoracic, abdominal, and pelvic cavities, cervical tissues, and organs were observed for any abnormalities. However, no abnormal lesions were found during necropsy examination, as shown a Figure 5.

3.6. Histopathology study

No morphological signs of toxicity were observed in internal organs such as the kidney, liver, lung, and spleen of experimental animals from the single- and repeated-dose toxicity studies (Figure 6). No lesions of pathological significance like degeneration or cellular necrosis were found on these internal organs in all treatment groups, including the group that received Eprex®.

4. Discussion

This study presents the overall results of the preclinical safety evaluation of GB*poietin*® in rats through a comparative acute toxicity study with Eprex®, following subcutaneous administration. The findings demonstrated that the toxicological profiles of GB*poietin*® and Eprex® were similar and aligned with the known pharmacology of EPO alfa, previously studied for a comprehensive safety and toxicity assessment of rhEPO.[23] No side effects or signs of toxicity were observed during daily observations of the animals inoculated with GB*poietin*® or Eprex® in the comparative acute toxicity assay. There were no changes in fur or pigmentation, and the eyes and mucosal surfaces appeared normal, as did the somatomotor activity and behavior. Proper responses to stimulation were obtained, and no deaths were reported during the study. Upon evaluation of the inoculation site, no signs of damage attributable to the administration of rhEPO.[24-26] were evidenced. The only abnormalities detected were hemorrhagic areas observed in the repeated-dose toxicity study, but these were attributed

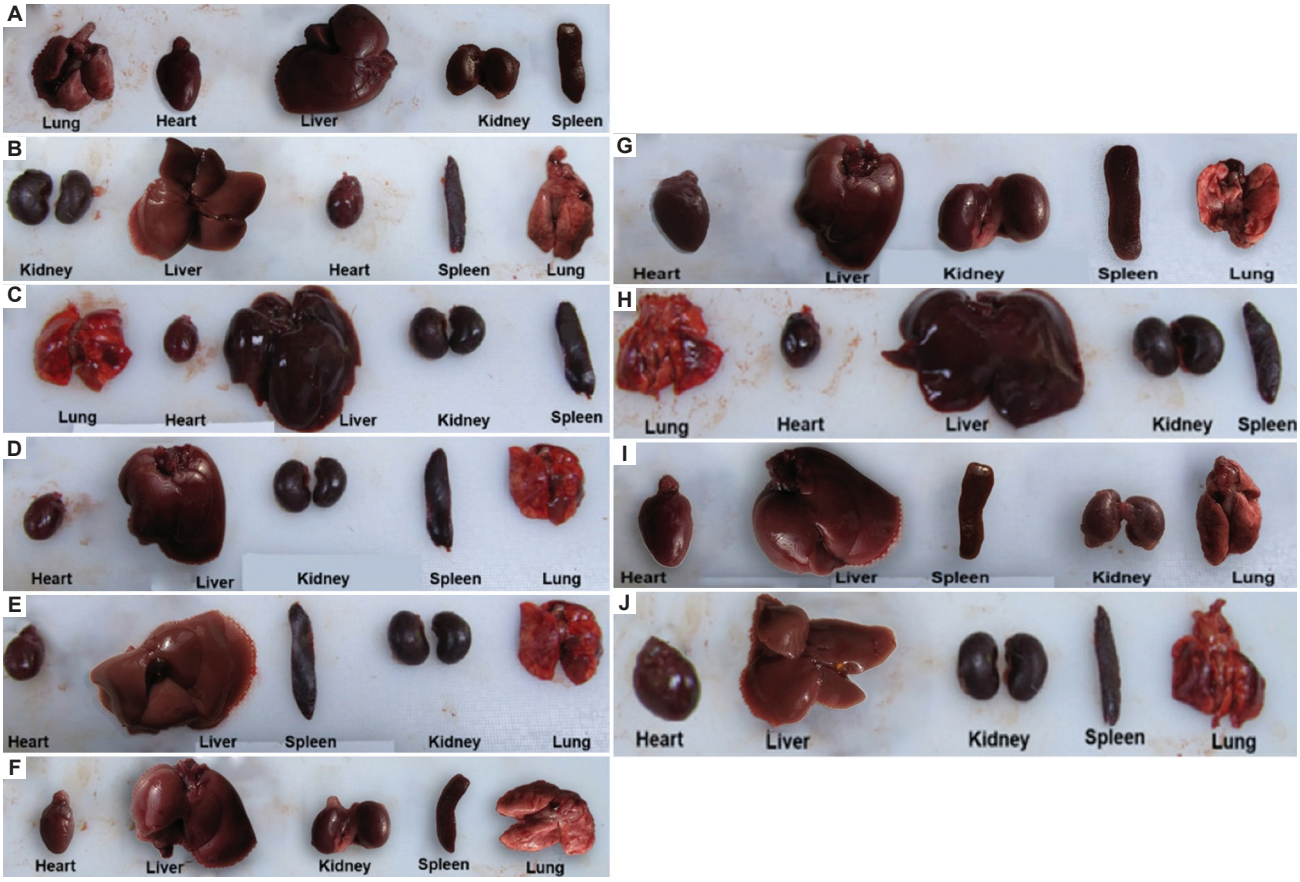


Figure 5. Representative necropsy findings of both single- and repeated-dose toxicity studies. (A) Control, (B) Placebo, (C) Treatment 1, (D) Treatment 2, (E) Treatment 3, and (F) Treatment 4 groups of single-dose toxicity study. (G) Control, (H) Placebo, (I) Treatment 1, and (J) Treatment 2 groups of repeated dose toxicity study.

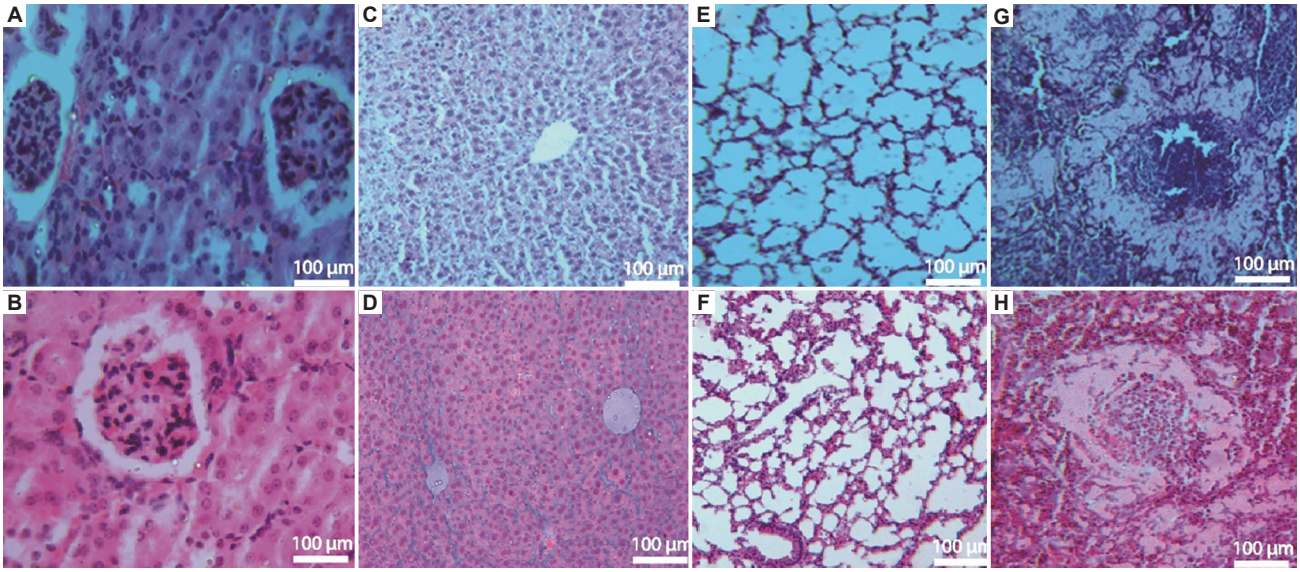


Figure 6. Representative histopathological findings. (A and B) kidney, (C and D) liver, (E and F) lung, (G and H) spleen, for both single- and repeated-dose toxicity studies, respectively.

to the method and the repetitive nature of the inoculation rather than to rhEPO itself,[27-29] as they appeared with the same intensity in the placebo group. The results of the hematological tests were normal when compared to their values before the start of the study.

Body weight increased steadily and significantly ($P < 0.05$) during the single-dose toxicity study; we found similar differences in body weight in all study groups at different evaluation time points (days 0, 3, and 7). The results were insignificant ($P > 0.05$) for the repeated-dose toxicity study as well. The increase of this parameter in all the study groups translates into a normal evolution of body weight for both genders indicating good health for the animals, further substantiating the non-toxicity of the GB*poietin*® in both studies. The absence of negative effects on body weight gain is favorable for evaluating the substance under study, as a decrease in body weight is one of the primary clinical symptoms of stress or illness in this rat strain.[30,31] Therefore, the increase in body weight is indirect evidence of non-toxicity for the substance under analysis. No significant changes in body temperature were observed among the treatment groups. This result remained similar when the same data were analyzed independently by gender or evaluation time points, which indicated normal health for the animals, further supporting the non-toxicity of the GB*poietin*®. Abnormal changes in body temperature under test conditions have been shown to indicate induced toxicity.[32,33] Accordingly, the absence of significant changes in body temperature outside the normal range suggests that severe toxicity was unlikely in the test animals during the experiment.

The results of the hematological tests were normal when compared to their values before the start of the study. Differences were found in the groups inoculated with GB*poietin*®, Eprex®, or the placebo in both single- and repeated-dose studies. The values stayed within the normal range for the species used in the study.[27,34] WBC levels decreased within the normal range across all study groups, including the control, with results being largely similar. The absence of WBC count changes beyond the normal range serves as evidence of the non-toxicity of the test item under analysis.[35] The high RBC count, HGB level, and HCT, together with the slight increase in PLT, confirm the therapeutic effect of EPO. These high counts of hematological parameters also reveal the therapeutical similarity of GB*poietin*® compared with Eprex®. These results are attributed to the mobilization of hematopoietic progenitor cells to the peripheral blood, which is the reported mechanism of action of the product under study.[36] On the other hand, MCV increased in all groups under study with Treatment-1 showing higher value than all other groups in the toxicity study. The differences in MCV levels among the groups were insignificant ($P < 0.05$). MCH levels increased in Treatment-2, Treatment-3, and Control groups, while they decreased in Treatment-1, Treatment-4, and Placebo groups, though the values were within the normal range ($P = 0.46$) in all study groups. In single-dose toxicity study, MCHC levels decreased in all animal groups, while they decreased in Treatment-2 and Control groups and increased in Treatment-1 and Placebo groups in the repeat-dose toxicity study. The absence of change in MCV, MCH, and MCHC levels outside the normal range demonstrates the non-toxicity of the test item under

analysis.[37] Collectively, these results also demonstrate the similarity between the two experimental EPO preparations, namely GB*poietin*® and Eprex®.

Our result is in agreement with the findings of other studies, where similar levels of biochemical parameters were reported (ALT/GPT, AST, and BUN).[38] In our study, we reported that serum ATL/GPT increased in all the treatment groups. Serum AST and BUN also increased beyond the normal level but the increases were less than that of the ALT/GPT levels in both single- and repeated-dose toxicity tests. Similar studies suggest that the upper level within the normal range of biochemical parameters indicates the uniformity of liver function.[39] It also serves as evidence for non-toxicity of the test item under analysis.[40] The observation suggests that GB*poietin*® is safe, with a toxicity profile similar to that of the reference product Eprex®.

The results of the necropsies for both experiments showed no indication of any abnormalities or anatomical changes. After two doses, the hemorrhagic regions were observed for single animal from each group (control, placebo, and treatment groups). Presumably, the trauma from repeated subcutaneous injections at the same location is associated with this symptom.[6] Thus, the macroscopic results support the clinical findings, indicating no changes attributable to EPO and no signs of local irritation or injury. Regardless of the dosage or volume of administration, the lack of macroscopic damage in the organs and tissues of the experimental animals is a crucial factor in assessing the safety of the tested product in both trials. One of the observed histopathological findings was the absence of lesions of pathological significance like degeneration or cellular necrosis in internal organs. These reactions have been described in the literature as a consequence of the intense metabolic activity of the organ, and their spontaneous appearance has been reported for this animal species.[41] The presence of these findings in the control groups indicates that their occurrence is not dependent on the effect of the different rhEPO doses in any of the assayed formulations. Notably, the histopathological results further support the safety of the test product GB*poietin*®, demonstrating similarity to the reference product Eprex®.

5. Conclusion

This study establishes that the toxicological profiles of GB*poietin*® and Eprex® are similar and align with the known pharmacology of EPO alfa. It is also important to note that the histopathological results preclude any noticeable toxicity effect of the test product, GB*poietin*®, in major organs and injection sites. Based on the experimental results, we conclude that the biological response of GB*poietin*® and Eprex® is essentially equivalent. Therefore, they can be considered biosimilar in pre-clinical settings, with a clinical study in humans potentially providing further insights.

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Conflict of interest

Kakon Nag is the CEO of Globe Biotech Limited, Dhaka, Bangladesh and R&D Management Solution Inc., Ontario, Canada. However, this has not influenced the content of the manuscript. The other authors declare that they have no competing interests.

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Ethics approval and consent to participate

The study plan and procedures for experiments were approved by the internal ethical review board (IECB-PCS: Internal Ethical Clearance Board for Pre-Clinical Study) of Globe Biotech Limited, which complies with local and international regulations. The ethical approval reference number is GB/EC/19/001.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available within the article or are available from the corresponding author upon reasonable request.

Further disclosure

The study was published in the preprint journal BioRxiv under the title "Preclinical study of the human recombinant Erythropoietin (GBPD002) compared with Eprex®" with a DOI number 10.1101/2024.06.05.597513.

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