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Oral iron intake compromises the integrity of the gastric mucosa and impairs gastrointestinal intestinal motility

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Abstract
Background: Iron is an essential nutrient for health and well-being. Mild gastric discomfort has however been reported following supplemental iron intake. This study was therefore designed to investigate the effects of chronic iron intake on gastric mucosal integrity and motility in male Wistar rats.
Methods: Forty animals (190-210g) were randomly divided into 2 equal groups and treated orally with either normal saline (0.2ml/day) or iron (3mg/kg/day) for 28 days respectively. Thereafter, gastric acid secretion, acidity, ulcer score, emptying and intestinal transit was evaluated. Retro-orbital blood samples were also collected for haematological (RBC count, PCV, haemoglobin, platelets, total and differential WBC counts) analysis, serum iron, transferrin, ferritin, and total iron-binding capacity (TIBC). Stomach samples were also harvested for histology, mucus and parietal cell counts, gastric antioxidant status (reduced glutathione, superoxide dismutase (SOD), catalase, nitric oxide, malondialdehyde), mucin concentration and total protein.
Results: The iron treated animals showed increased (P<0.05) monocytes, serum iron, ferritin, transferrin, total iron-binding capacity (TIBC), gastric juice pH, malondialdehyde and SOD compared to control. Iron treated group also exhibited increased gastric nitric oxide, mucus content, parietal and mucous cell counts compared to control. Reductions (P<0.05) in gastric catalase, reduced glutathione, gastric emptying and intestinal motility which was accompanied by gastric mucous cell hyperplasia, coagulation necrosis, and moderate atrophy of parietal and chief cells were also observed in the iron treated group.
Conclusion: Chronic iron intake impairs gastrointestinal motility, induces gastric oxidative stress, and causes gastric structurally aberrations that may predispose to increased acidity of gastric juice and ulceration.

Keywords: *Micronutrient, oral iron, gastrointestinal tract, stomach, intestinal motility, oxidative stress, iron-overload*

Résumé
Contexte : Le fer est un nutriment essentiel pour la santé et le bien-être. Une légère gêne gastrique a cependant été rapportée suite à un apport supplémentaire en fer. Cette étude a donc été conçue pour étudier les effets d'un apport chronique en fer sur l'intégrité et la motilité de la muqueuse gastrique chez des rats Wistar mâles.
Méthodes : Quarante animaux (190-210g) ont été répartis au hasard en 2 groupes égaux et traités par voie orale avec une solution saline normale (0,2 ml/jour) ou du fer (3 mg/kg/jour) pendant 28 jours respectivement. Par la suite, la sécrétion d'acide gastrique, l'acidité, le score d'ulcère, la vidange et le transit intestinal ont été évalués. Des échantillons de sang rétro-orbitaire ont également été prélevés pour analyse hématologique (nombre de globules rouges, PCV, hémoglobine, plaquettes, nombre de globules blancs total et différentiel), fer sérique, transferrine, ferritine et capacité totale de fixation du fer (TIBC). Des échantillons d'estomac ont également été prélevés pour l'histologie, la numération du mucus et des cellules pariétales, le statut antioxydant gastrique (glutathion réduit, superoxyde dismutase (SOD), catalase, oxyde nitrique, malondialdéhyde), la concentration de mucine et la protéine totale.
Résultats : Les animaux traités au fer ont montré une augmentation (P<0,05) des monocytes, du fer sérique, de la ferritine, de la transferrine, de la capacité totale de fixation du fer (TIBC), du pH du suc gastrique, du malondialdéhyde et de la SOD par rapport au témoin. Le groupe traité au fer présentait également une augmentation du monoxyde d'azote gastrique, de la teneur en mucus et des numérations des cellules pariétales et muqueuses par rapport au groupe témoin. Des réductions (P < 0,05) de la catalase gastrique, du glutathion réduit, de la vidange gastrique et de la motilité intestinale accompagnées d'une hyperplasie des muqueuses gastriques, d'une nécrose de la coagulation et d'une atrophie modérée des cellules pariétales et principales ont également été observées dans le groupe traité au fer.
Conclusion : L'apport chronique en fer altère la motilité gastro-intestinale, induit un stress oxydatif gastrique et provoque des aberrations structurelles gastriques qui peuvent prédisposer à une acidité accrue du suc gastrique et à une ulcération.

Mots clés : *Micronutriment, fer oral, tractus gastro-intestinal, estomac, motilité intestinale, stress oxydatif, surcharge en fer*

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Introduction

Iron is a micronutrient that is of crucial importance to living cells [1]. It is an essential component of hemoglobin (Hb), myoglobin, cytochromes, and iron–sulfur complexes of the electron transport chain. Iron also plays important roles in metabolic processes, DNA synthesis [2], cell growth, apoptosis, gene regulation [3], myelogenesis, and signal transduction [4]. Dietary iron is the principal source of iron for the body and the amount absorbed from diet ranges from 5% to 35% depending on the bodies need, health conditions and type of iron [1]. Iron is recycled from body stores, particularly the liver and senescent red blood cells (RBCs) and is thus conserved by the body [1]. In contrast with other minerals, the level of iron in the body is mainly controlled by its absorption from the gastrointestinal tract [5]. Furthermore, there seems to exist no precise active mechanism for iron excretion from the body and the limited excretion seen is an unregulated process that occurs through loss in sweat, menstruation, shedding of hair and skin cells, and through enterocyte rapid turnover and excretion [5]. There is thus a tendency for iron to be easily accumulated when exogenous iron is loaded by hereditary factors (such as hereditary hemochromatosis), repeated transfusions, diseased conditions [6], ascorbic acid [7] and diet [8]. Accumulating evidence indicates that too much iron can provide an easy accessible source of iron for pathogenic bacteria and can reduce the immune response to infection [9].

Previous studies have established that the gastrointestinal tract, which extends from the oral cavity to the anus, can be regarded as a first line defense organ against ingested bacteria [10,11,12]. Its mucosal lining exerts a plethora of functions ranging from barrier, protective digestive, propulsive to immune functions [10]. The gastric mucosa plays an essential role in maintaining the physiological functions of the stomach [11]. The mucosa acts as gastric barrier providing protection for deeper tissue against the damaging actions of the gastric juice components and ingested mucosal irritants [12]. Coordinated contractions and relaxations of the muscles in the various parts of the gut is responsible for motility within the gastrointestinal tract [13]. Though uncommon, gastric mucosal damage has been suggested to be a potential adverse effect of oral iron intake [14,15]. Its effects on gastric integrity, acid secretion and motility have scarcely been reported in the literature. This study was therefore designed to evaluate gastric integrity (structure, antioxidant status, parietal and mucous cell counts, mucous level, acid secretion and pH) and motility (gastric emptying rate and intestinal motility) in Wistar rats treated orally with iron.

Materials and methods

Animals and groupings

Forty male rats (190 - 210 g) were housed in well-aerated cages, fed on standard rat chow, had access to drinking water *ad libitum* and exposed to natural alternating day and night cycles. They were acclimatized for 14 days in the animal house prior to commencing experimental procedures. The animals were randomly divided into 2 equal groups. Group I was control and was treated with normal saline (0.2ml/day) while animals in group II received iron as ferrous sulphate (3mg/kg/day) [16] for 28 days respectively. The Applied and Environmental Physiology Unit, Department of Physiology approved this study and all procedures were carried out in accordance with guidelines laid down by the Animal Care and Use Research Ethics Committee, University of Ibadan and that of the Guide for the Care and Use of Laboratory Animals [17], published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA.

Analytical procedures

Haematology, and biochemical evaluations

After 28 days of treatment, blood samples were obtained into clean EDTA containing and plain sample bottles from the orbital sinus of five [5] rats in each group after light diethyl ether anesthesia using non-heparinized capillary tubes. From the EDTA containing blood samples, packed cell volume (PCV) were determined using a hematocrit tube, and read off a hematocrit reader [18]. Hemoglobin (Hb) concentration was assessed using the cynamet hemoglobin method. Red blood cell (RBC) and total white blood cell (WBC) counts were determined using the hemocytometer and the Wright's stain was used for differential WBC (lymphocytes, neutrophil, monocytes, eosinophil) counts. Platelet count was evaluated using the method of Rees and Ecker [19]. Blood samples collected into plain bottles were allowed to coagulate and centrifuged at 3000 g for 10 min at 4^{o}C to obtain serum. Aliquots of the clear serum obtained was analysed for serum iron (Centronics kits, Germany), ferritin, transferrin, and total iron-binding capacity (TIBC) (Fortress Diagnostics, United Kingdom) using commercially available assay kits. Stomach samples were also harvested from each animal, weighed and opened up by making an incision through the lesser curvature,

weighed and homogenized on ice in 1.15% KCl buffer (pH = 7.4) [20]. The gastric homogenates were centrifuged at 10,000 rmp for 10 min at 4°C. The clear supernatant obtained was analyzed for reduced glutathione [21], catalase [22], superoxide dismutase (SOD) [23], lipid peroxidation [24] and nitric oxide (Griess reaction as described by Green *et al*., [25].

Gastric acid secretion, pH and ulcer score

Animals (n=5/group) were fasted (18hours) after their respective treatments and anaesthetized using ketamine (35mg/kg body weight) and xylazine (5mg/kg body weight), and gastric effluents were collected using the Pyloric ligation method as described by Shay et al., [26]. The gastric effluent collected was measured, flow rate determined and centrifuged at 3000 rpm for 10 minutes. The pH of supernatant obtained was thereafter taken using a pH meter. The ulcer score of each animal also was scored using the method of Takagi and Okabe [27].

Motility, mucous content, histopathology, mucous and parietal cell counts

Gastric emptying rate (n=5/group) was evaluated as described by Droppleman *et al.,* [28]. Thereafter the stomachs were thereafter excised under ketamine-xylazine anaesthesia as above and the gastric barrier mucus was estimated by method of Corne *et al*, [29]. Mucous cell count, parietal cell count, histopathology and intestinally motility (n=5/group) was estimated using the Periodic Acid Schiff (PAS) reaction technique and Hematoxylin and Eosin (H and E) staining techniques respectively as described by Adewoye and Salami [30]. Intestinal motility was evaluated using activated charcoal meal method [31].

Statistical analysis

Results obtained are expressed as mean ± standard error of mean (SEM). Using the Student's t and Mann-Whitney tests statistical significance was taken at $p < 0.05$.

Results

Effect of oral iron intake on body weight

Body weight increased ($P<0.05$) in groups I and II on day 28 compared to initial body weights within same group. The percentage weight gain in the iron-treated group (5.52%), was significantly reduced compared to the percent weight gain seen in group I (11.06 %), the control group (Table 1)

Table 1: Body weight (g) changes in control and iron treated groups

	Initial body weight Day 0 (g)	Final body weight Day 28 (g)	% Change in Body Weight
Group I	193.4 ± 4.0	214.8 ± 8.0*	11.06
Group II	210.0 ± 2.2	221.6 ± 5.2*	5.52#

*Values are mean ± SEM for n=5 values. * indicates values that are significantly different from initial values within same group at P<0.05. # indicates values that are significantly different from group I at P<0.05.*

Effect of oral iron intake on haematological indices

No significant difference was observed between groups I and II for packed cell volume (%), red blood cells count ($10^6/mm^3$) and haemoglobin concentration (g/dL) (Table 2). Similarly, total white blood cell count (3220 ± 283.5 vs. 3320 ± 213.7 cells/mm^3), lymphocytes (74.8 ± 0.7 vs. 75.8 ± 0.4 %), neutrophils (22.0 ± 0.71 vs. 22.4 ± 0.25 %), platelets (121.60 ± 5.35 vs. 130.00 ± 6.57 (x10^3 mm^3/L) and the

Table 2: Red blood cell indices in control and iron treated groups

	Group I	Group II
Packed Cell Volume (%)	51.8 ± 0.8	52.4 ± 0.812
Red Blood Cell Count ($10^6/mm^3$)	8.36 ± 0.203	8.442 ± 0.242
Haemoglobin (g/dL)	16.82 ± 0.269	17.16 ± 0.366

Values are mean ± SEM for n=5 values.

neutrophil to lymphocyte ratio were also not significantly different between group I, control, and group II, the iron treated group (Table 3). However, monocyte level in group II (1.8 ± 0.2 %) was significantly increased (P<0.05), compared to group I (1.0 ± 0.0 %)(Table 3).

Effect of oral iron intake on serum iron indices

Values obtained in group II show a 56.5% increase (P<0.05) in serum iron level compared to group I (Fig 1A). Serum ferritin, transferrin and total iron binding capacity in group II also showed a 156.9%,

Table 3:. White blood cell and platelet counts in control and iron treated groups

	Group I	Group II
White Blood Cell Counts (cells per mm^3)	3220 ± 283.5	3320 ± 213.7
Lymphocytes (%)	74.8 ± 0.7	75.8 ± 0.4
Neutrophils (%)	22.0 ± 0.71	22.4 ± 0.25
Monocytes (%)	1.0 ± 0.0	1.8 ± 0.2 *
Neutrophil to Lymphocyte Ratio	0.2784 ± 0.02198	0.2987 ± 0.03011
Platelets x10^3 (mm^3/L)	121.60 ± 5.35	130.00 ± 6.57

*Values are mean ± SEM for n=5 values. * indicates values that are significantly different from group I (control) at P<0.05.*

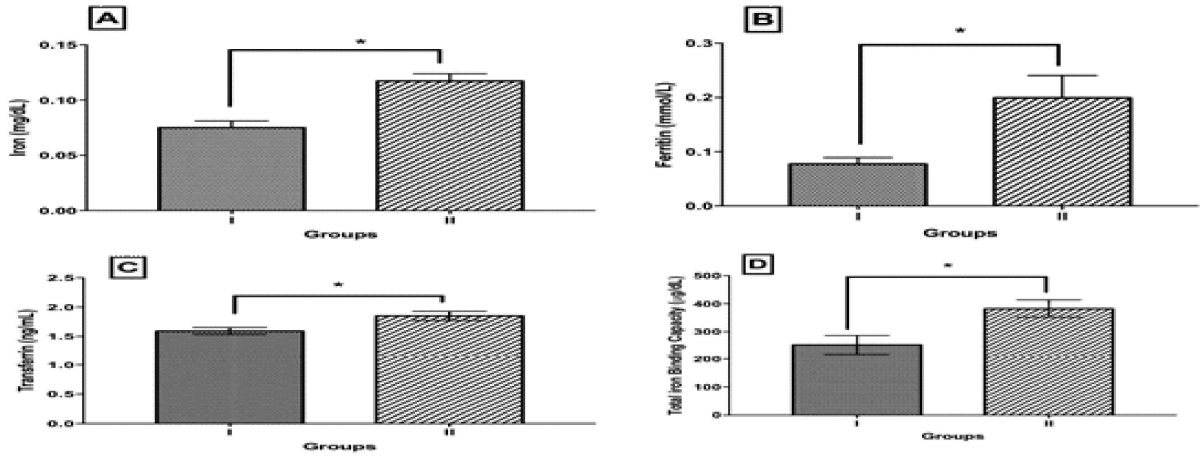

Fig 1. (A-D) Serum iron indices in control and iron treated groups**.** Values are mean ± SEM for n=5 values. * indicates values that are significantly different from group I (control) at P<0.05.

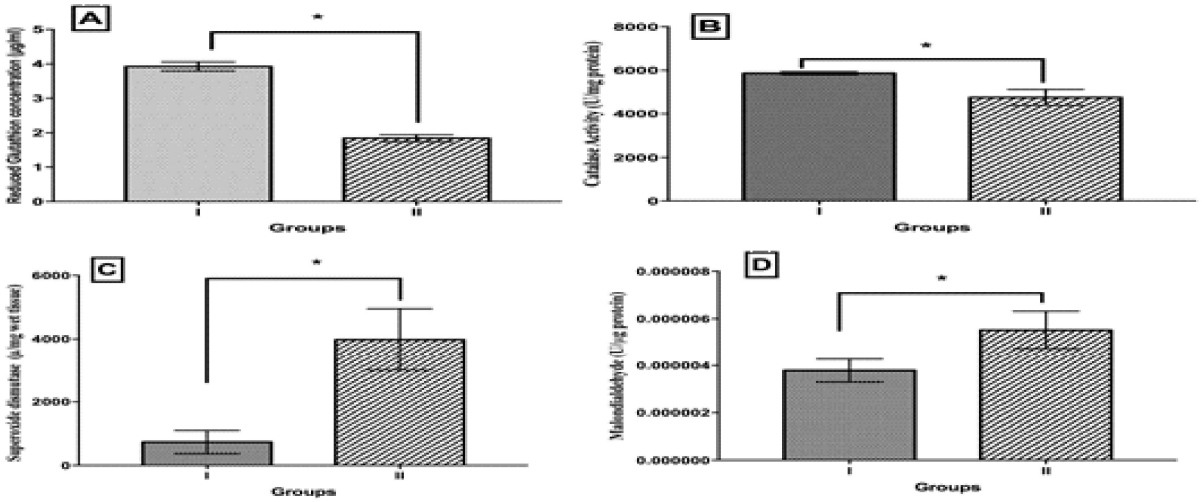

Fig 2. (A-D) Gastric oxidative stress status in control and iron treated groups. Values are mean ± SEM for n=5 values. * indicates values that are significantly different from group I (control) at P<0.05.

16.2%, and 51.4% increase (P<0.05) respectively compared to group I, the control group (Fig 1B-D).

Effect of oral iron intake on gastric integrity indices

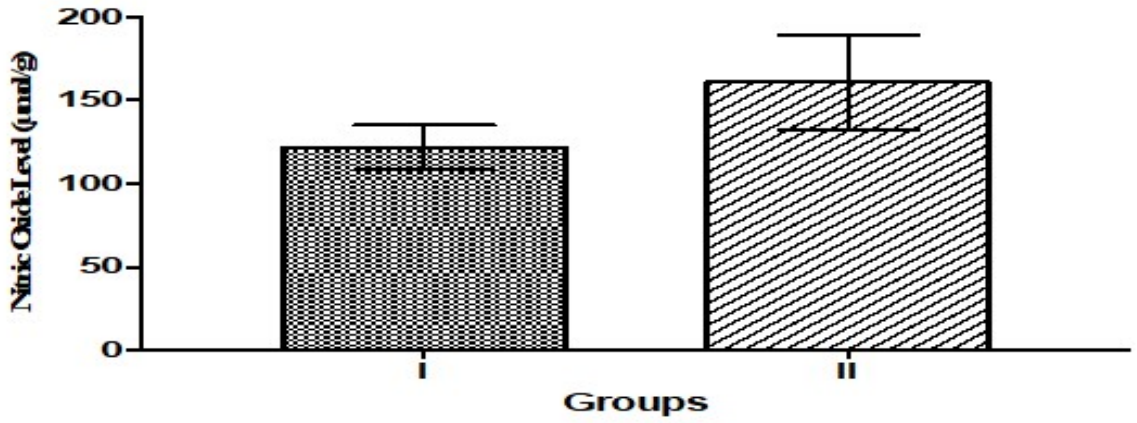

Fig 3. Gastric nitric oxide in control and iron treated groups. Values are mean ± SEM for n=5 values.

Effect of oral Iron intake on gastric antioxidant status and gastric nitric oxide level

Gastric reduced glutathione level showed a 52.9% reduction (P<0.05) in group II compared to control group (Fig 2A). Catalase activity was 18.9% decreased (P<0.05) (Fig 2B) while superoxide dismutase activity (Fig 2C) and malondialdehyde, a marker of lipid peroxidation, (Fig 2D) was significantly increased (P<0.05) in group II compared to group I, control. Gastric nitric oxide in the iron treated group was increased by 31.9% compared to control group (Fig 3).

Ulcer index in the iron treated group showed a 38.5% increase compared to group I, the control group. The pH of gastric juice collected in the iron treated group (1.09 ± 0.02) was significantly reduced compared to control (1.20 ± 0.03). Gastric mucous content, mucous cell count and parietal cell counts were 4.5%, 14.5% and 9.5% increased respectively in groups II compared to values obtained in control (Table 4).

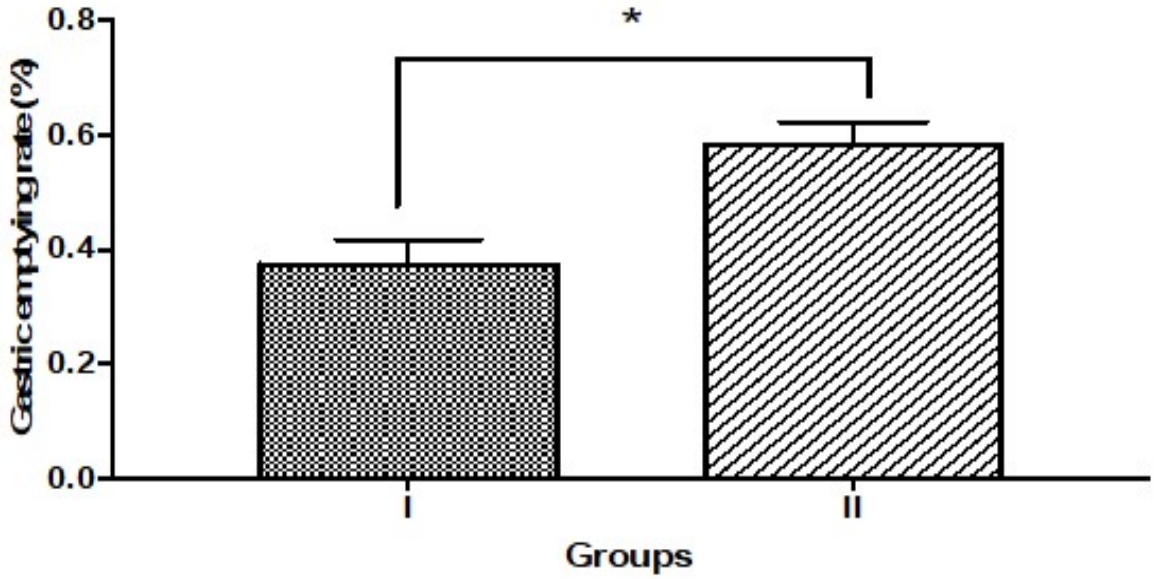

Fig 4. Gastric emptying in control and iron treated groups**.** Values are mean ± SEM for n=5 values. * indicates values that are significantly different from group I (control).

Table 4. Gastric integrity indices in control and iron treated groups

	Group I	Group II
Ulcer Index	3.25 ± 0.85	4.50 ± 1.32
Gastric Juice pH	1.20 ± 0.03	1.09 ± 0.02*
Gastric mucus content	11.77 ± 0.69	12.3 ± 0.73
Mucous cell count	192.1 ± 15.6	219.9 ± 25.2
Parietal cell count	279.5 ± 46.5	305.9 ± 29.4

*Values are mean ± SEM for n=5 values. * indicates values that are significantly different from group I (control) at P<0.05.*

Effect of oral iron intake on gastric emptying, intestinal motility and gastric histology

Gastric emptying duration in group II (0.58 ± 0.04 %) was increased (P<0.05) compared to control (0.38 ± 0.04 %) (Fig 4). Intestinal transit time in group II (41.37 ± 1.63 %) was also significantly increased (P<0.05) compared to values obtained in group I (26.49 ± 1.86 %), the control group (Fig 5).

a result of oversaturation of iron storage (as ferritin) and transport (iron bound to transferrin) thus leading to the presence in circulation of non–transferrin-bound iron (NTBI) and redox active labile plasma iron [33]. Some cells in the body absorb the NTBI's resulting in iron mediated cellular injury while the redox active plasma iron, through the Haber-Weiss and Fenton reactions [34], trigger a cascade that results in

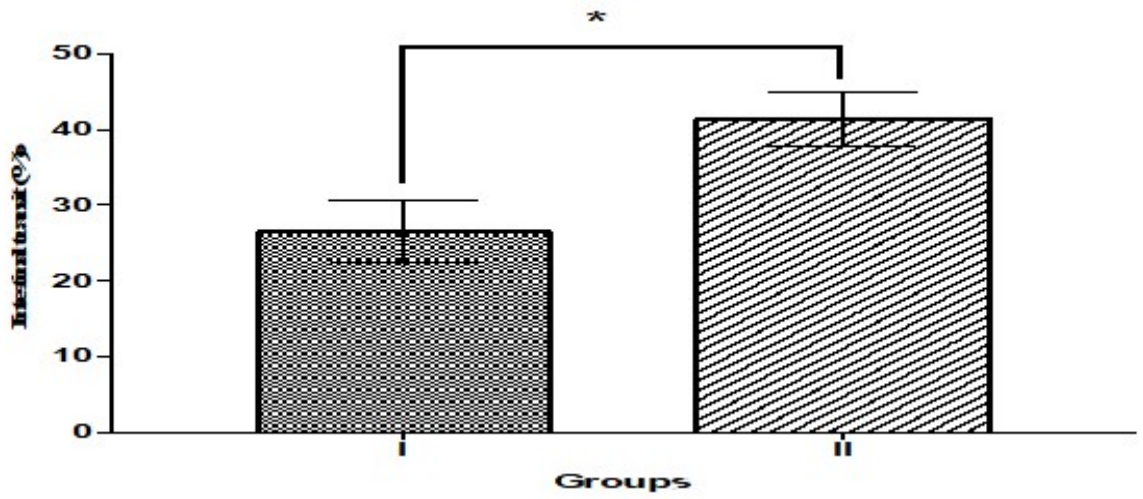

Fig 5: Intestinal motility in control and iron treated groups. Values are mean ± SEM for n=5 values. * indicates values that are significantly different from group I (control).

Histological evaluation

Histological evaluation of gastric sections in the control group, showed normal architecture of the stomach with no observable lesions (Fig 6A and B) while the iron treated group exhibited severe mucous cell hyperplasia (black arrow) and coagulation necrosis (blue arrows) (Fig 6C and D).

Discussion and conclusion

Iron is a nutrient that is mainly recycled by the human body. When dietary, supplemental or intravenous exposure to iron becomes excessive in the body, it results first in to iron-induced oxidative stress [32] and when chronic, iron overload [6]. This occurs as production of excessive free radicals. In this study, the iron treated group (group II) exhibited increased serum iron, ferritin, transferrin and total iron binding capacity suggesting the presence of iron-induced oxidative stress and increased inflammatory conditions [35] but not iron overload [36] in this group as total iron binding capacity was still elevated. This observation is in accordance with the report of Abd Allah *et al.*, [16] who also reported iron-induced oxidative stress in experimental animals treated orally with iron at 3mg/kg/day.

Clinically, haematological investigations are often carried out to evaluate the health status of an individual and deviations from normal values are

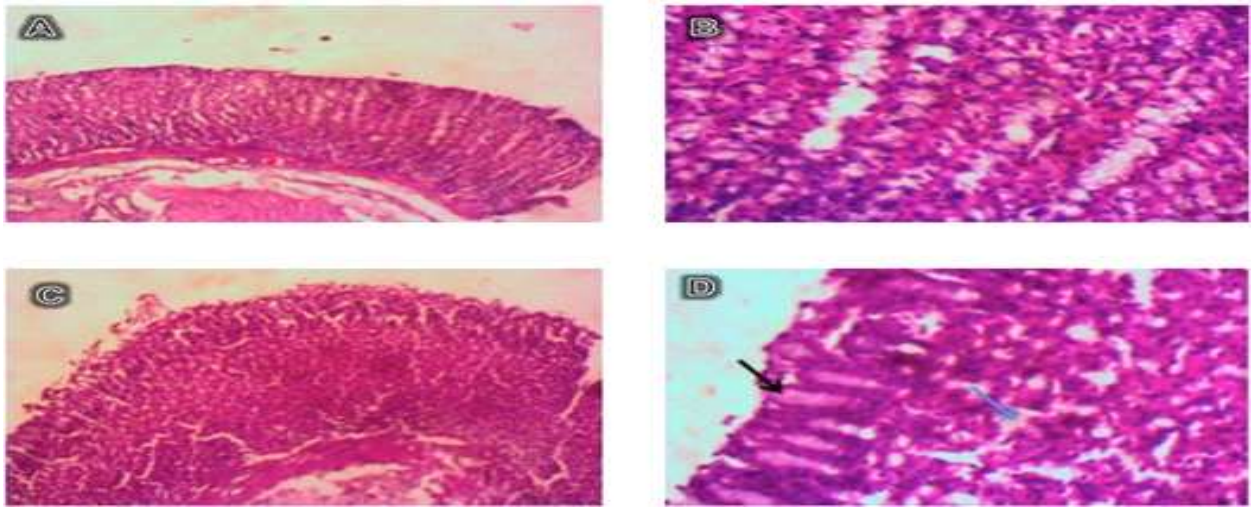

Fig 6. (A–D) Photomicrograph of stomach section in control and iron treated groups. x100 (A,C), x400(B, D). Sections show control animals with normal architecture, preserved mucosa epithelial cell layer, submucosal layer, circular muscle layers and no observable lesions (A and B). Iron treated group also appear normal at low magnification (x100) (C). However, at higher magnification (x400), mucous (foveolar) cell hyperplasia (black arrow) and coagulation necrosis (blue arrow) are observed (D).

usually taken as indicative of the likely presence of pathology in the body. This study shows marginal increases in red blood cell indices (red cell count, packed cell volume and haemoglobin concentration), which may be attributed to increased availability of iron, a red blood cell maturation factor, for erythropoetic processes [37]. Similarly, the marginal increase in white blood cell indices (white blood cell count, neutrophils, lymphocytes, ratio of neutrophil to lymphocytes) and platelet counts may suggest the beginning of haematological perturbations that can arise from iron-induced oxidative stress and inflammation [35]. More so, monocytes, a marker of injury and infection, was elevated in the iron treated group. The authors here speculate that the deleterious effect of iron-induced oxidative stress and perhaps iron overload on blood haematology may be duration dependent and become more exacerbated with age [38] and continuous expose to iron. It is also likely that the reduced percent weight gain observed in the iron treated group compared to control may be attributed to oxidative stress induced by prolonged oral iron intake that may have interrupted gut-neuronal signaling pathways and initiated changes in eating behavior, energy expenditure and maintenance of energy balance [39]. More so, that symptoms that have been reported to accompany iron stress or overload include fatigue, loss of appetite and weight loss [40].

Physiologically, a balance between free radicals and antioxidants is essential for normal functioning [41]. However, when free radicals' production overwhelms the bodies' antioxidant defense mechanisms, they adversely alter proteins, lipids and trigger a variety of disease conditions [34]. To prevent peroxidative tissue and cellular damage in the body is the antioxidant defense mechanism [42] that is made up of enzymatic defense systems (antioxidant enzymes e.g. superoxide dismutase, catalase, glutathione peroxidase, etc.) and free radical scavengers (antioxidants e.g. polyphenols, ascorbic acid, vitamin A, alpha-lipoic acid, glutathione, etc.). This study shows the likely presence of iron-induced gastric oxidative stress as gastric antioxidants, reduced glutathione and catalase, were reduced and gastric malodialdehyde, a marker of lipid peroxidation, was elevated in the iron treated group. However, gastric superoxide dismutase levels observed in the iron treated group were elevated. Superoxide dismutase (SOD) is often regarded as a first line endogenous defense antioxidant enzyme [42]. It catalyzes the dismutation of superoxide anion to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2).

Its elevation in the gastric tissue of the iron treated group may be due to its increased secretion as a result of iron-induced production of hydroxyl radicals, which would need to be neutralized to form hydrogen peroxide and molecular oxygen [34,42]. The hydrogen peroxide so produced would then be further decomposed by catalase, another first line defense antioxidant, to oxygen and water. This may thus account for the depletion of gastric catalase levels observed in the iron treated group. Reduced

glutathione has been described as a second line antioxidant defense mechanism [42]. It directly scavenges reactive hydroxyl free radicals, other oxygen-centered free radicals, and radical centers on DNA as well as other biomolecules [42]. Its depletion in the gastric tissue of the iron treated group suggests impairment in the ability of the cells to scavenge free radicals as they are being produced. The overall impairment in gastric antioxidant defense may account for the increased levels of malondialdehyde and hence lipid peroxidation observed in the iron treated group

Nitric oxide (NO) has been reported to exert various physiological roles due to its ability to induce vascular vasodilatation. Increased level of nitric oxide (NO), which is often associated with elevated expression and activity of inducible nitric oxide synthase (iNOS), is always evident during the inflammation process [43]. It is produced by numerous cells which are involved in the immune response that mediate inflammatory processes by enhancing cyclooxygenase (COX) enzymes which ultimately leads to an increase in the production of pro-inflammatory eicosanoids [43]. It is therefore likely that the increased activity of gastric NO seen in the iron treated maybe due to increased inflammation induced by (non-transferrin-bound iron) NTBI that might have been taken up by gastric cells

According to Sunkara *et al*., [14], iron pill gastritis is an elusive and under reported diagnosis in oral iron therapy. Ewing *et al.,* [44] has also reported gastric siderosis following intra venous iron supplementation. This study suggests that in addition to likely predisposition to gastritis and gastric siderosis, iron supplementation may also increase the acidity of gastric secretions as observed in the iron treated group. More so that parietal cell count, the acid secreting cells of the stomach, were increased. This may also account for the increase in mucous cell count and mucus secretion observed in the iron treated group. Furthermore, histological evaluation of the stomach sections in the iron treated group also shows gastric mucous cell proliferation, coagulation necrosis, and moderate atrophy of parietal and chief cells. The precise mechanism through which oral iron induces gastritis is at presently unknown however it has been suggested to be due to iron oxidation from ferrous to ferric state, which causes epithelial injury and inflammation to the esophagus and stomach [14]. Within the gut is an integral nervous system, the enteric nervous system, which controls both motility and secretory functions within the gut [45].

The neurons that integrate the gut signals necessary for these functions are located in the Auerbachs (myenteric) and Meissners (submucousal) plexuses. While the Auerbachs plexus provides motor innervation to the muscles of the gut wall, which are themselves innervated by both sympathetic and parasympathetic signals, the Meissners plexus regulates glandular secretions, alters electrolyte and water transport, and controls luminal blood flow. Hence, impairment in the function of these nerve plexuses would cause a dysfunction of both motility and secretory function with the gut. Excess non-transferrin bound iron (NTBI) and free labile iron in circulation in the iron treated group [46] could thus have been taken up by the enteric nervous cell, and following iron oxidation, gastric neuron oxidative stress results resulting in impaired function and cell death. This iron stress-induced impairment of gut signals and especially motility could therefore account for the reduction in gastric emptying and intestinal motility observed in the iron treated group.

In conclusion, this study suggests that chronic oral iron intake may comprise the integrity and function of the gastrointestinal tract by favoring gastric oxidative stress, inducing increased acidity of gastric juice, and decreasing both gastric emptying and intestinal motility. In so far as oral iron therapy remains the conventional treatment modality for iron deficiency anemia (IDA), there may be a need to constantly monitor iron status so as to prevent gastrointestinal secretory, motility and structural impairments that can arise from iron-induced systemic and gastric oxidative stress.

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