4. Introduction

Bioactive glasses (BGs) have immense hard tissue clinical applications (Hench, Splinter et al. 1971) and have received several FDA approvals for periodontal (Stanley, Hall et al. 1997), orthopedic (Jones, Brauer et al. 2016), and cranio-maxilo-facial applications (Bernardeschi, Nguyen et al. 2015). Later on, with improvement in the field of inorganic biomaterials, BGs have found their way to regenerating and repairing non-osseous and non-calcified tissues (Pires, Bonan et al. 2018, He, Ding et al. 2019, Qi, Zhu et al. 2020). Similarly, in **Chapter 2,** the anti-inflammatory and tissue regenerative potential of barium leached from BaBG was demonstrated. We also observed that there was dose-dependent release of network modifiers into the plasma after oral administration of BaBG (reported in **Chapter 3**). Despite the tremendous potential regenerative properties of BGs observed preclinically, there are no clinically translated products of BGs for oral and systemic administration. This is mainly due to inadequate preclinical toxicity studies that could prove their efficacy and safety. So, there is a need to perform oral toxicity studies of BGs to ascertain the therapeutic range for future pharmacological applications.

Further, the bioactivity of BGs is mainly due to the leaching of various doped ions from its framework which may enter the systemic circulation through the sublingual or buccal cavity and cross the blood-brain barrier, affecting the central nervous system (CNS) (i.e., brain), which controls most of the bodily functions. If BGs are used as implants then they are intended to remain *in situ* throughout the patient's life, so it is essential to perform long-term toxicity studies to assess their possible toxic effects on various vital organs, including the neurotoxic effects. Therefore, the toxicity study is essential for new drug discovery as it helps envisage the lethal dose (LD_{50}), a crucial

criterion for dose selection during systemic administration. Hence, an oral acute and sub-acute toxicity study for BaBG was performed according to the OECD (Organization for Economic Co-operation and Development) guidelines. Since 45S5 is the FDA-approved and commercialized BG for tissue engineering, it was used to compare the safety profile of BaBG in this study. In the present study, changes in the body weight and organ coefficients, hematological parameters, neuro-behavioral tests, biochemical estimation of various enzymes, and histological analysis were performed. Hence, the preclinical oral toxicity studies performed will help establish the safety and efficacy and will also help to estimate the dose of the developed BG when used for various pharmacological applications in not-too-distant future.

4.1. Materials and Methods

4.1.1. Materials

AUTOSPAN® Liquid Gold Calcium and MBK Alkaline Phosphatase Assay kit were purchased from ARKRAY Healthcare Pvt. Ltd., India. AST (GOT) and ALT (GPT)-Modified IFCC kits were procured from TARA Clinical Systems, India. We obtained creatinine and CK MB (NAC act.) assay kits from Coral Clinical Systems, India.

4.1.2. Animals

In the experiment, adult albino Wistar rats of 200±20 g were used (acquired from the Institutional animal house, IMS- BHU, Varanasi, India). All the rats were habituated for a week in a controlled temperature of 25±1°C and relative humidity of 45-55% (12/12 h light/dark cycle) with *ad libitum* supply of food (Paramount Laboratory Animal feed, Lanka, India) and water throughout the analysis. The current study was designed to curtail the number of animals used during the experiment, and all the studies were

performed as per the National Institute of Health Guidelines (publication number 85-23, revised 2013) on animal care experimentation. The experimental protocol was approved by the Central Animal Ethical Committee of Banaras Hindu University, Varanasi, India (Ref No. Dean/2021/IAEC/2557).

4.1.3. Acute oral toxicity study

The *in vivo* acute oral toxicity of BaBG was performed on healthy young adult female rats (as they are the most sensitive between the sexes) to determine the range of median lethal dose (LD_{50}) according to the OECD Guideline 423 (Acute Toxic Class Method) (OECD 2001). In the present study, 45S5 was used to compare the safety profile of BaBG. As per the experimental protocol, the animals were randomly allocated in groups with three animals in each group. Since there is no information on the toxicity profile of test substances (45S5 and BaBG), the starting dose of 300 mg/kg body weight (b.w.) was selected. If no or one animal is dead or in a moribund, then 300 mg/kg was administered in another three rats. Based on the result, higher or lower doses were tested. The detailed experimental protocol is depicted in **Figure 4.1.** 45S5 and BaBG was suspended in 0.5% carboxy methylcellulose (CMC), and the experimental protocol consists of a single-dose administration of the test substances by gavage in the overnight fasted rats. The control rats received 0.5 % CMC suspension, and all the rats were observed individually at least once during the first four hours and daily thereafter for 14 days. Various signs of toxicity like changes in the skin, fur, eyes, and mucous membranes were observed. Additionally, the rats were also monitored if any signs of tremor, convulsions, salivation, lethargy, diarrhea, sleep, and comatose conditions appeared. The individual body weight of the experimental animals in each group was also measured prior to and after administering the test substances weekly. After the 14th

day, the rats were weighed and anesthetized with 3% v/v isoflurane inhalation (Cat: R620 veterinary anesthesia machine, RWD Life Science, San Diego, USA). The animal was then killed by decapitation, and blood was collected for biochemical analysis. Various organs like the brain, heart, liver, kidney, spleen, and lungs were collected for a necropsy to observe any sign of test substance-induced pathological changes.

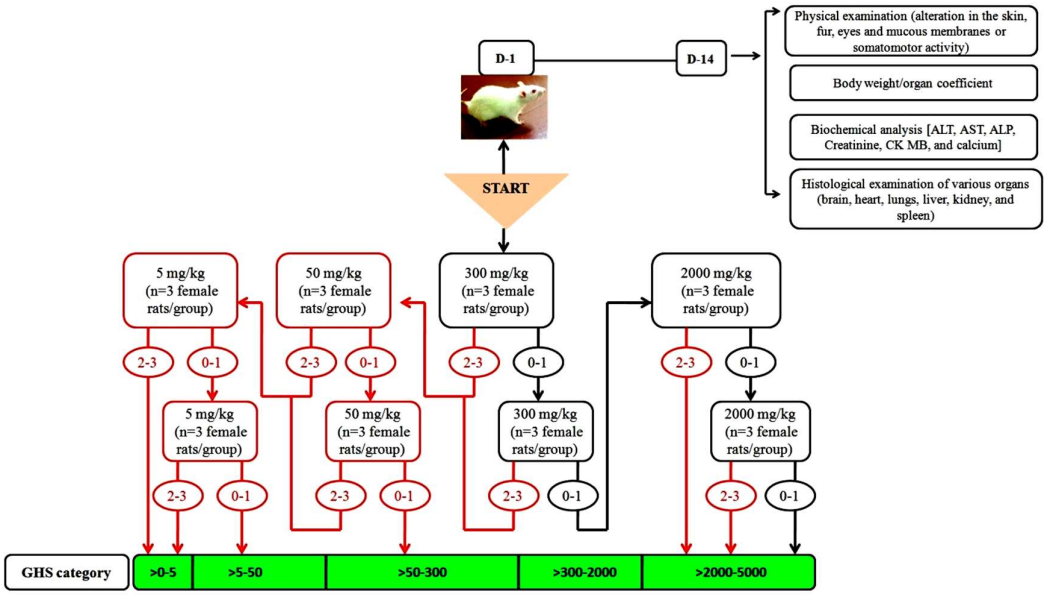

Figure 4.1: Schematic representation of the experimental protocol for single-dose acute toxicity study (OECD 423) to determine the LD_{50} cut-off (mg/kg b.w.) value. The starting dose of 300 mg/kg b.w. was selected. If 2-3 animals die, a lower test dose (i.e., 50 mg/kg) was tested and if no or one animal dies, the next higher dose (i.e., 2000 mg/kg) was tested. Black arrow indicates the test procedure followed in our study.

4.1.4. Subacute oral toxicity study

The repeated dose 28-day oral subacute toxicity study was performed in the Wistar rats according to the OECD 407 guidelines (Co-operation and Development 2008). The rats were randomly divided into groups: Control, 45S5 (50 mg/kg), 45S5 (500 mg/kg), 45S5 (1000 mg/kg), BaBG (50 mg/kg), BaBG (500 mg/kg), and BaBG (1000 mg/kg); each group containing 5 females and 5 males (n=10 rats/group). The doses were selected in such a manner that the highest dose would induce toxic effects without causing death

or severe suffering to the experimental animals and subsequently, the lowest dose would have no-observed-adverse effects (NOAEL). The control rats received 0.5% CMC suspension, while 45S5 and BaBG treatment groups were administered test substances (suspended in 0.5% CMC) daily for 28 days. All the experimental rats were observed daily for any abnormal behavior or changes in somatomotor activity. The behavioral assessment tests (like rotarod test, spontaneous locomotive activity, grip strength test, and Open-field test (OFT)) were performed on D-1, D-7, and D-28 to assess the progressive changes in motor activity and its effects on the central nervous system. Moreover, body weight and food and water intake were monitored throughout the experiment protocol. At the end of the 28^{th} day, the experimental rats were anesthetized and sacrificed. The blood was collected for hematological analysis, and to investigate the toxic effects of BaBG on various organs (brain, heart, lungs, liver, kidney, and spleen), histopathological analysis was performed **(Figure 4.2)**.

4.1.5. Behavioral assessments

4.1.5.1. Rotarod test

The rotarod test is performed to assess the motor coordination ability of the rats (Rozas, Guerra et al. 1997). The experimental animals were trained on the rotarod (IKON Instrument, India) twice for two consecutive days at a lowest rotation speed of 5 rpm to reach stable baseline performances. On D-1, D-7, and D-28, during the test session, the speed of the rotating rod was increased to 15 rpm, and the time spent by the rats on the rod was recorded with maximum cut-off time of 300 s. The moment rats fall from the rod, the counting is stopped and reported as the retention time on rod.

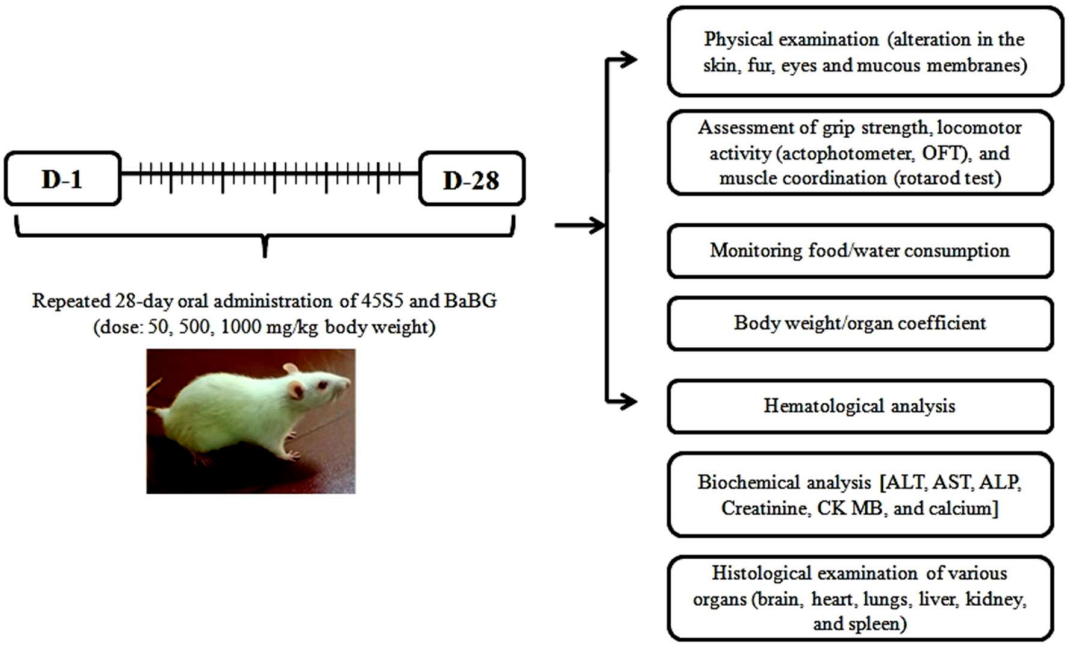

Figure 4.2: Schematic representation of the repeated 28-day oral toxicity study as per OECD 407 guidelines. In the subacute toxicity study, rats (n=10/group) were administered BaBG (dose: 50, 500, and 1000 mg/kg b.w.) and were observed daily for any sign of toxicity.

4.1.5.2. Open field test (OFT)

OFT, an important behavioral test performed to assess the locomotor activity of the experimental rats (Bronstein 1972). In this test, a square wooden box was fabricated (61x61 cm) representing an open field and is painted blank with enclosed high walls. The box is divided with white lines into 16 squares. An incandescent bulb dimly lights the apparatus suspended vertically in the central portion of the field. During the 5 min test, the animal was placed at the corner of the box facing towards the walls, and the parameters like the total number of rearing (rats stood on its hind limbs) and grooming (rats licked or scratched its face), total distance travelled, and time spent in the central region were recorded using ANY-maze^{TM} (version 3.72, USA). Prior to each experiment, the arena was cleaned with alcohol.

4.1.5.3. Grip strength test

Grip strength test is performed to determine the neuromuscular strength of the experimental animals and was performed on D-1, D-7, and D-28. The apparatus consists of a 90 cm long horizontal wire (diameter of 1mm) fixed between two vertical poles at the height of 50 cm from the flat surface. The rats were hung on the wire centrally with its forepaws and were scored as per the observations: 0-fall off; 1-hangs on the wire with both fore-paws; 2-same as 1 but also tries to climb on the wire; 3-hangs on the wire with both fore-paws and also with either of the two hind-paws; 4-hands on the wire with all four paws along with its tail wrapped around the wire; 5-escape the apparatus and falls on the flat surface (Meyer, Tilson et al. 1979, Prajapati, Garabadu et al. 2017). The cut-off time considered during this test was 90 s.

4.1.5.4. Spontaneous locomotor activity (Actophotometer)

Actophotometer is a square box (30 x 30 cm) with walls fitted with photocells and is used to measure the locomotor activity of the rats (IKON instruments, India) and was performed on D-1, D-7, and D-28. The rats were placed into the instrument, and their total activity counts were recorded for 5 min. The movement of the animal inside the box cut off the beam of light from the photocell and was recorded digitally as counts/5 min per animal (Reddy and Kulkarni 1998).

4.1.6. Organ coefficient

The organ coefficient is a vital parameter to evaluate the toxic effect of the test compounds by determining the changes in the organ's weight (Yang, Wu et al. 2019). The animals were sacrificed, and the organs were dissected and weighted properly. The organ coefficient of different organs was calculated using the following formulae:

$$\text{Organ coefficient} = \left[\frac{\text{Weight of the organ (g)}}{\text{Total body weight (g)}}\right] \text{X}100$$

4.1.7. Preclinical pathology

The hematological analysis was performed in the blood samples collected in tubes coated with anticoagulant (EDTA). For biochemical analysis, serum was collected from blood samples stored in tubes without any anticoagulant. The blood was allowed to clot at room temperature and centrifuged at 3000 rpm for 10 min; the supernatant was decanted and stored at -20°C for further analysis.

4.1.8. Hematology

To evaluate whether BaBG has any toxic effects on the blood cells, hematological blood parameters were measured using Coulter® LH750 Hematology Analyzer (Beckman Coulter Inc., CA, USA). The blood parameters like red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), red blood cell distribution width (RDW), blood platelets (PLT), and white blood cells (WBC) were measured.

4.1.9. Biochemical analysis

The biochemical analysis was performed in the serum to access the effects of BaBG on functioning of various organs using assay kits as per the manufacturer's instructions. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured to evaluate liver function. Similarly, the kidney and heart functions were detected by the level of creatinine (CRE) and CK-MB, respectively. Calcium (Ca^{2+}) level was also analyzed in the serum as excessive increase in its level is considered as an important pathological marker in various diseases.

4.1.10. Histological analysis

On the completion of the oral acute and subacute toxicity studies, various organs (brain, heart, lungs, liver, kidney, and spleen) were collected immediately and fixed in a 10% buffered formalin solution. The finely sliced tissues (5 μm) were stained with hematoxylin for 3 min and rinsed in running tap water. The samples were then counterstained with eosin for 1 min, dehydrated using graded series of alcohol, and mounted with dibutyl phthalate xylene (DPX). The slides prepared were observed under the microscope (Olympus DS-52, Japan) for any gross pathological aberrations (Fischer, Jacobson et al. 2008).

4.1.11. Statistical analysis

All the data were analyzed statistically by Graph Pad Prism 5.0 software (San Diego, RRID: SCR_002798). The behavioral parameters, the weight of the animals, and food consumed by them were analyzed by two-way ANOVA, while the biochemical studies, organ coefficient, and hematological parameters were assessed statistically by one-way ANOVA followed by Tukey's multiple comparison tests. $P<0.05$ was considered significant in all the analyses, and the values were presented as mean ± SD.

4.2. Results and discussion

4.2.1. Acute toxicity study

4.2.1.1. General observation and behavioral analysis

The animals were observed regularly after the oral administration of 45S5 and BaBG (300 and 2000 mg/kg b.w.), and there was no observed treatment-related mortality at any dose tested during the experimental protocol. Thus, the LD_{50} value of 45S5 and BaBG is more than 2000 mg/kg b.w. as per OECD 423, and the test compounds (45S5

and BaBG) fall under the GHS category (Globally Harmonized Classification System) 5 (non-toxic). Moreover, the animals in each group did not exhibit any treatment-related signs of toxicity like changes in the skin, fur, and mucous membrane or respiratory, circulatory, and somatosensory distress **(Table 4.1).** Additionally, there were no behavioral changes in the treated rats like salivation, convulsions, and tremors. This finding is supported by a previous report, where 45S5, when implanted into the peritoneal cavity of rats for two weeks, exhibited no toxicity like peritonitis or organ toxicity (Wilson, Pigott et al. 1981). Furthermore, in the present study, the oral administration of BaBG, as well as 45S5 at higher doses (2000 mg/kg b.w.), did not show any morphological changes in the vital organs like the liver, brain, lungs, kidneys, and spleen **(Figure 4.3).** A previous study also reported that the mesoporous bioactive glass spheres, when injected intravenously in rats, exhibited no pathological abnormalities in the internal organs (Mao, Chen et al. 2016). Thus, from these observed results, it can be affirmed that BaBG is non-toxic and can be used for various biomedical applications.

Table 4.1: General observation and behavioral analysis during the first 4 h and 24 h after single-dose administration of BaBG and 45S5 (300 and 2000 mg/kg b.w.) in rats (n=6 female rats/group).

Observation	Control		45S5(300 mg/kg)		45S5(2000mg/kg)		BaBG(300mg/kg)		BaBG(2000mg/kg)	
	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
Skin and fur	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Eyes	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Mucous membrane	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Salivation	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
Diarrhea	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Lethargy	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Tremors and convulsions	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
Behavior pattern and somatosensory activity	N	N	N	N	N	N	N	N	N	N

Note: N-Normal, NO-Not observed, and NC-No change

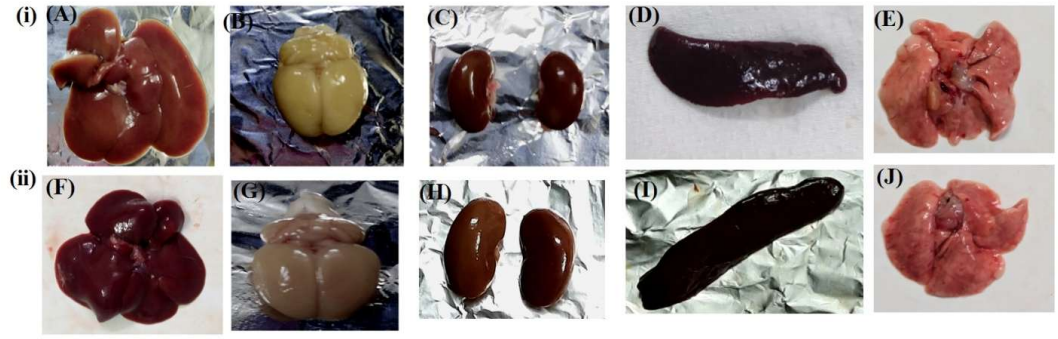

Figure 4.3: Representative macroscopic photographs showing normal morphology of (i) 45S5 [liver (A), brain (B), kidneys (C), spleen (D), and lungs (E)] and (ii) BaBG [liver (F) , brain (G), kidneys (H), spleen (I), and lungs (J)] treated rats after single-dose administration of highest dose (i.e., 2000 mg/kg b.w.) at the end of day 14.

4.2.1.2. Effect of BaBG on the body weight during the acute toxicity study

Body weight is an essential parameter to affirm the normal physiological functioning of the internal organs, and involuntary changes in it due to the administered test compounds are considered vital signs of severe pain, distress, or impending death of the experimental animals (OECD 2000, Chapman, Sewell et al. 2013). Thus, in the

present oral acute toxicity study, the body weight of the rats in each group was regularly monitored, which is depicted in **Figure 4.4.** Statistical analysis by two-way ANOVA revealed no significant differences in the body weight among the groups ([$F_{(4,75)}$ = 2.972; p >0.05]), time ([$F_{(2,75)}$ = 14.05; p > 0.05]) and their interaction ([$F_{(8,75)}$ = 0.0498; p > 0.05]). In support of our observation, previously, a study reported no differences in the body weight gain between the control and treated rodents even after the intravenous administration of mesoporous BG (Mao, Chen et al. 2016). Furthermore, Schwotzer et al. (Schwotzer, Ernst et al. 2017) also investigated the toxicity profile of barium sulfate nanoparticles. It was found that 90 days of exposure did not produce any changes in food/water consumption, with no significant differences in body weight among the groups. The above-observed result suggests that BaBG is safer and can be used for therapeutic purposes.

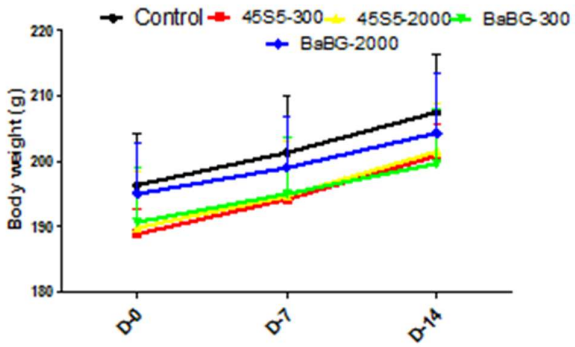

Figure 4.4: Effect of single-dose oral administration of BaBG and 45S5 on body weight of rats at various time points during the experimental protocol. All values are in mean ± SD (n=6 female rats/ group). (Two-way ANOVA followed by Bonferroni post hoc test).

4.2.1.3. Effect of BaBG on the organ coefficient during the acute toxicity study

Organ weight is an essential and sensitive parameter in experimental biology, and it often changes prior to any visible morphological changes; hence it is important to

measure it during the toxicological screening of any test compounds (Piao, Liu et al. 2013). The effect of single-dose oral administration of BaBG on the organ coefficient of vital organs (brain, heart, lungs, kidneys, liver, and spleen) during the acute toxicity study is depicted in **Figure 4.5**. Statistical analysis by one-way ANOVA revealed no significant changes in the organ coefficient of brain, heart, lungs, kidneys, liver, and spleen among the groups ([$F_{(4,29)} = 2.117$; $p>0.05$], [$F_{(4,29)} = 0.829$; $p>0.05$], [$F_{(4,29)} = 2.935$; $p>0.05$], [$F_{(4,29)} = 0.617$; $p>0.05$], [$F_{(4,29)} = 0.428$; $p>0.05$], and [$F_{(4,29)} = 2.662$; $p>0.05$] respectively). This confirms that the single-dose oral administration of BaBG had no apparent toxic effects on most of the highly perfused organs like in the 45S5 treated rats, which corroborates the biochemical estimation of various enzymes **(Figure 4.6)**. Moreover, previous studies have also similarly reported that oral administration of 45S5 in the stress-induced ulcer experimental rodents did not show evidence of any systemic toxicity due to minimal oral absorption (Ma, Gong et al. 2013). Similarly, the barium nanoparticles did not show any sign of toxicity in rats after 4-week exposure (Konduru, Keller et al. 2014) as the barium toxicity depends on their solubility (Oskarsson 2015). Previously, Majumdar et al. (Majumdar, Hira et al. 2021) reported that there is leaching out of the Ba^{2+} from the BaBG after it comes in contact with the simulated body fluid (SBF), and the free Ba^{2+} is generally deposited in the skeleton, essential for their calcification (Moore 1964, Oskarsson 2015). Therefore, it ascertains that the oral administration of BaBG does not have any toxic effects on the normal functioning of the vital organs.

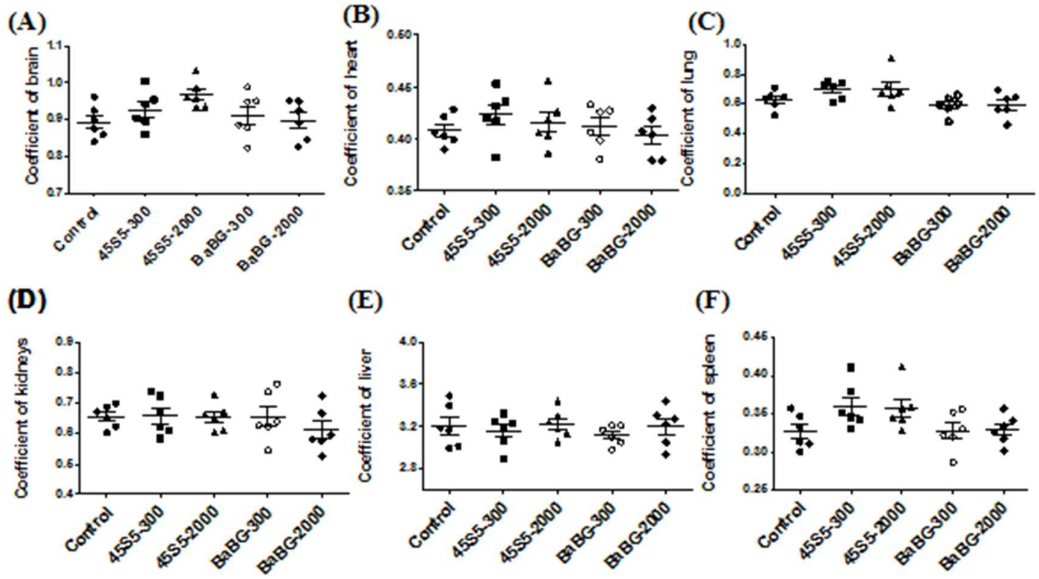

Figure 4.5: Effect of single-dose oral administration of BaBG and 45S5 on organ coefficient of the brain (A), heart (B), lung (C), kidneys (D), liver (E), and spleen (F) at the end of the experimental protocol. All values are in mean ± SD (n=6 female rats/ group). (One-way ANOVA followed by Tukey's multiple comparison post hoc test).

4.2.1.4. Effect of BaBG on various enzymes during the acute toxicity study

The assessment of the biochemical parameters (various enzymes) reflects the possible toxic effects of the test compounds on the functioning of vital organs of the body. In the present study, various enzymes were measured in the serum at the end of the experimental protocol and are shown in **Figure 4.6**. Normally, the vital organs have multifarious mechanisms to reduce their toxic load by eliminating the metabolites produced mainly through the liver and kidneys. Hence, it is essential to assess the liver and kidney functions during the acute toxicity study (**Figure 4.6A-D**) as they are mostly affected during the toxin purgation process (Liyanagamage, Jayasinghe et al. 2020). ALT, AST, and ALP are the important enzymes used clinically to screen the proper functioning of the liver (Lagarto, Bueno et al. 2011). AST is reported to be present in the mitochondria and cytosol of hepatocytes, while ALT is specifically present in the cytosol (Lagarto, Bueno et al. 2011). Thus, changes in the AST level are clinically

considered as an indication of hepatocellular necrosis. However, alteration in the serum ALT level is a specific sign of hypertrophy of hepatocytes or other liver disease conditions. Besides, ALP is another important enzyme present in the body, and an increase in its level indicates obstruction in the bile ducts (Giannini, Testa et al. 2005). In the present study, statistical analysis by one-way ANOVA revealed no significant changes in the serum level of ALT, AST, and ALP among the groups ([$F_{(4,29)} = 2.399$; $p>0.05$], [$F_{(4,29)} = 0.024$; $p>0.05$], and [$F_{(4,29)} = 2.258$; $p>0.05$] respectively) (**Figure 4.6A-C**). Earlier studies have also reported that short-term oral exposure to barium salts did not produce any significant adverse health effects in rats or any microscopic hepatic lesions (Borzelleca, Condie Jr et al. 1988). Moreover, the toxicology study performed in the rodents reported that administration of barium chloride at the dose of 200 mg/kg/day did not produce any barium-related neurobiological or reproductive toxicity (Program 1994).

Kidneys are the major excretory organ that eliminates metabolic wastes from the body, and damage to it leads to azotemia, where there is the accumulation of nitrogenous wastes in the blood. Usually, creatinine is considered an essential anthropometric parameter and is used as an index of renal functions (Casal, Nolin et al. 2019). In our study, serum creatinine level was measured (shown in **Figure 4.6D**), and statistical analysis by one-way ANOVA revealed no significant changes in the serum level of creatinine among the groups ([$F_{(4,29)} = 2.076$; $p>0.05$]. Therefore, this affirms that single-dose oral administration of 45S5 and BaBG did not affect kidney functions. Similarly, in a recent study, Hasan et al. (Hasan, Schaner et al. 2021) reported that 45S5 did not exhibit any changes in the creatinine level compared to the control rats when used to treat osteomyelitis. Further, another study also reported that bioglass, when

implanted into the bone of rabbits, did not show any changes in the creatinine level, indicating that kidney functions were not affected due to the leaching out of silicon from the glass framework (Lai, Garino et al. 2002). On the other hand, there are no reports on the oral acute toxicity profile of barium-doped BGs. Nevertheless, some studies have indicated an increase in kidney weight after 13 weeks of exposure which may be due to the long-term exposure of very high doses of barium salts (Program 1994).

The heart, a muscular organ that pumps blood to the entire body by the rhythmic contraction and relaxation of the involuntary cardiac muscle and blockage of blood flow, leads to myocardial infarction, one of the leading causes of death worldwide (Wu, Huang et al. 2019). Studies have reported that following myocardial infarction, the necrotic tissue releases enzymes like CK-MB after 4-6 h into the blood, which reaches a peak at 24-36 h and then declines rapidly (Grande, Hansen et al. 1982). Thus, estimating the serum CK-MB level is widely established clinically as a diagnostic marker for myocardial cell injury. In the present study, CK-MB level was measured in the serum and is represented in **Figure 4.6E**. Statistical analysis by one-way ANOVA revealed no significant changes in the serum level of CK-MB among the groups ([$F_{(4,29)}$ = 1.511; $p > 0.05$]. This strongly indicates that single-dose oral administration of 45S5 and BaBG did not exhibit any toxic effects on the cardiac muscle, which also substantiates the heart's histoarchitecture **(Figure 4.7)**. Our finding is consistent with the previous report where a 13-week daily administration of barium salts did not exhibit any chemical-related clinical signs of cardiovascular toxicity in the male and female rats (Program 1994). Moreover, it is also reported that 45S5, formulated as elastomeric nanocomposites, are used as cardiac patches *in vivo* without any cytotoxicity (Kargozar,

Hamzehlou et al. 2017). Hence, this study suggests that like 45S5, BaBG is safer without significant toxic effects and can be used for various biomedical applications.

Further, calcium was estimated in the serum at the end of the experimental protocol (shown in **Figure 4.6F)** as it is an important element present in the body, primarily in the bone and teeth, and plays a vital role in various physiological activities like nerve conduction, muscle contraction, blood clotting, and also regulates cell death by apoptosis (Orrenius, Gogvadze et al. 2015). However, an increase in calcium in the serum is reported to play a pathogenic role in various diseases (Verma, Wills et al. 2018). In the present study, statistical analysis by one-way ANOVA revealed no significant changes in the serum level of calcium among the groups ([$F_{(4,29)} = 2.451$; $p > 0.05$]. Therefore, from the results obtained, it can be affirmed that single-dose oral administration of both 45S5 and BaBG did not exhibit any toxic effects on the vital organs.

4.2.1.5. Histological analysis of various organs of BaBG-treated rats during the acute toxicity study

Mostly following the exposure to any toxic exogenous compounds, the primary organs like the brain, heart, liver, kidney, lungs, and spleen get affected due to their metabolic reactions. Hence histopathological studies need to be performed at the end of the experimental protocol. In the present study, the histological sections of the single-dose treated tissues of the experimental rats exhibited intact architecture with no histopathological changes in any of the highly perfused organs, as depicted in **Figure 4.7**. In the brain section of control and BaBG-treated rats, there was no sign of toxicity in the neurons of the cortical region (as shown in **Figure 4.7A)** which corroborates the

organ coefficient of the brain where there were no significant changes observed compared to the control group and 45S5 treated rats (Yu, Li et al. 2013). Likewise, Lai et al. (Liang, Xiang et al. 2018) performed the subchronic oral toxicity study for the silica nanoparticles at a very high dose of 1500 mg/kg b.w. in rats and reported no chemical-induced toxicity histological changes. Further, a previous study also reported that short-term oral administration of $BaCl_2$ did not produce any significant adverse effects in the rats (Borzelleca, Condie Jr et al. 1988). Furthermore, in the liver section of the control and the treatment groups (BaBG and 45S5) at high doses (2000 mg/kg b.w.), there were normal hepatocytes with acidophilic cytoplasm and intact vesicular nucleus radiating from the central vein surrounding the portal tract. Moreover, there were no significant changes in the appearance of sinusoids in the control and the treated group, which confirmed that the test compounds (BaBG) had no toxic effects on the liver. This finding corroborates the liver enzyme level (AST, ALT, and ALP) (**Figure 4.7 A-C**), and previous studies have also reported that 45S5 administration did not produce any histological changes in the internal organs (Lai, Ducheyne et al. 1999). Similarly, Anand et al. (Anand, Lalzawmliana et al. 2019) reported that after the implantation of the mesoporous BG, the liver section displayed healthy hepatic parenchyma with limited cellular infiltration after 45 days.

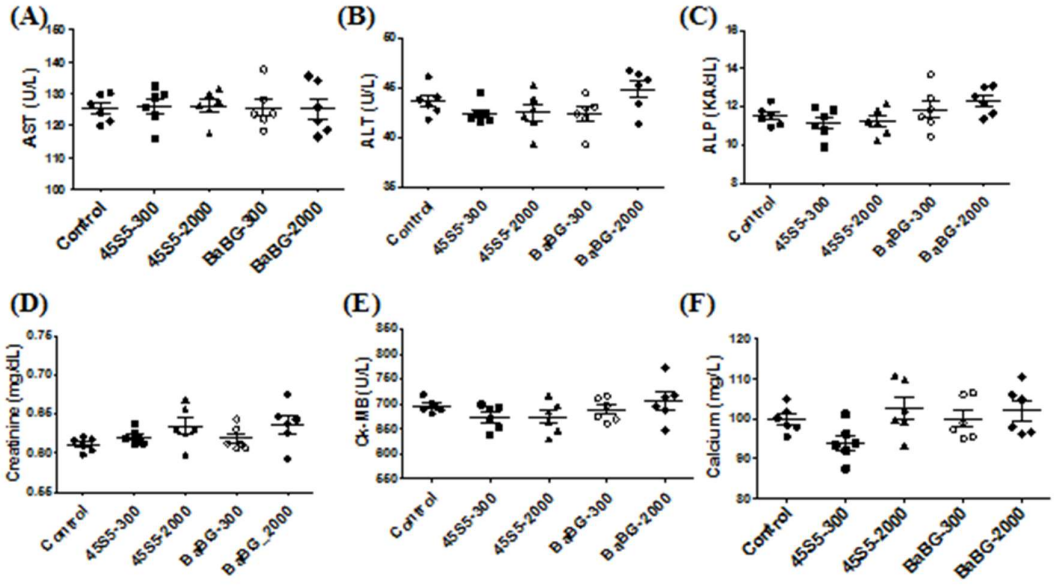

Figure 4.6: Effect of single-dose oral administration of BaBG and 45S5 on serum concentration of AST (A), ALT (B), ALP (C), creatinine (D), CK-MB (E), and calcium (F) at the end of the experimental protocol. All values are in mean ± SD (n=6 female rats/ group). (One-way ANOVA followed by Tukey's multiple comparison post hoc test)

Kidneys are the complex organs that excrete the wastes out of the body, and the hepatocytes have specialized ion channels and transporters that maintain the ionic concentration through their absorption and secretion into the urine. Hence, in the toxicity screening of BGs that leache out ions after coming in contact with the physiological fluid, it is essential to check its effect on the kidneys. At the end of the experimental protocol, the cross-section of the kidney of the control and treatment groups exhibited normal renal tubules in the present study. Moreover, it is also evident from the histological image of the renal cortical sections (shown in **Figure 4.7**) that the control and BaBG-treated groups had the typical structure of the glomeruli, bowman's capsule, and renal corpuscles like in 45S5 treated rats. Even the proximal and distal convulated tubules (PCT and DCT) showed normal architecture with no sign of toxicity which was similarly observed previously by De souza et al. (de Souza, Lopes et al.

2020) in 45S5 bioglass. Besides, fewer studies reported where the intravenous injection of mesoporous bioactive glass nanospheres did not produce any histological anomalies in the treatment groups like the test compounds in our present study, thus confirming the biological safety of BaBG (Sui, Zhong et al. 2016).

The spleen is the largest secondary lymphoid organ that contains two important structures, i.e., red pulp and white pulp. The red pulp is a blood filter that stores iron and removes foreign materials and older RBCs from the blood circulation. The white pulp is reported to contain a large number of lymphocytes that initiate immune responses in the presence of blood-borne antigens when blood flows into the spleen through the splenic artery (Cesta 2006). Thus, during the toxicity screening of any compounds, it is essential to evaluate their effect on the spleen as any compound that enters the systemic circulation after its administration reaches the spleen for elimination by phagocytosis. In the present study, the histological section of the spleen showed an indication of normal splenic tissue following the oral treatment with a single dose of 45S5 and BaBG. The cross-section exhibited a normal appearance of the lymphatic nodules of white pulp, splenic cords of red pulp, and the spleen trabecula. Previously Yang et al. (Yinhua, Jianbo et al. 1993) investigated the effect of barium chloride on the spleen and reported that daily administration of the barium salts for a month in drinking water did not produce any histological abnormalities in the structure of the spleen in the rodent model.

The heart, a muscular organ that pumps oxygenated blood to the body, and damage may lead to ischemic stroke due to reduced blood pumping to the brain. Thus it is essential to assess the effects of the administered compounds (45S5 and BaBG) on the cardiac muscle. In the present study, the photomicrograph of the heart of the control and treated

rats showed the presence of normal myocardial muscle bundles with thin fibro collagenous stroma, which substantiates the unchanged CK-MB level in the serum post-treatment with BaBG and 45S5 (**Figure 4.7E**). Further, the slice of the heart of all the groups showed single, oval, and centrally located nuclei in cardiomyocytes regularly arranged in myofibres. Similar to our observation, Fu et al. (Fu, Liu et al. 2012) examined the acute toxicity effects of silica nanorattle and reported no remarkable histological changes in the heart of the female mice after 14-day continuous administration. Correspondingly, the histological section of the lung of the control and the treated groups also exhibited normal architecture with no sign of edema, hemorrhage, and fibrosis. Moreover, there is the appearance of thinner interstitial matrix with the normal alveolar lumen and no evidence of inflammatory cells. Likewise, Loza et al. (Loza, Föhring et al. 2016) also reported that $BaSO_4$ nanoparticles produce no cytotoxic inflammatory response on the alveolar macrophages. Thus, from the above reports, it can be avowed that the single-dose oral administration of the barium-doped bioactive glass did not impart any toxic effects on the vital organs, and to our knowledge, this is the first time we are reporting the single-dose oral acute toxicity study of barium-doped BGs in rats.

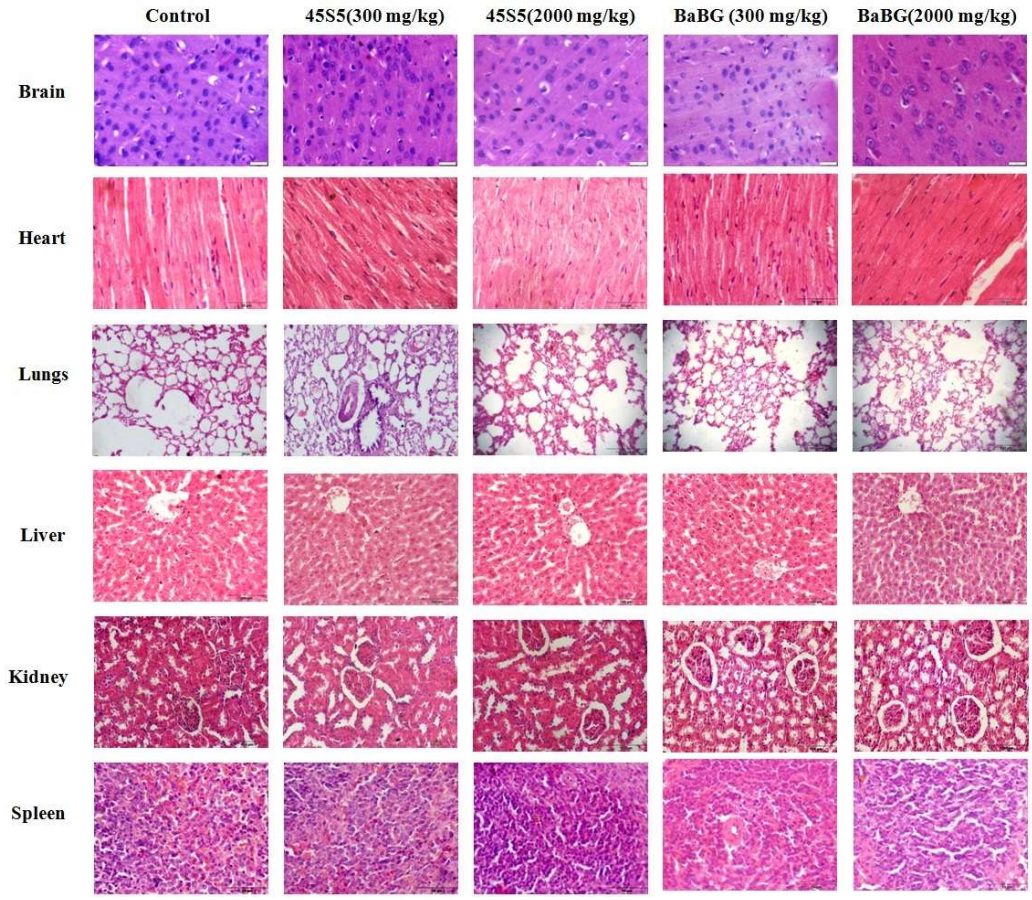

Figure 4.7: Effect of single-dose oral administration of BaBG and 45S5 (dose of 300 and 2000 mg/kg b.w.) on highly perfused organs like brain, heart, lung, liver, kidney, and spleen tissue stained with hematoxylin and eosin.

4.2.2. Sub-acute toxicity study

4.2.2.1. General observations

The animals were observed regularly following the repeated dose 28-day oral administration of 45S5 and BaBG (50, 500, and 1000 mg/kg b.w.) and the test compounds did not produce any treatment-related mortality at any dose tested during the experimental protocol. Further, no treatment-related signs of toxicity were observed, like changes in the skin, fur, and mucous membrane or any behavioral distress like salivation, convulsions, and tremors **(Table 4.2)** as previously observed (Program

1994). Moreover, the experimental rats did not exhibit any changes in the respiratory, circulatory, and somatosensory activities. Similar to our observations, a previous study reported no local or systemic toxicity in rodents following oral administration of silica nanoparticles at a dose of 1000 mg/kg b.w. (Cabellos, Gimeno-Benito et al. 2020). Besides, the toxicology study of barium chloride performed in rats reported did not produce any barium-related neurobiological toxicity (Program 1994). Thus, it can be affirmed that BaBG is safer to be used systemically for various biomedical applications like 45S5.

Table 4.2: General observation and behavioral analysis after repeated 28-day administration of 45S5 and BaBG (50, 500, and 1000 mg/kg b.w.) in rats (n=10 rats/group).

Observation	Control	45S5(50 mg/kg)	45S5 (500 mg/kg)	45S5 (1000 mg/kg)	BaBG (50 mg/kg)	BaBG (500 mg/kg)	BaBG (1000 mg/kg)
Changes in skin, fur, eyes, and mucous membrane	NC	NC	NC	NC	NC	NC	NC
Salivation	NC	NC	NC	NC	NC	NC	NC
Diarrhea	NO	NO	NO	NO	NO	NO	NO
Tonic-clonic movements	NO	NO	NO	NO	NO	NO	NO
Stereotypies (excessive grooming or repetitive circling)	NO	NO	NO	NO	NO	NO	NO
Behavior pattern and somatosensory activity	N	N	N	N	N	N	N
Sensory reactivity to auditory, visual, and proprioceptive stimuli)	N	N	N	N	N	N	N
Color and consistency of faces	N	N	N	N	N	N	N
Breathing pattern	N	N	N	N	N	N	N
Grip strength	NO	NO	NO	NO	NO	NO	NO

Note: N-Normal, NO-Not observed, and NC-No change

4.2.2.2. Effect of BaBG on the body weight and food consumption during the sub-acute toxicity study

In the sub-acute toxicity study, the effect of oral administration of 45S5 and BaBG at different doses (50, 500, and 1000 mg/kg b.w.) on body weight and food consumption is shown in **Figure 4.8.** Statistical analysis by two-way ANOVA revealed no significant changes in the body weight and food consumption among the groups ($p > 0.05$) which confirms that the test compounds did not affect the normal physiological functioning of the internal organs (Buesen, Landsiedel et al. 2014). Similarly, there was no observed significant change in the water consumption of the treated rats between all the groups recorded throughout the experimental protocol (data not shown), verifying the safety of BaBG like the 45S5.

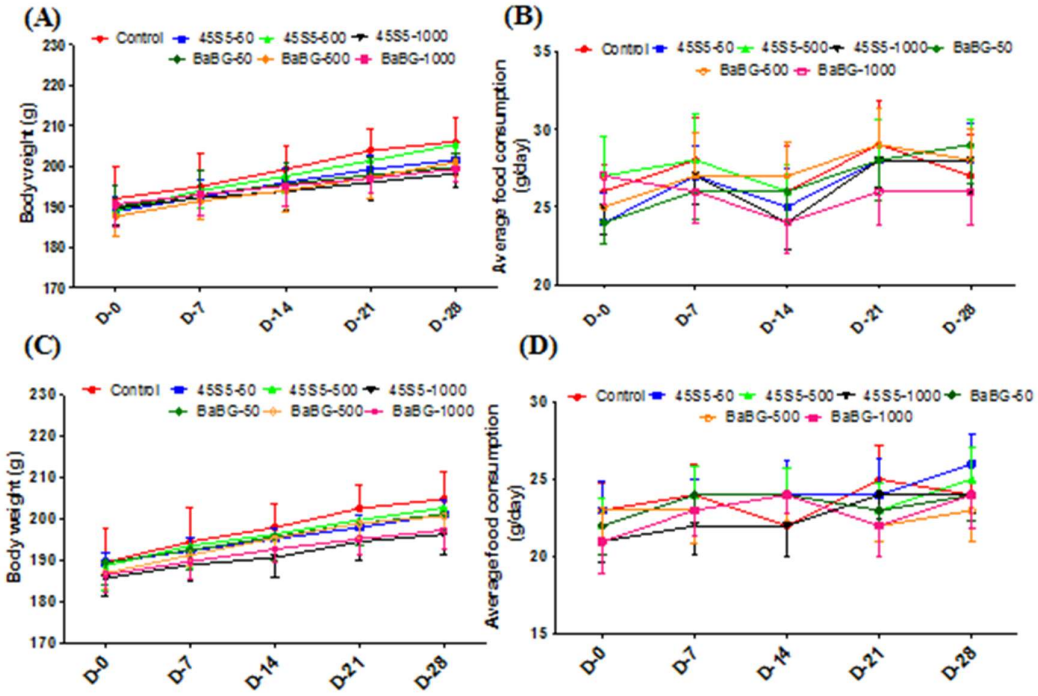

Figure 4.8: Effect of repeated-dose 28 days oral administration of BaBG and 45S5 (50, 500, and 1000 mg/kg) on body weight of male (A) and female (C) rats along with their food intake (B and D, respectively) during the experimental protocol. All values are in mean ± SD (n=5 rats/ group). (Two-way ANOVA followed by Bonferroni post -hoc test).

4.2.2.3. Effect of BaBG on hematological parameter during the sub-acute toxicity study

The hemotopoietic system is an important system of the body involved in producing the cellular blood components, and alteration in the hematological parameters indicates drug-induced toxicity in humans and animals (Buesen, Landsiedel et al. 2014). In the present study, the effects of oral administration of BaBG on the blood cells (RBC, WBC, platelets) and its associated parameters in male and female rats were evaluated and compared to the 45S5 treated rats (data not shown**)**. Statistical analysis by two-way ANOVA showed no significant differences in the blood parameters ($p > 0.05$) like RBC, Hb, HCT, MCH, MCHC, RDW, PLT, and WBC among the groups in treated rats (both male and female) when compared to the control group. Thus, it is evident that the test compounds did not interfere with hematopoiesis and leucopoiesis in the male and female experimental rats. This finding is supported by previous reports, where the oral administration of silica nanoparticles in rodents, up to a dose of 1000 mg/kg b.w. did not exhibit any dose-related adverse effects on blood cells (Yun, Kim et al. 2015). Furthermore, Majumdar et al. (Majumdar, Hira et al. 2021) reported the biocompatibility of barium-doped BG in an *in-vitro* cell-line-based study performed in a human leukemic cell line (K652). Hence, these results ascertain that BaBG does not impair erythropoiesis or have any toxic effects on the hematopoietic centers (kidney/spleen) that corroborate with the histological analysis (**Figure 4.14 & 4.15**).

4.2.2.4. Effect of BaBG on organ-coefficient during the sub-acute toxicity study

A comparative study of the effects of 45S5 and BaBG on the organ coefficient of male and female rats, 28 days post oral administration of the test substances (dose 50, 500, and 1000 mg/kg b.w.) is shown in **Table 4.3**. Statistical analysis by one-way ANOVA

showed no significant changes in the organ coefficient of the brain, heart, lungs, liver, kidneys, and spleen among the groups ($p > 0.05$) compared to the control rats. This strongly indicates that the oral administration of both 45S5 and BaBG did not disturb the normal functioning of the vital organs, hence confirming the safety profile of the BGs (45S5 and BaBG). This result also corroborates the no significant changes in the treated rat's body weight (**Figure 4.8**) and hematological parameters. Moreover, in support of our findings, previously, it has been reported that the 28-day oral exposure of $BaSO_4$ nanoparticles at a high dose of 1000 mg/kg did not exhibit any changes in the absolute organ weight of highly perfused organs of both male and female rats (Buesen, Landsiedel et al. 2014). Furthermore, Cabellos et al. (Cabellos, Gimeno-Benito et al. 2020) also reported that the short-term oral administration of mesoporous silica in mice did not significantly change body weight, hematology, and relative organ weights. Thus, we may conclude that doping of barium in the BG framework did not affect the safety profile of BG and BaBG can be considered to be safer for future therapeutic applications without any significant toxic effects.

Table 4.3: The organ coefficient of male and female rats after repeated 28 days oral administration of BaBG and 45S5 (50, 500, and 1000 mg/kg b.w.)

	Brain	Heart	Lungs	Liver	Kidneys	Spleen
(i) Male rats						
Control	0.946 ± 0.098	0.482 ± 0.0381	0.614 ± 0.056	3.371 ± 0.425	0.629 ± 0.038	0.373 ± 0.031
$45S5_{50}$	0.873 ± 0.062	0.471 ± 0.0547	0.602 ± 0.046	3.28 ± 0.334	0.582 ± 0.066	0.369 ± 0.040
$45S5_{500}$	0.834 ± 0.085	0.479 ± 0.0448	0.617 ± 0.089	3.572 ± 0.228	0.561 ± 0.041	0.379 ± 0.043
$45S5_{1000}$	0.915 ± 0.082	0.475 ± 0.0562	0.626 ± 0.084	3.723 ± 0.308	0.644 ± 0.048	0.381 ± 0.037
$BaBG_{50}$	0.979 ± 0.092	0.496 ± 0.0432	0.556 ± 0.092	3.421 ± 0.417	0.593 ± 0.075	0.346 ± 0.047
$BaBG_{500}$	0.861 ± 0.042	0.463 ± 0.0485	0.612 ± 0.063	3.521 ± 0.251	0.642 ± 0.080	0.352 ± 0.028
$BaBG_{1000}$	0.938 ± 0.079	0.476 ± 0.0703	0.632 ± 0.131	3.833 ± 0.413	0.613 ± 0.053	0.377 ± 0.036
(ii) Female rats						
Control	0.881 ± 0.011	0.463 ± 0.0235	0.581 ± 0.072	3.188 ± 0.278	0.616 ± 0.0731	0.358 ± 0.068
$45S5_{50}$	0.823 ± 0.063	0.465 ± 0.0395	0.597 ± 0.058	3.243 ± 0.388	0.611 ± 0.0636	0.344 ± 0.074
$45S5_{500}$	0.911 ± 0.098	0.438 ± 0.0162	0.604 ± 0.084	3.302 ± 0.315	0.609 ± 0.0427	0.352 ± 0.049
$45S5_{1000}$	0.904 ± 0.071	0.446 ± 0.0278	0.636 ± 0.048	3.397 ± 0.451	0.597 ± 0.0642	0.361 ± 0.052
$BaBG_{50}$	0.893 ± 0.088	0.475 ± 0.0473	0.601 ± 0.051	3.148 ± 0.294	0.612 ± 0.0209	0.353 ± 0.032
$BaBG_{500}$	0.923 ± 0.145	0.435 ± 0.0285	0.614 ± 0.044	3.177 ± 0.416	0.617 ± 0.0562	0.358 ± 0.061
$BaBG_{1000}$	0.906 ± 0.064	0.445 ± 0.0224	0.624 ± 0.068	3.355 ± 0.253	0.619 ± 0.0438	0.364 ± 0.047

All values are in mean ± SD (n=5 rats/ group). (One-way ANOVA followed by Tukey's multiple comparison post-hoc test)

4.2.2.5. Effect of BaBG on various enzymes during the sub-acute toxicity study

The biochemical analyses of various enzymes in serum are generally performed during the sub-acute toxicity study to monitor the effect of test compounds on the functioning of various organs. The liver, an essential metabolic clearance organ, acts as a biological filtration unit that sequesters most of the administered nanoparticles from the bloodstream. Thus, upon repeated administration of any compounds, their effects on liver functioning need to be assessed. In the present study, the effects of BaBG on hepatic functioning were evaluated by measuring the level of AST, ALT, and ALP in serum (represented in **Table 4.4)** and compared with the 45S5 treated rats. Statistical analysis by one-way ANOVA exhibited no significant differences in the level of AST, ALT, and ALP among the groups ($p > 0.05$) in 45S5-treated male and female rats when

compared to the control. This result substantiates the histological analysis of the liver section of 45S5 treated rats that showed intact architecture with normal hepatocytes (**Figure 4.11)**. Similar to our observation, it has been reported earlier that silica nanoparticles also did not cause any substance-induced toxicity, as evident from the unchanged level of liver enzymes in rodents (Yun, Kim et al. 2015, Cabellos, Gimeno-Benito et al. 2020). Likewise, in the case of BaBG, there were no significant differences in the level of AST, ALT, and ALP among the groups ($p > 0.05$) in male rats at all the doses tested. Thus, this confirms that BaBG did not lead to hepatotoxicity after their repeated oral administration in male rats (Program 1994). However, statistical analysis by one-way ANOVA showed a significant increase in the level of ALT among the groups ([$F_{(6,34)} = 3.436$; $p < 0.05$] when compared to the female control rats. This gender-specific effect may be because the liver is a sexually dimorphic organ responsive to sex hormones (Buzzetti, Parikh et al. 2017).

Further, creatinine, a metabolic byproduct generated in the body to maintain a continuous supply of energy to the muscles, and these nitrogenous waste products are eliminated by the kidneys. Thus, measuring creatinine level is considered an advocated index to determine the efficiency of kidneys to eliminate metal ions and uremic toxins. In the present study, there were no significant differences in the serum creatinine level of BaBG-treated rats compared to control rats and 45S5-treated group ($p > 0.05$). This finding is supported by another study where the leaching of silicon from 45S5 did not exhibit any significant changes in the creatinine level (Lai, Garino et al. 2002), indicating that kidney functions were not affected in the experimental animals. Therefore, we may conclude that repeated-dose oral administration of BaBG did not affect the renal functions in male and female rats.

CK-MB, an important clinically used diagnostic marker for cardiac muscle damage, and in the present study, statistical analysis by one-way ANOVA exhibited no significant differences in its level among the groups ($p > 0.05$). This confirms that the synthesized inorganic biomaterial (BaBG) is not cardiotoxic which substantiates the normal cardiac architecture observed after repeated-dose oral administration of BaBG (**Figure 4.12**). Furthermore, calcium, an essential element present in the body, helps in various physiological functions, and administration of BaBG did not exhibit any significant changes in the serum calcium level in male and female rats like 45S5. Thus, it can be affirmed that BGs did not affect the calcium homeostasis post-oral administration of BaBG. Hence, BGs can be considered an advanced biomaterial for effective treatment purposes with no toxic effects on the highly perfused vital organs.

Table 4.4: The effect of repeated-dose 28 days oral administration of BaBG and 45S5 (50, 500, and 1000 mg/kg b.w.) on serum concentration of calcium, creatinine, CK-MB, ALP, ALT, and AST of male and female rats at the end of the experimental protocol.

Enzymes	ALP (KA/dL)	ALT (U/L)	AST (U/L)	Creatinine (md/dL)	Calcium (mg/L)	CK-MB (U/L)
(A) Male rats						
Control	12.231 ± 1.56	43.592 ± 2.453	125.893 ± 5.940	0.715 ± 0.0805	102.431 ± 6.689	696.204± 13.152
$45S5_{50}$	12.729 ± 1.799	44.220 ± 4.003	129.471 ± 4.992	0.708 ± 0.0457	105.202 ± 3.201	714.315 ± 19.237
$45S5_{500}$	12.817 ± 0.948	46.013 ± 3.177	132.760 ± 7.801	0.742 ± 0.0931	106.11 ± 5.316	722.082 ± 17.438
$45S5_{1000}$	12.173 ± 0.656	46.946 ± 1.351	135.616 ± 5.073	0.757 ± 0.069	109.887 ± 5.912	729.734 ± 20.123
$BaBG_{50}$	12.652 ± 1.648	42.289 ± 1.705	127.466 ± 4.831	0.698 ± 0.0742	103.461 ± 8.236	705.126 ± 20.231
$BaBG_{500}$	13.143 ± 0.991	45.501 ± 2.927	130.037 ± 2.316	0.718 ± 0.0624	104.115 ± 7.103	713.441 ± 14.284
$BaBG_{1000}$	13.094 ± 1.401	43.747 ± 2.461	134.188 ± 4.668	0.732 ± 0.0873	106.783 ± 9.592	718.189 ± 13.567
(B) Female rats						
Control	12.106 ± 1.248	41.127 ± 3.176	120.983 ± 3.167	0.694 ± 0.0703	105.112 ± 8.117	682.124 ± 15.462
$45S5_{50}$	12.004 ± 0.972	40.826 ± 2.851	122.015 ± 1.445	0.685 ± 0.0814	107.347 ± 7.236	684.573 ± 16.192
$45S5_{500}$	12.49 ± 1.521	44.213 ± 2.127	125.747 ± 4.448	0.702 ± 0.0469	104. 231 ± 9.297	689.403 ± 17.703
$45S5_{1000}$	11.788 ± 1.362	46.034 ± 1.186	127.105 ± 6.149	0.722 ± 0.0602	109. 678 ± 6.167	704. 865 ± 19.042
$BaBG_{50}$	12.267 ± 0.813	42.267 ± 1.935	123.028 ± 4.002	0.690 ± 0.0832	105.526 ± 7.205	691.904 ± 11.984
$BaBG_{500}$	12.089 ± 1.308	43.139 ± 4.061	126.991 ± 3.141	0.711 ± 0.0790	109.561 ± 3.648	702.706 ± 19.301
$BaBG_{1000}$	12.105 ± 0.847	$47.025 \pm 3.514^{a,b}$	127.191 ± 2.203	0.719 ± 0.0822	113.812 ± 6.875	709.911 ± 20.443

All values are in mean ± SD (n=5 rats/ group). $^{a}p<0.05$ and $^{b}p < 0.05$ compared to control and $45S5_{50}$ respectively. (One-way ANOVA followed by Tukey's multiple comparison post-hoc tes

4.2.2.6. Effect of BaBG on the neurobehavioral activity during sub-acute toxicity study

The central nervous system (CNS) is an integral part of the body that controls most of the bodily functions like movement, sensations, memory, speech and appetite, and slight alteration in the nervous system can profoundly affect other body activities. Usually, the phenotypic changes in motor activity are considered the first sign of neurotoxicity in animals, and there is very little information available on the neurotoxic effects of BGs. Thus, in the present study, we have also tested the effect of oral administration of BaBG on the neurobehavioral aspects in male and female rats that directly corroborates with their effects on CNS. Statistical analysis by two-way ANOVA showed no significant differences in the grip strength, locomotor activity, retention time on the rotarod, rearing, grooming, total distance travelled, and time spent in the central space of OFT ($p > 0.05$) among groups in male and female rats compared to control (**Figure 4.7**). Evaluating motor coordination or muscle strength is essential in experimental animals during toxicity testing of newly synthesized compounds. It is generally evaluated by testing the ability of the rats to retain on the revolving rod at a fixed rpm in the rotarod apparatus (Prajapati, Garabadu et al. 2017). In the present study, it was observed that BaBG did not impair the muscle coordination like 45S5 after the sub-acute exposure in both male and female rats during the rota-rod test (data not shown); hence confirming no neurotoxic effects of 45S5 and BaBG as the loss of muscle coordination is considered as an index of neurotoxicity. Similar to our observation, Tuusa et al. (Tuusa, Peltola et al. 2008) also reported that the BG fiber, did not produce any neurological damage in the rabbits when used for the calvarial bone defect,. Furthermore, the change in neuromuscular strength is as an important indicator

of neurotoxicity and was evaluated using the grip strength test. It was observed that repeated-dose 28-days oral administration of both 45S5 and BaBG did not produce any significant changes in the grip strength score of both male and female rats at all the doses tested (data not shown). Previously, studies have also reported that oral exposure to SiO_2 and $BaSO_4$ nanomaterials did not produce any substance-related adverse effects (Buesen, Landsiedel et al. 2014). Together with the rota-rod test observation, these findings confirm that BaBG did not produce any neurobehavioral deficits in the treated rats of both sexes.

Moreover, the neurological safety profile of BaBG was further evaluated by analyzing their effects on spontaneous locomotor activity using an actophotometer. Spontaneous locomotor activity is also considered an index of wakefulness or alertness of mental activity, and changes in it can be observed as motor dysfunction (Prajapati, Garabadu et al. 2017). In the present study, there was no significant change in the locomotor activity of the treated rats at all doses tested compared to the control rats and 45S5-treated rats ($p > 0.05$) (data not shown). Similarly, in the OFT, the total distance covered by the experimental rats (BaBG) indicates their voluntary locomotor activity, and statistical analysis by two-way ANOVA showed no significant changes in it among the groups compared to control and 45S5. Thus, the total distance covered by the male and female rats (track plot shown in **Figure 4.9**) corroborates the spontaneous locomotor activity observed in the actophotometer test.

Further, anxiety induced by substances can be evaluated by the increase in grooming behavior and aversion from the central area in OFT (Bronstein 1972), and in the present study, sub-acute oral administration of BaBG did not produce anxiety-like symptoms in both male and female rats. There were no significant differences in the grooming

behavior and time spent in the central square among the groups compared to the control animals and 45S5 treated rats in both male and female rats. Additionally, rearing (standing on hind limbs) in a novel environment is an important parameter that signifies learning and memory (Lever, Burton et al. 2006) were not affected by BaBG. Hence, based on the neurobehavioral studies, the neurological safety profile of BaBG as well as 45S5 can be affirmed. To the best of our knowledge, this is the first time the effect of inorganic biomaterials (45S5 and BaBG) on neurobehavioral paradigms has been studied preclinically.

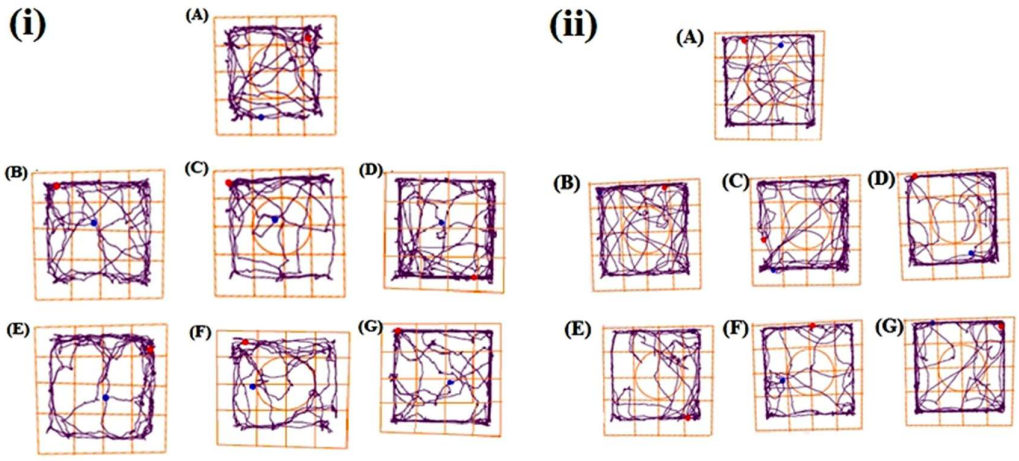

Figure 4.9: Schematic representation of the track plot of (i) male and (ii) female rats respectively divided into following groups: control (A), 45S5 (B, C, D; dose: 50, 500, and 1000 mg/kg b.w. respectively), and BaBG (E, F, G; dose: 50, 500, and 1000 mg/kg b.w. respectively) recorded during 5 min test sessions on day 28 (ANYMAZE).

4.2.3.7. Histological analysis of various organs of BaBG treated rats during the sub-acute toxicity study

Nanomaterials are widely used in biomedicine; however, most inorganic biomaterials tend to accumulate in the liver and spleen with delayed clearance. Hence, histopathological analyses were performed in vital organs like the brain, lungs, heart, spleen, and metabolic and excretory organs, such as the liver and kidneys. In the present

study, the histological section of brain tissues of repeated-dose treated experimental rats exhibited intact architecture with no histopathological changes, as depicted in **Figure 4.10**. The photomicrographs exhibited pyramidal cells with basophilic cytoplasm and a larger nucleus. Furthermore, there was the presence of granular cells having large vesicular nuclei as observed similarly in the control group. Thus, it can be affirmed that BaBG had no adverse effect on the brain which was also substantiated from the neuro-behavioral studies performed during the sub-acute toxicity study. Likewise, light microscopic examination of the lungs of the control and treated groups (BaBG and 45S5) exhibited oval-shaped alveoli with very thin epithelial walls surrounded by capillaries (as shown in **Figure 4.11**) that aid in gaseous exchange. Further, no edema or alveolar hemorrhage was observed without any sign of infiltration of the inflammatory cells in the alveolar cavities of both 45S5 and BaBG-treated groups. Besides, 28-days repeated dose oral administration of BaBG also did not impart any toxic effects on the highly perfused organs like the heart, as evident from the normal histological structure of the cardiac muscle (**Figure 4.12)** (Fu, Liu et al. 2012). Moreover, **Figure 4.12** exhibited the presence of oval nuclei without any granular or vacuolar degeneration of the myofibrils. Hence, it can be confirmed from the histopathological studies that BaBG did not impart any toxic effects on the vital organs, which acts as a supporting piece of evidence for biochemical and hematological observations.

In addition, the liver is a metabolic organ essential for the metabolism of drugs and may cause infiltration of leukocytes if the accumulated nanoparticles are not excreted effectively (Sui, Zhong et al. 2016). Thus in the present sub-acute toxicity study, the effects of BGs on the microscopic structure of the liver were analyzed. The 45S5-treated

male and female rats showed normal hepatocytes with intact nuclei arranged in the form of branched lobules embedded in connective tissue without any congestion of the central vein or dilation of the sinusoid (**Figure 4.13**). This corroborates with the serum enzyme level of hepatic markers, i.e., AST, ALT, and ALP (**Table 4.4**), and thus indicates the non-toxic nature of 45S5 on hepatocytes (de Souza, Lopes et al. 2020) as similarly observed in BaBG treated male rats (Buesen, Landsiedel et al. 2014). However, in the BaBG-treated female rats, there was very mild lymphoid infiltration in the portal areas without any sinusoid dilation or changes in the structure of hepatocytes.

Kidneys are the filtering organs that remove the wastes (toxins) from the body; hence they are susceptible to various toxicants (Krishna, Jaiswal et al. 2020). In the sub-acute toxicity study of BaBG, there were no substance-induced histological anomalies observed (**Figure 4.14**) as in the case of 45S5 treated rats (de Souza, Lopes et al. 2020). The treatment groups (45S5 and BaBG) and the control animals of both sexes exhibited normal histological architecture of the glomeruli and Bowman's capsule. Further, the PCT and DCT were intact without any sign of renal toxicity in all groups, supported by the biochemical parameter (creatinine), an important indicator of renal function. Similarly, there were no abnormal histopathological changes in the splenic tissues post-repeated dose administration of 45S5 and BaBG in male and female rats compared to the control rats **(Figure 4.18)** (Yinhua, Jianbo et al. 1993). Therefore, we can deduce that BaBG are non-toxic similar to 45S5 and do not affect the functioning of vital organs.

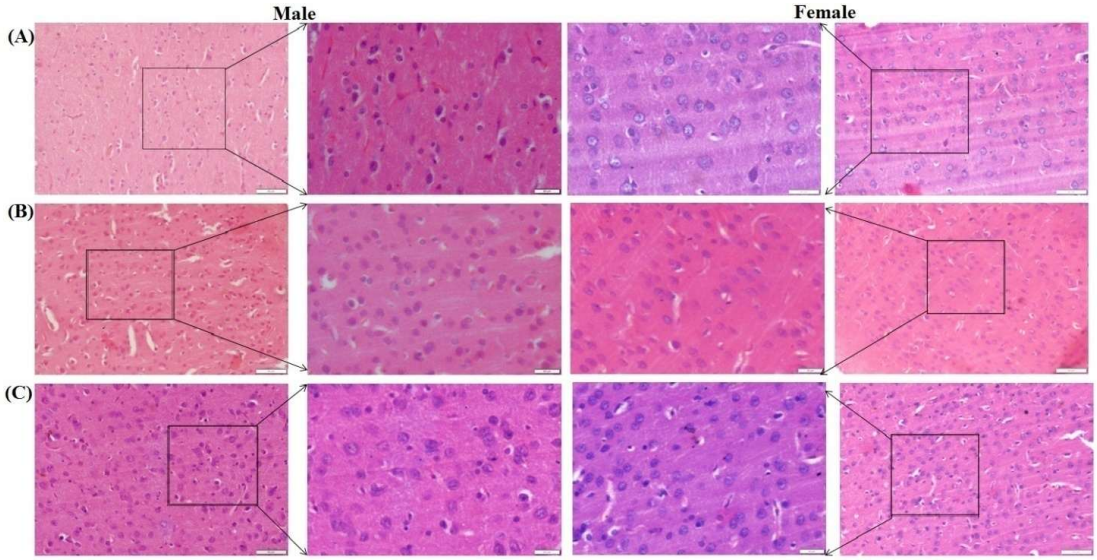

Figure 4.10: Histological analyses of the brain tissue of control (A), 45S5, and BaBG (B and C respectively; dose: 1000 mg/kg b.w.) treated male and female rats after repeated 28 days oral administation. Bar: 50 µm and 20 µm. Hematoxylin and eosin staining.

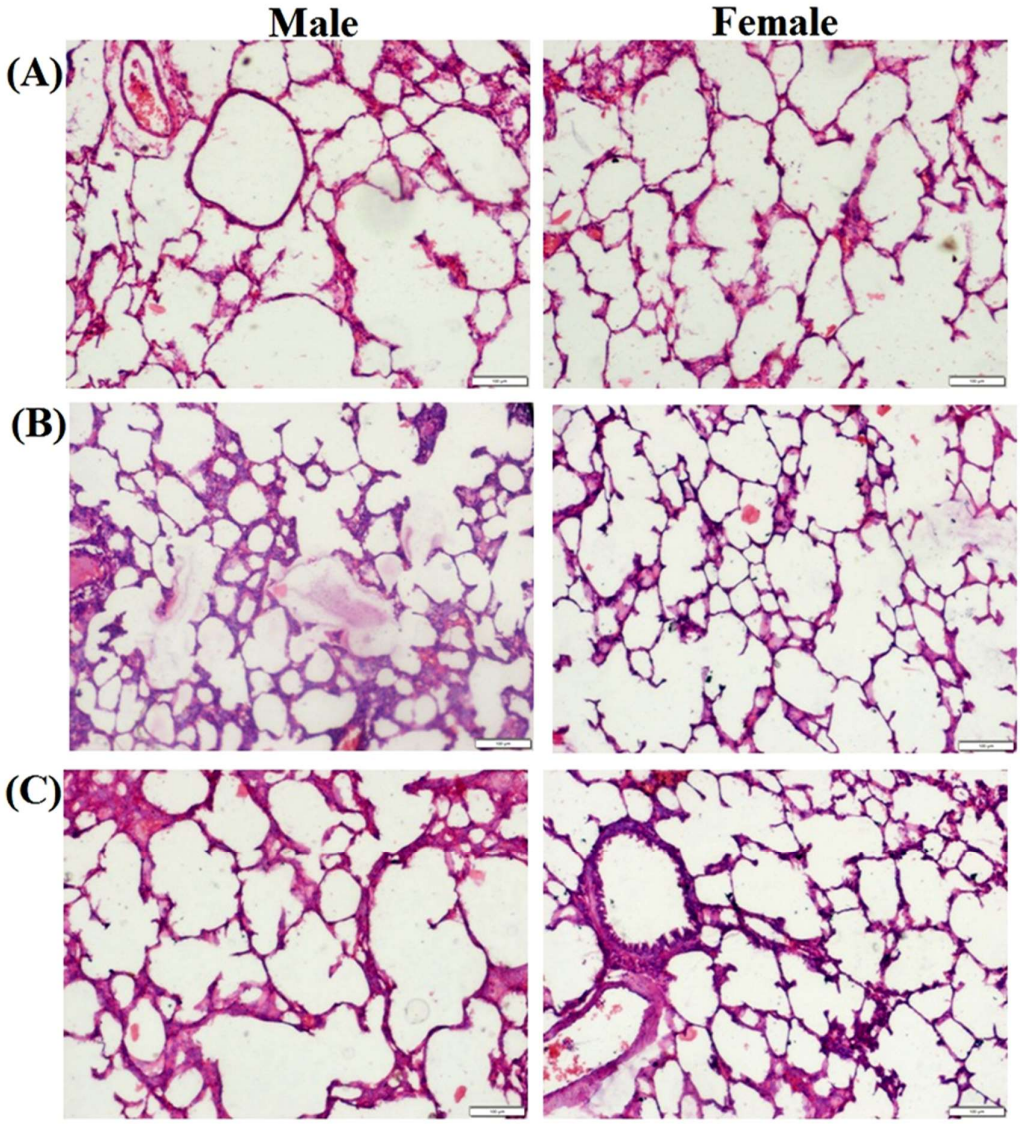

Figure 4.11: Histological analyses of the lungs of control (A), 45S5, and BaBG (B and C respectively; dose: 1000 mg/kg b.w.) treated male and female rats in subacute toxicity study. 45S5 and BaBG treated rats exhibited normal appearance of alveoli with thin epithelial walls surrounded by capillaries similar to the control rats. Edema or alveolar hemorrhage in the alveolar cavities was also not observed in the treatment groups. Bar: 100 μm. Hematoxylin and eosin staining.

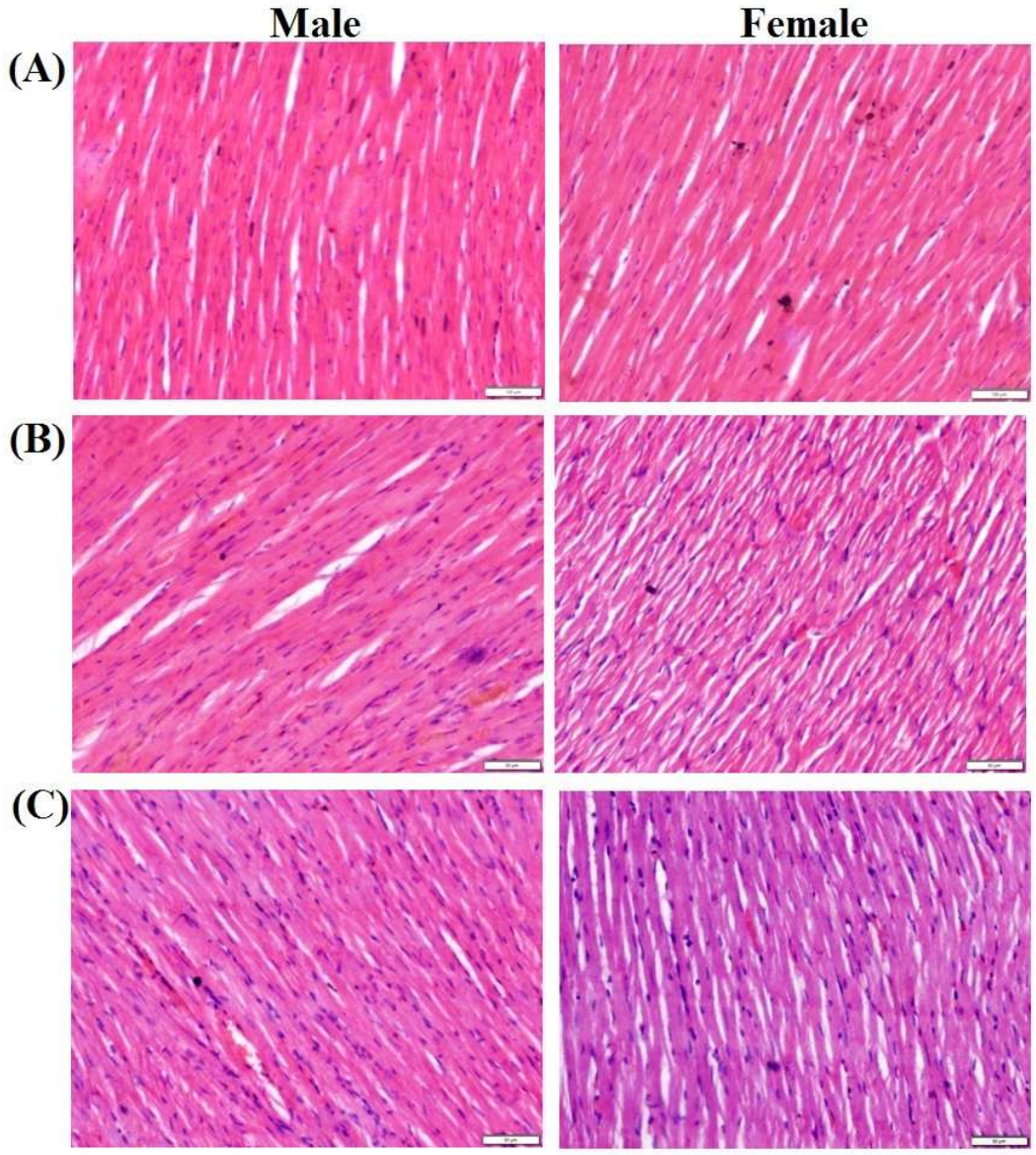

Figure 4.12: Histological analyses of the heart of control (A), 45S5, and BaBG (B and C respectively; dose: 1000 mg/kg b.w.) treated male and female rats. The heart of 45S5 and BaBG treated rats exhibited normal morphology with oval and centrally located nuclei in cardiomyocytes regularly arranged in myofibres. No vacuolar degeneration of the myofibrils was observed in 455S5 and BaBG rats. Bar: 50 µm. Hematoxylin and eosin staining.

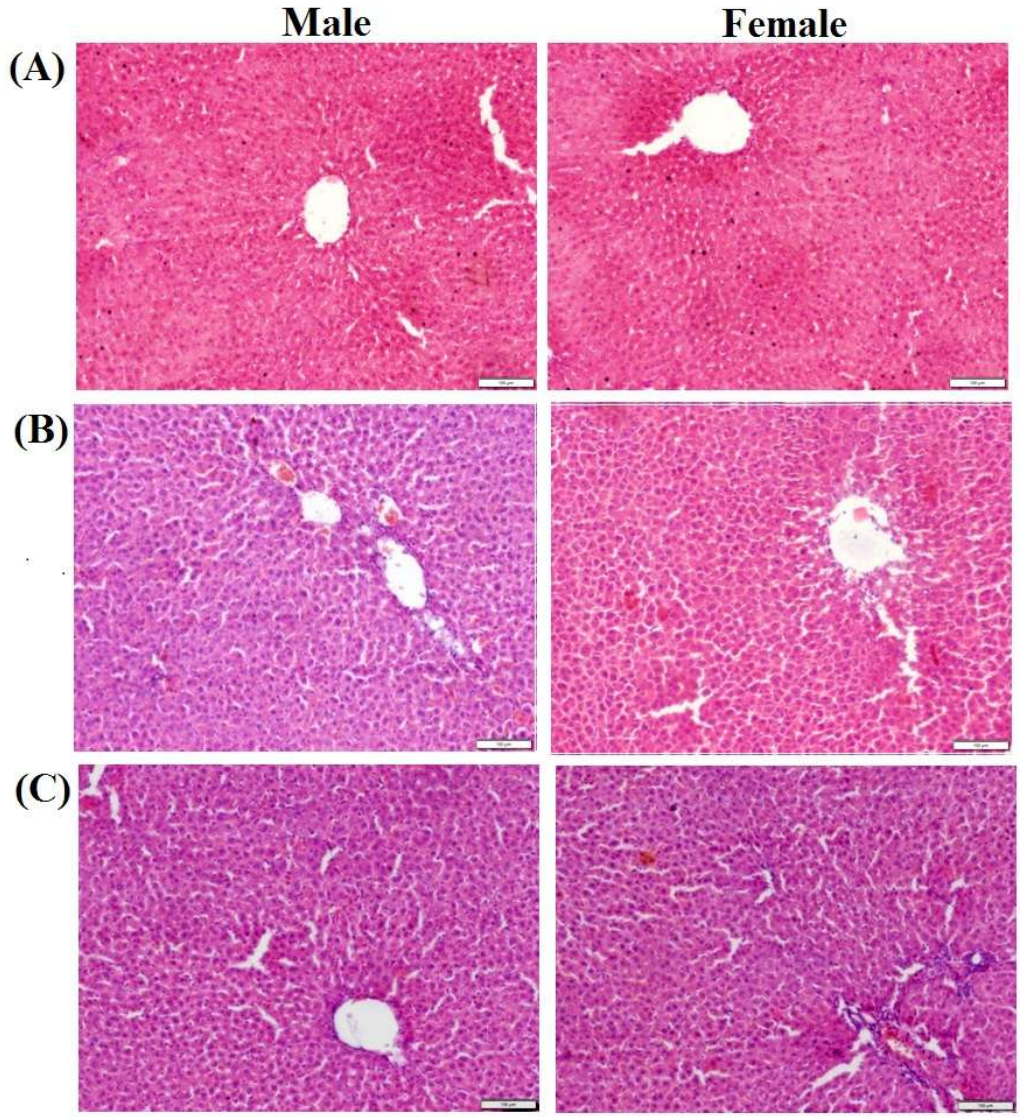

Figure 4.13: Effect of repeated-dose 28 day oral administation of BaBG and 45S5 on microstructure of liver (dose: 1000 mg/kg b.w.) in male and female rats. (A) Control and (B) 45S5 treated male and female rats exhibited normal hepatocytes with intact vesicular nucleus radiating from the central vein surrounding the portal tract. No congestion of the central vein or dilation of the sinusoid was observed. (C) The female BaBG treated rats showed mild lymphoid infiltration in the portal areas without any sinusoid dilation. Bar: 100 μm. Hematoxylin and eosin staining.

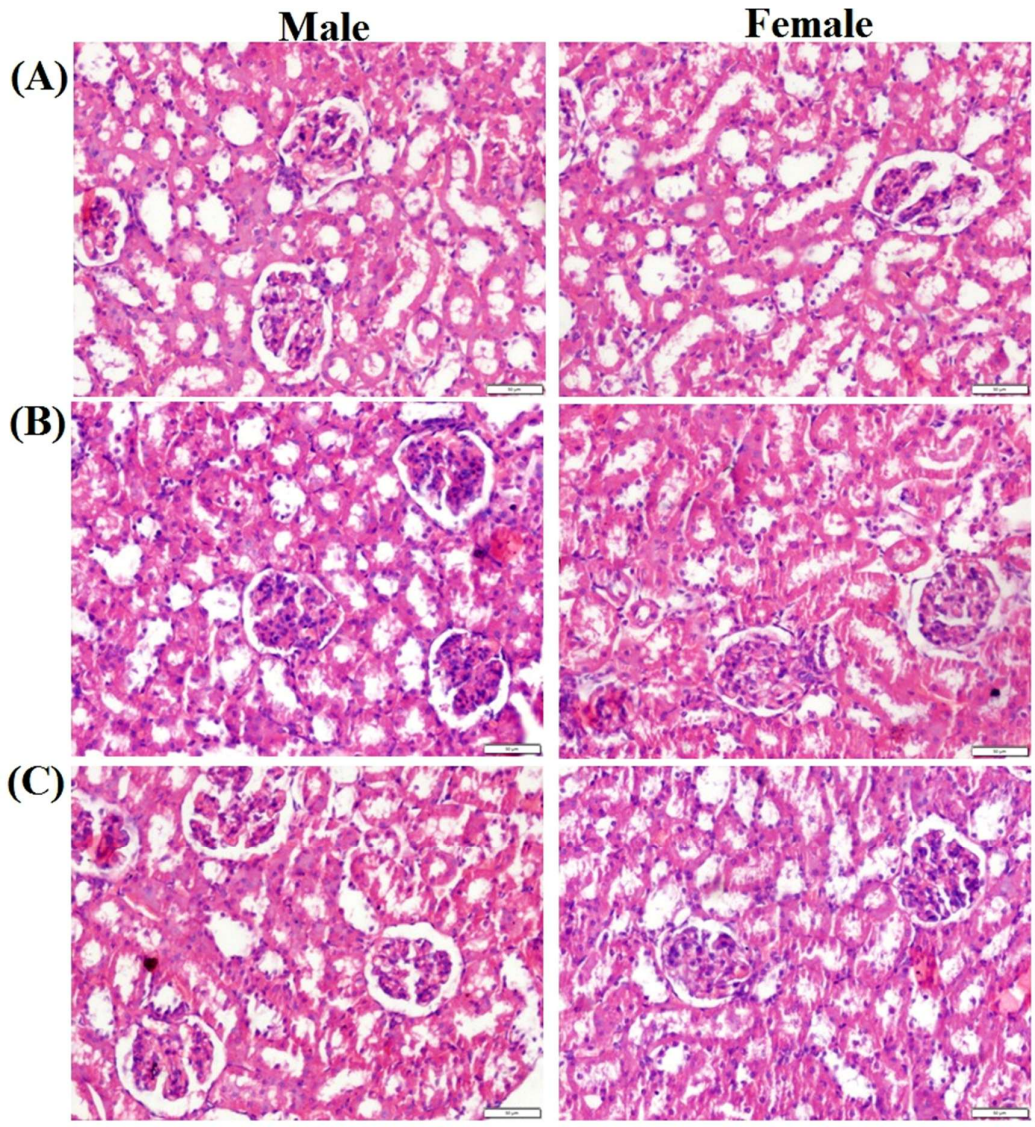

Figure 4.14: Effect of repeated-dose 28 day oral administation of BaBG and 45S5 on microstructures of kidney (dose: 1000 mg/kg b.w.) in male and female rats. The cross-section of (A) control, (B) 45S5, and (C) BaBG treated male and female rats exhibited normal architecture of glomeruli, bowman's capsule, and renal corpuscles with intact proximal and distal convulated tubules. Bar: 50 μm. Hematoxylin and eosin staining.

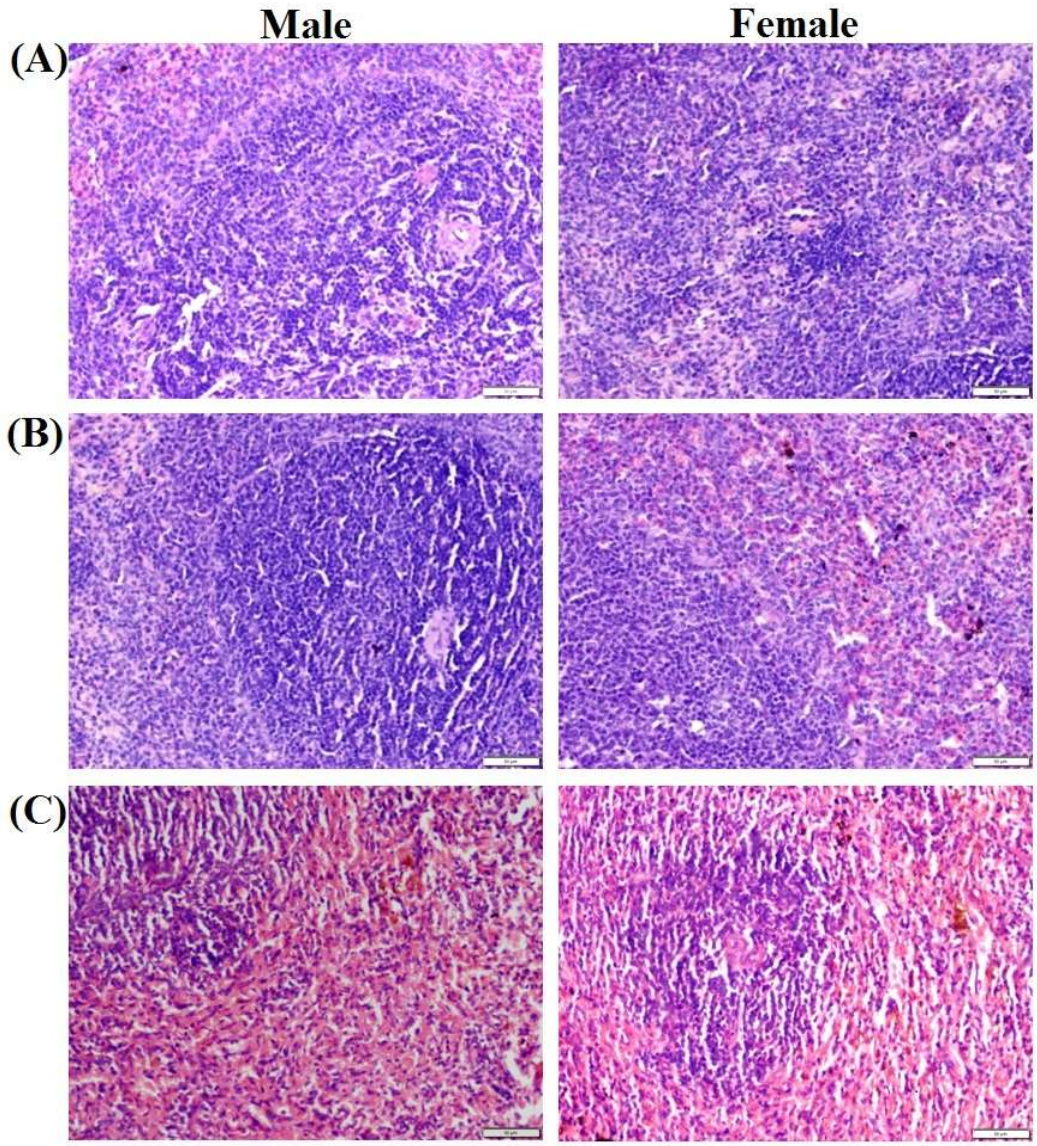

Figure 4.15: Effect of repeated-dose oral administration of BaBG and 45S5 (dose: 1000 mg/kg b.w.) on spleen of male and female rats. The cross-section of (A) control, (B) 45S5, and (C) BaBG treated male and female rats showed normal appearance of the lymphatic nodules of white pulp, splenic cords of red pulp, and the spleen trabecula. Bar: 50 µm. Hematoxylin and eosin staining.

4.3. Summary

In the current study, the acute and sub-acute toxicity of BaBG was evaluated orally in rats according to the OECD guidelines and compared to 45S5 treated rats. There was no observed mortality without any signs of toxicity during the acute toxicity study, and the LD_{50} of both the BGs was more than 2000 mg/kg b.w. Further, the organ coefficients and the biochemical parameters (AST, ALT, ALP, creatinine, and CK-MB) revealed no possible toxic effects of BaBG on the functioning of vital organs. Similarly, the safety profile of BaBG was further confirmed by the hematological parameters and histological examinations during the sub-acute toxicity study. Moreover, the neurological safety profile of BaBG was also established from the neurobehavioral studies as there were no changes in muscle coordination, spontaneous locomotion, or observed anxiety-like behavior in the treated rats as observed in the 45S5 group. Therefore, BaBG is safer and biocompatible without any significant toxic effects observed preclinically.