4.1 Abstract

Bioactive glass has the potential to repair of hard as well as soft tissue. However, few reports are available regarding the toxicity profile of bioactive glass within the body. In the current study, we have performed 28 days repeated dose toxicity study using barium containing bioactive glass (BaBG) and silver-containing bioactive glass (AgBG) at a dose of 1, 10 and 50 mg/kg and 5, 10 and 50 mg/kg respectively in rats. The animal were observed for changes in physical appearance, cardiovascular, pathological and complete blood parameters. BaBG at 10 mg/kg dose and AgBG at 5 mg/kg dose are found to be safe if given intravenously for 28 days daily. BaBG at the highest dose (50 mg/kg) while AgBG at a middle dose (10) and highest dose (50 mg/kg) showed liver deposition and low *in vivo* liver toxicity as confirmed by pathological, blood flow and the liver weight/body weight coefficients. Further, BaBG at 50 mg/kg and AgBG at 10 & 50mg/kg increase the blood flow of the lungs significantly compared to the control animal indicating mild pulmonary vascular defect. Further, the LD50 of the BaBG was found to be more than 2000 mg/kg while for AgBG it was found to be less than 300 mg/kg representing the toxic nature of AgBG. Finally, the present study gave information regarding the safe dose of BaBG and AgBG, which can be utilized in future.

Keywords

BaBG; AgBG; ECG; blood pressure; blood flow; hematology; histology

4.2 Introduction

Bioactive glass is a biologically active bioceramic material that can regenerate hard as well as soft tissue (Day et al. 2004). Hench and Wilson were the first to discover the bioactive glass in 1969, termed as 45S5 bioglass[®]. Based on the compositions, bioactive glasses are of different types such as 45S5 bioglass[®] (45 % silicate) and 70S30C (70 % silicate). Several patients have successfully used bioactive glass-based product to repair bone and dental defects (Baino et al. 2018). Several bioactive glass compositions have been produced for novel biomedical applications, including soft tissue repair and drug delivery (Baino et al. 2018). Moreover, bioactive glass has been the choice of interest in the field of drug delivery (Dai et al. 2015). Earlier reports suggest the potential of bioactive glass in the delivery of therapeutic molecules (El et al. 2012) with the benefit of a drug-carrying property with sustained-release effect (Wu et al. 2013). Bioactive glass possesses large pore volume, high surface area, mesoporous size, bioactivity (Vallet et al. 2011; Fan et al. 2012), cytocompatibility and bactericidal property (Liu et al. 2014). It is reported to possess anti-inflammatory and antimicrobial activities (Greenspan et al. 2003). Bioactive glass promotes wound healing of the burn tissue and gastroduodenal ulcer healing by stimulating the secretion of angiogenic growth factors and promotes angiogenesis (Day et al. 2005; Keshaw et al. 2005; Paliwal et al. 2018). Further, bioactive glass can cause nerve regeneration in peripheral nerve injury (Bunting et al. 2005). Thus, the availability of bioactive glass in the brain facilitates the regeneration of damaged nerve and the formation of a new blood vessel.

Furthermore, the barium oxide substitution for SiO_2 content in 45S5 bioactive glass markedly enhances its biocompatibility and bioactivity by enhancing crystallization and hydroxycarbonate apatite (HCA) layer formation (Arepalli et al. 2015; Paliwal et al. 2018).

These studies indicate a higher potential of barium containing bioactive glass (BaBG) as biomaterials than 45S5 bioactive glass. Moreover, Silver containing bioactive showed excellent antibacterial property (Kawashita et al. 2000). Silver particles have an intrinsic antiplatelet property and effectively prevent integrin-mediated platelet responses, both in vivo and in vitro, in a concentration-dependent manner. Ultrastructural studies show that silver accumulates within platelet granules and reduces interplatelet proximity (Shrivastava et al. 2009), indicating a higher potential of silver containing bioactive glass (AgBG) antithrombotic biomaterial than 45S5 bioactive glass. Thus we have prepared a bioactive glass containing barium and silver and tested its safety profile.

Despite the huge application of bioactive glasses in the biological system, its long-term effect has not been assessed (Sui et al. 2016). Recent studies have been performed to check the risk assessment of bioactive glass after iv administration, but these studies are acute toxicity studies at a high dose with a single administration of bioactive glass (Sui et al. 2016; Mao et al. 2016). Therefore, there is a lack of information on the long term effect of repeated-dose of bioactive glass.

The liver metabolizes most of the drug and responsible for the removal of foreign particulates. Further, it has been noticed that if the particles not excreted effectively and accumulated in the liver, then these particles leads to inflammatory infiltrate. Infiltration causes necrosis in the liver cell (Xie et al. 2010). Therefore, it is important to know the effect of the BaBG & AgBG particulates on liver cell. Oral exposure and inhalation have a limitation of the relatively low systemic exposure due to less absorption of bioactive glass from the gastrointestinal tract (GI-tract) and lung; therefore, the data of systemic toxicity are inadequate. To avoid this limitation, we have given BaBG & AgBG via the intravenous

route. Studies provided evidence of silica-based nanoparticles-induced cytotoxicity (Rabolli et al. 2010). Further, a previous studies reported in vitro toxicity of silica nanoparticles in myocardial cells (Ye et al. 2010; Guerrero et al. 2017). Thus, it is essential to determine the effect of the BaBG & AgBG on heart tissue and cardiovascular functioning.

In the present study, BaBG and AgBG toxicity were assessed for both single and repeated doses. BaBG and AgBG were synthesized through the melt quenching method. The acute and subacute toxicity of bioactive glasses was evaluated according to OECD guidelines with slight modifications. We have evaluated ECG parameters, blood pressure, haematology, histology and mean blood flow of the highly perfused organ including heart, brain, lungs, kidney, liver and spleen. Histology study was done for all the highly perfused organs as these are the organ which undergoes maximum exposure of bioactive glasses and are most susceptible to injury.

4.3 Materials and methods

4.3.1 Animals

Experimentation, transportation and care of the animals were performed in compliance with guidelines of animal care (National Research Council US Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011). Male albino Wistar rats of 250-300 gm were procured from IMS, BHU after taking ethical committee approval (Ref No. Dean/2015/CAEC/1421).

4.3.2 Experiment design

The present experiment was performed as per the OECD guideline 407 ("Repeated dose 28-day study in rodents") with minor modifications. Fine powder of bioactive glasses was prepared by melt quenching method as per the published protocol (Paliwal et al. 2018). Male

Wistar rats were administered via intravenous administration at three-dose group's low (1), mid (10) and high (50 mg/kg) for BaBG experiment while low (5), mid (10) and high (50 mg/kg) for AgBG experiment and one control group for both BaBG and AgBG (n=10/group). Similarly, bioactive glass (BG) dispersion and vehicle (PEG-400, 30 % v,v in saline) were administered intravenously through tail vein injection once daily for 28 days. On 28th day after recording the ECG and blood pressure, the animals were weighed and killed by cervical dislocation. All the highly perfused organ including liver, brain, kidney, lungs, heart, spleen, stomach and femur bone for bone marrow were taken out and immediately put into formalin for histopathological examination. The whole blood was collected in an EDTA-coated tube for the haematological studies.
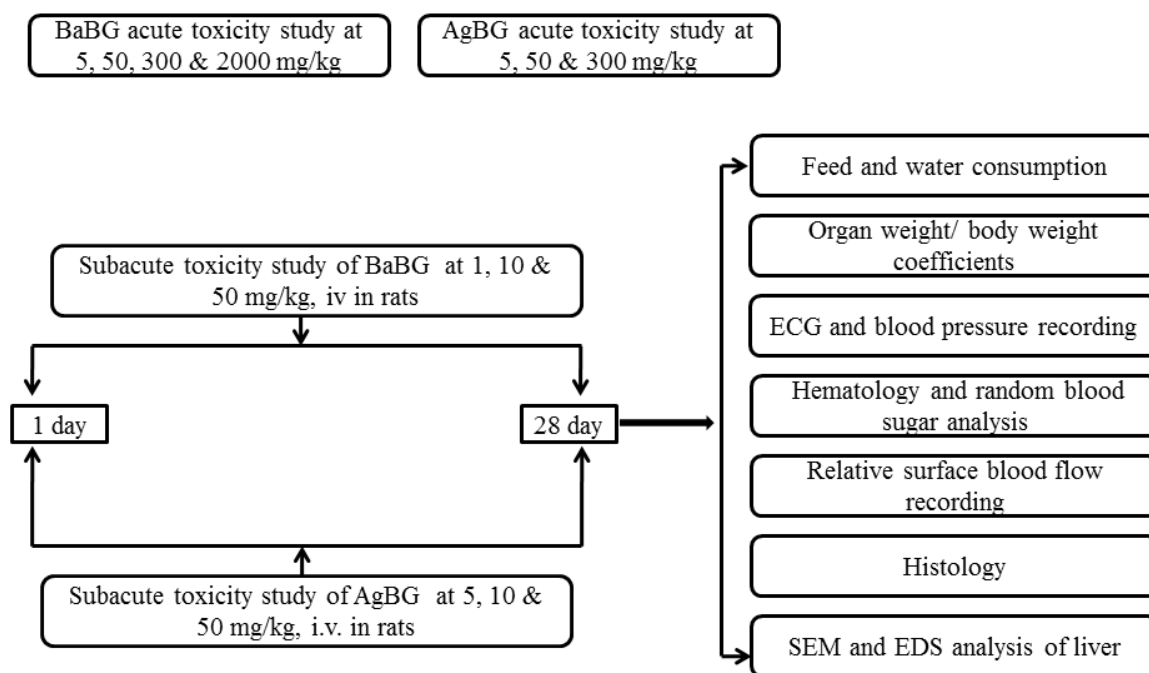

Figure 4.1 Schematic representation of the experimental design where i.v.: intravenous and mg/kg: milligram/kilogram.

4.3.3 General observation, food intake, water intake, body weight and mortality

The general appearance, feed and water consumption, animal body weight of entire animals were documented during the experiment. The gain in body weight was calculated by subtracting the animal initial body weight from final body weight (Luo et al. 2016).

4.3.4 Organ weight/body weight coefficients

All the highly perfused organs such as heart, brain, lungs, liver, kidney and spleen were isolated on the last day of the experiment, washed with saline and weighed. The organ weight/ body weight coefficients were calculated as organ weight (wet weight, g)/animal body weight (g) X 100% (Xu et al. 2013).

4.3.5 ECG and Blood Pressure

ECG and blood pressure of the animal were recorded on the last day of the experiment to determine the effect of BG on the cardiovascular system. Mean arterial blood pressure was recorded in awaked animals by using a non-invasive tail-cuff method using NIBP200A. ECG was recorded to determine heart rate, QRS complex, Q-T interval, P wave and S-T interval in anaesthetized animals (pentobarbitone sodium 35 mg/kg, *i.p.*) using MP45, BIOPAC System, INC, USA (Asdaq et al. 2009).

4.3.6   Haematology and random blood sugar test

On the last day of dosing i.e 28th day, blood was collected in EDTA-coated tubes for haematology analysis. Red blood cell count (RBC), haemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT), mean platelet volume (MPV), red blood cell distribution width (RDW), haemoglobin distribution width (HDW) and white blood cell count (WBC) were recorded by using Celltac E MEK-7222 automatic analyzer

(Mao et al. 2016; Rabolli et al. 2010; Devi et al. 2014). In addition, random blood sugar (RBS) is measured by Accu-Chek active blood glucose monitoring system (Zachwieja et al. 1997; Buesen et al. 2014).

4.3.7 Relative surface blood flow recording

The surface blood flow of the highly perfused organs was measured by using a laser speckle blood flow imaging system using omegazone OZ-2 STD as per the previously published protocol (Paliwal et al. 2018). Rats were anaesthetized by pentobarbitone (35 mg/kg, i.p.) and surface blood flow of the heart, brain, lungs, kidney, liver, and spleen was recorded. Image pixels were analyzed to produce average perfusion values using the software OZ-2 (LSIv3.3.1 and LIAv3.3.0). The unit of mean blood flow was an arbitrary unit (AU).

4.3.8 Histology

Rats were anaesthetized with pentobarbitone sodium on the last day of the experiment; organs were isolated and immediately fixed with 10% formalin solution. Then the formalin-fixed tissue specimens were dehydrated and embedded in paraffin. Again the tissue specimens were sectioned by 6 μm, and stained with hematoxylin and eosin and analyzed with an Olympus BH2 light microscope (Paliwal et al. 2018).

4.3.9 Scanning electron microscope (SEM) and energy dispersive x-ray spectroscopy (EDS) analysis of liver section.

The liver was isolated and subsequently transferred to 2.5% glutaraldehyde and stored at 4 °C for 24 hr. Thereafter, the specimens were washed by phosphate buffer for 15 min. The washing procedure was repeated for three times. Then the specimens were dehydration in a graded series of acetone solutions and vacuum coated with gold-palladium. Then the samples were examined in a scanning electron microscope coupled with energy dispersive

spectrometry (SEM, EVO LS 10, Carl Zeiss, Germany) to investigate elemental analysis in the specimens (Paliwal et al. 2018).

4.3.10 Acute toxicity study

The single-dose acute toxicity of BG formulation was carried out based on OECD guidelines 423. BG formulation was administered by an intravenous route at 5, 50, 300 and 2000 mg/kg in rats (n=3). Possible death of animals was monitored for 14 days to ascertain the median lethal dose (LD50) of the BaBG and AgBG formulation.

4.3.11 Statistical analysis

All the experimental data were presented as Mean ± SEM. One-way ANOVA followed by Student Newman-Keuls post-hoc test was done for the data analysis. A level of $p < 0.05$ was regarded as significant in all the data analysis.

4.4 Results

4.4.1 Animal observation and effect on feed, water consumption and body weights of BaBG study

Body-weight of control and BaBG treated animals were recorded over a period of 28 days, as shown in Table 4.1. The body-weight of the rats augmented significantly in a pattern parallel to control rats during the experiment period, injected with BaBG at all three doses of 1, 10 and 50 mg/kg, representing that the rats constantly grown-up with no any major toxic effects. There was no mortality in the normal and BaBG treatment groups. Likewise, BaBG rats demonstrated no major alteration in feed [$F_{(3, 39)} = 74.91$; $P > 0.05$] and water utilization [$F_{(3, 39)} = 24.68$; $P > 0.05$] among the group as revealed by one-way-ANOVA, indicating that BaBG has no major impact on the food and water consumption.

Table 4.1 Effect of BaBG treatment on feed consumption, water consumption and changes in body weight

Treatment	Food intake (g/rat/day)	Water intake (ml/rat/day)	Initial Body weight (g)	Final Body weight (g)	Body weight gain (g)
Control	17.19 ± 2.83	20.85 ± 1.75	263.01 ± 5.37	294.85 ± 7.98	30.46 ± 2.04
BaBG 1	16.26 ± 3.26	19.48 ± 1.26	266.53 ± 6.80	293.84 ± 8.02	28.88 ± 1.93
BaBG 10	19.67 ± 1.94	21.25 ± 1.47	267.36 ± 6.35	296.85 ± 5.79	29.81 ± 1.87
BaBG 50	17.95 ± 2.75	21.99 ± 1.75	263.31 ± 6.17	297.85 ± 6.35	32.32 ± 1.53

Units g = grams, ml = millilitre

Data represented as mean $\pm$ SEM (N = 10). No significant difference was found against control group: $p > 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test)

4.1.2 Animal observation and effect on feed, water consumption and body weights of AgBG study

Body weight of control and AgBG treated animals were recorded over a period of 28 days as shown in table 4.2. The body weight gain of the rats significantly decreased at 10 and 50 mg/kg of AgBG during the experiment period. There was no mortality in the normal and AgBG treatment groups. AgBG rats demonstrated no major alteration in feed and water utilization among the group revealed by one-way-ANOVA [F $(18, 252) = 1.77$; P>0.05].

Table 4.2 Effect of AgBG treatment on feed consumption, water consumption and changes in body weight

Treatment	Food intake (g/rat/day)	Water intake (ml/rat/day)	Initial Body weight (g)	Final Body weight (g)	Body weight gain (g)
Control	19.65 ± 3.25	20.25± 2.54	264.36 ± 3.54	297.54 ± 6.65	33.79 ± 4.41
AgBG 5	17.35 ± 2.95	18.57 ± 2.64	265.35 ± 3.71	290.26 ± 5.84	24.54 ± 3.55
AgBG 10	16.35 ± 1.65	16.44 ± 1.65	262.74 ± 4.64	281.45 ± 5.54	19.45 ± 3.12*
AgBG 50	12.35 ± 2.35	13.35 ± 2.35	264.55 ± 3.65	272.35 ± 6.35*	8.54 ±1.53*

Units g = grams, ml = milliliter

Data represented as mean ± SEM (N = 10). A significant difference was found against the control group: * $p \leq 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test)

4.4.3 Organ weight/body weight coefficients of BaBG study

On the last day of the experiment, the rats were weighed and sacrificed. Highly perfused organs, including the heart, lung, liver, spleen, kidneys and brain, were isolated and weighed. Organ weight coefficients value were represented as means ± SEM (n = 10) as seen in Table 4.3. No major changes were seen in the coefficients of the heart, lung, kidney, spleen and brain in BaBG treated rats as revealed by one-way-ANOVA test. Furthermore, the coefficients of the liver in BaBG treated rat at the dose of 50 mg/kg increased significantly against control group [F (3, 39) = 0.41; P<0.05]. This indicates the mild liver toxicity of BaBG at 50 mg/kg when administered intravenously once daily for 28 days.

Table 4.3 Effect of BaBG treatment on organ weight / body weight coefficients

Treatment	Heart	Lungs	Liver	Spleen	Kidney	Brain
Control	0.378±0.060	0.512±0.128	2.568±0.220	0.199±0.042	0.726±0.108	0.652±0.092
BaBG 1	0.352±0.056	0.528±0.124	2.652±0.204	0.203±0.044	0.734±0.096	0.684±0.111
BaBG 10	0.362±0.047	0.524±0.116	2.712±0.210	0.209±0.039	0.712±0.112	0.640±0.084
BaBG 50	0.384±0.054	0.546±0.108	3.349±0.180*	0.212±0.048	0.746±0.076	0.656±0.840

Data represented as mean ± SEM (N = 10). Significant difference was found against control group: * $p < 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test)

4.4.4 Organ weight/body weight coefficients of AgBG study

Organ weight coefficients value were represented as means ± SEM (n = 10) as seen in table 4.4. Coefficients of the liver in AgBG treated rat at the dose of 10 and 50 mg/kg increased significantly against control group. No major changes were seen in the coefficients of the heart, lung, kidney, stomach and brain in AgBG treated rats as revealed by one-way-ANOVA test [$F_{(15, 216)} = 0.39$; $P < 0.05$].

Table 4.4 Effect of AgBG treatment on organ weight / body weight coefficients

Treatment	Heart	Lungs	Liver	Spleen	Kidney	Brain
Control	0.339±0.057	0.506±0.063	2.427±0.241	0.189±0.038	0.717±0.073	0.638±0.062
AgBG 5	0.342±0.049	0.532±0.084	2.683±0.284	0.213±0.042	0.721±0.083	0.642±0.085
AgBG 10	0.354±0.038	0.558±0.091	3.341±0.318*	0.228±0.048	0.731±0.103	0.649±0.104
AgBG 50	0.361±0.049	0.564±0.098	3.935±0.328*	0.241±0.068	0.732±0.115	0.653±0.094

Data expressed as mean ± SEM (N=10). Significance difference was found against control group: * $p < 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test).

4.4.5 Effect of BaBG on ECG parameters and blood pressure

Electrocardiograph abnormalities are the main criteria generally used for the determination of possible myocardial injury. Table 4.5 shows the effect of BaBG (1, 10, and 50 mg/kg) on changes in blood pressure and ECG parameters including heart rate, P wave, QRS complex, S-T interval and Q-T interval in rats. One-way-ANOVA stated that 50 mg/kg of BaBG significantly altered heart rate compared to vehicle group [$F_{(3, 39)} = 456.8$; $P<0.05$], indicating that BaBG at higher dose induced cardiovascular disorder.

Table 4.5 Effect of BaBG on ECG parameters and blood pressure

Treatment	Heart Rate (Beats/Min)	P wave (sec)	QRS complex (sec)	Q-T interval (sec)	S-T interval (sec)	Blood Pressure (mmHg)
Control	391.62 ± 6.23	0.036± 0.003	0.042 ± 0.001	0.064 ± 0.006	0.020 ± 0.002	112.4 ± 5.97
BaBG (1)	391.37 ± 7.74	0.034± 0.001	0.041 ± 0.001	0.063 ± 0.005	0.020 ± 0.005	110.4 ± 4.96
BaBG (10)	394.61 ± 6.85	0.033 ± 0.004	0.040 ± 0.001	0.060 ± 0.003	0.019 ± 0.004	112.2 ± 3.75
BaBG (50)	418.27 ± 6.09*	0.031± 0.003	0.038 ± 0.002	0.055 ± 0.003	0.016 ± 0.004	118.5 ± 4.86

Data expressed as mean ± SEM (N=10). Significant difference was found against control group: * $p < 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test).

4.4.6 Effect of AgBG on ECG parameters and blood pressure

Electrocardiograph abnormalities are the main criteria generally used for the determination of possible myocardial injury. Table 4.6 shows the effect of AgBG (5, 10, and 50 mg/kg) on changes in blood pressure and ECG parameters including heart rate, P wave, QRS complex, S-T interval and Q-T interval in rats. One-way-ANOVA stated that 50 mg/kg of AgBG significantly altered heart rate compared to vehicle group [$F_{(3, 39)} = 354.45$; $P<0.05$], indicating that AgBG at higher dose induced cardiovascular disorder.

Table 4.6 Effect of AgBG on ECG parameters and blood pressure

Treatment	Heart Rate (Beats/Min)	P wave (sec)	QRS complex (sec)	Q-T interval (sec)	S-T interval (sec)	Blood Pressure (mmHg)
Control	390.55 ± 6.05	0.037± 0.003	0.041 ± 0.001	0.065 ± 0.007	0.021 ± 0.002	114.74 ± 7.87
AgBG (5)	395.68 ± 8.65	0.035± 0.002	0.042 ± 0.002	0.062 ± 0.006	0.021 ± 0.005	115.76 ± 8.90
AgBG (10)	398.92 ± 8.05	0.034 ± 0.003	0.041 ± 0.001	0.061 ± 0.006	0.020 ± 0.005	117.78 ± 5.78
AgBG (50)	**421.51 ± 7.97***	0.033± 0.004	0.043 ± 0.002	0.063 ± 0.005	0.022 ± 0.005	118.6 3± 7.54

Data expressed as mean ± SEM (N=10). Significant difference was found against control group: * $p < 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test)

4.4.7 Effect of BaBG on haematology parameters and blood random glucose level

The hematology results including WBC count, RBC count, Hb, Ht, MCV, MCH, MCHC, RDW, HDW, PLT count and MPV were within normal ranges as shown in table 4.7. We also detected the blood random glucose level on the last day of the experiment. One-way-ANOVA confirmed that there were no significant alteration between groups of BaBG (1, 10,

50 mg/kg) and control after 28 days dosing. This indicate that BaBG have no significant impact on the vascular component even if administered at 50 mg/kg intravenously daily for 28 days.

Table 4.7 Effect of BaBG on hematological parameters

Parameters	Control group	BaBG 1 mg/kg	BaBG 10 mg/kg	BaBG 50 mg/kg
RBC; tera/L	7.93 ± 0.51	8.15± 0.47	8.29 ± 0.56	8.23 ± 0.49
HGB; mmol/L	9.01 ± 0.46	9.26 ± 0.54	9.49 ± 0.61	9.20 ± 0.55
HCT; L/L	0.45 ± 0.04	0.43 ± 0.03	0.47 ± 0.04	0.47 ± 0.03
MCV; fL	52.34 ± 4.57	53.96 ± 5.18	56.25 ± 5.28	54.27 ± 5.15
MCH;fmol	1.04 ± 0.16	1.13 ± 0.12	1.09 ± 0.07	1.11 ± 0.09
MCHC; mmol/L	20.53 ± 1.93	23.17 ± 1.53	21.87 ±1.05	24.34 ± 2.1
RDW; %	12.52 ± 0.73	12.94 ± 0.48	13.57 ± 1.01	13.01 ± 0.52
PLT; giga/L	984.65 ± 175.65	990.45 ± 190.67	1022.01 ± 180.28	987.29 ± 169.00
PCT;%	0.62 ± 0.04	0.57 ± 0.03	0.54 ± 0.04	0.50 ± 0.03
MPV;fL	5.24 ± 0.31	4.73 ± 0.27	4.09 ± 0.32	4.65 ± 0.36
PDW; %	42.85 ± 6.21	38.97 ± 4.95	43.59 ± 6.44	43.95 ± 5.97
WBC; giga/L	6.73 ± 0.56	6.91 ± 0.52	7.15 ± 0.62	8.21 ± 0.89

Neut; %	36.45 ± 4.61	35.53 ± 5.1	37.28 ± 3.9	35.19 ± 4.8
Lymph; %	59.15 ± 5.34	60.36 ± 4.92	61.94 ± 6.42	63.25 ± 4.89
Mono;%	1.57 ± 0.09	1.23 ± 0.12	1.42 ± 0.14	1.46 ± 0.05
Eos; %	2.34 ± 0.11	2.59 ± 0.15	2.43 ± 0.19	2.83 ± 0.21
Baso; %	0.46 ± 0.01	0.45 ± 0.01	0.42 ± 0.02	0.40 ± 0.02
RBG; mmol/L	5.67 ± 0.57	5.85 ± 0.48	5.92 ± 0.42	5.69 ± 0.39

RBC; red blood cells, HGB; hemoglobin, HCT; hematocrit, MCV; mean corpuscular volume, MCH; mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration, RDW; red cell volume distribution width, PLT; platelets, PCT; plateletcrit, MPV; mean platelet volume, PDW; platelet cell volume distribution width, WBC; white blood cells, Neut; neutrophils, Lymph;.lymphocytes, Mono;.monocytes, Eos;.eosinophils, Baso;.basophils, RBG; Random blood glucose. Units giga/L = 109/liter; tera/L = 1012/liter; fL = fentoliter; mmol/L = millimole/liter; fmol = fentomole; L/L = liter/liter. Data represented as mean ± SEM (N = 10). No significant difference was found against control group: $p > 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test)

4.4.8 Effect of AgBG on haematology parameters and blood random glucose level

The haematology results, including WBC count, RBC count, Hb, Ht, MCV, MCH, MCHC, RDW, HDW, PLT count and MPV were within normal ranges shown in table 4.8. We also detected the blood random glucose level on the last day of the experiment. One-way-ANOVA confirmed no significant alterations between groups of AgBG (5, 10, 50 mg/kg) and control after 28 days dosing. This indicates that AgBG has no significant impact on the vascular component, even if administered at 50 mg/kg intravenously daily for 28 days.

Table 4.8 Effect of AgBG on haematological parameters

Parameters	Control group	AgBG 5 mg/kg	AgBG 10 mg/kg	AgBG 50 mg/kg
RBC; tera/L	7.87 ± 0.43	8.14± 0.52	8.42 ± 0.61	8.06 ± 0.58
HGB; mmol/L	9.27 ± 0.52	9.39 ± 0.37	9.19 ± 0.58	9.36 ± 0.62
HCT; L/L	0.43 ± 0.03	0.45 ± 0.04	0.41 ± 0.03	0.46 ± 0.04
MCV; fL	53.97 ± 5.26	54.19 ± 4.91	55.74 ± 6.02	53.96 ± 5.72
MCH;fmol	1.03 ± 0.14	1.08 ± 0.10	1.02 ± 0.06	1.19 ± 0.16
MCHC; mmol/L	21.04 ± 1.06	22.90 ± 1.15	20.81 ±1.17	22.84 ± 1.74
RDW; %	13.64 ± 0.85	13.86 ± 0.21	12.94 ± 0.59	13.36 ± 0.39
PLT; giga/L	998.14 ± 141.97	989.65 ± 151.87	1007.74 ± 159.98	996.79 ± 170.98
PCT;%	0.57 ± 0.02	0.55 ± 0.04	0.53 ± 0.03	0.55 ± 0.04
MPV;fL	5.04 ± 0.76	4.94 ± 0.53	5.54 ± 0.42	5.05 ± 0.74
PDW; %	43.75 ± 4.93	39.99 ± 3.93	41.26 ± 3.97	44.24 ± 4.09
WBC; giga/L	6.57 ± 0.48	6.27 ± 0.39	7.01 ± 0.49	7.02 ± 0.26
Neut; %	35.36 ± 4.26	35.49 ± 3.98	36.95 ± 2.91	34.95 ± 4.38
Lymph; %	62.76 ± 4.93	61.02 ± 3.86	62.14 ± 4.98	61.93 ± 4.06
Mono;%	1.49 ± 0.08	1.38 ± 0.15	1.39 ± 0.11	1.44 ± 0.16

Eos; %	2.27 ± 0.19	2.38 ± 0.27	2.27 ± 0.14	2.31 ± 0.16
Baso; %	0.44 ± 0.03	0.39 ± 0.02	0.41 ± 0.05	0.43 ± 0.06
RBG; mmol/L	5.51 ± 0.51	5.53 ± 0.49	5.73 ± 0.26	5.70 ± 0.41

RBC; red blood cells, HGB; hemoglobin, HCT; hematocrit, MCV; mean corpuscular volume, MCH; mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration, RDW; red cell volume distribution width, PLT; platelets, PCT; plateletcrit, MPV; mean platelet volume, PDW; platelet cell volume distribution width, WBC; white blood cells, Neut; neutrophils, Lymph;.lymphocytes, Mono;.monocytes, Eos;.eosinophils, Baso;.basophils, RBG; Random blood glucose. Units giga/L = 10^9/liter; tera/L = 10^{12}/liter; fL = fentoliter; mmol/L = millimole/liter; fmol = fentomole; L/L = liter/liter. Data represented as mean ± SEM (N = 10). No significant difference was found against control group: $p > 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test)

4.4.9 Effect of BaBG on surface blood flow of highly perfused organs

Table 4.9 illustrates the effect of BaBG (1, 10 and 50 mg/kg) on changes in surface blood flow of highly perfused organ including brain, liver, spleen, kidney, heart and lungs in rats. One-way-ANOVA proved that the changes in the blood flow of heart, kidney, spleen, and brain were not significant compared to the vehicle group at all the three doses of BaBG. However, 50 mg/kg of BaBG significantly altered the liver [$F_{(3, 39)} = 15.24$; $P<0.05$] and lung [$F_{(3, 39)} = 17.47$; $P<0.05$] blood flow compared to vehicle groups. This indicates that BaBG has a mild toxic effect on the liver and lungs at a high dose of 50 mg/kg in rats.

Table 4.9 Effect of BaBG in surface blood flow of highly perfused organ

Treatment	Heart	Lungs	Liver	Spleen	Kidney	Brain
Control	29.56±1.24	24.75±1.38	13.04±1.23	10.23±1.22	21.16±1.29	26.84±1.37
BaBG 1	29.93±1.36	24.95±1.24	13.98±1.19	10.24±1.06	21.33±1.36	27.19±1.20
BaBG 10	30.48±1.29	26.80±1.41	15.85±1.22	10.23±1.38	21.87±1.27	27.46±1.41
BaBG 50	30.72±1.57	29.84±1.25*	18.01±1.30*	11.42±1.09	22.14±1.37	27.83±1.23

Data expressed as mean ± SEM (N=10). A significant difference was found against the control group: * $p < 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test)

4.4.10 Effect of AgBG on surface blood flow of highly perfused organs

Table 4.10 illustrates the effect of AgBG (5, 10 and 50 mg/kg) on changes in surface blood flow of highly perfused organ including brain, liver, spleen, kidney, heart and lungs in rats. One-way-ANOVA shows the changes in the blood flow of the all highly perfused organs at 50 mg/kg, indicating the toxic nature of AgBG at the higher doses.

Table 4.10 Effect of AgBG in surface blood flow of highly perfused organ

Treatment	Heart	Lungs	Liver	Spleen	Kidney	Brain
Control	29.25±1.27	24.65±1.28	13.07±1.26	10.17±1.13	21.26±1.41	26.76±1.29
AgBG 5	30.16±1.71	25.95±1.44	14.54±1.35	11.24±1.21	22.15±1.54	27.21±1.37
AgBG 10	32.15±1.56	29.54±1.34*	19.94±1.38*	13.24±1.26	24.53±1.47	28.64±1.56
AgBG 50	35.34±1.85*	32.75±1.55*	19.46±1.57*	15.29±1.38*	28.63±1.96*	33.24±1.84*

Data expressed as mean ± SEM (N=10). Significant differences were found against the control group: * $p < 0.05$, (One-way ANOVA followed by Student Newman-Keuls post-hoc test)

4.4.11 Histology of BaBG study

Figure 4.2 show the histological section of highly perfused organs after continuous bolus intravenous injection of BaBG for 28 days daily once. The control rat liver samples illustrated normal hepatic cells with an intact nucleus, nucleolus and central vein. However, at the dose of 50 mg/kg BaBG, moderate lymphocytic infiltration in the parenchyma with focal hepatocytic necrosis and chronic active hepatitis was observed, representing the main target organ of BaBG was liver at the higher dose. In spleen at 50 mg/kg doses lymphocytes were detected in the red pulp of the spleen, indicating that BaBG can be mildly toxic for the spleen. Other pathophysiological observations were recorded at higher doses as detailed below. Mild congestion, intra-alveolar haemorrhage with focal intra-alveolar congestion and lymphocytic infiltration in the interstitium was observed in the lungs. Stomach showed mild infiltration of neutrophils, eosinophils and lymphocytes at the basal part of the stomach with mild appearance of eosinophilic esophagitis. Heart examination shows mild infiltration of

lymphocyte with focal myonecrosis. Kidney examination shows mild lymphocytic infiltration at interstitial tubule as well as in the glomerulus. Number of cells with vacuole in Figure 4.2 are followings Kidney-control (0), BaBG 1.0 mg/kg (3), BaBG 10 mg/kg (8), BaBG 50 mg/kg (19); Liver-control (1), BaBG 1.0 mg/kg (6), BaBG 10 mg/kg (12), BaBG 50 mg/kg (29); Lungs-control (0), BaBG 1.0 mg/kg (1), BaBG 10 mg/kg (4), BaBG 50 mg/kg (11); Spleen-control (1), BaBG 1.0 mg/kg (4), BaBG 10 mg/kg (8), BaBG 50 mg/kg (21); Stomach-control (2), BaBG 1.0 mg/kg (2), BaBG 10 mg/kg (5), BaBG 50 mg/kg (10); Brain-control (0), BaBG 1.0 mg/kg (2), BaBG 10 mg/kg (5), BaBG 50 mg/kg (12);. Heart-control (0), BaBG 1.0 mg/kg (2), BaBG 10 mg/kg (3), BaBG 50 mg/kg (8); Bone marrow-control (1), BaBG 1.0 mg/kg (8), BaBG 10 mg/kg (14), BaBG 50 mg/kg (26). Thus, BaBG median and lower doses were safe and did not induce significant alteration in the morphology of highly perfused organs including kidney, liver, lungs, spleen, brain, heart and bone marrow in rats.
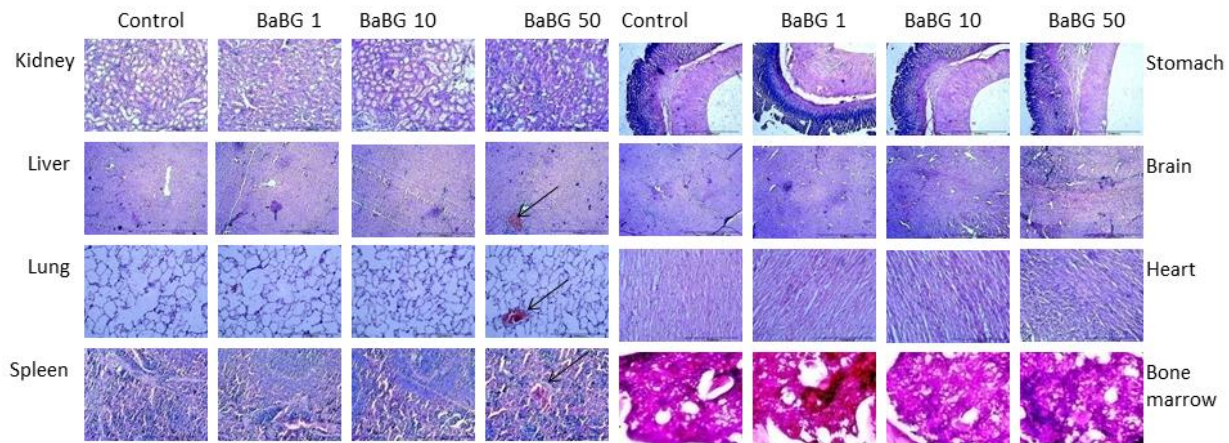

Figure 4.2 Effect of 28 days daily intravenous administration of BaBG at the dose of 1, 10, and 50 mg/kg in histology stained with (H.E., haematoxylin and eosin) of the highly perfused organ including kidney, liver, lung, spleen, stomach, brain, heart and bone marrow in rats.

4.4.12 Histology of AgBG study

Figure 4.3 shows histological section of highly perfused organs after continuous bolus intravenous injection of AgBG for 28 days daily once. The control rat liver samples showed normal hepatic cells with an intact nucleus, nucleolus and central vein. However, at the dose of 10 and 50 mg/kg AgBG, moderate lymphocytic infiltration in the parenchyma with focal hepatocytic necrosis and chronic active hepatitis was observed, representing the main target organ of AgBG was liver at middle and higher doses. In spleen at 50 mg/kg doses lymphocytes were detected in the red pulp of the spleen, indicating that AgBG can be mild toxic for the spleen. Other pathophysiological observations were recorded at higher doses as detailed below. Mild congestion, intra-alveolar haemorrhage with focal intra-alveolar congestion and lymphocytic infiltration in the interstitium was observed in the lungs. Stomach showed mild infiltration of neutrophils, eosinophils and lymphocytes at the basal part of the stomach with mild appearance of eosinophilic esophagitis. Heart examination shows mild infiltration of lymphocyte with focal myonecrosis. Kidney examination shows mild lymphocytic infiltration at interstitial tubule as well as in the glomerulus. Number of cells with vacuole in Figure 4.3 are followings Kidney-control (0), AgBG 5.0 mg/kg (5), AgBG 10 mg/kg (13), AgBG 50 mg/kg (21); Liver-control (2), AgBG 5.0 mg/kg (11), AgBG 10 mg/kg (25), AgBG 50 mg/kg (31); Lungs-control (1), AgBG 5.0 mg/kg (6), AgBG 10 mg/kg (9), AgBG 50 mg/kg (11); Spleen-control (2), AgBG 5.0 mg/kg (6), AgBG 10 mg/kg (11), AgBG 50 mg/kg (16); Stomach-control (2), AgBG 5.0 mg/kg (8), AgBG 10 mg/kg (16), AgBG 50 mg/kg (19); Brain-control (0), AgBG 5.0 mg/kg (8), AgBG 10 mg/kg (16), AgBG 50 mg/kg (9);. Heart-control (0), AgBG 5.0 mg/kg (5), AgBG 10 mg/kg (16), AgBG 50 mg/kg (11); Bone marrow-control (1), AgBG 5.0 mg/kg (6), AgBG 10 mg/kg (13), AgBG

50 mg/kg (17);Thus, AgBG lower doses were safe and did not induce significant alteration in the morphology of highly perfused organs including kidney, liver, lungs, spleen, stomach, brain, heart and bone marrow in rats.
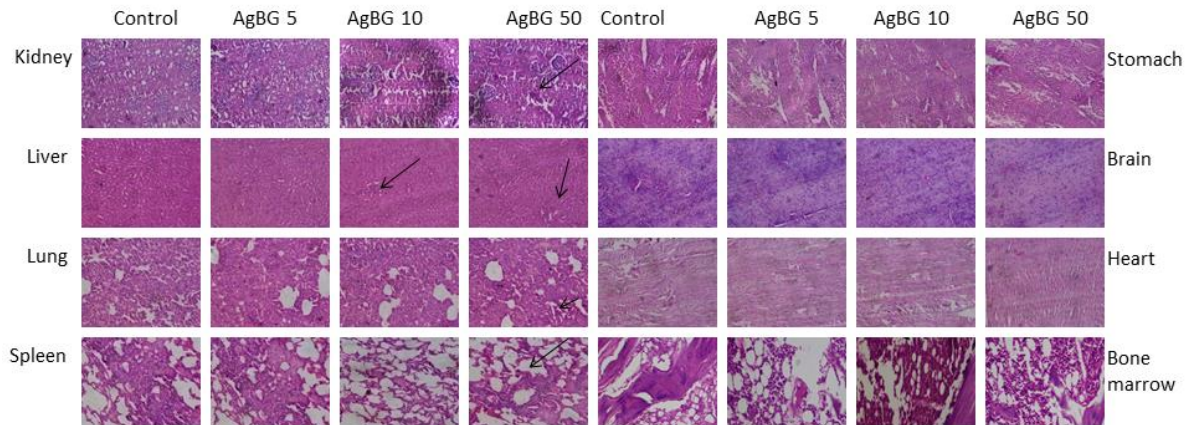

Figure 4.3 Effect of 28 days daily intravenous administration of AgBG at the dose of 5, 10, and 50 mg/kg in histology stained with (H.E., haematoxylin and eosin) of the highly perfused organ including kidney, liver, lung, spleen, stomach, brain, heart and bone marrow in rats.

4.4.13 Liver SEM and EDS analysis of BaBG study

The SEM images (Figure 4.4A) and EDS spectra (5.3B) of the group (50 mg/kg; BaBG) shows BaBG in the liver. The image was captured at a magnification of 200X which show the presence of particles in the liver. Further, image B represented that the particle present on the liver section after 28 days repeated exposure of BaBG exposure has peaks of barium as well as silica. This indicates that BaBG is accumulated in the liver at the higher dose of 50 mg/kg in rats
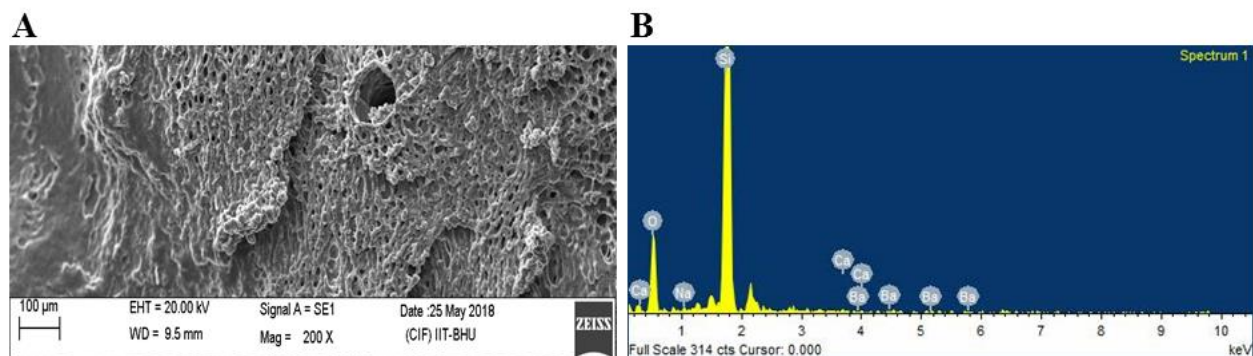
Figure 4.4 Deposition of BaBG in the liver. Liver image A was captured at a magnification of 200X shows the presence of particles, while image B shows particles present on the liver specimen have barium and silica peaks that confirm the particles are of BaBG

4.4.14 Liver SEM and EDS analysis of AgBG study

The SEM images (Figure 4.5A) and EDS spectra (B) of the group (50 mg/kg; AgBG) shows AgBG in the liver. The image was captured at a magnification of 200X which show the presence of particles in the liver. Further, image B represented that the particle present on the liver section after 28 days repeated exposure of AgBG exposure has peaks of silver as well as silica. This indicates that AgBG is accumulated in the liver at a higher dose of 50 mg/kg in rats.
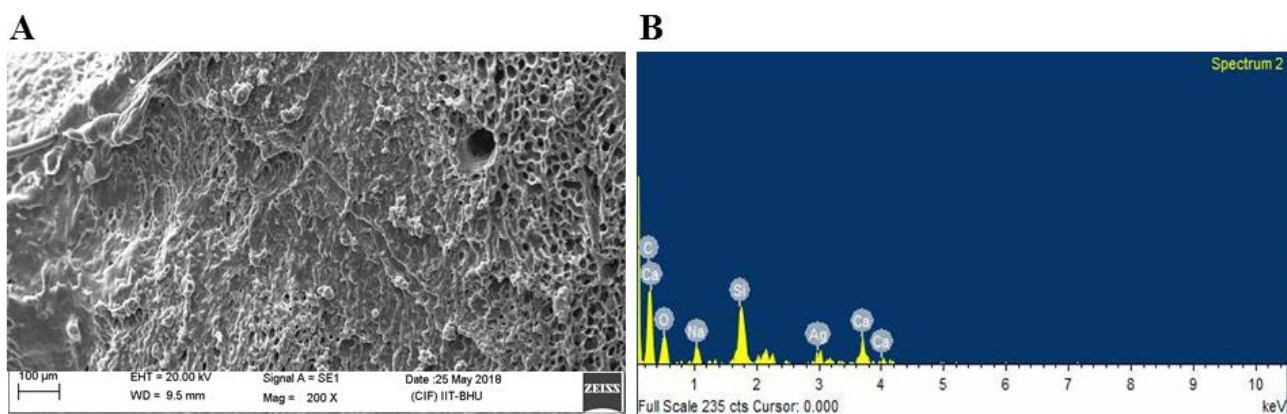

Figure 4.5 Deposition of AgBG in the liver. Liver image (A) was captured at a magnification of 200X shows the presence of particles, while image (B) shows particle present on the liver specimen have silver and silica peaks that confirm the particles are of AgBG

4.4.15 Acute toxicity study of BaBG

After the exposure of BaBG, the symptoms and mortality in each dose group were observed for 14 days. No mortality was observed at 5, 50 and 300 mg/kg body wt. doses of BaBG with soft or liquid faeces at 300 and 2000 mg/kg. However, one rat died at 2000 mg/kg out of the three rats therefore based on the OECD guideline 423, the LD50 value of single dose of the BaBG formulation was found to be more than 2000 mg/kg body weight.

4.4.16 Acute toxicity study of AgBG

No mortality was observed at 5 and 50 mg/kg body wt. doses of AgBG with soft or liquid faeces. However, two rats died at 300 mg/kg out of the three. Therefore, based on the OECD guideline 423, the LD50 value of the single dose of the AgBG formulation was found to be 300 mg/kg body weight.

4.5 Discussion

The present study revealed that 10 mg/kg dose of BaBG and 5 mg/kg of AgBG is safe dose if given continuously for 28 days daily. LD50 of the single-dose parenteral BaBG formulation was found to be more than 2000 mg/kg while LD50 of the single-dose parenteral AgBG formulation was found to be less than 300 mg/kg. However, BaBG at the dose of 50 mg/kg and AgBG at 10 mg/kg were found to have low *in vivo* toxicity as revealed by pathological examination, blood flow and weight/body weight coefficients of the liver. Further, both BaBG & AgBG at 50 mg/kg significantly increases the blood flow to the lungs.

The bioactive glass has multipurpose applications including drug delivery (El et al. 2012) cytocompatibility and bactericidal property (Liu et al. 2014), anti-inflammatory and antimicrobial activities (Greenspan et al. 2003), angiogenesis (Day et al. 2005; Keshaw et al.

2005) and neurogenesis (Bunting et al. 2005). However, there are no reports available with regard to repeated intravenous dosed toxicity study of bioactive glass. Therefore, in the present study, we have assessed the 28 days repeated dose subacute safety of BG in rats. The BaBG were administered at three doses (1, 10 and 50 mg/kg) and AgBG at (5, 10 and 50 mg/kg) by the tail vein injection continuously daily once for 28 days. No mortality or histological defects were seen at the doses of 1 and 10 mg/kg of BaBG doses and 5 mg/kg of AgBG. The haematology, P wave, QRS complex S-T interval, Q-T interval, blood pressure, food and water consumption showed no significant changes among the group. However the coefficient of liver weight, heart rate, lungs blood flow and histology of all most all the highly perfused organ shows the significant changes compared to the control animals at a dose of 50 mg/kg of BaBG and AgBG. Further, no significant alterations were seen in haematological data after 28 days repeated intravenous administration of the BG. Furthermore, LD50 of the single-dose parenteral BaBG formulation was found to be more than 2000 mg/kg, and for AgBG formulation, it was found to be less than 300 mg/kg.

An earlier study showed 20-25 nm silica-based nanoparticles completely clear from nude mice through hepatobiliary excretion with no sign of organ toxicity within 15 days followed by single intravenous infusion (Kumar et al. 2010). In contrast, another study demonstrated extensive liver injury by silica-based nanoparticles (Xie et al. 2010). A similar hepatotoxic effect was also reported for both single as well as the repeated dose of silica-based nanoparticles (Ye et al. 2010; Nishimori et al. 2009). Similarly, in the present study, EDS analysis confirms that BG accumulated in the liver when the dose was high enough to cause mild toxicity. Since the BG was administered by intravenous route even though the particulates of BG was observed in liver confirm that BG undergoes hepatobiliary excretion.

Further, the earlier report stated that phagocytosis of bioactive glass particulate plays a critical role in removing particles from the systemic circulation and transport them to the liver for excretion through faeces by bile (Dai et al. 2015). Moreover, mild lymphocytic infiltration, focal hepatocytic necrosis and chronic active hepatitis of the liver were seen at 50 mg/kg of BaBG and 10 & 5 mg/kg of AgBG. In support, another study demonstrated extensive liver injury by silica-based nanoparticles (Xie et al. 2010). A similar hepatotoxic effect was also reported for both single as well as the repeated dose of silica-based nanoparticles (Ye et al. 2010; Nishimori et al. 2009). Spleen examination confirms the presence of the megakaryocytes at the higher dose of 50 mg/kg of BaBG and AgBG. The microscopic examination of liver and spleen verified that the BG targets macrophages in liver and spleen, which is further supported by earlier studies (Dai et al. 2015). Further, a significant increase of liver weight coefficient at the higher dose of 50 mg/kg of BaBG and AgBG supported the fact that liver is the targeted organ for BaBG & AgBG mild toxic effect. Earlier reports also indicated that the silica has toxic effects on mitochondria-associated energy metabolism in hepatocytes causes swelling of the mitochondria, and then hinder cellular function (Mao et al. 2014). The macrophage has a tendency to quickly detect foreign particles (Brown et al. 2012) and is responsible for their removal through phagocytosis (Liu et al. 2014). Silica-based particles penetrate to liver cells. These particles activate liver specialized macrophage, also known as kupffer cell. These kupffer cells engulf the foreign particles and remove out from the body (Liu et al. 2014). Proinflammatory cytokines encourage chemotaxis and neutrophils aggregation, which help in the engulfment of the particles by liver specialized macrophage cells. In spite of this, the chemotaxis and

neutrophils aggregation results in the production of granulomas and causes liver injury (Yu et al. 2013).

Histology of lung showed that the dose of 50 mg/kg has a mild toxic effect as slight congestion, intra-alveolar haemorrhage with focal intra-alveolar congestion and lymphocytic infiltration in the interstitium. Inflammation in the lungs was reported after silica treatment in mice by intratracheal instillation (Roursgaard et al. 2011), which is in support of our observations. The mean blood flow of lungs significantly increases at the dose of 50 mg/kg of BaBG and 10 & 50 mg/kg of AgBG. However, all the test doses of BaBG and AgBG did not reveal any toxic effect through the lung weight coefficient.

In the current study, ECG data indicated a significant increase of heart rate at the dose of 50 mg/kg of BaBG and 10 & 50 mg/kg of AgBG, microscopic examination of heart revealed mild infiltration of lymphocyte with focal myonecrosis. The above information confirmed that BaBG at 50 mg/kg and AgBG at 10 & 50 mg/kg have a mild toxic effect in the heart which was supported by the earlier study that reported the toxic effect of silica nanoparticles in myocardial cells (Ye et al. 2010). Histological examination of kidney shows lymphocytic infiltration at interstitial tubule as well as glomerulus at the higher dose of 50 mg/kg of BaBG and AgBG. Earlier, silica nanoparticles were reported to induce cytotoxicity in human embryonic kidney cells (Wang et al. 2009). Phosphorus pentoxide is one of the lead components of the BG, and kennel reported that the bisphosphonate causes gastroesophageal irritation with esophagitis (Kennel et al. 2009). This may be the reason that microscopic examination of the stomach shows infiltration of neutrophil, eosinophil and lymphocyte at the basal part of the stomach with mild appearance of eosinophilic esophagitis at the dose of 50 mg/kg of BaBG and 10 & 50 mg/kg of AgBG.

In our study, subacute toxicity assessment was systematically investigated for BaBG at (1, 10, and 50 mg/kg) and AgBG at (5, 10, and 50 mg/kg) doses, iv in rats. The animal observation, organ weight coefficient, ECG, blood pressure, haematology, random blood glucose, mean blood flow and pathological examination of the highly perfused organ revealed that the dose of 10 mg/kg of BaBG and 5 mg/kg of AgBG is safe if given continuously for 28 days daily. Further, LD50 of the single parenteral dose of BaBG was found to be more than 2000 mg/kg while for AgBG it was found to be less than 300 mg/kg. In spite of that, the histological, heart rate, blood flow and organ weight/body weight coefficients of the liver revealed that 50 mg/kg dose of BaBG and 10 mg/kg of AgBG have low in vivo hepatotoxicity. Further, the blood flow of the lungs was found to be increased significantly at the higher dose of BaBG and AgBG, demonstrated their mild toxic effect in the liver and lungs.