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Cobalt Modulates Healing of Gastric Ulceration in Male Wistar Rats: iNOS/COX-2 Crosstalk

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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 article.

Abstract

Background: Information on the interplay between gastric iNOS and COX-2 expression by Cobalt ($CoCl_2$) during gastro-protection is unclear.

Objective: Cobalt activities on iNOS and COX-2 interplay was investigated using Reserpine (RGU), and Pyloric-ligation (PLGU) gastric ulcer models.

Materials and Methods: In RGU, 50 Wistar rats grouped; 1-control, 2-ulcerated untreated (RUU), 3, 4, and 5 were ulcerated pre-treated with 62mg/kg, 25mg/kg $CoCl_2$ and 3.6mg/kg omeprazole for 5 days and sacrificed 3 and 8 hours after reserpine administration. In PLGU, 25 Wistar rats grouped; A-25mg/kg $Cocl_2$, B-62mg/kg $Cocl_2$, C-Cimetidine (36.88mg/kg), D-ulcerated untreated (UU), and E–control, were pretreated for 8 days prior 18 hours pyloric-ligation. Thereafter, macroscopic gastric ulceration (GU), biochemical analysis with histology, and immunohistochemistry were evaluated on excised stomach. Data were expressed as Mean±SEM, and $p \leq 0.05$ was significant.

Results: In *RGU* model, significant increase in *GU* score, nitric oxide, lipid peroxidation, and degranulated mast cells but a decrease in sulfhydryl, hydrogen peroxide levels in RUU by 3H and 8H were ameliorated by *$CoCl_2$*. Histology revealed increased degranulated mast cells in the RUU compared with *$CoCl_2$*-treated groups. $CoCl_2$ treatment significantly decreased gastric acid secretion, ulcer index, parietal cell count, H^+/K^+ATPase and Na^+/K^+ATPase activities compared with UU. Gastric sulfhydryl, mucin, pepsin, with mucous cell count significantly increased in $CoCl_2$-treated groups compared with UU. Gastric iNOS positive reactivity was upregulated while COX-2 positive reactivity was downregulated in CoCl2-treated groups compared with UU .

Conclusion: Cobalt chloride exerted gastrointestinal cytoprotective action via mast cell stabilization, mucus production, with modulated expressions of gastric iNOS and COX-2 reactivity at the ulcerated site.

Keywords: Reserpine induced Gastric ulcer, Pyloric ligation gastric ulcer model, Cobalt chloride, antioxidant assay, mast cells

INTRODUCTION

The normal stomach mucosa strives to maintain a constant homeostatic balance between protective and aggressive factors in a bid to prevent gastric ulceration (Oncel and Basson 2022). However, during gastric ulceration, the stomach tries to heal itself in a sequential manner involving several stages: haemostasis and inflammatory phase, angiogenesis in granulation tissue at ulcer base, re-epithelisation starting from ulcer margins and subsequent re-establishment of glandular architecture. Healing of ulcers at the ulcer margin involves contraction of wounds which is facilitated by regulated release of some substances namely histamine, serotonin, prostaglandin etc. at ulcer beds of the wound-site most times released from mast cells. Rizzi *et al.*, 2016). These phases are also mediated by cytokines and growth factors through a coordinated signaling pathway expressed at ulcerated sites.

Mast cells are found in abundance in the gastrointestinal tract and are involved in a variety of physiological processes including tissue repair, wound healing and likely adaptive immunity, fibroblast proliferation, regulation of GI barrier functions (mucus production, secretory functions, defense release, etc.) (Albert-Bayo *et al.*, 2019). They are activated in inflammatory reactions but are also important for proper wound healing and regeneration of tissues damaged (Komi *et al.*, 2020. However, its secretion of tryptase and histamine (which are also important mast cell markers, are keys to fibroblast production of collagen and αSMA contraction. They maintain angiogenesis to supply vital nutrients to repairing cells, without the functioning of mast cells, wound healing is impaired in animal models (Bacci 2021). Mast cells can either be beneficial or harmful depending on the mediator triggering its release. During inflammation of the gut especially ulceration, a coordinated interplay between cyclooxygenase-2 (COX-2) and inducible, nitric oxide synthase (iNOS) proteins is grossly expressed at the ulcer sites and margins which facilitates healing through the release of prostaglandins (Fujiwara *et al.*, 2023). Prostaglandins promote and modulate epithelial defense mechanism by stimulating the gastric cells to release mucous, and bicarbonate, and enhance d blood flow to ulcerated sites. Vasodilatation is achieved mostly by release of nitric oxide which is also mediated by COX-2 (Ilari et al., 2020) to achieve gut homeostasis and reduction of pro-inflammatory reactions (Salvemini et al., 2013). Regulated nitric oxide release from iNOS also facilitates inflammatory cells apoptosis during inflammation thus enhancing gastric healing (Guo et al., 2006). This leads to a cascade of event of release and secretion of growth factors essential for cell proliferation and wound healing .

It has been archived that reserpine produces gastric ulceration by depleting catecholamine, serotonin and histamine stores at both peripheral level and central nervous system (Zhou et al., 2014). Reserpine was initially used in managing hypertension but discontinued due to certain adverse complications from long term use one of which is gastric lesion. Various reports indicated that reserpine causes the degranulation of mast cells with increase in the gastric acid secretion via sympathetic activation (Cho *et al.*, 1985), and inhibits the prostaglandin synthesis. However, the ulcerogenic mechanisms of reserpine due to mast cell degranulation are not clear. A similar sequence of events has been observed in stressed animals where appreciable falls in stomach wall mast cell counts are causally related to increases in gastric histamine release (Cho and Ogle, 1979). Various reports indicated that reserpine causes the degranulation of mast cells with increase in the gastric acid secretion via sympathetic activation (Cho *et al.*, 1985). Reserpine increases gastric acidity, cholinergic tone, motility, with altered gastric- mucosa blood vessels.

Pyloric ligation ulcer model is based on induction or stimulation of the histamine-2 receptor leading to the production of gastric hydrochloric acid (Kim *et al.*, 2020) from increased gastric reflux. Histamine-2 receptor is upregulated during gastric ulceration but is down-regulated during gastroprotection or healing activities

It is evident that most gastro-protection and gastric ulcer healing activities of treatments are through modulation of the COX-2/iNOS pathways (Sing *et al.*, 2019). Expressions of COX-2 and iNOS are paramount in all gut inflammatory processes signaling pathways that focus on restoring gut epithelial defense mechanisms (Guo et al., 2006). Activated mast cells causes a transient production of COX-2 while Zhao et al 2022 emphasized the regulatory role of COX-2 during inflammatory response and its ability to mediate nitric oxide production for defense during microbial infection inferring a COX-2/iNOS crosstalk within the enterocyte mainly through MAPK/NF-κB signaling (Mendes *et al*., 2017). Both COX-2 and iNOS are signaled or expressed through secretions of certain gut cells like mast, parietal, mucus, or enterochromaffin cells that modulate gastric inflammation responses.

Recently, metals have been reported to stimulate allergen-mediated mast cells even at less cyotoxic levels (Walczak-Drzewiecka *et al*., 2023). Heavy metals have been documented to exacerbate activities

and signalling of mast cells especially during infection and environmental air pollution. Certain heavy metals when in low doses such as vanadium (Omayone *et al.,* 2020), cobalt (Salami *et al.,* 2018) and have been documented to be gastroprotective. Still, there is dearth of information as regards their activities on gastric mast cells stimulation nor COX-2/iNOS signaling activation linked to mast cells stimulation either during gastric ulcer formation or healing.

Albeit, cobalt chloride has been documented to be toxic at higher doses and expressed in cardiac and renal tissues (Oyagbemi *et al.,* 2019), CNS cells (Gomez-Arnaiz *et al.,* 2022). Cobalt chloride at lower doses in a previous study (Salami *et al.,* 2018) was documented to facilitate acetic acid-induced gastric ulcer healing without reoccurrence by day 21 post-ulceration (and 7 days post-treatment). The mechanism of observed healing is unknown but postulated to probably be via conferring repair at the gastric epithelial and mucous gel layers. The precise mechanism of action of gastro-protection conferred by cobalt chloride at lower doses remains vague which was unraveled using reserpine and pyloric ligation induced gastric ulcer models.

METHODOLOGY

Animal

Healthy male Wistar rats (140-160g) obtained from the Central Animal House, Department of Physiology, College of Medicine, University of Ibadan, Nigeria, were used for this study. They were acclimatized for a period of 2 weeks and housed in cages at standard laboratory condition of room temperature (23 ±2^{o}C), humidity (55 ± 15%) with natural environmental 12 hours light and dark cycle. They were allowed free access to water and standard commercial rat pellets (Ladokun Feeds Nigeria Limited, Ibadan, Nigeria). The rats were handled according to the ethics of animal handling in compliance with the institution's guideline and criteria for human care (National institute of Health Guidelines for the care and Use of Laboratory Animals. University of Ibadan Ethical approval was obtained and assigned a number UI-ACUREC/100-1021/9.

Reserpine experimentally induced ulceration

A total of 50 adult male rats were grouped into 5 groups as follows. Group 1 (Normal control; non ulcerated untreated, Group II ($HCoCl_2$) pre-treated 62mg/kg $CoCl_2$, Group III ($LCoCl_2$) pre-treated 25mg/kg $CoCl_2$, Group IV (Omeprazole) pre-treated omeprazole and Group V (ulcerated untreated). Prior to reserpine induced ulceration, cobalt chloride and omeprazole treatments were given orally, once daily for 5 days and 30 min before reserpine treatment on the fifth day. Gastric lesions was induced as previously reported by (Gupta *et al.,* 1974). Briefly, 0.1mLs of glacial acetic acid was added to reserpine, and then dissolved with distilled water and administered intraperitoneally (5mg/kg i.p). In each groups five animals were sacrificed after 3 hours and 8 hours post-ulcer induction. Each stomach was removed for macroscopic and microscopic evaluation as well as gastric biochemical estimation. Microscopic scoring of stomach ulcer in reserpine-induced ulcers was according to (Valcayi *et al.,* 1982) which place ulcer severity in a scale of 0 to 10. The following formula calculated the percent protection with each treatment:

% Protection = Uc – Ux/Uc × 100

Pyloric Ligation Induced Gastric Ulceration

A total 25 adult male Wistar rats were divided into 5 groups as follows: Group A (Low Cobalt, 25mg/kg b.w of Cobalt chloride), Group B (High Cobalt, 62mg/kg b.w of Cobalt chloride), Group C (36.875mg/kg b.w Cimetidine, CIME), Group D (Ulcer Untreated), Group E (Normal control; no ulceration and drug was administered). Groups A, B, and C were pretreated for seven days before pyloric-ligation gastric ulceration induction. Wistar rats were fasted overnight before pyloric ligation, but had free access to water. The rats were put with wide-mesh wire bottoms to prevent coprophagia during the experiment. Pyloric ligation was performed in groups A to E rats for the induction of gastric ulcers 1 hr after the last administration of the respective test solutions on fasted rats. The abdomen was opened by using a small incision below the xiphoid process after induction of anesthesia by cocktail of xylazine (10mg/kg) and ketamine HCl (75mg/kg) (intraperitoneal). The stomach was exposed, a thread was placed around the pyloric sphincter and tied in a tight knot, after which the abdomen was sutured back. Eighteen (18) hours after ligation the animals were euthanized, engorged stomach was carefully excised and gastric effluent was collected into clean centrifuging tubes and centrifuged at 1000 rpm for 10 minutes, and the volume of gastric juice void of sediments was noted. An aliquot of 1 mL gastric juice was diluted with 1 mL of distilled water, and pH of the solution was measured using a pH meter. The clean stomach was scored for gastric ulceration before being processed for biochemical, histological and immunohistochemical analysis. The ulcer index and

percentage of ulcer inhibition were determined as follows: macroscopic ulcer score (gross gastric mucosal lesions) was assessed and scored for number and severity of erosions using the scoring method as 0 = No lesion, 0.5 = Hemorrhage, 1 = 1–3 small lesions < 1 mm length, 2 = 1 – 3 large lesions > 1 mm length, 3= 1–3 thickened lesions, 4 = more than 3 small lesions, 5 = more than 3 large lesions, 6 = more than 3 thickened lesions. The results were expressed as ulcer index (UI) and the percentage protection was calculated using the following formula: Protection % = [(UI control − UI treated group) / (UI control)] x 100

Biochemical assay

A larger section of the excised stomach samples (in all induced gastric ulcer models) were weighed and homogenized in 10 volume (weight) of 0.1M iced cold phosphate buffer, pH 7.4 using a Tefflon Homogenizer. The resulting homogenates were centrifuged at 3600 rpm (1452g) at 4°C for 15 minutes. The supernatant fraction was collected, well labeled and kept frozen until use for biochemical estimations.

Gastric tissue protein concentration was determined by means of Biuret method according to Gornall *et al.*, (1949), protein carbonyl content was determined using the method proposed by Swarnakar *et al.*,while gastric malondialdehyde (MDA) and Hydrogen peroxide (H_2O_2) estimations were quantified using the method of (Vashney and Kale, 1990; and Wolff, 1994) respectively. Gastric Superoxide dismutase (SOD) activity was determined by the method of Misra and Fridovich, 1972), Catalase activity was determined according to the method of Claiborne (1985) and Sulfhydryl level was assayed according to the method of (Ellman, 1959). Gastric Nitric oxide (NO) was quantified indirectly as total nitrite (NO_2^-) using Griess reagent. Gastric mucin content was determined according to the method of Winzle (1995) and gastric Na^+K^+ and H^+K^+ATPase activities were spectrophotometrically determined according to the method of Bewaji *et al.*, (1985)

Histological procedures

The section of the harvested stomach in 10% formalin was used for histological evaluation. Sections of the stomach with a thickness of 5 µm were prepared from strips removed from the stomach fundic area and stained using, using PAS, Hematoxylin and Eosin stain. The various gastric mucosal secretory cells were clearly differentiated, taking up different colours. The nuclei of the parietal cells were stained deep blue while the mucous cells were clearly vacuolated with red patches. Microscopic evaluation of gastric mucosal lesions was performed by an experienced pathologist who was unaware of the groupings. Identification, characterization and counting of both mucous and parietal cells were carried out using motic image software. Gastric mast cell count was by counting the number of gastric cells that stain for Toludine blue using motic image software.

Immunohistochemistry method

Immunohistochemistry procedure was as described by Oyagbemi *et al*. (2021) for iNOS and COX-2 with slight modification using 2-step plus Poly-HRP Anti Mouse/Rabbit IgG Detection System with DAB solution (Catalog number: E-IR-R217 from Elabscience Biotechnology®, China). The following antibody was used viz: Synthetic peptide of human NOS2 Polyclonal Antibody (E-AB-70051 1:500), COX-2 Polyclonal Antibody (E-AB-62884 1:200) for the iNOS and COX-2 respectively. Slide sections were observed with a light microscope equipped with a digital camera [(Leica LAS-EZ®) using Leica software application suite version 3.4]

Statistical analysis

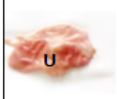
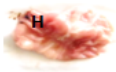
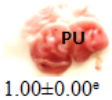
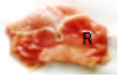
Data was analysed by Graphpad prism statistical package using descriptive statistics, ANOVA and t-test at $p = 0.5$.

RESULTS

Effect of Cobalt on Ulcer Index, Percentage Protection and Relative stomach weight in Reserpine induced gastric ulcer.

Figure 1 present the effect of Cobalt Chloride on Ulcer Index, Percentage Protection, and Relative stomach weight in Reserpine induced gastric ulcer. Reserpine induced gastric ulcer presented as pin-point ulcer, deep tissue ulceration and Haemorrahage. There was a significant decrease in the ulcer index of all the treated groups compared with ulcer untreated group both by 3 hours and 8 hours. Omeprazole, low and high dose cobalt chloride protected the mucosa against Reserpine-induced gastric ulcer and was highest in this order by 3 hours (Omeprazole> low $CoCl_2$ > high $CoCl_2$) and this by 8 hours (high $CoCl_2$ > low $CoCl_2$ > Omeprazole).There was a significant increase in the relative stomach weight in all the treated groups; except for 62 mg/kg High $CoCl_2$ treated group, when compared with the

untreated group by 3 hours and by 8 hours, relative stomach weight significantly decreased in all the treated groups; except for the 3.6 mg/kg Omeprazole treated group, when compared with the untreated group.

Treatment	Ulcer index (Mean ± SEM)	
	3 hours	8 hours
Normal Control	0.00±0.00^{b,c,d,e}	0.00±0.00^{b,c,d,e}
Reserpine (5mg/kg)	 5.00±0.00^{c,d,e}	 10.00±00^{c,d,e}
High $CoCl_2$ (62mg/kg) + Reserpine	 2.00±0.00^{e}	 1.00±0.00^{e}
Low $CoCl_2$ (25mg/kg) + Reserpine	 1.33±0.33	 4.00±1.00
Omeprazole (3.6mg/kg) + Reserpine	 1.00±0.00	 4.33±0.67

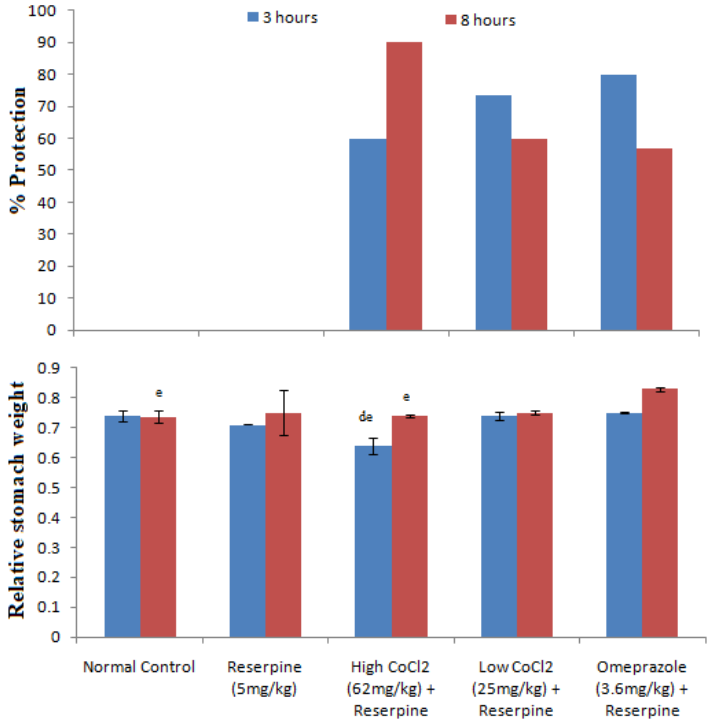


Figure 1. Effect of Cobalt Chloride on Ulcer Index, Percentage Protection, and Relative stomach weight in Reserpine induced gastric ulcer.

Values are expressed as Mean±SEM.Values are significant when p≤0.05. Keys of significance :[a] compared with Baseline (normal control),[b] compared with 5mg/kg of Reserpine,[c] compared with 62mg/kg High $CoCl_2$,[d] compared with 25mg/kg of Low $CoCl_2$,[e] compared with 3.6mg/kg of Omeprazole. NOTE: U: Ulcer, PU: Pin point Ulcer; H:Haemorrahage

Plate 1 is a photomicrograph of the stomach section showing histological alterations by 3 and 8 hours in reserpine-induced gastric ulcer. The reserpine treated showed slight edema, slight mucosa and submucosa infiltration of inflammatory cells, mild mucosa hemorrhagic and submucosa fibroblasts (*Fbc*) by 3 hours and including mild submucosa vessel congestion and mild angiogenesis by the 8th hour. The low cobalt treated presented mild infiltration of inflammatory cells into the mucosa and submucosa, mild angiogenesis (*Ang*) and mild presence of fibroblasts (*Fbc*) in the submucosa by the 3rs hour and scanty fibroblasts *(Fbc)* in the submucosa by the 8th hour. The high cobalt treated showed mild edema (dashed arrow), mild infiltration of inflammatory cells into the mucosa, submucosa, and muscularis, mild haemorrhagic lesion in the mucosa (*HL*), mild angiogenesis (*Ang*) and mild fibroblasts (*Fbc*) in the submucosa by the 3rd hour and normal parietal and mucous glands with no significant lesion by the 8th hour. The omeprazole treated showed mild infiltration of inflammatory cells to the mucosa by the 3rd hour and moderate edema, mild congestion, moderate infiltration of inflammatory cells, and mild presence of fibroblasts (*Fbc*) in the submucosa by the 8th hour.

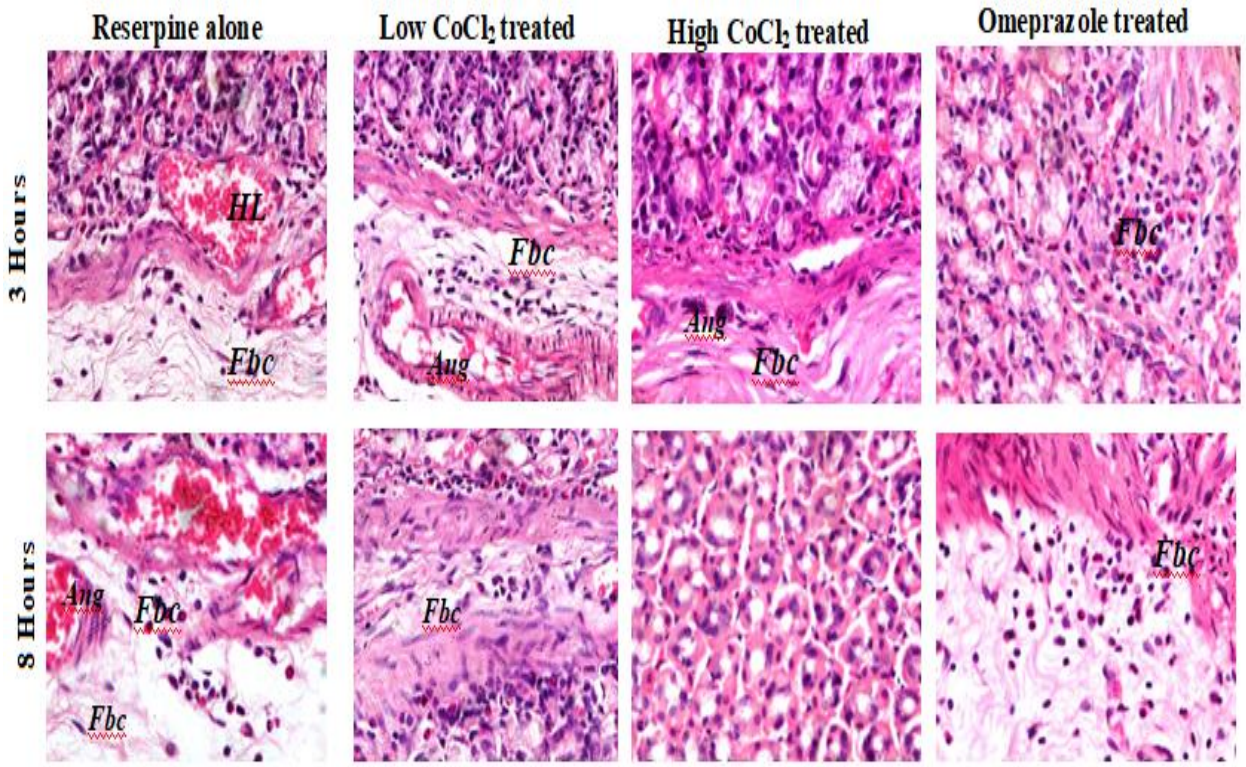


Plate 1. Photomicrograph of stomach section histological alteration by 3 and 8 hours in reserpine induced gastric ulcer (H & E STAIN, MAG.X 400)

Effect of cobalt on gastric biochemical assay (redox status; MDA, Sulfhydryl; NO and Hydrogen Peroxide levels) during reserpine induced Ulcer

Figure 2 presents the effect of cobalt on gastric biochemical assay (redox status; MDA, Sulfhydryl; NO and Hydrogen Peroxide levels) during reserpine induced Ulcer. There was a significant increase in the mucosa MDA level of the Reserpine group when compared with the control group and treated groups (High $CoCl_2$group, Low $CoCl_2$ group, Omeprazole group) by 3 and 8 hours. Gastric sulfhydryl level of the reserpine group was significantly decrease when compared with the control group and treated groups, (High $CoCl_2$ group, Low $CoCl_2$ group, Omeprazole group) by 3 and 8 hours. Also, there was a significant increase in the gastric nitric oxide level of the reserpine group when compared with the treated groups (High $CoCl_2$ group, Low $CoCl_2$group, Omeprazole group) by 3 and 8 hours. Gastric hydrogen peroxide level was significantly increase in the reserpine groups when compared with the control groups and omeprazole group by 3 and 8 hours except for Cobalt treated groups (High $CoCl_2$ and Low $CoCl_2$) which increase when compared with the reserpine induced group by 3 hours.

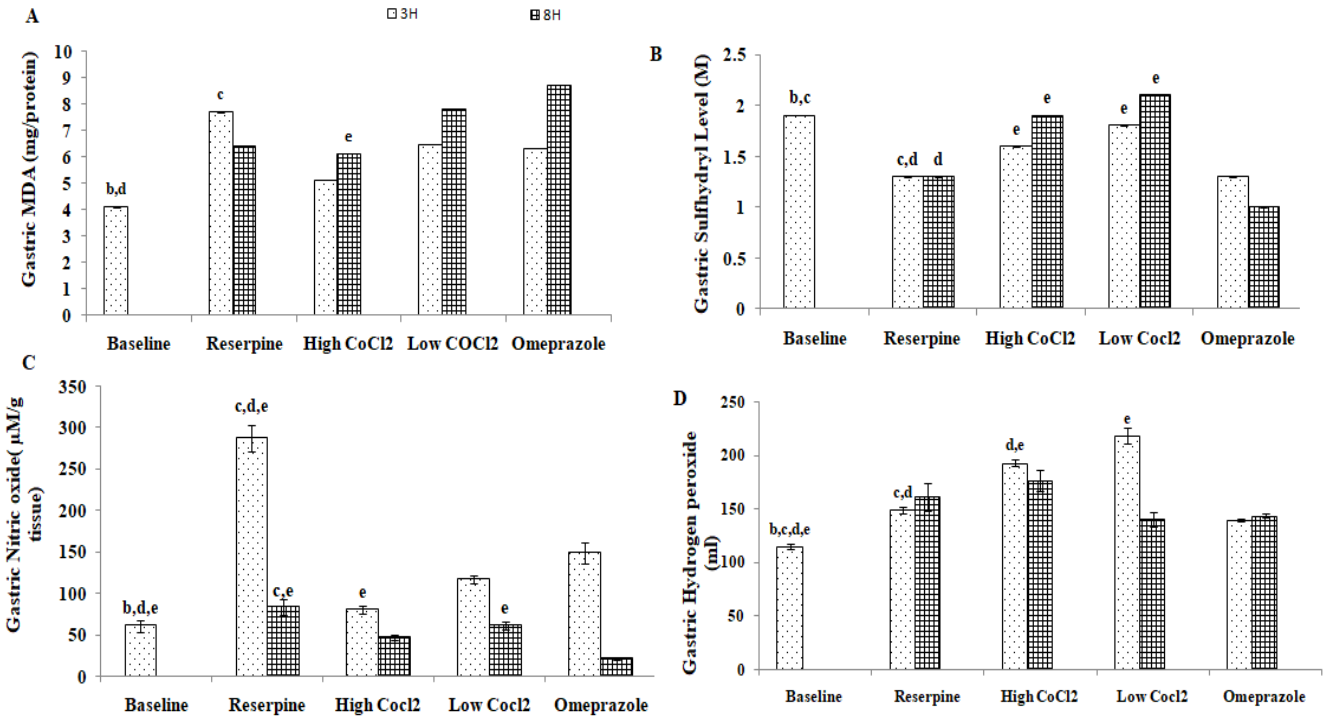


Figure 2. Effect of cobalt on gastric biochemical assay (redox status; MDA, Sulfhydryl; NO and Hydrogen Peroxide levels) during Reserpine induced Ulcer

Values are expressed as Mean±SEM. Values are significant when p≤0.05. Keys of significance :[a] compared with Baseline (control),[b] compared with 5mg/kg of Reserpine,[c] compared with 62mg/kg High $CoCl_2$,[d] compared with 25mg/kg of Low $CoCl_2$,[e] compared with 3.6mg/kg of Omeprazole.

Effect of Cobalt on Intact and De-granulated Gastric Mast cells count in Reserpine induced gastric ulcer

Plate 2 is a photomicrograph of stomach section showing mast cell by 3 and 8 hours in reserpine induced gastric ulcer. There were scanty mast cells in the submucosa of reserpine along while the low cobalt treated showed mild presence of mast cells in the submucosa and high cobalt treated showed mild presence of mast cells in the submucosa and muscularis layers and the omeprazole treated showed marked absence of mast cells by 3 hours. However, by 8 hours, reseprine treated group showed moderate presence of mast cells in the submucosa and muscularis, the low Cobalt treated showed scanty mast cells in the submucosa while the high Cobalt treated showing normal parietal cells and mucous glands and the Omeprazole treated showed moderate mast cells in the submucosa and mid presence of in the muscularis.

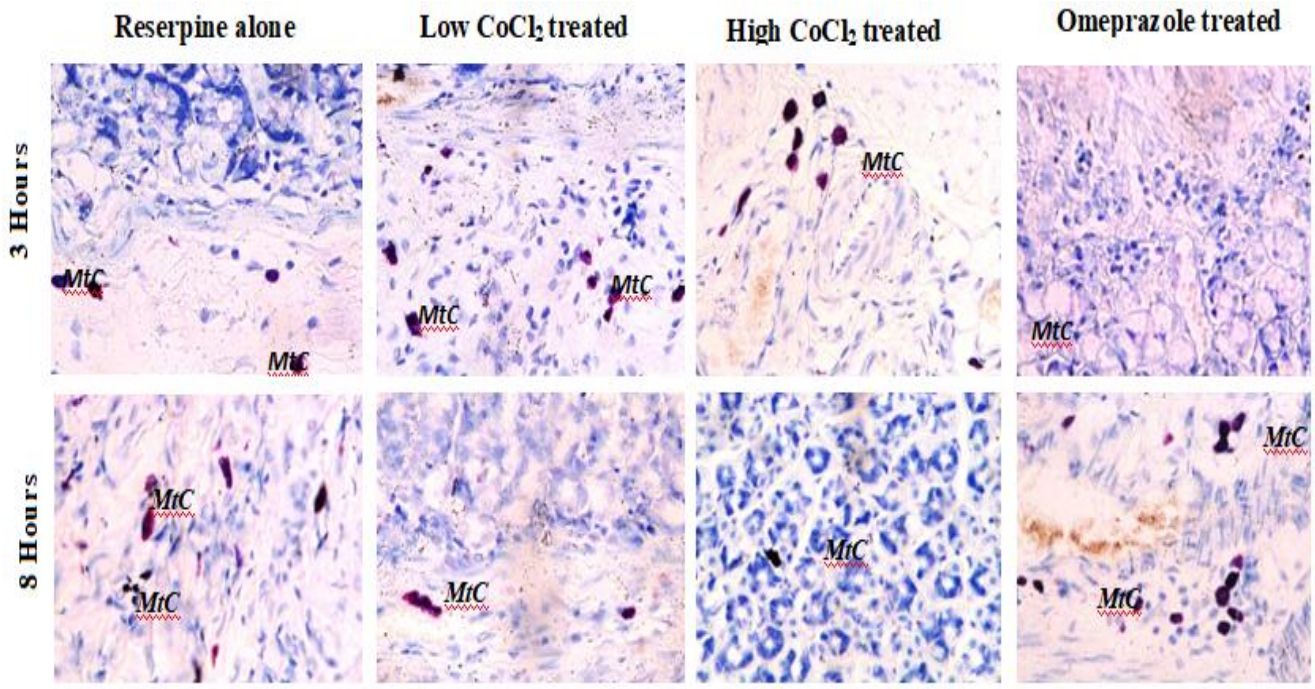


Plate 2. Photomicrograph of stomach section showing mast cell by 3 and 8 hours in reserpine induced gastric ulcer (Toluidine blue stain, MAG. x 400). Mast cells **(*MtC*)**

Table 1 shows the effect of cobalt on intact and de-granulated gastric mast cells count in reserpine induced gastric ulcer. There was a significant increase in the intact mast cell count in the High Cobalt treated groups compared with other groups by 3 hours while the low cobalt treated groups had significantly lower intact mast cell count compared with other groups by 8 hours. Reserpine alone group had significantly increased degranulated mast cell count by 3 hours compared with other groups while the omeprazole treated groups had a significantly reduced degranulated mast cell by 8 hours compared with other groups.

Table 1. Effect of cobalt on intact and de-granulated gastric mast cells count in reserpine induced gastric ulcer

Groups	**Non-ulcerated untreated**		**Reserpine (Ulcerated Untreated) alone**		**Low $CoCl_2$ (25mg/kg) treated**		**High $CoCl_2$ (62mg/kg) treated**		**Omeprazole treated**	
Duration	**3 hours**	**8 hours**	**3 hours**	**8 hours**	**3 hours**	**8 hours**	**3 hours**	**8 hours**	**3 hours**	**8 hours**
Intact mast cells	0.00 ± 0.00	0.00 ± 0.00	6.33 ± 2.33c	23.33 ± 4.167	0.00 ± 0.00^{c}	3.33 ± 1.67^{c}	3.33 ± 1.67^{c}	25.00 ± 0.00	3.33 ± 1.67^{c}	26.00 ± 0.00
Degranulated mast cells	0.00 ± 0.00	0.00 ± 0.00	27.50 ± 6.640	7.50 ± 1.443	3.33 ± 1.667	3.33 ± 1.67^{a}	3.33 ± 1.67^{a}	2.50 ± 1.44^{a}	3.33 ± 1.67^{a}	11.67 ± 2.33

Values are expressed as Mean ± SEM. Values are significant when $p \leq 0.05$.
Keys of significance ^{a} compared with 5mg/kg of Reserpine,^{b} compared with 62mg/kg of High $CoCl_2$,^{c} compared with 25mg/kg of Low $CoCl_2$,^{d} compared with 3.6mg/kg of Omeprazole.

Effect of cobalt chloride on the gastric effluence volume, acid concentration, pH, and pepsin activity.
Table 2 shows the gastric effluence volume, acid concentration, pH and pepsin activity in all the treatment groups. The volume and concentration of gastric effluence in the cimetidine treated was significantly lower when compared

to all groups while those of cobalt treated were lower than the untreated group. The cobalt treated and cimetidine treated groups had a significant decrease in the gastric effluence pH when compared to the untreated group. Gastric effluence pepsin activity was significantly reduced in the cobalt treated groups compared with cimetidine treated, however, the cimetidine and cobalt treated groups had a significantly increased pepsin activity compared with the untreated group.

Table 2. Effect of cobalt chloride on gastric effluence volume, acid concentration, pH and pepsin activity during Pyloric ligation Induced gastric ulcer model.

Groups	Gastric Effluence Volume (mLs)	Gastric Effluence Concentration x10^{-4}	Gastric Effluence pH	Pepsin Activity ()
A 25mg/kg $CoCl_2$	13.5 ± 1.26	4.5 ± 0.048	3.39 ± 0.02	1442.4 ± 14.89
B 62mg/kg $CoCl_2$	13.16 ± 0.75	4.7 ± 0.024	3.37 ± 0.02	1500.94 ± 45.72^{d}
Cimetidine (36.88mg/kg)	**9.5 ± 0.35^{abd}**	4.3 ± 0.127	3.36 ± 0.01	1532.9 ± 10.79^{d}
Untreated	16.67 ± 1.01	4.8 ± 0.048	3.32 ± 0.01	1431.3 ± 10.16

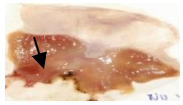
Values are expressed as Mean ± SEM. Values are significant when p≤0.05.

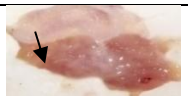
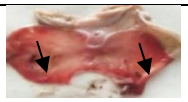
Keys: . [a] significant compared with A, 25mg/kg $Cocl_2$, [b] compared with B, 62mg/kg $Cocl_2$, [c] compared with CIM, 36.875 mg/kg Cimetidine [d] compared with Ulcerated Untreated (UNT),, [e]Control (CON).

Effect of Cobalt chloride on Gross Ulcer Score and Percentage Ulcer Inhibition during Pyloric ligation Induced gastric ulcer model

Table 3 shows the effect of Cobalt chloride on Gross Ulcer Score and Percentage Ulcer Inhibition during pyloric ligation induced gastric ulcer model. It shows that there is significant increase in percentage ulcer inhibition in the group treated with low dose of cobalt chloride and cimetidine when compared to the untreated group and ulcer index was significantly decrease in the treated groups when compared with the untreated group.

Table 3. Effect of Cobalt chloride on Gross Ulcer Score and Percentage Ulcer Inhibition during Pyloric ligation Induced gastric ulcer model

Groups	Ulcer Index	% Ulcer Inhibition
Control	 **0**	**100**
25 mg/kg $CoCl_2$	 **2.42 ± 0.04**	**56^{c}**
62 mg/kg $CoCl_2$	 **3.00 ± 0.24**	**45.45^{e}**
Cimetidine (36.88mg/kg)		**71.27 ^{abde}**

	 **1.58 ± 0.04**	
Untreated	 **5.50 ± 0.14**	**0**

Values are expressed as Mean ± SEM. Values are significant when p≤0.05.
Keys: . [a] significant compared with A, 25mg/kg Cocl2, [b] compared with B, 62mg/kg Cocl2, [c] compared with CIM, 36.875 mg/kg Cimetidine [d] compared with Ulcerated Untreated (UNT),, [e]Control (CON).

Effect of cobalt on gastric biochemical assay (redox status; MDA, carbonyl, Sulfhydryl, and catalase activity) during Pyloric ligation Induced gastric ulcer model

Figures 3 show the effect of cobalt chloride on gastric biochemical assay (redox status; MDA, carbonyl, Sulfhydryl, and catalase activity) during pyloric ligation induced gastric ulcer model. All treated groups show no significant difference in gastric tissue MDA concentration compared with the untreated and control groups. There was a significant decrease in gastric tissue carbonyl concentration of the untreated ulcerated group compared with the treated and control groups.

Gastric tissue sulfhydryl concentration was not significantly different between the treated and untreated or control groups. However, the Cimetidine treated had a significant increase gastric tissue catalase activity when compared with the Untreated. Low dose cobalt treated chloride had a significant increase in gastric tissue catalase activity compared with the Control, Untreated and all other groups.

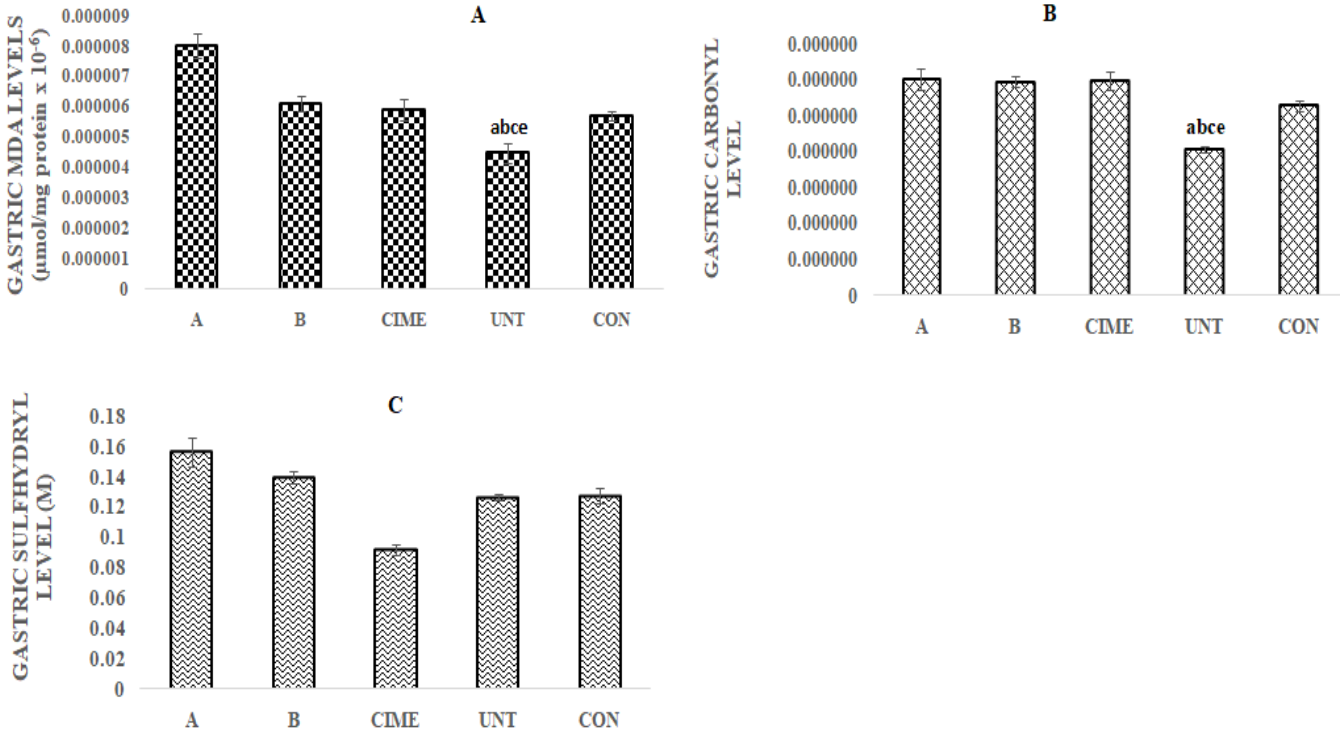


Figure 3. Effect of cobalt on gastric biochemical assay (redox status; MDA, carbonyl, Sulfhydryl and catalase) during Pyloric ligation Induced gastric ulcer model

Values are expressed as Mean±SEM. Values are significant when p≤0.05. Keys of significance Keys: . [a] significant compared with A, 25mg/kg Cocl2, [b] compared with B, 62mg/kg Cocl2, [c] compared with CIM, 36.875 mg/kg Cimetidine [d] compared with Ulcerated Untreated (UNT),, [e]Control (CON).

Effect of cobalt on gastric biochemical assay (mucin, Nitric oxide, H^+K^+ ATPase and Na^+K^+ ATPase activities) during Pyloric ligation Induced gastric ulcer model

Figures 4 show the effect of cobalt chloride on gastric biochemical assay (mucin, nitric oxide, H^+K^+ ATPase and Na^+K^+ ATPase activities) during pyloric ligation induced gastric ulcer model.

Gastric tissue mucin level was significantly increased in control and cobalt treated groups compared with the untreated groups. Cimetidine treated group had a significant decrease gastric tissue mucin concentration compared with the groups treated with cobalt chloride.

Gastric tissue nitric oxide level was significantly increased in the cobalt treated groups compared with the cimetidine and untreated group. The control and cimetidine treated groups had a significant decrease gastric tissue nitric oxide compared with the groups treated with cobalt chloride.

Gastric tissue H^+K^+ and Na^+K^+ATPase pump activities were significantly increased in the ulcerated untreated group compared with the control, cobalt, and cimetidine treated groups. The control and cimetidine treated groups had a significant decrease gastric tissue nitric oxide compared with the groups treated with cobalt chloride

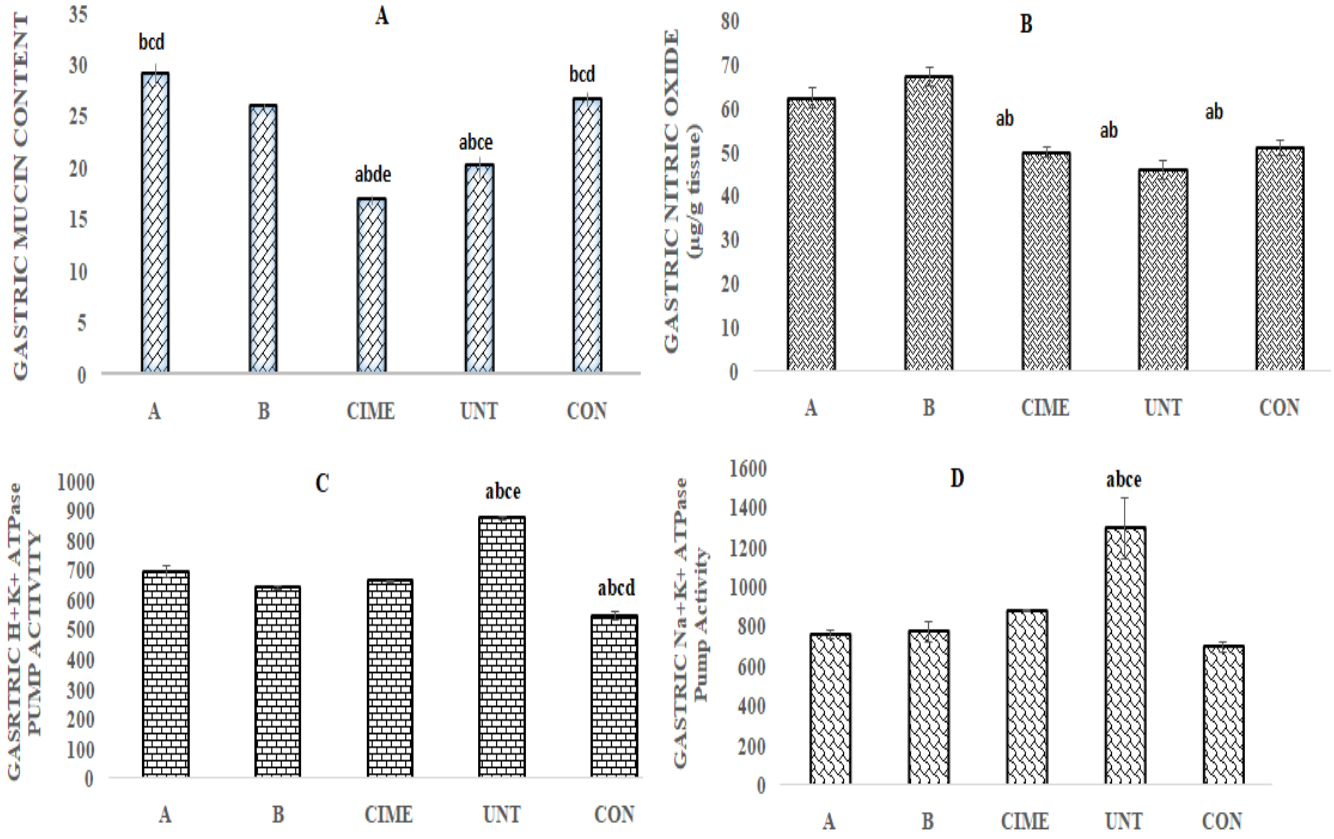


Figure 4. Effect of cobalt on gastric biochemical assay (mucin, Nitric oxide, H^+K^+ ATPase and Na^+K^+ ATPase activities) during Pyloric ligation Induced gastric ulcer model

Values are expressed as Mean±SEM. Values are significant when p≤0.05. Keys of significance Keys: . [a] significant compared with A, 25mg/kg $Cocl_2$, [b] compared with B, 62mg/kg $Cocl_2$, [c] compared with CIM, 36.875 mg/kg Cimetidine [d] compared with Ulcerated Untreated (UNT),, [e]Control (CON).

Effect of Varying doses of Cobalt chloride on Parietal and Mucous cell counts in Stomach tissue

Quantifying the total parietal cell count in gastric tissue of all the groups showed the low and high $CoCl_2$ treated groups had significantly reduced parietal cell count and density compared with the ulcerated untreated groups (Table 4). Mucous cell count and density quantification showed a significant increase in the group treated with the low dose of cobalt chloride compared with the untreated group (Table 4).

Table 4. Effect of Cobalt chloride on Mucous cell count, density with Parietal cell count and density during Pyloric ligation Induced gastric ulcer model

Groups	Mucous Cell Count	Mucous Cell Density	Parietal Cell Count	Parietal Cell Density
Control	384.1 ± 0.06	408.71 ± 0.01	4.2E-05 ± 1.07E-06	15.8 ± 0.46
Untreated	146.67 ± 3.33^{abce}	156.03 ± 3.55 ^{abce}	9.43E-06 ± 3.04E-07 ^{abce}	24.8 ± 0.93 ^{abce}
25 mg/kg $CoCl_2$	519.667 ± 64.33^{de}	552.837 ± 68.44	3.31E-06 ± 2.12E-07	13.2 ± 0.95
62 mg/kg $CoCl_2$	385.83 ± 21.67	409.93 ± 23.05	5.6E-06 ± 1.97E-07	8.8 ± 0.17
Cimetidine (36.88mg/kg)	475.0 ± 35.00	505.32 ± 37.23	2.6E-06 ± 7.99E-08	6.4 ± 0.46

Values are expressed as Mean ± SEM. Values are significant when p≤0.05.

Keys: . [a] significant compared with A, 25mg/kg $Cocl_2$, [b] compared with B, 62mg/kg $Cocl_2$, [c] compared with CIM, 36.875 mg/kg Cimetidine [d] compared with Ulcerated Untreated (UNT),, [e]Control (CON).

Effect of cobalt chloride on percentage parietal and mucous cells count in stomach tissue during pyloric ligation induced gastric ulcer model

Plate 3 showed photomicrograph of stomach section showing parietal cell population in pyloric-ligation induced gastric ulcer. The low and high $CoCl_2$ treated groups presented showed reduced surface epithelia parietal production with no lesion while the cimetidine treated group showed abundant surface epithelia parietal production and the ulcerated untreated presented abundant surface epithelia parietal production with severe mucosa lesions.

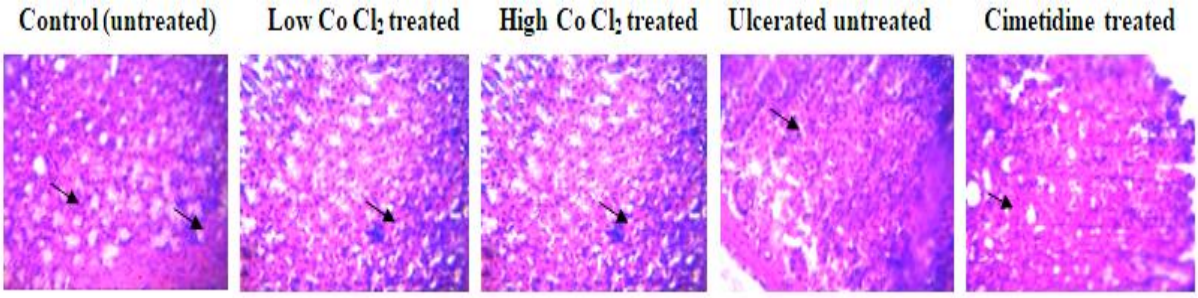


Plate 3. Photomicrograph of stomach section showing parietal cell population in pyloric-ligation induced gastric ulcer (H & E stain, MAG. x 400). Arrow = epithelia parietal cells

Plate 4 show the photomicrograph of stomach section showing mucus cell population in pyloric-ligation induced gastric ulcer. There were abundant surface epithelia mucin production cells, weakly stained foveolar and mucus neck cells in the low and high $CoCl_2$ as well as the cimetidine treated groups. The ulcerated untreated group showed scanty surface epithelia mucin production, weakly stained foveolar and mucus neck cells.

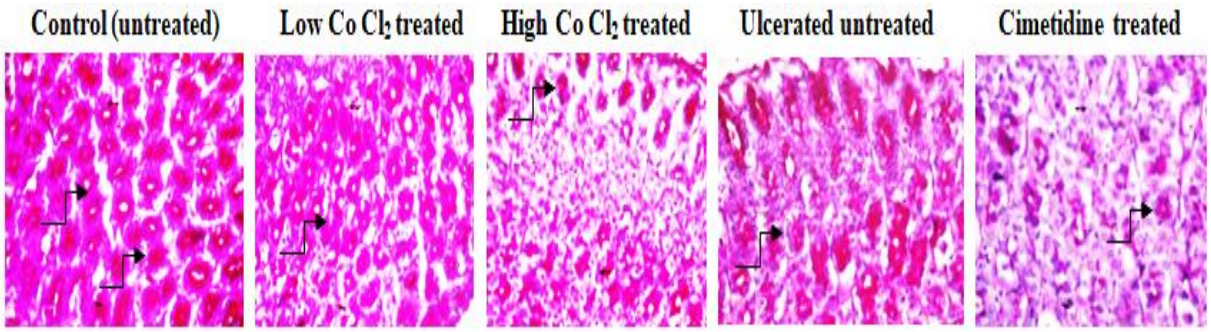


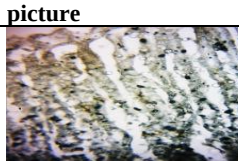
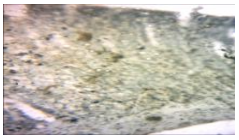
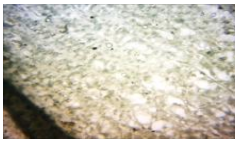
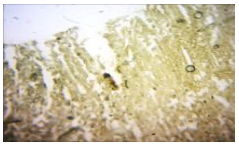
Plate 4. Photomicrograph of stomach section showing mucus cell population in pyloric-ligation induced gastric ulcer (PAS stain, MAG. x 400). Arrow = epithelia mucin producing cells

Effect of Cobalt chloride ($CoCl_2$) treatment on gastric iNOS and COX-2 immunoreactivity.

Table 5 shows the effect of Cobalt chloride on gastric iNOS immunoreactivity during pyloric ligation induced gastric ulcer model. It shows that there is significant increase in the gastric iNOS positive immunoreactivity in the cimetidine and cobalt treated groups compared with the control group.

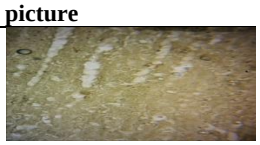
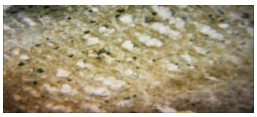
Table 6 shows the effect of Cobalt chloride on gastric COX-2 immunoreactivity during pyloric ligation induced gastric ulcer model. It shows that there is significant increase in the gastric COX-2 negative immunoreactivity in the cimetidine and cobalt treated groups compared with the untreated group.

Table **5: Effect of Cobalt chloride ($CoCl_2$) treatment on gastric iNOS immunoreactivity**

Groups	Percentage contribution of high positive	Percentage contribution of positive	Percentage contribution of low positive	Percentage contribution of all positive	Percentage contribution of negative	Microscopic gastric iNOS immunoreactivity picture
Con	0.95 ± 0.088 ^{e}	25.68 ± 1.96	69.70 ± 0.81*	96.33 ± 2.86	3.67 ± 1.61 ^{abcd}	
Unt	0.69 ± 0.072 ^{e}	33.11 ± 2.57 ^{e}	66.16 ± 2.57*	99.96 ± 5.21 ^{e}	0.041 ± 0.009 ^{ce}	
A (25mg/kg) $CoCl_2$	2.00 ± 0.788	32.00 ± 3.17 ^{e}	65.00 ± 2.34	99.79 ± 6.29 ^{e}	0.00 ± 0.12	
B (62mg/kg) $CoCl_2$	0.91 ± 0.16 ^{a}	32.64 ± 4.51 ^{e}	65.88 ± 4.64	99.43 ±9.30 ^{e}	0.57 ± 0.31 ^{e}	
Cimetidine (36.88 mg/kg)	0.37 ± 0.004 ^{e}	30.37 ± 2.21^{e}	69.25 ± 2.21	99.99 ± 4.42 ^{e}	0.015 ± 0.001 ^{e}	

Keys: . [a] significant compared with A, 25mg/kg $Cocl_2$, [b] compared with B, 62mg/kg $Cocl_2$, [c] compared with CIM, 36.88 mg/kg Cimetidine [d] compared with Ulcerated Untreated (UNT),, [e]Control (CON). Microscopic picture of representation from the groups showing immunoreactivity quantified i.e area stained brown

Table 6: Effect of Cobalt chloride ($CoCl_2$) treatment on Gastric COX-2 Immunoreactivity

Groups	Percentage contribution of high positive	Percentage contribution of low positive	Percentage contribution of positive	Percentage contribution of positive	Percentage contribution of negative	Microscopic gastric COX-2 immunoreactivity picture
CON	0.52 ± 0.043^{abcd}	57.68 ± 0.451	41.79 ± 0.48	99.99 ± 0.0032 ^{abc}	0.0055 ± 0.0032 ^{abc}	
UNT	0.66 ± 0.17 ^{ab}	62.317 ± 4.126	36.97 ± 4.13	99.946 ± 0.0311 ^{abc}	0.0539 ± 0.0311 ^{abc}	
A (25mg/kg) $CoCl_2$	0.65 ± 0.004 ^{b}	54.59 ± 3.95	39.13 ± 4.66	94.367 ± 1.926	5.63 ± 1.926	
B (62mg/kg) $CoCl_2$	2.65 ± 1.18	56.99 ± 3.036	35.11 ± 2.69	94.744 ± 1.202	5.256 ± 1.202	
Cimetidine (36.88 mg/kg)	0.74 ± 0.10 ^{ab}	62.48 ± 2.971	34.05 ± 2.88	97.266 ± 0.437	2.734 ± 0.437 ^{ab}	

Values are expressed as Mean ± SEM. Values are significant when p≤0.05.

Keys: [a] significant compared with A, 25mg/kg $Cocl_2$, [b] compared with B, 62mg/kg $Cocl_2$, [c] compared with CIM, 36.88 mg/kg Cimetidine [d] compared with Ulcerated Untreated (UNT),, [e]Control (CON).

Microscopic picture of representation from the groups showing immunoreactivity quantified i.e area-stained brown,

DISCUSSION

In this study (reserpine and pyloric induced gastric ulcers models), cobalt treated group had higher gastric ulcer healing percentages and gastro-protection index respectively in similar manner as the previous study Salami *et al*., 2018.

Mast cells have been shown to contribute to wound healing in the skin by modifying neutrophil trafficking, angiogenesis and re-modelling of the extracellular matrix. Tellechea *et al*., (2019) reported that intact mast cells and reduced degranulated mast cells aid healing of wounds in diabetes. However, some treatments can modulate the de-granulating activities of mast cells (found around the ulcerated sites) to secrete some of these substances in minute amounts thus initiating wound healing, especially at the inflammatory phase of gastric ulcer healing.

In the reserpine-induced gastric ulcer, mast cells were abundant around the ulcerated site of the ulcerated untreated, unlike the cobalt-treated groups (with fewer mast cells). Increased degranulation of mast cells found at the ulcerated site in the ulcerated untreated consequently increased gastric ulceration (reduced or no percentages of gastric ulcer healing). This could probably be that cobalt maximally modulated or stabilized degranulation of the mast cells thus releasing its substances (needed for wound contraction at the ulcer beds) which aided gastric ulcer healing observed in reserpine.

There has been reports of increase gastric acid secretion (Cho *et al.*, 1985) and acidity during (reserpine stimulation of) mast cell degranulation. In this study there was an increase in the number of parietal cell counts in the ulcerated untreated groups of the pyloric ligation gastric ulcer model and increased gastric acidity as well as volume. One of the mediators released from mast cells when stimulated or degranulated is histamine. This Histamine from de-granulated mast cells has been documented to excite the parietal cells, however, there is a direct relationship between the amount of circulating histamine and parietal cell activation. Increased acidity and volume are instrumental to gastric ulceration. The gastro-protective activities of misoprostol is by stabilizing the mast cells.

Histamine is the final chemo-stimulator of parietal cells and also its elicited responses

In the pyloric ligation ulcer model, there was increased gastric acidity towards alkalinity, reduced gastric effluent volume, and pepsin activity in cobalt chloride treated group unlike the ulcerated untreated. The secretion of gastric juice which is important for pepsin activity in an acidic medium is modulated by

the gastric proton pump (H^+K^+ATPase) activity. The H^+K^+ (proton) and Na^+K^+ ATPase pump activities were reduced in the pyloric ligation model in the cobalt-treated groups unlike the ulcerated untreated groups. This reduction is suggestive of the modulatory activities of parietal cells by cobalt chloride at low doses. However, histological evaluations revealed fibroblasts and angiogenesis in the cobalt chloride-treated groups in all the ulcer models. The parietal cell counts in all the cobalt chloride-treated groups of all gastric ulcer models were reduced but had increased mucous cells counts. The increased mucous cell count and consequently release of some of its secretion might be another probable mechanism by which cobalt elicited its healing activities in this study.

It has been documented that gastric ulceration is mostly accompanied with increased generation of free radical release. Increased mobilization of superoxide (O_2) and hydroxyl radicals (OH) are known to be responsible for inhibition of mucus release and stimulation of surface mucus breakdown via β-adrenoreceptor stimulation which have been assigned to reserpine induced gastric ulcer. Increased lipid peroxidation was ameliorated in the cobalt treated group in a dose dependent manner in all the gastric ulcer model studies. It may be that cobalt exerted anti-inflammatory activities as observed in other studies.

Inherent within most body cells are antioxidant defense systems Superoxide dismutase; sulfhydryl which ameliorate adverse effects of released free radicals, enhancing gastro-protection and epithelial integrity as well as protecting the regenerating tissue (Kwon *et al*., 2022). Treatment with cobalt caused an increased production of superoxide dismutase (SOD), and catalase levels which was dose and duration dependent. However, administration of cobalt increased both the sulfhydryl (NP-SH) and protein content of the gastric mucosa irrespective of the increased free radical generation during reserpine and pyloric ligated ulceration. It is well documented that increase in sulfhydryl compounds (de Nascimento *et al*., 2020) and protein contribute to protecting the stomach from ulcerogens. This increase in antioxidant observation suggests another probable mechanism by which cobalt chloride might be exerting its gastro-protective activities in the gastric ulcer models studied.

There has been conflicting believes about the importance and benefits of hydrogen peroxide, some are of the opinion that it aggravates inflammation thus harming the cells while others uphold its importance in facilitating vascular endothelial growth factor at wound sites. In the reserpine gastric ulcer, there was increased in the hydrogen peroxide level of both high and low $CoCl_2$ treated groups which might have facilitated the actions of vascular endothelial growth factor. This might have resulted in the appearance of fibroblast in the cobalt treated groups gastric tissue from histological evaluation in all gastric ulcer models.

Nitric oxide (NO) is thought to be mediator, not only of gastrointestinal mucosal defense via mNOS, Constitutive NOS (cNOS) and eNOS but also of its epithelial damage via iNOS (Ishii *et al*., 2000). During inflammatory phase of gastric ulcer healing nitric oxide has been documented to enhance vasodilatation thus facilitating healing or prevent ulceration. Nitric oxide causes vasodilatation resulting to increased supply of nutrients and oxygen to the ulcerated stomach thus prompting angiogenesis. However, a relatively high nitric oxide level was observed in the pyloric ligation cobalt chloride treated group, implicative of enhanced vasodilatation and tissue oxygenation. In the reserpine induced gastric ulcer model, ulceration is as a result of untamed overproduction of nitric oxide. However, cobalt inhibits nitric oxide overproduction in reserpine induced gastric ulcers in a dose-dependent manner thus preventing gastric epithelium damage. Increased NO has also been documented to prevent uncontrolled mast cells degranulation, this might be responsible for the observed reduced mast cell degranulation observed in the cobalt treated group. Indicative of cobalt chloride stabilizing mast cells. Nitric oxide has also been implicated to be involved the COX2 pathway and production of prostaglandin during gastric ulcer healing.

Inflammation has been documented to occur due to expressions and an interplay between iNOS and COX-2, however, impairment of both COX-2 and iNOS expression at ulcerated site have been documented to delay gastric ulcer healing (Tiwari *et al.,* 2019). However, COX-2 is important for wound contraction and angiogenesis especially at the early phase of ulcer healing (days 3 to 7) basically due to COX-2 derived prostaglandins (PGs). The interplay between these two (iNOS and COX-2) enhances and facilitates gastric ulcer healing

Cobalt chloride increased the expressions of gastric iNOS but moderated the expression of COX-2 at the gastric ulcer margins in the pyloric ligation ulcer model of this study. These increased expressions of iNOS might have facilitated the release of PGs only through expressions of COX-2 at the ulcerated margins. It could be that cobalt enhanced the interplay between iNOS and COX-2 for the observed gastroprotection in the various ulcer model. Knowing that NO is important for sustained expressions of COX-2 and increased production of PG . Studies have documented that COX-2 does not elicit mast cells secretions, but increased expressions of COX-2 are observed in mast cells tumour and is a notable marker

in clinical practice. However, COX-2 expression was downregulated in this study, indicative of gastric ulcer healing and not tumour growth.

However, iNOS has been documented to modulate activities of mast cells during inflammatory processes (Salvemini et al., 1991). In the pyloric ligation model the expression of gastric iNOS was increased in the cobalt chloride treated groups. It might probably be that cobalt chloride ameliorated gastric ulceration through modulated activities of nitric oxide, iNOS and COX-2 pathway.

Reserpine induced gastric ulcer model is an easily inducible model of gastric ulcer in rats and share many of the characteristics of human ulcers in terms of histopathological appearances. It causes intracellular acidification resulting in massive epithelial damage (Cho *et al.*, 1985) as well as uncontrolled degranulation of mast cells. In this present study, an increase in the number of inflammatory cells infiltrations, hemorrhage and mast cells degranulation were detected in the reserpine alone treated (animals') stomach by microscopic evaluation. Compare with the reserpine control group, orally administered cobalt in the present study, dose dependently reduced the severity and the extent of hemorrhages and mast cell degranulation in the involved tissue which was associated with reduced microscopic stomach ulcer lesion. It may probably be that administered doses of cobalt chloride in this study attenuated the mechanism of action of reserpine gastric lesion via reduced cholinergic tone which is worth investigating in further studies.

CONCLUSION

It can therefore be concluded that cobalt mimicks being a potential mast cell stabilizer hence inhibiting mast cell degranulation during reserpine induced gastric ulceration and enhancing release of mast cell substances needed for vasoconstriction (of gastric ulcer bed). The interplay and modulated expressions of gastric COX-2 and iNOS in the pyloric ligation ulcer model is also suggestive of the mechanism by which cobalt chloride ameliorated or modulated gastric ulcer healing and gastro protection. Cobalt invariably confers increased protection of gastric tissue probably by exerting modulatory activities on the mast cells during gastric ulceration and or healing with facilitated interplay between iNOS and COX2.

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