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Manganese Chloride Attenuates Gut Dysmotility, Dysbiosis, and Ileal Bile Acid Malabsorption during Diabetes-Induced Cholesterol Gallstone in Swiss Mice

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article.

Abstract

Background: Accumulation of bile acids (BA) from intestinal BA malabsorption causes gut dysmotility, leading to gut dysbiosis, which is evident in obesity and diabetes mellitus. Diabetes mellitus has been reported to facilitate progression of Cholesterol Gallstone (CGS) pathogenesis in patients.

Objectives: Manganese Chloride ($MnCl_2$) attenuates CGS formation but there is paucity of information regarding its activities on BA malabsorption during CGS formation in diabetic Swiss mice, which this study design investigated

Materials and Methods: One hundred and forty female Swiss mice (n=20) were grouped into 7: I-normal control, **II-**untreated CGS, **III**-normal+0.37 mg/kg or **IV-**normal+0.74 mg/kg $MnCl_2$, 5-CGS+0.37 mg/kg or **VI-**CGS+0.74 mg/kg $MnCl_2$, and **VII-**CGS+75mg/kg aspirin. Alloxan-induced diabetic mice were fed with cholesterol-rich diets to induce CGS. Blood glucose level (BGL) was monitored and intestinal motility, fecal cholesterol, BA, biochemical analysis of excised ileum, total bacterial load of ileum homogenate and stool samples, ileum histopathology and morphometric evaluations were determined at 2 and 4-weeks. Data was analyzed using ANOVA, expressed as Mean±SEM and significant at $p \leq 0.05$.

Results: Weekly BGL significantly decreased in CGS+$MnCl_2$ groups. Intestinal motility significantly increased in normal and CGS+0.37mg/kg $MnCl_2$ compared with untreated CGS groups at 2 and 4-weeks. Intestinal malondialdehyde, mast cell counts, and fecal BA levels were significantly decreased while nitric-oxide, mucin level, Na^+/K^+-ATPase activity, villi/crypt, villi absorption area and commensal bacteria were increased in CGS+$MnCl_2$ compared with untreated CGS groups at 2 and 4-weeks.

Conclusion: Manganese chloride attenuated diabetic induced cholesterol gallstone gut dysmotility through stimulated bile acid absorption, reduced intestinal mast cell count and pathogenic gut bacteria.

Keywords: Cholesterol gallstone, manganese chloride, small intestinal motility, gut dysbiosis, ileum bile acids malabsorption.

INTRODUCTION

Diabetes mellitus is a disorder of glucose and lipid metabolism through decreased pancreatic beta cells' production of insulin (type-1 diabetes) or impaired secretion (type-2 diabetes). Type-2 diabetes has been implicated in increasing the risk for gallstone development, because of its association with metabolic syndrome (Ratheesh *et al*., 2023), with about 25% of the general diabetic population Lv *et al*., (2017) reported have cholesterol gallstones (CGS). Cholesterol gallstone

(Fan *et al*., 2017) often develop when bile cholesterol concentration exceeds bile solubility, leading to the formation of small crystals that coalesce into large stones over time.

Bile acids (BAs) are synthesized in the liver, facilitating lipid digestion and absorption in the small intestine (Marasco *et al*., 2022). In normal conditions, about 90–95% of the synthesized BAs are reabsorbed in the terminal ileum and recycled via enterohepatic cycling, while the unabsorbed BAs reach the large intestine (Camilleri *et al*., 2022). Here, the conjugated primary BAs synthesized in the liver are deconjugated by the actions of microbiota into secondary BAs, thereby modifying the composition of the total BA pool.

Gut dysbiosis has been linked with gastrointestinal composition and total content of Bas. Under physiologic conditions, the normal population of beneficial microbiota interacts with pathogens to prevent them from crossing the barriers. Gut bacteria, in turn, control the circulation of BA composition and pool size; hence, any alteration in BA homeostasis and microbiota dysbiosis leads to metabolic disease pathogenesis. The GM helps to maintain normal intestinal motility by providing beneficial nutrients, such as vitamins and Short-Chain Fatty Acids (SCFAs). Hence, interaction between the microbiome and the intestinal motility system is essential to maintain normal intestinal function.

Small intestine motility is a physiologic parameter that determines nutrition and overall gut health, such as impacting glucose absorption, which is a target for pharmacological manipulations. Multiple factors contribute to small intestinal dysmotility, such as gut dysbiosis and mast cell activation. Mast cells are important immune cells that, when activated, release mediators associated with disturbed motility (Gasbarrini *et al.,* 2008). Mast cell-derived mediators can also modify the microbial community by influencing the survival and growth of certain microbial species, thus promoting health by supporting a balanced microbiome (Bosveld *et al.,* 2023).

Manganese is an essential trace element that is involved in the metabolic regulation of protein, cholesterol, and polysaccharides synthesis, as well as the activation and regulation of several enzymes, endocrine and immune function. Its activities in oxidative stress protection (Azadmanesh and Borgstahl, 2018), blood sugar regulation, cellular energy, and homeostasis have been reported. Moreover, the gastro-protective actions of manganese against acetic- (Salami *et al*., 2016), and indomethacin-induced (Tijani *et al*., 2023) gastric ulcerations and attenuated cholesterol gallstone in Swiss mice (Salami *et al*., 2024) have been documented. Even though manganese is known for its beneficial role in cholesterol metabolism (Bae *et al*., 2011), there is a paucity of reports on its role in ileum bile acids absorption and gut dysbiosis during CGS pathogenesis due to diabetes complications, which this study seeks to investigate.

MATERIALS AND METHODS

Experimental Animals

One hundred and forty (140) healthy female Swiss mice (20-27g) were obtained from Central Animal House, University of Ibadan, Nigeria. The study was approved by the University of Ibadan Animal Care and Use Research Ethics Committee (ACUREC) with an assigned number (UI-ACUREC/18/0103). The mice were maintained under standard environmental conditions with 12/12-hour light/dark cycle at room temperature in a well-ventilated animal house at the Department of Physiology. They were housed in plastic cages with access to drinking water and basal feed *ad libitum*. The study adheres to the experimental procedure and Guidelines for Care and Use of Laboratory Animals for Biomedical Research (National Research Council, 1985).

Induction of cholesterol gallstones as a result of diabetes complication

Diabetes was induced in overnight-fasted mice using alloxan monohydrate (150 mg/kg intraperitoneal) as previously reported by Abayomi *et al*. (2011). Seventy-two (72) hours after achieving sustained hyperglycemia, diabetic mice were then subjected to cholesterol gallstone induction following the modified method of Salami *et al*. (2021; 2024). The diabetic mice were fed a lithogenic diet (basal diet plus 0.5% cholesterol) for 4 weeks alongside their respective treatments.

Study design

One hundred and forty (140) healthy female Swiss mice (20-27g) were evenly distributed into seven (7) groups of 20 animals each as follows: a set of the animals (70) was used for the small intestinal motility study, while the second set of animals (70) was used for biochemical assay and microflora studies. The choice of the doses of manganese was based on the daily recommended dose (EFSA, 2013), route of treatment was orally for four weeks.

Baseline- Normal Control (free access to basal chow and drinking water)

GU– CGS alone (hyperglycemic + basal chow + 0.5% cholesterol)

HM– $MnCl_2$ high dose(Normal animals + basal chow $MnCl_2$ 0.74mg/kg)

LM- $MnCl_2$ low dose (Normal animals + basal chow + $MnCl_2$ 0.37mg/kg)

GHM- CGS+$MnCl_2$ high dose (hyperglycemic+basal chow+0.5% cholesterol + $MnCl_2$ 0.74mg/kg).

GLM – CGS+$MnCl_2$ low dose (hyperglycemic basalchow+0.5%cholesterol+$MnCl_2$ 0.37mg/kg).

GA – CGS+Aspirin (hyperglycemic+basal chow + 0.5% cholesterol + treated with aspirin 75mg/7kg)

Intestinal transit study

Small intestinal transit ~~w~~as studied using charcoal meal as previously reported by Otamere *et al*. (2016). Briefly, the distance travelled by food in 30 minutes was determined by measuring the distance from the pylorus to the leading edge of the charcoal meal and to the distal edge of the ileum. The quotient of the charcoal progression distance divided by the small intestinal length was calculated, providing an index of small intestinal transit.

Sample collection

Blood glucose level were monitored weekly after an overnight fast. Briefly, the tail was pricked and blood glucose was measured using an ACUU-CHECK ACTIVE glucometer (S/N GB30555855; REF 06993770001) with glucose test strips. Abdominal incision was made along the mid line; the gallbladder and the small intestine were harvested, weighed and stored in phosphate buffer for biochemical evaluation and in 10% phosphate buffer formalin for histological processing and microscopic evaluation.

Biochemical processing and evaluation

Small intestinal tissues were excised from the other Swiss mice (70) not used for gut motility and homogenized in phosphate buffer (pH adjusted to 7.4 with 1M hydrochloric acid; 10% weight/volume) using a Tefflon homogenizer. The homogenates of the small intestine were cold centrifuged for 15 minutes at 3600 rpm and at 4 ºC the supernatants were stored for biochemical estimations.

Total protein concentration in the small intestinal homogenate was estimated using the method of Gornal *et al*., (1949). Lipid peroxidation was spectrophotometrically estimated as described by Varshney *et al*., (1949). Tissue mucin concentration was assessed using the method of Miner-Williams *et al*., (2009). The concentration of nitrite in the small intestinal supernatant was measured as an indicator for production of nitric oxide (NO) as described by Ignarro *et al*., (1987). Intestinal tissue ATPase activities were estimated by the method described by Bewaji *et al*., (1985).

Histological procedure

A section~~s~~ of small intestine fixed in phosphate buffer formalin were histologically prepared into slides following the standard histological procedures. Haematoxylin and Eosin (H&E) staining was performed, and the slides were viewed under light microscope.

The intestinal mast cells were stained using toluidene blue and their numbers were evaluated using Image J software.

Histomorphometry of the small intestine: The villi height, depth, cryptal height, and depth were measured using Motic Image Plus software. The area of the villi's absorption surface was calculated by the method described by Marchewka *et al*. (2021).

Gut Microfloral Tests

Terminal ileum and fecal samples collected from the cecum of each animal were used for microbial study. They were dissolved in sterile water; the pH was measured before microflora biochemical analysis. Biochemical tests assayed for the microflora included oxidase, catalase, indole, urease, coagulase, citrate, glucose, lactose, and sucrose. (APHA, 1992).

Fecal analysis

Fecal samples were pooled together and dried before carrying out cholesterol, bile acids, and phospholipids assays.

Statistical analysis

The results were expressed as Mean ± SEM. One-way ANOVA was used to analyze the differences among the groups, and significant at $p<0.05$

RESULTS

Effect of manganese treatment on blood glucose levels (BGL) at weeks 1, 2, 3, and 4

The GU increased significantly compared with other groups. In the manganese treatment groups, BGL significantly decreased compared with the untreated group (Table 1).

TABLE 1: Effect of Manganese Treatment on Blood Glucose Levels from Weeks 1–4

GROUPS	Blood glucose (mg/dl)			
	Week 1	**Week 2**	**Week 3**	**Week 4**
BASELINE	40.8 ± 3.60	37.20±1.59	39.00±0.60	43.80±4.20
GU	436.80 ± 14.85^{o}	222.47 ± 9.70^{o}	230.4 ± 5.40^{o}	117.87 ± 5.00 ^{o}
LM	66.13 ± 4.37^{p}	52.20 ± 3.11^{p}	57 ± 5.12^{p}	73.8 ± 8.11^{p}
HM	61.80± 2.62^{p}	57.00 ± 2.62 ^{p}	60.6 ± 7.8 ^{p}	71.27 ± 5.05 ^{o,p}
GLM	346.80 ± 13.56^{o,p,q,r}	172.80 ± 11.00^{o,p,q,r}	122.40 ± 3.60 ^{o,p,q,r}	109.20 ± 4.80 ^{o,p,q}
GHM	321.00 ± 3.17^{o,p,q,r,s}	177.60 ± 7.23^{o,p,q,r}	114.47 ± 6.37^{o,p,q,r}	103.2 ± 11.45^{o,p,q}
GA	387.00 ± 7.84^{o,p,q,r,s,t}	205.80 ± 16.67^{o,q,r,s}	94.80 ± 2.40^{o,p,q,r}	72.00 ± 9.50^{o,p,q,r}

*Values are expressed as Mean ± SEM and S**ignificance** $p \leq 0.05$ when compared**: w**ith baseline -^{o}, with GU -^{p},-with LM -^{q}, with HM -^{r}, with GLM -^{s}, with GHM -^{t}, with GA -^{u}.*

Effect of manganese treatment on intestinal transit time at weeks 2 and 4

Small intestinal transit decreased significantly in GU compared with other experimental groups at 2 weeks. In contrast, at 4 weeks, a significant increase was observed in LM, GLM, and GA groups compared with other experimental groups (Figure 1).

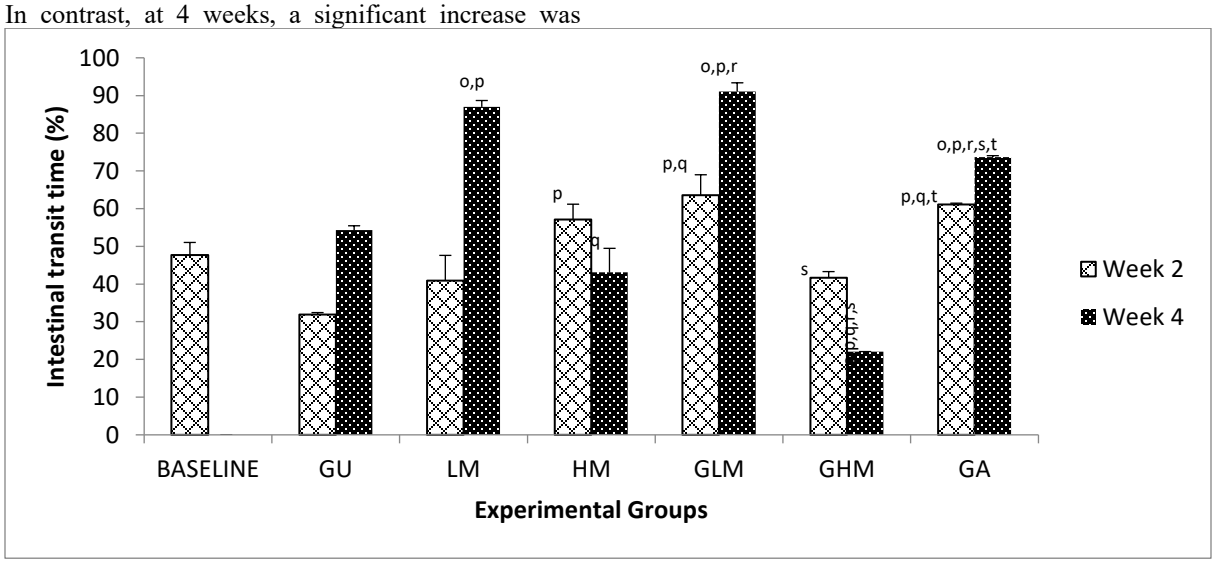

Figure 1: Effect of manganese treatment on intestinal transit time at week 2 and 4.

*Cortical bar represents Mean ± SEM and S**ignificance** when compared**: w**ith baseline -^{o}, with GU -^{p},-with LM -^{q}, with HM -^{r}, with GLM -^{s}, with GHM -^{t}, with GA -^{u}..*

Effect of manganese treatment on biochemical parameters of the small intestine at weeks 2 and 4

The lipid peroxidation (MDA) level of GLM, GHM, and GA groups at week 4 was significantly reduced compared with other experimental groups (Figure 2A). A significant reduction in small intestinal nitric oxide (NO) levels was observed in all the groups compared with baseline, except the LM group. At week 4, small intestinal NO levels was significantly increased in all manganese treatment groups compared with GU. All treatment groups significantly increase NO levels at week 2 compared with week 4, but in the GU group, there was a significant decrease in the NO level at week 2 was compared with week 4 (Figure 2C).
A significant increase in mucin concentration in the LM group at week 2 compared with other experimental groups while at week 4, GHM and GA group decreased significantly compared with other experimental groups. The LM significantly increases mucin concentration while HM significantly reduces it (Figure 2D).
At week 2, small intestinal Na^+/K^+ pump activity in GHM and GA and groups were significantly increased compared with other experimental groups while at 4 weeks, the activity of Na^+/K^+ pump activity decreased significantly compared with other experimental groups (Figure 3).

there was a reduction in the V:C of the GU group compared with manganese treatment groups (Table 3).
Villi absorption area: A significant increase in the absorptive area was observed in HM, GLM and GHM at both weeks 2 and 4 compared with other experimental groups (Figure 3).

Effect of manganese treatment on Fecal Cholesterol, Phospholipids and Bile acids at weeks 2 and 4

There was a significant reduction in the amount of cholesterol excreted in the $MnCl_2$-treated groups compared with control and GU groups.
Bile acids excreted was reduced in the $MnCl_2$-treated groups while phospholipid levels were increased
Fecal Cholesterol Phospholipid ratio was significantly reduced compared with GU group (Table 2).

Effect of manganese treatment on histo-morphometry of the villi at weeks 2 and 4

Villi height: The LM, GHM, and GA ~~have~~ significantly reduced villi height at week 2 while at week 4 GHM had reduced villi height (Table 3).
Villi width: a significant increase in villi width was observed in HM, GLM, and GA groups compared to baseline, GU and LM group at week 2 while at week 4 LM villi width was significantly lower compared with other experimental groups.
Crypt depth: at week 2, there was a significant reduction in cryptal depth of LM group while at week 4, an increase was observed in all treatment groups compared with GA.
Villi cryptal ratio: at week 2, Control, LM and GLM groups had significantly increased V:C while at week 4

Effect of manganese treatment on ileum mast cell count at week 4

Mast cell count: A significant decrease was observed in the mast cell count of LM compared with other experimental groups (Figure 4; Plate 1).

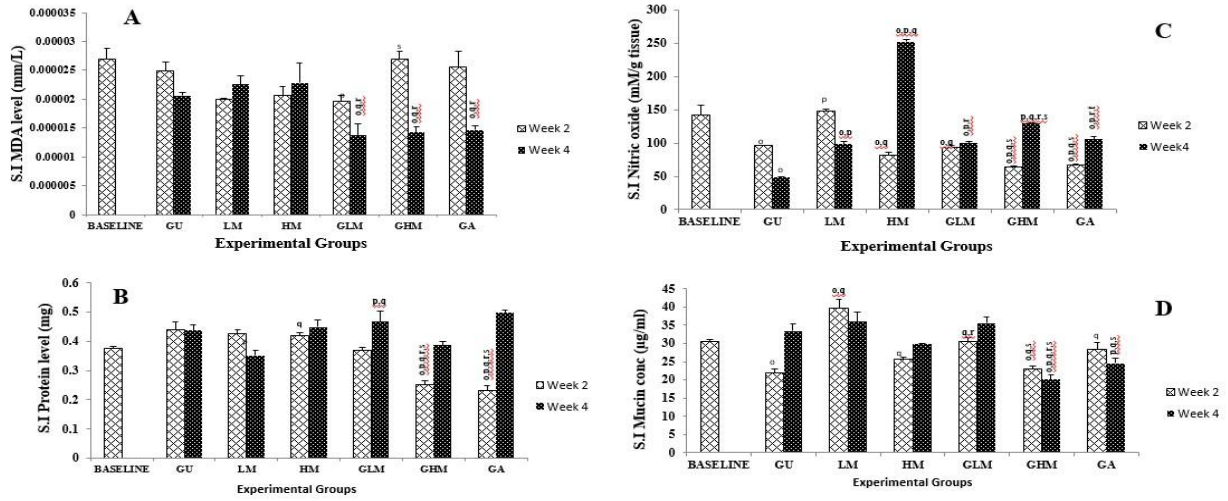

Figure 2: Effect of manganese treatment on small intestine MDA (A); protein (B), Nitric oxide (C), and Mucin (D) levels at weeks 2 and 4. *Cortical bar represents Mean ± SEM. Values are significant when $p \leq 0.05$. **Significance:** [a]- compared with baseline, [p]- compared with GU, [q]- compared with LM, [r]- compared with HM, [s]- compared with GLM, [t]- compared with GHM and [u]- compared with GA.*

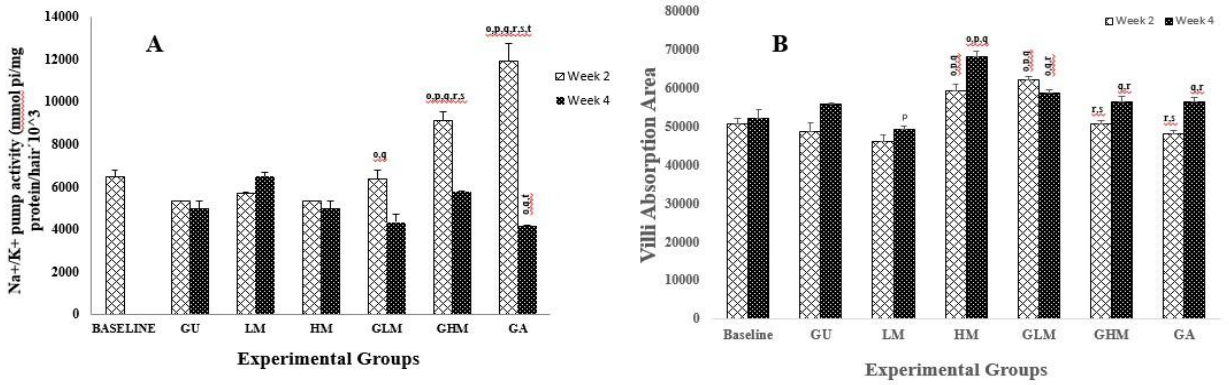

Figure 3: Effect of manganese treatment on small intestine Na+/K+ pump activity (A) and Villi Absorptive Area (B) at weeks 2 and 4.

*Cortical bar represents Mean ± SEM. Values are significant when $p \leq 0.05$. **Significance:** ^{o}- compared with baseline, ^{p}- compared with GU, ^{q}- compared with LM, ^{r}- compared with HM, ^{s}- compared with GLM, ^{t}- compared with GHM and ^{u}- compared with GA.*

TABLE 2: EFFECT OF MANGANESE TREATMENT ON FECAL EXCRETION RATE OF CHOLESTEROL, BILE ACIDS AND PHOSPHOLIPIDS

GROUPS	Cholesterol (mg/dl)		Bile acids (mg/g)		Phospholipids (mg/100g)		Cholesterol: Phospholipid	
	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
BASELINE	86.16±15.39		20.75 ±2.05		2.40±0.7		36.7± 2.60	
GU	86.92±11.54	68.2 ±0.1	19.17±6.27	23.13±0.28	2.90±0.2	2.42±0.02	30.25±3.51	28.18±0.16^{o}
LM	53.18±2.97	44.75±0.25	23.28±3.18	19.17±0.17	3.38±0.18	3.75±0.25	15.76±0.04^{o} ,p	11.97±0.42^{o} ,p
HM	35.325±1.48^{o,} p	31.1±0.5^{o}	24.94±3.74	13.475±1.0 3	4.15±0.14^{o}	2.18±0.08^{e}	8.54±0.37^{a,b,} c	14.30±0.15^{a,} b
GLM	17.69±2.31^{o,p}	22.08±0.38 o,p	22.66±1.0	21.00±0.7	4.17±0.17^{o}	2.725±0.08	4.24±0.22^{o,p} ,q	8.10±0.04^{o,p}
GHM	43.85±11.54^{o}	51.53 ±0.78	13.875±1.4 3^{r}	13.7±0.5	2.91±0.69	3.95±0.15^{o,} q,r	15.00±0.24 q,r,s	13.06±0.40^{o} ,p
GA	58.46±27.69	29.42±0.77 o,p	19.61±3.28	15.83±0.18	2.49±0.51^{r,s}	2.11±0.05^{q,} t	22.58±3.83^{o} ,p,rs,t	13.98±0.04^{a,} b

*Values are expressed as Mean ± SEM and **Significance** $p \leq 0.05$ when compared**:with** baseline -^{o}, with GU -^{p},-with LM -^{q}, with HM -^{r}, with GLM -^{s}, with GHM -^{t}, with GA -^{u}.*

TABLE 3: EFFECT OF MANGANESE TREATMENT ON ILEAL VILLI HISTOMORPHOMETRY

GRPS	Villi Height		Villi width		Crypt depth		Crypt width		V:C (villi height:crypt depth)	
	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
Baseline	275.13±3.44	216.77±4.09	58.60±2.19	76.47±2.19	91.33±2.85	71.00±4.93	38.80±0.30	46.47±0.67	3.02±0.06	3.09±0.26
GU	241.17±3.03^{o}	242.27±2.95^{o}	64.37±2.30	73.30±0.76	92.33±1.45	76.67±2.91	38.33±0.33	45.67±0.67	2.16±0.04	1.87±0.05^{o}
LM	214.57±2.80^{o,p}	254.37±5.21^{o}	60.93±2.63	64.70±1.14^{o,p}	72.00±4.93^{o,p}	90.67±1.86^{o,p}	50.33±2.03^{o,p}	41.67±1.20	3.00±0.20^{p}	2.81±0.07^{p}
HM	240.20±2.82^{o,q}	246.87±1.72^{o}	88.00±2.08^{o,p,q}	85.27±0.23^{o,p,q}	122.67±2.96^{o,p,q}	91.97±0.30^{o,p}	64.33±0.88^{o,p,q}	49.67±0.33^{q}	2.03±0.02^{o,q}	2.88±0.03^{p}
GLM	241.83±3.21^{o,q}	241.83±3.21^{o}	82.33±2.03^{o,p,q}	75.67±0.88^{q,r,}	85.67±3.48 ^{q,r}	100.5±0.50^{o,p}	54.33±0.33^{o,p,r}	55.67±3.71^{o,p,q}	2.84±0.14^{p,r}	2.42±0.04^{o,r}
GHM	210.47±0.47^{o,q,r,s}	219.73±2.51^{p,q,r,s}	66.70±1.55^{o,p,r,s}	74.33±0.88^{q,r,}	94.00±3.51^{q,r}	96.00±1.53^{o,p}	50.67±2.85^{o,p,r}	47.00±0.58^{t}	2.21±0.06^{o,q,s}	2.23±0.05^{o,q,r}
GA	204.33±4.14^{o,q,r,s}	239.43±1.45^{o,q,t}	73.00±1.16^{o,p,q,r,s}	81.67±0.88^{p,q}	93.87±2.03^{q,r}	101.00±2.00^{o,p}	58.67±0.67	43.67±0.88^{t}	2.18±0.09^{o,q,s}	2.12±0.05^{o,q,r}

*Values are expressed as Mean ± SEM and s**ignificance** $p \leq 0.05$ when compared**:w**ith baseline -^{o}, with GU -^{p},-with LM -^{q}, with HM -^{r}, with GLM -^{s}, with GHM -^{t}, with GA -^{u}.*

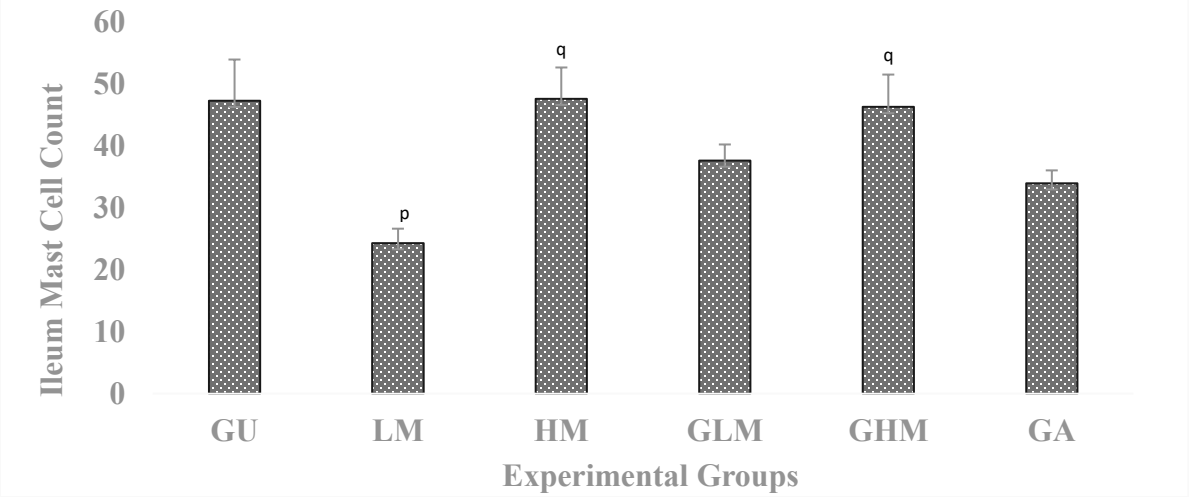

FIGURE 4: EFFECT OF MANGANESE TREATMENT ON ILEUM MAST CELL COUNT AT WEEK 4.

Cortical bar represents Mean ± SEM and ***Significance*** *$p \leq 0.05$ when compared****: w****ith baseline -^{o}, with GU -^{p},-with LM -^{q}, with HM -^{r}, with GLM -^{s}, with GHM -^{t}, with GA -^{u}.*

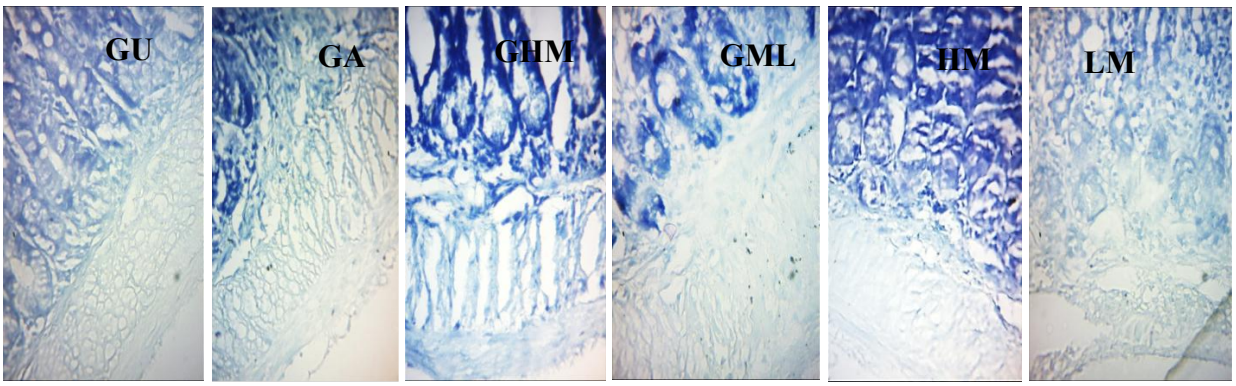

Plate 1: PHOTOMICROGRAPH SECTION OF THE INTESTINAL TISSUE BY WEEK 4 STAINED WITH TOLUIDENE BLUE (MAGX400) SHOWING MAST CELLS: ***GU****-Cholesterol gallstone untreated ,* ***GA****–Cholesterol gallstone+Aspirin,* ***GHM****-Cholesterol gallstone+HM,* ***GML****- Cholesterol gallstone+LM ,* ***HM****- Normal+High dose* $MnCl_2$*,* ***LM****–Normal+low dose* $MnCl_2$*,*

Effect of manganese treatment on ileal villi histology at weeks 2 and 4

The histology observations of each groups are below: **Control (C):** showed moderately preserved mucosal epithelium with normal villi (white arrow), lamina propria and glands show no infiltration of inflammatory cells (slender arrow). **Gallstone untreated (GU):** Showed poorly preserved mucosal epithelium, some villi show edema at the apical part (white arrow), The lamina propria and glands show mild infiltration of inflammatory cells (slender arrow). **Manganese chloride 0.34 mg/kg (ML):** Showed well preserved mucosal epithelium with normal villi (white arrow), The lamina propria and glands show scanty infiltration of inflammatory cells (slender arrow). **Manganese chloride 0.74 mg/kg (MH):** The lamina propria and glands show mild to moderate infiltration of inflammatory cells (slender arrow). **Gallstone+Manganese chloride 0.74 mg/kg (GMH):** showed that the lamina propria and glands show mild infiltration of inflammatory cells (slender arrow). The mucosal layer appear mildly infiltrated (blue arrow). **Gallstone+Manganese chloride 0.34mg/kg (GML):** The lamina propria and glands show mild infiltration of inflammatory cells (slender arrow). The sub mucosal layer appears mildly infiltrated (blue arrow). **Gallstone+Aspirin (GA):** showed mildly preserved mucosal epithelium with some loss of villi (white arrow), The lamina propria and glands show mild infiltration of inflammatory cells (slender arrow). (Plate 2)

At week 4:

Gallstone alone (4JG): showed moderately preserved mucosal epithelium with mild loss of villi (white arrow), The lamina propria and glands show very mild infiltration of inflammatory cells (slender arrow). The sub mucosal layer mildly inflamed (blue arrow). The muscularis layer appear normal (red arrow). **Manganese chloride 0.34mg/kg (4ML):** Showed moderately preserved mucosal epithelium with normal villi (white arrow), The lamina propria and glands show severe infiltration of inflammatory cells (slender arrow). The sub mucosal layer appears mildly infiltrated (blue arrow). **Manganese chloride 0.74mg/kg (4MH):** Showed well preserved mucosal epithelium with normal villi (white arrow), The lamina propria and glands show mild infiltration of inflammatory cells (slender arrow). The sub mucosal layer show focal area of mild inflammatory cells aggregated (blue arrow). The muscular layer appear normal (red arrow). **Gallstone+Manganese chloride 0.74 mg/kg (4GMH):** Showed moderately preserved mucosal epithelium with mildly eroded villi (white arrow), The lamina propria and glands show severe infiltration of inflammatory cells (slender arrow). **Gallstone+Manganese chloride 0.34mg/kg (4GML):** Showed moderately preserved mucosal epithelium with mildly infiltration of inflammatory cells and normal villi (white arrow), The lamina propria and glands show very mild infiltration of inflammatory cells (slender arrow). **Gallstone+Aspirin (4GA):** Showed moderately to severe infiltrated mucosal epithelium with severely infiltrated villi by inflammatory cells (white arrow), The lamina propria and glands show moderate infiltration of inflammatory cells (slender arrow). The sub mucosal layer appear severely infiltrated (blue arrow). (Plate 3).

Effect of manganese treatment on bacteria study in the ileum and stool at weeks 2 and 4

Total Bacteria Count (TBC): A significant increase in the TBC was observed in GU group compared with manganese treatment groups (Figure 5)

Compared with GU groups, there was a significant increase in the total *Enterobactericae* count in the ileum of the manganese treatment groups (Table 4)

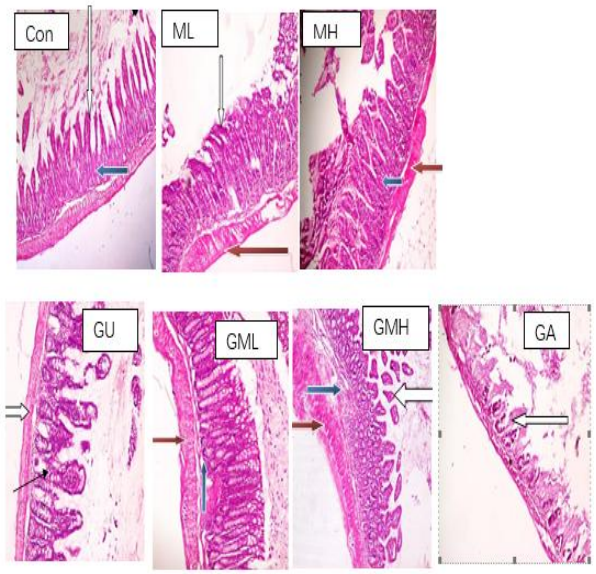

Plate 2: PHOTOMICROGRAPH SECTION OF THE INTESTINAL TISSUE BY WEEK 2 STAINED WITH H&E (MAGX100) SHOWING ILEAL VILLI: Con: Baseline, Control normal, MH- Normal+High dose $MnCl_2$, ML– Normal+ low dose $MnCl_2$, GU- Cholesterol gallstone untreated, GML- Cholesterol gallstone+LM, GHM- Cholesterol gallstone+HM, GA– Cholesterol gallstone+Aspirin.

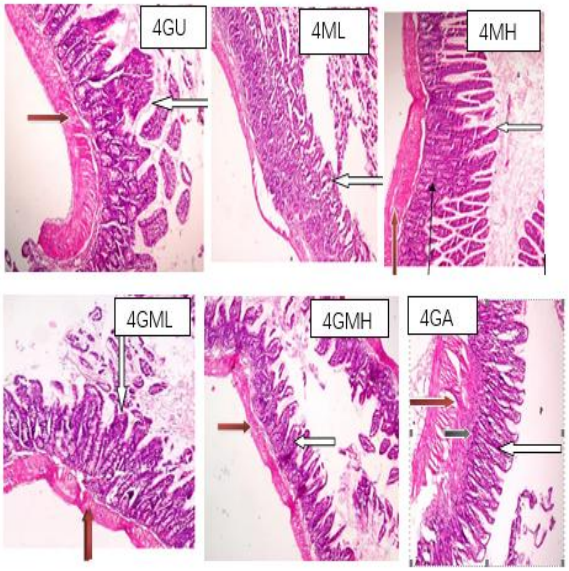

Plate 3: PHOTOMICROGRAPH SECTION OF THE INTESTINAL TISSUE BY WEEK 4 STAINED WITH H&E (MAGX100) SHOWING ILEAL VILLI: **Con:** Baseline, Control normal, ***MH****- Normal+High dose* $MnCl_2$, ***ML****– Normal+ low dose* $MnCl_2$, ***GU****- Cholesterol gallstone untreated,* ***GML****- Cholesterol gallstone+LM,* ***GHM****- Cholesterol gallstone+HM,* ***GA****– Cholesterol gallstone+Aspirin,*

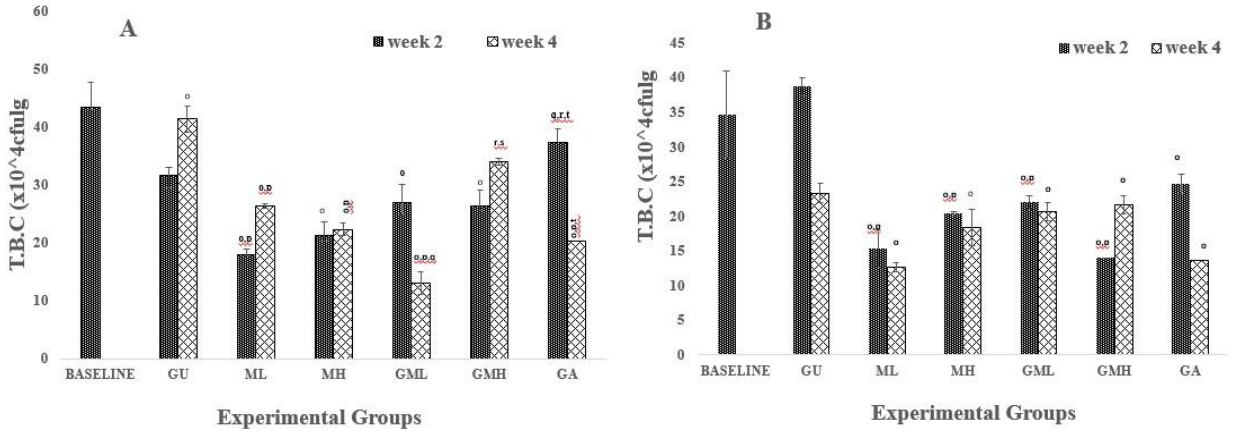

Figure 5: Effect of manganese treatment on Total bacteria count in the ileum (A) and stool (B) at weeks 2 and 4
Cortical bar represents Mean ± SEM. Values are significant when $p \leq 0.05$. ***Significance:*** *[o]- compared with baseline, [p]- compared with GU,* ***[q]****- compared with LM, [r]- compared with HM, [s]- compared with GLM, [t]- compared with GHM and [u]- compared with GA.*

TABLE 4: EFFECT OF MANGANESE TREATMENT ON MICROBIAL CLASSIFICATION TESTS.

	Gram reaction	**Catalase**	**Indole**	**Citrate**	**Oxidase**	**Metylred**	**V.P**	**Urease**	**Glucose**	**Fructose**	**Lactose**	**Maltose**	**Sucrose**	**Mannitol**
Kleibsella sp.	-Rod	+	_	+	_	_	+	+	+	+	+	_	+	_
Proteus sp.	-Rod	+	_	_	_	+	_	+	_	_	_	+	+	+
E. coli	- Rod	+	+	_	+	+	_	_	+	_	+	+	_	_
s sp.	+Cocci	_	_	+	_	_	+	_	+	+	+	+	_	+
Pseudomonas sp.	-Rod	+	_	+	+	_	_	_	+	_	_	_	+	_
Enterobacter sp.	-Rod	+	_	+	+	_	+	_	+	_	+	+	+	+
Citrobacter sp.	-Rod	+	+	+	+	+	_	+	+	_	+	+	_	+
Salmonella sp.	-Rod	+	_	+	_	+	_	_	+	_	_	+	_	+
Shigella sp.	-Rod	+	_	_	_	+	_	_	_	_	_	+	_	+

pH Value: There was a decrease in the pH in the ileum and stool of GU group (Table 5)
Relating the ratio of commensal (good) to aggressive (bad) organisms, there was an increase in the commensal compared with the aggressive organisms in the ileum, while in the stool, there was an increase in the ratio of the aggressive organisms to the commensal ones (Figures 6 and 7).

TABLE 5: EFFECT OF MANGANESE TREATMENT ON PH VALUES IN ILEUM AND STOOL AT WEEKS 2 AND 4

GROUPS	pH Value in Ileum		pH Value in Stool	
	WEEK 2	WEEK 4	WEEK 2	WEEK 4
BASELINE	7.10±0.06	-	6.80±0.06	-
GU	6.97±0.03	6.97±0.12	6.63±0.12	6.80±0.06
ML	7.07±0.09	6.83±0.09	6.77±0.15	6.87±0.03
MH	7.07±0.07	7.07±0.09	6.77±0.03	6.80±0.1
GML	7.07±0.09	6.93±0.09	6.87±0.03	6.83±0.09
GMH	7.07±0.09	6.90±0.06	6.87±0.09	6.87±0.03
GA	7.17±0.09	7.07±0.09	6.90±0.06	6.90±0.06

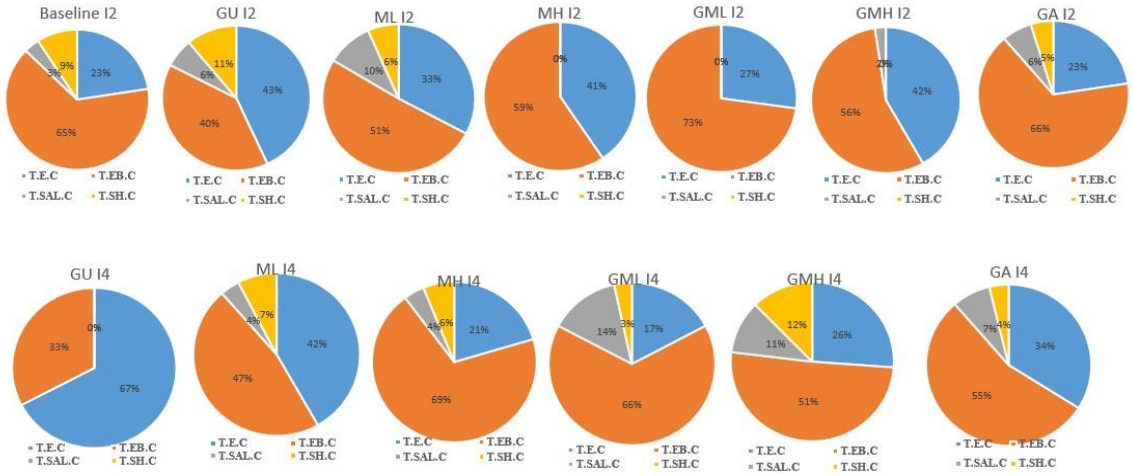

Figure 6: Effect of manganese treatment on some percentage bacteria load in the ileum at weeks 2 (I2) and 4 (I4)
TEC = Total Esherishia coli count T.EB.C = Total Enterobactericae count T.SAL.C = Total Salmonella count T.SH.C = Total shigella count

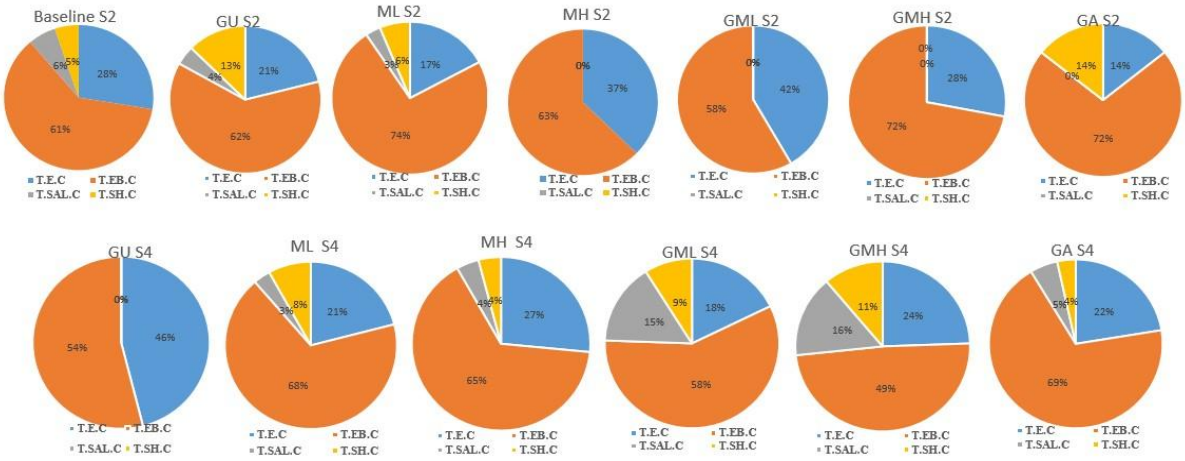

Figure 7: Effect of manganese treatment on some percentage bacteria load in the stool at weeks 2 and 4.
***TEC* = Total *Esherishia coli* count *T.Eb.C* = Total *Enterobactericae* count *T.Sal.C* = Total *Salmonella* count *T.Sh.C* = Total *shigella* count**

DISCUSSION

In the pathophysiology of gallstones, small intestinal dysmotility has been noted and patients with cholesterol gallstones have been shown to experience a decline in the function of intestinal transit. In this present study, a decrease in the small intestinal transit time of the gallstone untreated groups was observed, which agrees with the study of Fan *et al*., (2007) who used animal subjects. Feeding lithogenic diets to mice promotes greater cholesterol absorption from the intestine, and this causes an increase in biliary cholesterol secretion. The increase or enhancement of intestinal absorption of cholesterol has been linked to the delay in small intestinal transit time in animals. This prolongs bile resident/transit time in the small intestine and thus increases levels of deoxycholic acid (DCA) and lithocholic acid (LCA), which are obtained from the action of intestinal bacteria on primary bile acid (Cholic acids). Moreover, DCA is positively linked with an increase in cholesterol saturation index and cholesterol crystallization rate. Intestinal transit decline causes impairments in the rate at which the gallbladder empties, and this impairment causes the concentration of bile to increase. Manganese at a low dose was able to increase intestinal transit time both in normal and animals with gallstones, while $MnCl_2$ at a high dose prolonged intestinal transit time both in normal and gallstone animals.

However, BAs are amphipathic molecules synthesized in the liver, and their pool is regulated by enterohepatic circulation. Bile acids malabsorption (BAM) usually results from the dysregulation of bile acids' enterohepatic recirculation (shrinking bile acid pool) and the eventual negative alteration in the production of bile acid (Marasco *et al*., 2022), in a positive-feedback mechanism via inflammation. Manganese from this study was able to prevent excess loss of bile acids into feces; this regulation also helps in ameliorating dysbiosis that could result from DM complications and cholesterol gallstones.

Prolonged enterohepatic circulation of bile acids has been directly linked with delayed intestinal transit time; this delay in bile salt circulation causes a decline in the rate at which bile salts are reabsorbed in the ileum and thus leads to a decrease in bile salt content and consequently promotes supersaturation and crystallization of cholesterol. Manganese at low doses probably increased enterohepatic circulation of bile acids, thereby mitigating the increase in cholesterol supersaturation and crystallization, as well as decreasing the rate at which DCA is formed from the action of intestinal microbiota.

A strong association has been observed to exist between type-2-diabetes mellitus (T2DM) and CGD. Diabetes induces insulin resistance and hyperinsulinemia, gastrointestinal hormone, fat metabolism disorder, bile bacterial infections, and other factors. These factors affect and promote each other, resulting in the eventual formation of stones (Tan and Kou, 2019). Manganese was observed from this study to cause a reduction in the blood glucose level of the animals that are diabetic.

Gastrointestinal system motility is controlled by the actions of enteric excitatory and inhibitory motor

neurons that innervate the smooth muscle layer; approximately 50% of the enteric nerves contain NOS. nNOS, assists in smooth muscle relaxation through its inhibitory factor and this is essential for coordinated intestinal motility. It is also involved in intestinal water transit by directly acting on the intestinal epithelium and blood flow or indirectly by the stimulation of neuronal reflexes. Manganese from this study was able to increase nitric oxide level in the small intestine, which might have been responsible for the enhanced intestinal motility.

In the pathogenesis of gallstones, gallbladder mucin has been well known to be implicated as it plays an important role as a nucleating factor. However, in the small intestine, mucin protects against bacteria by providing a diffusion barrier, and bacteria would need to travel against mucus flow to penetrate the tissue; this diffusion barrier is due to its lipophilicity. In this present study, manganese at a low dose was able to increase mucin concentration; the diffusion barrier provided by mucin would help to reduce intestinal bacteria load, thus reducing the rate at which primary bile acids are converted to secondary bile acids (lithogenic bile acids) by these bacteria in the intestine, thereby reducing the rate of gallstone formation. Commensal microbiota is usually tolerated by normal mucosal epithelium but not pathogenic ones because it could distinguish them by their molecular pattern. Any invasion by an enteric pathogen circumvents the mucous layer protective function, causing the penetration and degradation of the mucus layers and its subsequent attachment, whereas probiotics like *Lactobacillus Spp* induce mucin production and prevent pathogen invasion (Frank *et al*., 2007). From our findings, manganese increased the quantity of commensal bacteria in the ileum.

Reports have it that Na^+-K^+ ATPase stimulates cholesterol metabolism (Sherman *et al*., 2009) and, as such, will cause a decrease in serum level of cholesterol. In this present study, the untreated gallstone group presented a reduced level of Na^+-K^+ ATPase activity. This finding suggests that in gallstone condition Na^+-K^+ ATPase activity is inhibited and by implication, results in the observed reduced cholesterol metabolism. Treatments with manganese chloride were observed to cause a dose-dependent stimulation in Na^+-K^+ ATPase activities. This implies that there was also an increased metabolism of cholesterol, which was also observed in this study by manganese chloride treatments.

The intestinal epithelium consists of 2 regions known as the villus, at the tip, and the crypt, at the base. The crypt-villus axis provides the necessary structure for optimal functioning of the intestinal epithelium, which includes absorption, protection, and secretion. The villi of the intestine are functional structures that are involved mainly in the absorption of nutrients; an increase in villi height directly affects nutrient absorption capability by increasing absorptive surface area. A positive association was found between an increase in villi height and an increase in animal body weight (Kovler and Hackam, 2019) as observed in this study. Villi crypt ratio, an important parameter associated with nutrient absorption and body weight was increased in our findings and an increase in body weight was also observed in our previous study (Salami *et al*., 2024). This increase is positively associated with an increase ileum absorption area and this could also be linked to the fecal cholesterol level excreted; the higher the absorption, the lesser the cholesterol level excreted in feces.

Mast cells are important cells of the immune system that play an important role in different physiological functions. Mast cells usually remain at a low level in a healthy state to regulate water balance and electrolytes, but are elevated in tissue inflammation (Chen *et al*., 2021). From our study, the observed increase in the mast cell count showed an associated decrease in gastrointestinal motility, while a decrease in the mast cell count showed an increase in intestinal motility as observed in the low-dose $MnCl_2$-treated group. This study agrees with the study of Kodani *et al*. (2018), who observed prolonged gastrointestinal motility with an increase in the number of mast cells. Moreover, interplay between mast cells and microbiome has been noted by research. In a physiological state, mast cells contribute to mucosal immunity and epithelial integrity, while in a pathological condition, they exacerbate inflammation and dysbiosis. This is because the release of excessive inflammatory mediators during mast cell activation disrupts the epithelial barrier, thus promoting a favourable environment for the growth of harmful microbes (Papa *et al.,* 2025). Results from our study showed that decreased mast cell count was associated with a decrease in total bacteria count; this could be another mechanism via which $MnCl_2$ prevented gut dysbiosis associated with cholesterol gallstones in diabetic conditions.

Conclusion

Manganese chloride attenuated intestinal dysmotility during **c**holesterol gallstone formation through reduction in small intestinal oxidative stress and mast cell count with increased ileum bile acid absorption, intestinal Na^+/K^+ATPase activities, and reduced aggressive gut bacteria load.

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