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Research Article

Manganese Chloride Modulated Bone Homeostasis and Haematological Parameters during Lithogenic Diet-Induced Cholesterol Gallstone in Female Swiss Mice

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Abstract

Body mass index and hormonal imbalances are hallmarks of obesity prevalent in the female population. Obesity is positively correlated with gallstones and osteoporosis due to decreased bone mineral density. Manganese is reported to ameliorate gallstone formation in diabetes; however, its effects on haematological variables and bone health during cholesterol gallstone formation remain limited, which this study design aimed to investigate. Ninety female Swiss mice (18-22g) were divided into two studies. In study one, naive female mice were grouped into: Control and $MnCl_2$ (0.37, 0.74 and 2.0 mg/kg), while in study two, cholesterol gallstone was induced using a lithogenic diet and treated with $MnCl_2$ doses (GS+$MnCl_2$) as in study one, GS-alone and GS+Aspirin (350mg/kg). By weeks 4 and 8, Body and liver weights, Full blood count, plasma proteins, Alkaline Phosphatase (ALP), Femoral Bone morphology, and marrow Myeloid to Erythroid ratio (M:E) were evaluated. In study one, higher manganese doses significantly reduced PCV and haemoglobin levels; however, WBC and lymphocyte count significantly decreased in $MnCl_2$-treated groups compared with the control. Total serum protein, globulin, albumin, and ALP decreased significantly in the 0.37mg/kg MnCl2-treated group compared with the control group at weeks 4 and 8. Femur weight significantly increased at week 4, while M:E increased in all $MnCl_2$-treated groups at weeks 4 and 8 compared with the control. During gallstone formation (study two), PCV, haemoglobin, and RBC count were significantly increased, while neutrophil and platelet count decreased in $MnCl_2$-treated groups compared with the GS-alone group. Serum total protein and albumin significantly increase in GS+0.37mg/kg, while globulin increases in GS+2.0mg/kg. Liver ALP was reduced significantly in GS+0.37mg/kg and GS+0.74mg/kg compared with GS-alone. Femur weight significantly increased while M:E decreased in GS+2.0mg/kg $MnCl_2$ at week 8 compared with GS-alone.

Manganese attenuated osteoporosis associated with cholesterol gallstones by modulating bone homeostasis and hematopoiesis.

Key Words: *Gallstone, Bone homeostasis, Haematology, Manganese chloride*

INTRODUCTION

Gallstones are a common gastrointestinal disorder with associated health and economic burden. It predisposes to other severe conditions such as pancreatitis, cardiovascular disease, and cancer, with increased mortality (Zheng *et al*., 2018). It is formed as a result of an imbalance among bile components, leading to bile that is supersaturated with cholesterol and microcrystals that eventually grow into pathologic stones (Di Ciaula *et al*., 2018). Depending on whether the primary constituent of the stone is cholesterol, bilirubin, or deposits of calcium, gallstone can be classified into cholesterol, pigment, or mixed gallstones (Klahan *et al*., 2014). The mechanism of formation of gallstone has been attributed to an interplay between environmental and genetic factors, including female sex and age (Kazi *et al*., 2022). Several risk factors, such as dyslipidemia, diabetes mellitus, obesity, age, and female gender, due to the effect of estrogen hormone, have been noted to contribute to the development of gallstones (Dooley *et al*., 2018).

Excessive body fat accumulation, as seen in obesity, is a predisposing factor for gallstone formation and has also been noted to influence bone homeostasis. Traditionally, obesity was believed to have a protective role on bones (Compston *et al.,* 2011); however, recent research has challenged this belief. Bones are connective tissues that provide structural support, an environment for haematopoiesis within the bone marrow (Kamel-ElSayed *et al*., 2024). The continuous process through which bones are renewed is known as bone remodelling. (Epsley *et al*., 2021; Rowe *et al*., 2023; Janssens *et al*., 2005). There is a well-documented, intricate relationship between bone metabolism and haematopoiesis. Various proteins, such as chemokines and Insulin-like Growth Factor-1 (IGF-1), have been shown to positively influence erythropoiesis, which is also linked to bone metabolism (Ho *et al*., 2024). The balance of haematopoiesis between the myeloid and the erythroid cell

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series is measured by the myeloid/erythroid ratio (Bain *et al*., 2011). An increase in the myeloid/erythroid ratio reflects either a decrease in erythropoiesis relative to myelopoiesis or an increased myelopoiesis relative to erythropoiesis. Animal studies have shown that bone cells interact with haematopoietic cells to maintain erythropoiesis and myelopoiesis (Valderrábano and Wu, 2019).

Osteoporosis is a common bone remodelling disorder characterized by reduced bone mass, deterioration of bone tissue microarchitecture, and an increased vulnerability to fractures (Compston *et al*., 2019). It occurs frequently in postmenopausal women, of which 1 out of 3 women above the age of 50 is affected (Gosset *et al*., 2021), and considering the aging population, it is estimated that osteoporotic fractures incidence might increase by 25% in another decade (Li *et al*., 2022). Risk factors associated with osteoporosis, such as low body mass index and renal disease, are also linked with blood cells (Ho *et al*., 2022).

Gallstones and osteoporosis are both prevalent in older adults, with females having a higher prevalence. Recently, a strong association between osteoporosis and gallstones has been identified (Li and He, 2025). Klahan *et al*. (2014) noted in a Taiwan population study that osteoporotic patients have a higher risk of developing gallstones relative to the general population. Inflammation has been noted to be the common cause of osteoporosis and gallstone. Bone formation is inhibited by inflammation in pathological conditions, leading to the overproduction of pro-inflammatory mediators like nitric oxides, cytokines, and prostaglandins (Javier *et al*., 2023). Inflammation can also alter bile components, resulting in bile supersaturated with cholesterol. The inflammatory mediators released during inflammation can damage gallbladder epithelial cells, leading to bile stasis. Prolonged inflammation can damage tissue, and scars from continuous tissue damage can impact the liver, alter enterohepatic circulation, and this could lead to stone formation (Lin *et al*., 2017). This is evident in higher level of Alanine transaminase, Aspartate transaminase, and Alkaline phosphatase that is seen in patient with gallstone and the increase in these enzymes are also frequently reported in people with obesity (Choi, 2003; Jalili *et al*., 2022). Alkaline phosphatase (ALP), an enzyme that hydrolyses phosphate in alkaline medium is expressed in various organ such as liver, bile duct, intestine, bones and adipocytes (Ali *et al*., 2013). In the bones, ALP is a key indicator for bone metabolism and its alteration could reflect growth, repair and bone remodelling (Le *et al*., 2022). Elevated level of ALP has been observed in obese persons (Ali *et al*., 2006), during bone metastasis (Huang *et al*., 2017) and liver disease conditions.

Manganese is an essential element present in a variety of leafy vegetables and nuts. Of which its role in the maintenance of bone function and integrity has been noted (Wang *et al*., 2022; Yang *et al*., 2024). Recent research has identified a complex relationship between Mn and bone biology (Wei *et al*., 2022). It has been noted to play an important role in the formation of collagens, cartilage, as well as bone mineralization (Barrioni *et al*., 2014) and remodelling, which is crucial in adult bone homeostasis (Taskozhina *et al*., 2024). Studies on Mn deficiency presented adverse effects on the development and integrity of the skeletal system, such as the inhibition of cartilage formation and induction of osteopenia (Pepa *et al*., 2016).

Considering the prevalence of gallstones and osteoporosis in older adults, especially females and the importance of manganese in bone health, this study design therefore investigated the activity of manganese chloride ($MnCl_2$) on haematological changes and bone morphology during cholesterol gallstone formation in female Swiss mice.

MATERIALS AND METHODS

Animals: A total of ninety (90) healthy female Swiss mice of 18-22 g were purchased from the Animal House of the College of Medicine, University of Ibadan, Nigeria and were housed under standard conditions with access to food and water ad libitum. The research was approved by the University of Ibadan, Nigeria, Animal Ethics Committee under the designated number UI-ACUREC/007-0122/20.

Animal Groupings: The experiment was divided into 2; study one was carried out on normal animals, while study 2 was on animals with induced cholesterol gallstones.

Study One: 40 female Swiss mice were randomly distributed into 4 groups of 10 animals;

Group 1 - Control

Group 2 – 0.37 mg/kg $MnCl_2$

Group 3 – 0.74 mg/kg $MnCl_2$

Group 4 - 2.00 mg/kg $MnCl_2$

Study Two: Fifty female Swiss mice were randomized into 5 groups of 10 animals each;

Group 1 – Gallstone-alone

Group 2 – Gallstone + 0.37 mg/kg $MnCl_2$

Group 3 – Gallstone + 0.74 mg/kg $MnCl_2$

Group 4 - Gallstone + 2.00 mg/kg $MnCl_2$

Group 5 – Gallstone + Aspirin 350 mg/kg

Induction of cholesterol gallstone: Experimental cholesterol gallstone was induced with a lithogenic diet that contains 15% butter fat, 0.5% cholic acids and 1% cholesterol according to the methods of Han *et al*., (2019). Lithogenic diet feeding and manganese chloride ($MnCl_2$) treatment administration were carried out co-currently to the mice for a duration of 8 weeks.

Body weight Assessment: The daily weight of each animal in the nine groups of the two studies was using a standard weighing scale throughout the experiment and their respective weights were noted down.

Relative Liver Weight: The liver weight was noted after it was excised from the mice, rinsed in cold phosphate buffer saline, blotted cleaned on a filter paper on both 4 and 8 weeks of sacrifice. The relative weight was calculated as a fraction of the mouse body weight per treatment/experimental group.

Blood collection and evaluation of haematological parameters: At weeks 4 and 8, blood was collected using a heparinized capillary tube via ocular puncture into a lithium-heparinized bottle, after ketamine (75mg/kg b.w) anesthesia, before the animals were sacrificed. Full blood count and

differential white blood cell count were evaluated using standard laboratory method.

Serum biochemistry and Liver enzymes: Total serum protein, albumin, globulin, Blood Urea Nitrogen (BUN), and creatinine were assessed using the method of Dacie and Lewis, (1991). Alkaline phosphatase was estimated via a specified assay kit (Randox laboratory limited).

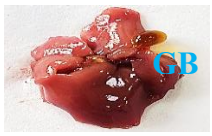
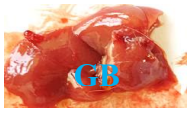
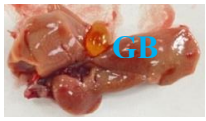
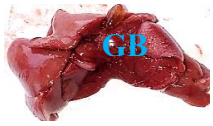
Bone collection and assessment: On sacrificing the animals the femur of each animal was collected and the soft tissues removed carefully using a scalpel blade. A smear of the bone marrow was made on a cleaned sterilized glass slide and allowed to air dry. The dried smeared slides were fixed with methanol after which it is stained with Wright's-Giemsa stain. The haematopoietic cell morphology was assessed under a light microscope and the myeloid and erythroid cells were counted. The myeloid to erythroid ratio was calculated from the proportion of total myeloid and erythroid lineage cells.

The right femur after the flesh has been teased out was preserved in 10% formalin. The weight of the femur was measured using a sensitive digital scale while the length, width and distance between the greater and lower trochanter (GT-LT) were measured using a digital vernier calliper.

Statistical Analysis: Data were analysed using one way analysis of variance and results were presents as Mean ± SEM. Difference between mean were considered significant at $p<0.05$ using Bonferroni post-hoc. A Graph-pad prism version 8.0.2 statistical package was used for analysis.

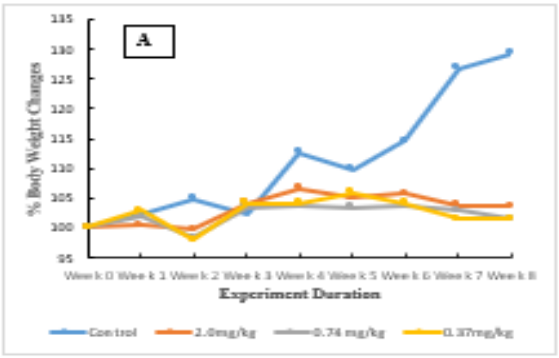
RESULTS

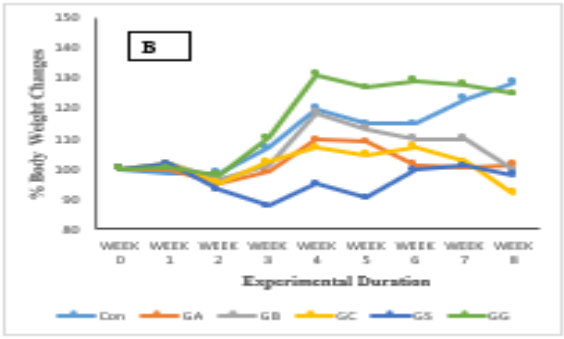
Table 1a:
Effect of $MnCl_2$ treatment on Macroscopic Liver and Gallbladder tissues in Naive Female Swiss and in Lithogen Diet Fed Mice During Cholesterol Gallstone Formation.

Groups	Week 4	Week 8	Groups	Week 4	Week 8
Control			Control		
	-	-	GS Alone		
0.37mg/kg $MnCl_2$			GS + 0.37mg/kg $MnCl_2$		
0.74mg/kg $MnCl_2$			GS + 0.74mg/kg $MnCl_2$		
2.00mg/kg $MnCl_2$			GS + 2.00mg/kg $MnCl_2$		
-	-	-	GS +ASP		

Note: GB: Gall bladder; FL: Fatty Liver

Effect of $MnCl_2$ Treatment on Percentage Weight Gain and Liver Relative Weight in Naive Female Swiss and Lithogenic Diet Fed Mice During Cholesterol Gallstone Formation: In experiment one, carried out using the normal/naive animals, the weekly percentage weight gain was observed to significantly decrease in the $MnCl_2$-treated groups compared with the control, Figure 1a. During cholesterol gallstone formation (experimental study 2), the GS-alone group had a significant increase in percentage weight gain, while the GS+$MnCl_2$-treated groups had a significant decrease in percentage weight gain compared with the GS-alone group. This showed that the lithogenic diet increased body weight gain, which was mitigated by $MnCl_2$, as shown in Figure 1b. The lithogenic diet caused hepatomegaly and fatty liver in the GS-alone group compared with the GS+$MnCl_2$-treated groups, as shown in Table 1a, 1b and 1c.





Figures 1A and B:
Effect of $MnCl_2$ treatment on Body Weight Changes in Naive Female Swiss Mice. (A) and in Lithogen Diet Fed Mice during CGS Formation (B)

Table 1b:
Effect of $MnCl_2$ treatment on Macroscopic Relative Liver Weight at 4 and 8 weeks in Naive Female Swiss Mice

Groups	Relative Liver weight	
	Week 4	Week 8
Control	4.50±0.30	5.10±0.10
0.37mg/kg $MnCl_2$	4.10±0.20	3.90±0.10
0.74mg/kg $MnCl_2$	5.00±0.20[b]	4.30±0.20[a]
2.0mg/kg $MnCl_2$	4.80±0.10	4.20±0.10[a]

Values are expressed Mean ± SEM significant at $p \leq 0.05$. [a]- compared with control, [b]- compared with 0.37mg/kg, [c]- compared with 0.74mg/kg, [d]- compared with 2.0mg/kg

Table 1c:
Effect of $MnCl_2$ treatment on Macroscopic Relative Liver Weight at 4 and 8 weeks in Lithogenic Diet Fed Mice During Cholesterol Gallstone Formation

Groups	Relative Liver weight	
	Week 4	Week 8
Control	5.20±0.05	4.51±0.09
GS-Alone	7.06±0.12[a]	5.56±0.25[a]
GS + 0.37mg/kg $MnCl_2$	5.77±0.03[b]	4.55±0.29
GS + 0.74mg/kg $MnCl_2$	5.43±0.06[b]	4.54±0.41[b]
GS + 2.0mg/kg $MnCl_2$	6.11±0.26[a,b]	4.79±0.12
GS +ASP	4.88±0.07[b,c,e]	5.73±0.12[a,c,d,e]

Values are expressed Mean ± SEM significant at $p \leq 0.05$. **Significance:** [a]- compared with control, [b]- compared with GS-alone, [c]- compared with GS+0.37, [d]- compared with GS+0.74, [e]- compared with GS+2.0, [f]- compared with GS+Asp.

Table 2a:
Effect of $MnCl_2$ treatment on Packed Cell Volume (PCV), Haemoglobin (Hb) level, Red Blood Cell (RBC) and Platelet counts In Naive Female Swiss Mice

GROUPS	PCV (%)		Hb (g/dL)		RBC (*10^6µL)		Platelet Count	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Control	41.00 ± 1.0	41.00 ± 0.88	13.97 ± 0.38	13.30 ± 0.17	6.40 ± 0.10	7.08 ± 0.23	74666.67 ± 4666.67	68000.00 ± 1154.70
0.37mg/kg $MnCl_2$	41.33 ± 0.67	42.67 ± 0.33	13.50 ± 0.06	14.17 ± 0.19	6.73 ± 0.06	7.30 ± 0.17	89666.00 ± 2185.81[a]	76666.67 ± 7055.33
0.74mg/kg $MnCl_2$	39.67 ± 0.33	35.33 ± 0.33[a,b]	13.30 ± 0.06	11.7 ± 0.27[a,b]	6.50 ± 0.04	5.63 ± 0.15[a,b]	110000.00 ± 3464.10[a]	107666.70 ± 2333.33[a,b]
2.0 mg/kg $MnCl_2$	37.33 ± 1.45[a,b]	36.33 ± 0.67[a,b]	11.43 ± 0.30[a,b,c]	12.00 ± 0.29[ab]	6.68 ± 0.39	6.56 ± 0.09[c]	95000.00 ± 2645.75[a]	87333.33 ± 4807.40[a,c]

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus 0.37mg/kg, [c]- versus 0.74mg/kg, [d]- versus 2.00mg/kg

Table 3a:
Effect of $MnCl_2$ treatment on White Blood Cell (WBC), Lymphocytes, Neutrophil, Monocytes and Eosinophil Counts in Naive Female Swiss Mice

GROUPS	WBC Count (*10^3µl)		Lymphocytes (%)		Neutrophils (%)		Monocytes (%)		Eosinophils (%)	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Control	4450.00 ± 175.59	4450.00 ± 175.59	71.00 ± 1.53	72.33 ± 0.33	28.33 ± 1.20	25.33 ± 0.67	2.67 ± 0.33	2.67 ± 0.33	3.00 ± 0.00	3.00 ± 0.00
0.37mg/kg $MnCl_2$	4283.00 ± 109.29	3916.67 ± 116. 67[a]	71.67 ± 0.67	72.33 ± 0.33	24.33 ± 0.33[a]	25.00 ± 0.58	1.67 ± 0.33	1.33 ± 0.33[a]	3.00 ± 0.00	2.33 ± 0.67
0.74mg/kg $MnCl_2$	4533.33 ± 88.19	4166.67 ± 305.96[a]	71.67 ± 0.67	66.67 ± 0.33[a,b]	25.33 ± 0.88	31.00 ± 1.16[a,b]	2.00 ± 0.00	2.00 ± 0.00	1.00 ± 0.00[a,b]	3.0 ± 0.00
2.0mg/kg $MnCl_2$	4433.33 ± 360.94	3600.00 ± 152.75[a]	68.00 ± 0.57[a,b,c]	67.33 ± 0.67[a,b]	28.00 ± 0.00[b]	31.67 ± 0.88[a,b]	2.33 ± 0.33	2.00 ± 0.00	2.00 ± 0.00[a,b,c]	2.00 ± 0.0[a,c]

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus 0.37mg/kg, [c]- versus 0.74mg/kg, [d]- versus 2.00mg/kg

Effect of $MnCl_2$ Treatment on Haematological Variables in Naive Female Swiss Mice: The packed cell volume and haemoglobin level was significantly reduced in the 0.74 mg/kg $MnCl_2$-treated group at week 8 only in experiment 1, while there was a significant reduction of these variables in the 2.0 mg/kg $MnCl_2$-treated group at both weeks 4 and 8 when compared with the control. Meanwhile, the red blood cell count was reduced significantly in 0.74mg/kg $MnCl_2$-treated group at week 8, and the platelet count was observed to significantly increase in all the $MnCl_2$-treated groups at both 4 and 8 weeks, as summarized in Table 2a.

The white blood cells at week 8 reduced significantly in all the $MnCl_2$-treated groups compare with the control. The

lymphocyte count was noted to significantly reduce in 0.74 mg/kg at week 8 and 2.0 mg/kg $MnCl_2$-treated group at weeks 4 and 8 compared with the control, while neutrophil counts increased in 0.74 and 2.0 mg/kg $MnCl_2$-treated at week 8 compared with the control. The eosinophils count at week 4 was reduced significantly in 0.74 and 2.0mg/kg $MnCl_2$-treated group while at week 8, there was a significant reduction in 2.0mg/kg group compared with control, as shown in Table 3a. The serum total protein, globulin, albumin, and blood urea nitrogen levels was observed to decrease significantly in the 0.37mg/kg $MnCl_2$-treated compared with control and other doses at both weeks 4 and 8, as displayed in Table 4a.

Table 4a:
Effect of $MnCl_2$ treatment on Total Protein, Globulin, Albumin, Albumin to Globulin Ratio, Blood Urea Nitrogen (BUN), and Creatinine Levels in Naive Female Swiss Mice

GROUPS	Total Protein (g/dL)		Globulin (g/dL)		Albumin (g/dL)		Alb:Glo		BUN (mg/dL)		Creatinine (mg/dL)	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Control	9.17 ± 0.33	8.83 ± 0.09	5.17 ± 0.03	5.13 ± 0.03	4.33 ± 0.22	3.27 ± 0.15	0.69 ± 0.02	0.69 ± 0.02	18.33 ± 0.37	17.77 ± 0.12	0.77 ± 0.03	1.00 ± 0.10
0.37mg/kg $MnCl_2$	7.60 ± 0.38[a]	7.80 ± 0.10	4.53 ± 0.34	4.47 ± 0.19	3.07 ± 0.09[a]	3.33 ± 0.09	0.74 ± 0.02	0.72 ± 0.02	16.63 ± 0.23[a]	16.50 ± 0.10[a]	0.63 ± 0.03	0.80 ± 0.00
0.74mg/kg $MnCl_2$	9.60 ± 0.15[b]	9.17 ± 0.54	5.23 ± 0.09	5.93 ± 0.22[a,b]	3.30 ± 0.06[a]	3.70 ± 0.15	0.73 ± 0.01	0.72 ± 0.03	18.30 ± 0.15[b]	18.57 ± 0.23[b]	0.77 ± 0.03	0.90 ± 0.1
2.0 mg/kg $MnCl_2$	9.53 ± 0.59[b]	9.30 ± 0.29[b]	5.87 ± 0.24[b]	5.30 ± 0.06[b]	4.70 ± 0.30[b,c]	4.27 ± 0.07[a,b]	0.75 ± 0.03	0.67 ± 0.01	17.73 ± 0.48	18.17 ± 0.39[b]	0.70 ± 0.06	1.17 ± 0.12[b]

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus 0.37mg/kg, [c]- versus 0.74mg/kg, [d]- versus 2.00mg/kg

Effect of $MnCl_2$ Treatment on Haematological Variables in Lithogenic Diet Fed Mice During Cholesterol Gallstone Formation: The GS-alone group was observed to have a significant reduction in Packed cell volume, Haemoglobin and red blood cell count. However, treatment with $MnCl_2$ resulted in a significant increase in the aforementioned variables especially at week 4 of treatment compared with the GS-alone group. However, the platelet count was significantly reduced at week 4 in 0.37mg/kg $MnCl_2$ compared with GS-alone, while at week 8, the 0.74mg/kg $MnCl_2$-treated group had a significant decreased platelet count, as summarized in Table 2b.

There was no significant difference in the white blood cell and lymphocytes counts when the $MnCl_2$-treated groups were compared with GS-alone however, neutrophils count was observed to decrease significantly in all $MnCl_2$-treated groups compared with GS-alone at both weeks 4 and 8, as shown in Table 3b.

The total protein, albumin level, and albumin to globulin ratio at week 4 was increased significantly in GS+0.37mg/kg $MnCl_2$-treated group compared with GS-alone. The globulin level was significantly increased in GS+2.0mg/kg at week 8 compared with GS-alone. The albumin to globulin ratio was observed to significantly decrease in all the $MnCl_2$-treated group at week 8 compared with GS-alone group. The aspirin treated group had a significant increase in total protein, albumin, globulin, and albumin to globulin ratio, as displayed in Table 4b.

Table 2b:
Effect of $MnCl_2$ treatment on Packed Cell Volume (PCV), Haemoglobin (Hb) level, Red Blood Cell count (RBC) and Platelet count in Lithogen Diet Fed Mice during Cholesterol Gallstone Formation

GROUPS	PCV (%)		Hb (g/dL)		RBC (*10^6 µL)		Platelet Count	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Control	40.33 ± 1.45	41.33 ± 0.88	12.80 ± 0.47	13.43 ± 0.30	6.67 ± 0.22	6.72 ± 0.25	71666.67 ± 1666.67	68000.00 ± 1154.70
GS Alone	34. 67 ± 0.67[a]	38.0 ± 1.53	11.43 ± 0.13	12.43 ± 0.62	5.57 ± 0.13[a]	6.29 ± 0.28	107666.67 ± 6227. 18[a]	99333. 33 ± 4055.18[a]
GS + 0.37 mg/kg $MnCl_2$	37.67 ± 1.20	37.00 ± 0.57	12.17 ± 0.38	12.03 ± 0.20	6.29 ± 0.27	6.79 ± 0.35	85333.33 ± 6009.25[b]	102000.00 ± 2309.40[a]
GS + 0.74 mg/kg $MnCl_2$	41.67 ± 1.45[b]	39.00 ± 0.57	13.50 ± 0.44[b]	12.47 ± 0.23	6.58 ± 0.17[b]	6.56 ± 0.09	104666.67 ± 2403.70[a,c]	72333.33 ± 2333.33[b,c]
GS + 2.0 mg/kg $MnCl_2$	42.33 ± 1.45[b,c]	40.33 ± 0.33	14.50 ± 0.61[a,b,c]	12.90 ±0.06	7.37 ± 0.28[b,c]	6.74 ± 0.09	121666.67 ± 3480.10[a,c]	94000.00 ± 5291.50[a,c,d]
GS +ASP	39.00 ± 0.57	38.33 ± 0.67	12.70 ± 0.06[e]	12.40 ± 0.12	6.36 ± 0.14[e]	6.57 ± 0.04	106666.67 ± 4055. 18[a,c]	92000.00 ± 4725.82[a,d]

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus GS-alone, [c]- versus GS+0.37, [d]- versus GS+0.74, [e]- versus GS+2.00, [f]- versus GS+Asp

Table 3b:
Effect of $MnCl_2$ treatment on White Blood Cell (WBC), Lymphocytes, Neutrophil, Monocytes and Eosinophil Counts in Lithogen Diet Fed Mice During Cholesterol Gallstone Formation

GROUPS	WBC Count (*10^3µl)		Lymphocytes (%)		Neutrophils (%)		Monocytes (%)		Eosinophils (%)	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Control	3033.33 ± 317.98	3244. 33 ± 109.54	64.67 ± 2.19	66.67 ± 0.67	25.67 ± 0.88	24.33 ± 0.67	2.33 ± 0.67	2.00 ± 0.57	2.67± 0.33	2.33± 0.33
GS Alone	3625.00 ± 72.17	3500.00 ± 104.08	70.00 ± 1.16	71.00 ± 2.08	28.33 ± 0.67	35.67 ± 0.33[a]	2.33 ± 0.33	2.00 ± 0.00	2.00 ± 0.57	2.00 ± 0.57
GS + 0.37mg/kg $MnCl_2$	3550 ± 144.34	3783. 33 ± 130.17	66.33 ± 0.88	66.00 ± 0.57	28.33 ± 0.33	30.67 ± 0.33[a,b]	1.67 ± 0.33	1.67 ± 0.33	1.33± 0.33	1.67± 0.33
GS + 0.74mg/kg $MnCl_2$	4225.00 ± 418.58[a]	3316. 67 ± 101.38	68.67 ± 0.88	66.67 ± 0.88	25.33 ± 1.33	30.67 ± 0.67[a,b]	1.67 ± 0.33	1.33 ± 0.33	1.33± 0.33	1.33± 0.33
GS + 2.0mg/kg $MnCl_2$	3033. 33[d] ± 130.17	4016. 67 ± 33.33	69.67 ± 1.86	68.33 ± 0.88	24.33 ± 1.20[b,c]	25.33 ± 0.33[b,d]	2.00 ± 0.57	2.00 ± 0.58	1.33± 0.33	1.33± 0.33
GS +ASP	3406.67 ± 229.95	3050.00 ± 57.74[e]	67.00 ± 1.53	64.67 ± 0.33[b]	30.67 ±0.88[a,d,e]	29.33 ± 0.882[a,b,e]	1.67 ± 0.33	1.33 ± 0.33	1.00 ± 0.00	1.67± 0.67

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus GS-alone, [c]- versus GS+0.37, [d]- versus GS+0.74, [e]- versus GS+2.00, [f]- versus GS+Asp.

Table 4b:
Effect of $MnCl_2$ treatment on Total Protein, Albumin, Globulin, Albumin to Globulin Ratio, Blood Urea Nitrogen (BUN) and Creatinine level in Lithogen Diet Fed Mice During Cholesterol Gallstone Formation

Groups	Total Protein (g/dL)		Albumin (g/dL)		Globulin (g/dL)		Alb:Glo		BUN (mg/dL)		Creatinine (mg/dL)	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Control	7.13 ± 0.44	7.13 ± 0.44	2.90 ± 0.21	2.90 ± 0.21	4.23 ± 0.23	4.23 ± 0.23	0.68 ± 0.01	0.67 ± 0.01	21.47 ± 1.26	21.47 ± 1.26	0.60 ± 0.06	0.60 ± 0.06
GS Alone	6.67 ± 0.17	7.50 ± 0.06	2.83 ± 0.03	3.23 ± 0.07	4.10 ± 0.15	4.33 ± 0.03	0.68 ± 0.01	0.76 ± 0.02[a]	20.53 ± 0.09	23.13 ± 0.15	0.60 ± 0.0	0.67 ± 0.03
GS + 0.37mg/kg $MnCl_2$	7.83 ± 0.03[b]	7.37 ± 0.09	3.37 ± 0.03[a,b]	3.13 ± 0.03	4.43 ± 0.03	4.23 ± 0.03	0.76 ± 0.01[a,b]	0.71 ± 0.01	22.80 ± 0.10	23.17 ± 0.30	0.67 ± 0.03	0.67 ± 0.03
GS + 0.74mg/kg $MnCl_2$	6.93 ± 0.18	7.63 ± 0.18	3.03 ± 0.09	3.20 ± 0.12	4.13 ± 0.03	4.30 ± 0.00	0.71 ± 0.00	0.68 ± 0.02[b]	22.80 ± 0.31	22.77 ± 0.20	0.60 ± 0.00	0.67 ± 0.03
GS + 2.0mg/kg $MnCl_2$	6.80 ± 0.06	7.77 ± 0.38	2.67 ± 0.03[c]	3.43 ± 0.03[a]	4.13 ± 0.03	5.13 ± 0.03[a,b,c,d]	0.65 ± 0.01[c,d]	0.67 ± 0.0[b]	21.47 ± 0.09	22.40 ± 0.21	0.63 ± 0.03	0.70 ± 0.06
GS +ASP	8.13 ± 0.17[b,d,e]	9.10 ± 0.35[a,b,c,d,e]	3.40 ± 0.06[a,b,e]	3.17 ± 0.09	4.73 ± 0.12[b,d,e]	5.27 ± 0.09[a,b,c,d]	0.74 ± 0.00[a,b,e]	0.75 ± 0.01[a,d,e]	23.10 ± 0.06	24.27 ± 1.10[a]	0.60 ± 0.00	0.67 ± 0.03

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus GS-alone, [c]- versus GS+0.37, [d]- versus GS+0.74, [e]- versus GS+2.00, [f]- versus GS+Asp.

Effect of $MnCl_2$ Treatment on Femur Morphology in Naive Female Swiss and Lithogenic Diet Fed Mice During Cholesterol Gallstone Formation: In the normal/naive animals, the femur weight was observed to increase significantly in all the $MnCl_2$-treated groups at week 4 compared with control while the distance between the greater and lesser trochanter was noted to decrease compared with control at week 4. The femur bone mass density in the normal animals was significantly increased in the $MnCl_2$-treated groups relative to the control especially at week 4 as summarized in Table 5a. However, during cholesterol gallstone formation, the femur weight at week 8 was observed to increase, with a significant increase seen in GS+0.74mg/kg $MnCl_2$. The femoral bone mass density of the GS+0.74mg/kg group was significantly increased compare with other groups including control at week 8, as shown in Table 5b.

Table 5a:
Effect of $MnCl_2$ treatment on Femur Morphology in Naive Female Swiss Mice

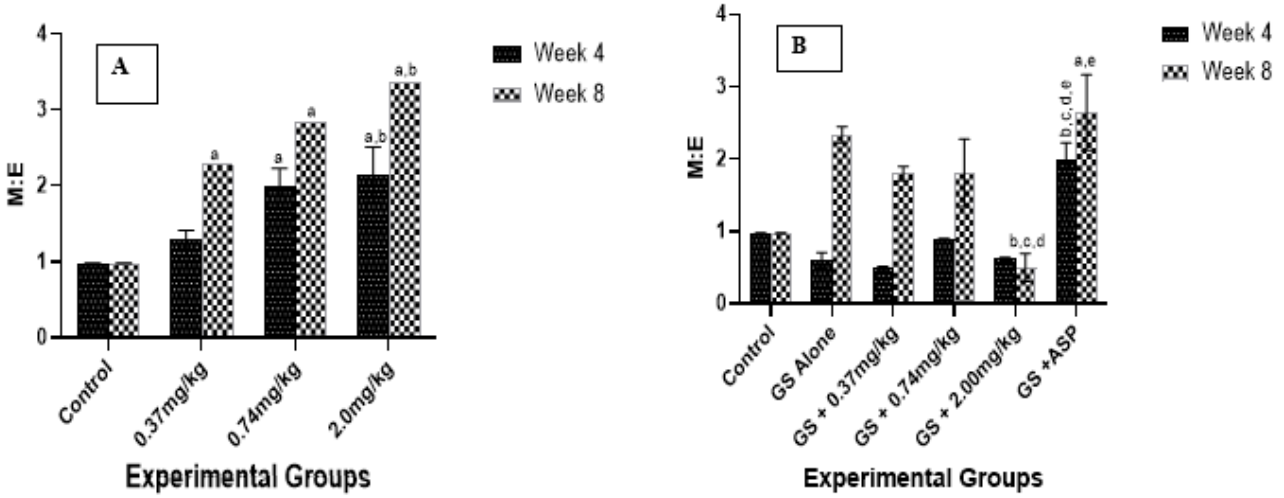
Groups	Femur weight *10^-2(g)		Femur length (In)		Femur width (In)		Bone Mass Density (g/in)*10^-1		GT-LT*10^-1(In)	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Control	3.00±0.56	3.10±0.56	0.53±0.05	0.57±0.10	0.07±0.01	0.07±0.01	0.58±0.05	0.58±0.05	1.37±0.06	1.34±0.04
0.37mg/kg $MnCl_2$	4.53±0.31[a]	3.47±0.09	0.57±0.02	0.57±0.0	0.07±0.00	0.06±0.0	0.76±0.02[a]	0.61±0.02	1.28±0.04	1.33±0.02
0.74mg/kg $MnCl_2$	4.67±0.47[a]	3.56±0.03	0.53±0.00	0.40±0.17	0.06±0.00	0.06±0.0	0.78±0.07[a]	0.63±0.00	1.29±0.02	1.42±0.01
2.00 mg/kg $MnCl_2$	4.55±0.14[a]	3.00±0.0	0.56±0.01	0.53±0.00	0.06±0.00	0.07±0.00	0.82±0.04[a]	0.56±0.00	1.20±0.06[a]	1.25±0.03[c]

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus 0.37mg/kg, [c]- versus 0.74mg/kg, [d]- versus 2.00mg/kg

Table 5b:
Effect of $MnCl_2$ Treatment on Femur Morphology in Lithogen Diet Fed Mice During Cholesterol Gallstone Formation

Groups	Femur weight *10^-2 (g)		Femur length (In)		Femur width (In)		Bone Mass Density (g/in)*10^-1		GT-LT*10^-1 (In)	
	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Control	3.00±0.00	3.00±0.00	0.57±0.10	0.50±0.05	0.07±0.01	0.06±0.01	0.58±0.05	0.58±0.05	1.37±0.06	1.31±0.04
GS Alone	3.90±0.00	3.70±0.15	0.57±0.02	0.57±0.00	0.07±0.00	0.06±0.0	0.59±0.01	0.64±0.02	1.34±0.08	1.26±0.05
GS + 0.37mg/kg $MnCl_2$	3.50±0.28	4.00±0.30	0.57±0.01	0.52±0.01	0.06±0.01	0.05±0.01	0.61±0.02	0.66±0.01	1.30±0.07	1.24±0.02
GS + 0.74mg/kg $MnCl_2$	3.13±0.23	4.80±0.73[a]	0.55±0.01	0.56±0.02	0.07±0.00	0.06±0.00	0.57±0.04	1.02±0.04[a,b,c]	1.24±0.07[a]	1.35±0.01
GS + 2.0mg/kg $MnCl_2$	3.33±0.12	0.35±0.40	0.54±0.02	0.47±0.03[b]	0.08±0.00[c]	0.05±0.0	0.62±0.00	0.59±0.04[d]	1.17±0.15[ab]	1.26±0.01
GS +ASP	3.33±0.30	4.10±0.50	0.56±0.01	0.56±0.00[e]	0.08±0.00[c]	0.06±0.0	0.53±0.03	0.65±0.01[d]	1.30±0.05	1.27±0.01

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus GS-alone, [c]- versus GS+0.37, [d]- versus GS+0.74, [e]- versus GS+2.00, [f]- versus GS+Asp.



Figures 2a and b:
Effect of $MnCl_2$ treatment on Myeloid to Erythroid series ratio in Naive Female Swiss Mice. (A) and in Lithogen Diet Fed Mice during cholesterol gallstone (B) formation
In A: *Significant at $p \leq 0.05$; [a]- versus control, [b]- versus 0.37mg/kg, [c]- versus 0.74mg/kg, [d]- versus 2.00mg/kg,* ***in B:*** *Significant at $p \leq 0.05$; [a]- versus control, [b]- versus GS-alone, [c]- versus GS+0.37, [d]- versus GS+0.74, [e]- versus GS+2.00, [f]- versus GS+Asp.*

Effect of $MnCl_2$ Treatment on Myeloid to Erythroid Series Ratio in Naïve Female Swiss and Lithogenic Diet Fed Animals During Cholesterol Gallstone Formation: In the normal/naive animals, a significant increase in the myeloid to erythroid series ratio was observed in all $MnCl_2$-treated groups at both weeks 4 and 8 compared with control, as displayed in Figure 2a. However, during cholesterol gallstone formation, the aspirin treated group had a significant increase in M:E ratio compared with GS-alone and the $MnCl_2$-treated groups at week 4, while at week 8 a significant reduction in M:E ratio was noted in 2.0mg/kg $MnCl_2$-treated group compared with GS-alone and the lesser doses of $MnCl_2$-treated groups, as shown in Figure 2b.

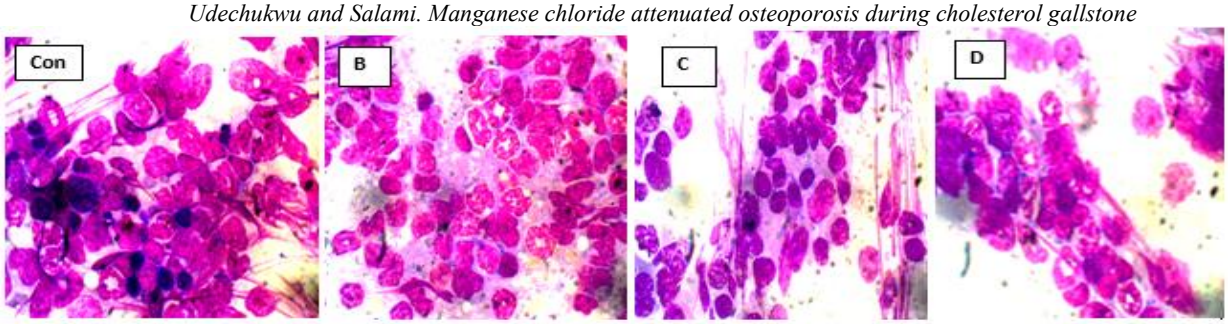


PLATE 1: PHOTOMICROGRAPH OF HEMATOPOIETIC CELLS IN BONE MARROW OF $MnCl_2$ TREATED ANIMALS AT 4WEEKS (USING GIEMSA STAIN, MAG. X 1000): Con (Control): The marrow is hypercellular with a hazy background. There is erythroid hyperplasia characterized by the abundant rubriblasts and rubricytes when compared to the myeloid series. **B (0.37mg/kg $MnCl_2$):** Normo-cellular- Marrow shows more of mature cell series for both myeloid and erythroid cells, **C (0.74mg/kg $MnCl_2$):** Marrow is normocellular, characterized by balanced maturation (orderly and proportional) of myeloid and erythroid cells. **D (2.00mg/kg $MnCl_2$):** Marrow is normocellular with less adipocytes. There are erythroid hypoplasia and more myeloid cells characterized by neutrophilic maturation.

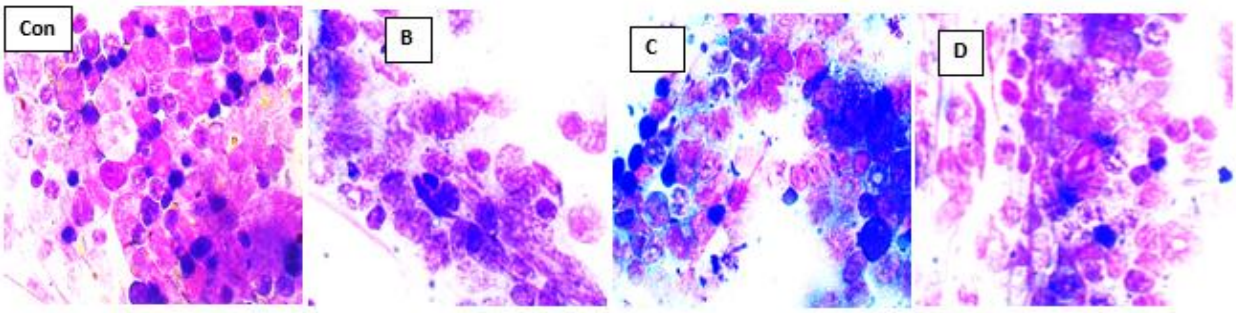


PLATE 2: PHOTOMICROGRAPH OF HEMATOPOIETIC CELLS IN BONE MARROW OF $MnCl_2$ TREATED ANIMALS AT 8WEEKS (USING GIEMSA STAIN, MAG. X. 1000): Con (Control): The marrow is hypercellular with a clear background. There is erythroid hyperplasia characterized by the abundant rubriblasts and rubricytes when compared to the myeloid series; **B (0.37mg/kg $MnCl_2$):** The marrow is normocellular. There are more myeloid cells. **C (0.74mg/kg $MnCl_2$):** The marrow is hypercellular. There are more erythroid cells. **D (2.00mg/kg $MnCl_2$):** The marrow is hypercellular. There are more myeloid cells.

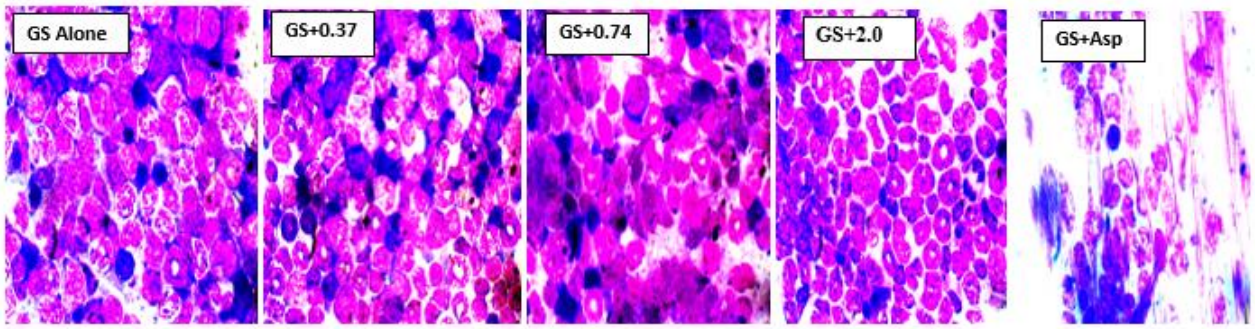


PLATE 3: PHOTOMICROGRAPH OF HEMATOPOIETIC CELLS IN BONE MARROW OF $MnCl_2$ TREATED ANIMALS DURING CHOLESTEROL GALLSTONE FORMATION AT 4WEEKS (USING GIEMSA STAIN, MAG. X 1000): Gallstone Untreated (GS ALONE): The marrow is hypercellular with a hazy background. There is erythroid hyperplasia characterized by the abundant rubriblasts and rubricytes compared with the myeloid series. **GS+0.37 mg/kg $MnCl_2$ (GS+0.37):** The marrow is hypercellular with a hazy background. There is erythroid hyperplasia characterized by the abundant rubriblasts and rubricytes compared with the myeloid series. **GS+0.74 mg/kg $MnCl_2$ (GS+0.74):** The marrow is hypercellular with a hazy background. There is erythroid hyperplasia characterized by the abundant rubriblasts and rubricytes compared with the myeloid series. **GS+2.00 mg/kg $MnCl_2$ (GS+2.0):** marrow is hypercellular with a clear background. There is erythroid hyperplasia characterized by the abundant rubriblasts and rubricytes compared with the myeloid series. There is also evidence of mitosis. **Gallstone+Aspirin (GS+Asp):** The marrow is hypocellular with a hazy background. There is erythroid hypoplasia compared with the myeloid series.

Effect of $MnCl_2$ Treatment on Serum Alkaline Phosphatase Level in Naive Female Swiss and Lithogenic Diet Fed Animals During Cholesterol Gallstone Formation: In the normal/naive animals, the level of alkaline phosphatase was significantly reduced in 0.37 mg/kg $MnCl_2$-treated group compared with control at both weeks 4 and 8 as shown in Figure 3a. During cholesterol gallstone, the level of alkaline phosphatase was observed to reduce significantly in GS+0.37mg/kg, GS+0.74mg/kg and GS+Aspirin groups compared with GS-alone group at week 8 while no significant difference was seen at week 4, as displayed in Figure 3b.

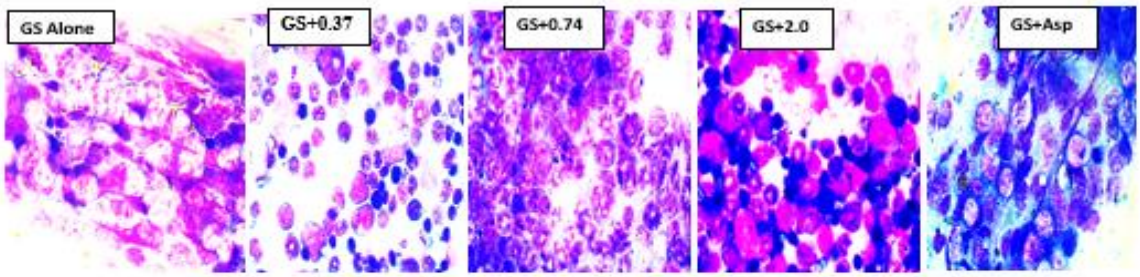
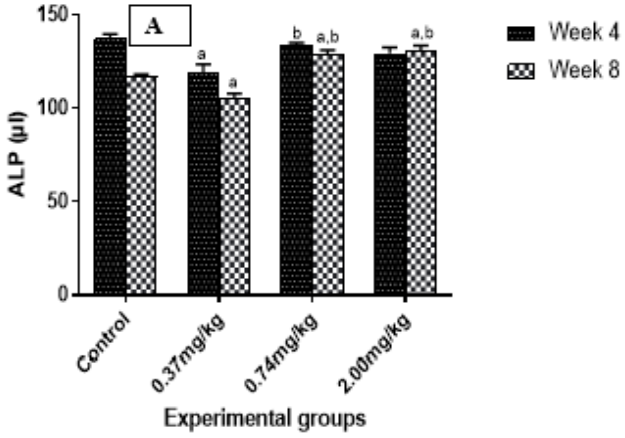
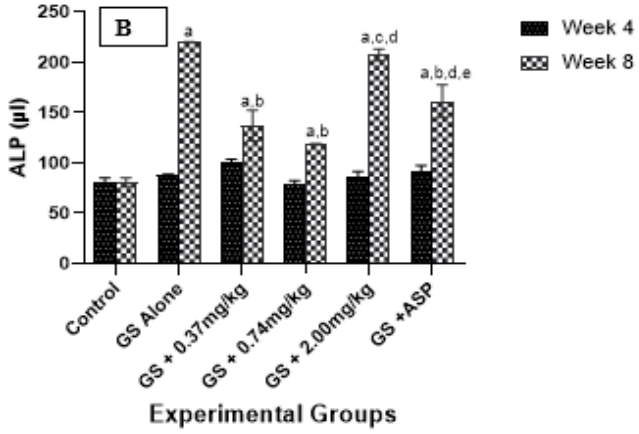


PLATE 4: PHOTOMICROGRAPH OF HEMATOPOIETIC CELLS IN BONE MARROW OF $MnCl_2$ TREATED ANIMALS DURING CHOLESTEROL GALLSTONE FORMATION AT 8WEEKS (USING GIEMSA STAIN, MAG. X 1000): GS Alone (Gallstone Untreated): The marrow is hypercellular with a hazy background. There is myeloid hyperplasia. **GS+0.37 mg/kg $MnCl_2$ (GS+0.37):** The marrow is normocellular. The cellular proportions are normal. **GS+0.74 mg/kg $MnCl_2$ (GS+0.74):** The marrow is hypercellular. There are more erythroid cells. **GS+2.00 mg/kg $MnCl_2$ (GS+2.0):** The marrow is hypercellular. There are more erythroid cells. **Gallstone+Aspirin (GS+Asp):** The marrow is normocellular. There are more myeloid cells.





Figures 3a and b:
Effect of $MnCl_2$ treatment on Serum Alkaline Phosphatase (ALP) Level in Naive Female Swiss Mice. (A) and in Lithogen Diet Fed Mice During Cholesterol Gallstone (B) Formation

***In A**: Significant at $p \leq 0.05$; [a]- versus control, [b]- versus 0.37mg/kg, [c]- versus 0.74mg/kg, [d]- versus 2.00mg/kg, **In B**: Significant at $p \leq 0.05$; [a]- versus control, [b]- versus GS-alone, [c]- versus GS+0.37, [d]- versus GS+0.74, [e]- versus GS+2.00, [f]- versus GS+Asp.*

Table 6a:
Effect of $MnCl_2$ treatment on the correlation between ALP and femur mass density in the naïve Female Swiss Mice

GROUPS	WEEK 4		WEEK 8	
	Correlation Coefficient	p-Value	Correlation Coefficient	p-Value
Control	0.74	0.47		
0.37mg/kg $MnCl_2$	0.88 [acd]	0.32	0.51	0.66
0.74mg/kg $MnCl_2$	-0.55	0.63	1.00 [bd]	0.00
2.00mg/kg $MnCl_2$	-0.99	0.10	0.50	0.67

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus 0.37mg/kg, [c]- versus 0.74mg/kg, [d]- versus 2.00mg/kg

Table 6b:
Effect of $MnCl_2$ treatment on the correlation between ALP and femur mass density in Lithogen diet fed mice during cholesterol gallstone formation

GROUPS	WEEK 4		WEEK 8	
	Correlation Coefficient	p-Value	Correlation Coefficient	p-Value
Control	0.74	0.47		
GS Alone	0.95 [acdef]	0.20	-0.81	0.40
GS + 0.37mg/kg $MnCl_2$	-0.06	0.96	-0.79	0.43
GS + 0.74mg/kg $MnCl_2$	-0.94	0.22	0.50 [bce]	0.67
GS + 2.00mg/kg $MnCl_2$	0.79 [cd]	0.43	0.03	0.98
GS +ASP	0.72 [cd]	0.49	1.00 [bcde]	0.02

Significant at $p \leq 0.05$; [a]- versus control, [b]- versus GS-alone, [c]- versus GS+0.37, [d]- versus GS+0.74, [e]- versus GS+2.00, [f]- versus GS+Asp.

Effect of $MnCl_2$ Treatment on the Correlation Between ALP and Femur Mass Density in Naive Female Swiss and

Lithogenic Diet Fed Mice During Cholesterol Gallstone Formation: In the normal/naive animals, a negative correlation was observed in all treatment groups at week 4 except in 0.37mg/kg, but were all reversed at 8 weeks, Table 6a. During cholesterol gallstone formation, a negative correlation was observed in GS+0.37mg/kg, GS+0.74mg/kg at week 4 which was reversed at week 8 except in the GS+0.37mg/kg group having negative correlation alongside the GS-alone group, Table 6b.

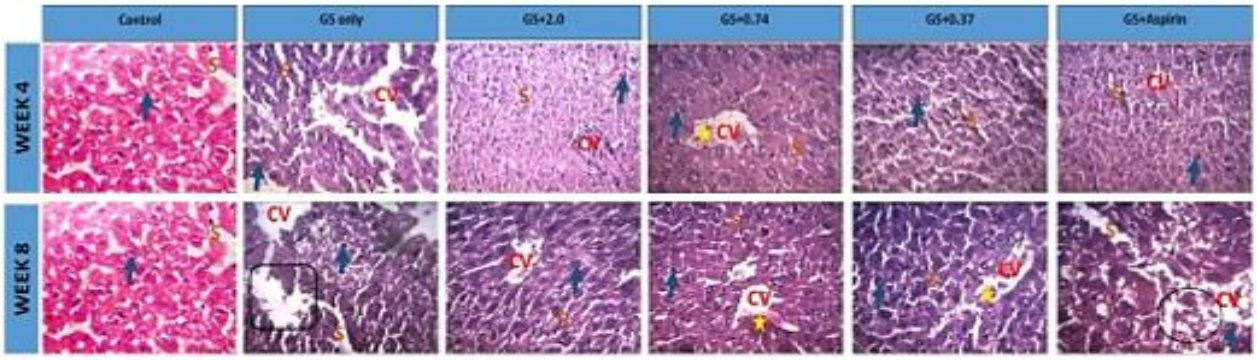


PLATE 5: PHOTOMICROGRAPH OF LIVER CELLS IN $MnCl_2$ TREATED ANIMALS BY WEEKS 4 AND 8 DURING CHOLESTEROL GALLSTONE FORMATION (MAGX100 USING HAEMATOXYLIN AND EOSIN STAIN): Control: Normal lobular architecture is preserved. Hepatocytes are seen radiating from the central vein with no evidence of necrosis, fatty change, or fibrosis at both week 4 and week 8. **Gallstone Untreated (GS only):** The central vein appears congested. Hepatocytes are sparsely distributed, with widened hepatic sinusoids around the central vein (rectangular-marked area) and loss of cellular integrity at both week 4 and week 8. **GS + 2.0 mg/kg $MnCl_2$ (GS+2.0):** Normal histological structure is observed. Hepatocytes are preserved with intact architecture, and hepatic sinusoids are not prominently dilated at both week 4 and week 8. **GS + 0.74 mg/kg $MnCl_2$ (GS+0.74):** Hepatocytes are large and polygonal with preserved hepatic cords and a central vein showing mild congestion at both week 4 and week 8. **GS + 0.37 mg/kg $MnCl_2$ (GS+0.37):** Hepatocytes are large and polygonal with preserved hepatic cords. The central vein shows mild congestion at week 8. **Gallstone+Aspirin (GS + Aspirin):** Hepatocytes are large and polygonal with preserved hepatic cords and central vein. Cellular infiltration is noted at week 8 (black circled area). *CV = central vein; S = hepatic sinusoid; yellow star = congested capillaries; blue arrow = hepatocytes.*

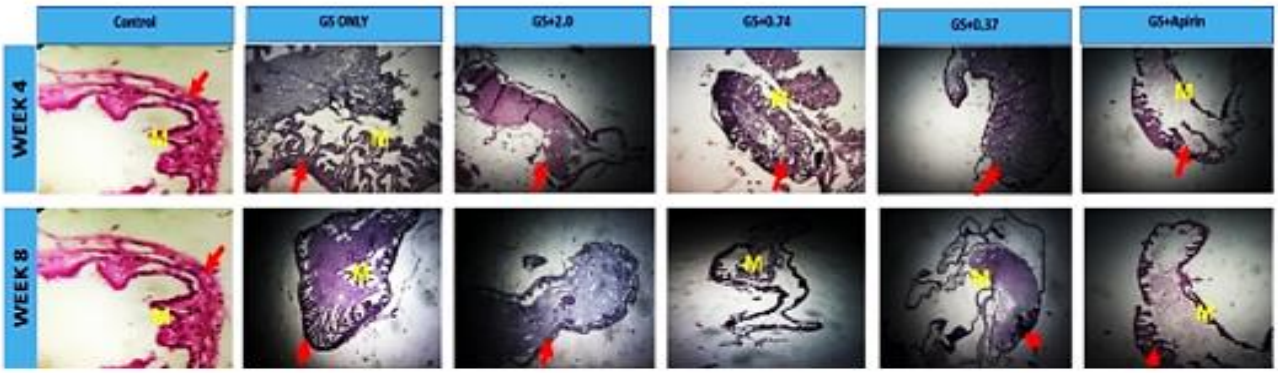


PLATE 6: PHOTOMICROGRAPH OF GALL BLADDER IN $MnCl_2$ TREATED ANIMALS BY WEEKS 4 AND 8 DURING CHOLESTEROL GALLSTONE FORMATION (MAGX100 USING HAEMATOXYLIN AND EOSIN STAIN): Control: Normal histological architecture is observed. The muscularis externa and mucosal lining appear intact with preserved epithelial lining at both week 4 and week 8. **Gallstone Untreated (GS only):** At week 4, the muscularis externa and mucosa are intact with well-preserved epithelial lining. By week 8, degenerative changes are evident in the epithelial lining, and the muscularis externa appears thinned compared to week 4. **GS + 2.0 mg/kg $MnCl_2$ (GS+2.0):** The muscularis externa is poorly defined, and the mucosal lining shows complete degeneration at both week 4 and week 8. **GS + 0.74 mg/kg $MnCl_2$(GS+0.74):** At week 4, the mucosal lining shows erosion with focal thinning of the muscularis externa. By week 8, only remnants of mucosa with attenuated epithelial lining are observed, while the muscularis externa is completely absent. Overall, the histoarchitecture is markedly distorted. **GS + 0.37 mg/kg $MnCl_2$ (GS+0.37):** A thin layer of muscularis externa is present, while the mucosal layer lacks rugae and shows loss of epithelial lining at both week 4 and week 8. **Gallstone+Aspirin (GS + Aspirin):** The muscularis externa is relatively preserved, and the mucosal layer is appreciable; however, the epithelial lining is eroded, and the underlying lamina propria appears compromised at both week 4 and week 8. *Red arrow = Muscularis externa and M= Mucosa layer.*

DISCUSSION

Increased body weight is an established risk factor for the development of gallstones, and the risk of symptomatic stones has been linked to an increase in BMI (Stokes and Lammert, 2021), which is a predictive marker for obesity. Obesity is a medical condition characterized by excessive body fat in the adipose tissues, impacting health negatively (Hendarto *et al*., 2023). Obesity is known to increase intra hepatic cholesterol secretion (Parra-Landazury *et al*., 2021), and this has been associated with reduced gallbladder motility, which also contributes to gallstone formation (Di Ciaula *et al*., 2012). Weekly changes in body weight were monitored, and from our study, the gallstone animal model had an increased body weight gain, this probably enhanced their hepatic response to fat accumulation (Kharga *et al*., 2016). Intervention with $MnCl_2$ caused a decrease in body weight gain that was induced by a lithogenic diet during cholesterol gall stone (CGS) formation, thereby reducing body fat distribution that play a role in gallstone formation. There was a reduction in weight gain in the normal animals treated with $MnCl_2$. However,

higher doses of $MnCl_2$, as reported by Salami *et al* (2016), resulted in increased body weight gain during gastric ulcer healing. Feeding animals with a lithogenic di*et al*so resulted in increased gallbladder and liver size in CGS-untreated animals. The gallbladder bile was darker and more turbid, but treatment with $MnCl_2$ mitigated the formation of lithogenic bile.

Haematological profiles are measurable parameters that can be assessed to identify and monitor physiological or pathological conditions, and several pathological situations that could alter haematopoietic physiology can influence these profiles (Tekle *et al*., 2022). A number of molecular and clinical evidence have suggested an interconnection between bone metabolism and haematopoiesis (Valderrabano *et al*., 2021). Haematopoiesis, under normal conditions, occurs in the bone marrow, which depends on the supportive environment of the bone. However, a deteriorating bone would provide a less supportive environment for haematopoiesis, causing anaemia (Valderrabano *et al*., 2017). Anaemia, characterized by low haemoglobin concentration, is often caused by nutritional deficiency, of which over 50% is due to iron deficiency (Valderrabano and Wu, 2019). Haemoglobin is an iron-containing oxygen-binding protein located in erythrocytes (Farid, 2023); iron in relation to nitric oxide synthetases (NOS) acts as a catalyst that helps in the formation of nitric oxide, which is effective in improving gallbladder function (Prasad *et al*., 2015). A population-based study revealed anaemia as a significant risk factor to fractures, falls, osteoporosis, as well as decline in physical activity especially in the elderly (Hirani *et al*., 2016; Bani Hassan *et al*., 2020).

Alteration in haematological indices is seen during CGS (Channa *et al*., 2005; Rasheed, 2014) with observed reduced Hb concentration. Several studies have shown an association between serum iron levels and the risk of gallstones (Pamuk *et al*., 2009; Prasad *et al*., 2015); this depleted iron store is reflected in the decreased Hb concentration that is seen in the CGS untreated animals. Iron deficiency has been shown to alter hepatic enzymes and decrease cholesterol-7a-hydroxylase activity (the rate-limiting enzyme for the synthesis of bile acids from cholesterol), causing a decrease in bile salt secretion and promoting cholesterol crystal formation (Johnston *et al*., 1997). Manganese, especially the 2.0 mg/kg, prevented anaemia in gallstone animals by increasing PCV, haemoglobin concentration, and RBC count. This may be attributed to an increase in iron absorption, as manganese and iron share the same binding site, thereby improving gallbladder function and also mitigating the fractures and falls that might be associated with osteoporosis. This observed intervention was further confirmed by the marked decrease in the myeloid/erythroid ratio of the 2.0 mg/kg group. However, in the normal animals, there was an increase in myeloid/erythroid ratio with a concomitant decrease in PCV, haemoglobin concentration in the 0.74 and 2.0 mg/kg groups. This could be because there was no pathological condition to combat with, and also due to the competition for the binding site between manganese and iron.

Inflammation has been noted to be a common cause of gallstones (Liu *et al*., 2018) and osteoporosis (Li *et al*., 2022); also, anaemia has been documented to arise from chronic inflammation (Dyer *et al*., 1972). In the pathogenesis of gallstones, studies have revealed inflammation as an important contributor whose inflammatory mechanisms and immune responses associated with them are essential to the disease pathogenesis (Sadri *et al*., 2022; Gu *et al*., 2025). During the inflammatory response, interactions occur between platelets, neutrophils, and lymphocytes, leading to monocyte adhesion and the release of various inflammatory mediators (Schrottmaier *et al*., 2020; Yang *et al*., 2023). Platelets are actively involved in systemic inflammation, contributing to vascular inflammation when activated (Smyth *et al*., 2009; Smith *et al*., 2011). Blood inflammatory markers, such as the ratios of Neutrophil to Lymphocyte (NLR) and Platelet to Lymphocyte (PLR), according to Li *et al* (2022), were observed to increase in osteoporotic patients compared with healthy subjects. As earlier reported in our previous study, the aforementioned blood inflammatory markers were also noted to increase in the gallstone model that was not treated. Intervention with manganese brought about a decrease in these inflammatory mediators, thereby mitigating against the inflammatory response seen during gallstone formation and osteoporosis as well. It is also worth noting that in the normal animals, there was increased platelet and neutrophil activity in the higher doses of manganese, especially at 8weeks

Alkaline phosphatase (ALP) is a homodimer protein with phosphorylation properties and exists as many isozymes (Tariq *et al*., 2019), and it changes can be evaluated in a range of disease such as cholestatic jaundice (Shah and John, 2021), liver disease, as well as cognitive impairment (Boccardi *et al*., 2021). The level of ALP can also be used to indicate the rate of bone turnover (Williams and Sapra, 2021); the level of serum ALP is considered a potential biomarker for bone formation (Tariq *et al*., 2021), helping to track the absence or presence of osteoporosis (Ponzano *et al*., 2021). A strong negative association has been observed to exist between ALP and Bone Mineral Density (BMD) of lumbar (Shu *et al*., 2022) and pelvic bones (Xiaosong and Chengjin, 2023), of which females had more significant association. A Japanese study also documented that females with more severe osteoporosis have elevated level of ALP (Iki *et al*., 2004). Osteoporosis is characterized by increased loss of bone mass which is reflected by BMD. During normal bone metabolism, to ensure stable bone mass, a balance between osteoblastic bone formation and osteoclastic bone resorption is maintained. However, when BMD is low as seen in osteoporotic conditions, the quiescent osteoblasts are activated, resulting in unmineralized bone-like tissue and undifferentiated osteoblasts. These osteoblasts proliferate in a feedback manner and synthesize a large amount of bone alkaline phosphatase (BALP), significantly increasing serum ALP levels (Shu *et al*., 2022). The level of ALP is also elevated in obese individuals (Jalili *et al*., 2022) and obesity increases the risk of falls (GR Neri *et al*., 2019) especially in the elderly due to reduced ability and postural stability that results from excessive body weight (Mitchell *et al*., 2015). Although initial belief has it that obesity has a favourable role in bone metabolism, protecting it from fractures. However, studies have shown obesity induced by high fat diet to cause a reduction in bone quality (Ionova-Martin *et al*., 2010), increase bone resorption (Patsch *et al*., 2011), and also increase bone marrow adiposity (Halade *et al*., 2011). In our study, the gallstone model animals had an increased serum ALP level with a concomitant decrease in femur bone mass. Low bone mass is a hallmark for osteoporosis leading to reduced bone strength and an increased risk of fracture (Anam and Insogna, 2021). Furthermore, a strong negative correlation was observed to exist between serum ALP and femur bone mass density of cholesterol gallstone animals in this study; which is a biomarker for osteoporosis. Higher level of ALP is associated with decreased bone mass density suggesting bone resorption to outpace bone formation. This association

(negative correlation) was reversed by $MnCl_2$ during lithogenic diet induced cholesterol gallstone formation in a dose dependent manner especially at week 8 indicative of good bone health and bone cell formation. In normal condition (no gallstone induced), the negative correction observed at week 4 at higher doses of $MnCl_2$ treatment was reversed by $MnCl_2$ at week 8 indicative of reversed osteoporosis. This is similar to the reports observed by Salami *et al*., (2019) during colitis healing when manganese is used as a treatment. It may also indicate that when manganese is taken at higher doses in healthy people, it may predispose to osteoporosis. This implies that caution should be adhered to while using manganese especially in healthy people.

Intervention with manganese resulted in a decrease in the level of serum ALP as well as increasing femur bone mass. Manganese probably regulated the balance between bone formation and resorption as reflected in the bone mass, facilitating the regulation of the number of osteoblasts and osteoclasts (Chang *et al*., 2022). Manganese Superoxide Dismutase (MnSOD), located within the bone mitochondria has been noted to be essential in osteoclasts formation and function (Kim *et al*., 2017; Ma *et al*., 2024); protecting the osteoclasts from extracellular superoxide anion released during bone resorption; thus, conferring cytoprotective function against oxidative damage (Winterbourn, 2017).

Conclusion

Manganese attenuated osteoporosis associated with lithogenic diet induced gallstones by modulating bone homeostasis and haematopoiesis.

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