IJLSR (2019), Vol. 5, Issue 12 **Research Article**

IJLSR INTERNATIONAL JOURNAL OF LIFE SCIENCES AND REVIEW

Received on 23 October 2019; received in revised form, 24 December 2019; accepted, 27 December 2019; published 31 December 2019

OESTROGEN AND THYROXINE MODULATE BLOOD FLOW MECHANISMS IN TADALAFIL-TREATED WISTAR RATS

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ABSTRACT: We earlier established the anti-secretory and mucogenic activities of tadalafil in indomethacin induced ulceration in rats and that these properties are modulated by the presence of sex hormones and thyroxine. The current study investigated the influence of these hormones on gastric blood flow, prostaglandin secretion and nitric oxide production in rats. Adult Wistar rats (140-160 g) were used. Gastric ulcer was induced in fasted rats using oral indomethacin (40 mg/Kg). Direct gastric blood flow was measured with a transonic T206 blood flow meter. Animals were sacrificed 4 h after ulcer induction. ELISA technique was used to evaluate the concentration of prostaglandin E2 (PGE2) and nitrite in the gastric homogenate. Data were analysed by ANOVA followed by Newman Keul's post-hoc test at α0.05. Blood flow, gastric PGE2 and nitric oxide contents were significantly elevated by tadalafil and by co-administration of tadalafil with oestrogen or thyroxine. We conclude that the gastro protective effect of tadalafil was mediated by increased PGE2 and nitric oxide generation and that this protective effect is enhanced by the presence of exogenous thyroxine and oestrogen in Wistar rats.

Keywords: Gastric ulcer, Thyroxine, Sex hormones, Blood flow, Prostaglandin, Vasodilation

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INTRODUCTION: A more recent approach directed at tackling the gastric ulcer problem focused on improving microcirculation around the ulcerated gastrum. A great deal of focus is still being directed at agents that improves blood flow to tissues. One of such agents is nitric oxide and its donors [1, 2]. Phosphodiesterase V inhibitors are drugs currently used to treat pulmonary hypertension and erectile dysfunction.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.IJLSR.5(12).172-79

Article can be accessed online on: www.ijlsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.IJLSR.5(12).172-79

These group of drugs work by increasing the regional blood flow in response to increased cyclic guanosine monophosphate (cGMP) synthesis (PDE V enzymes break down cGMP). cGMP mediates many of the biological actions attributed to nitric oxide (NO) which is a proven vasodilator that increases blood flow in tissues.

We have previously established that Tadalafil at high doses ameliorated the effects of indomethacin-induced ulcerations in rats [3]. We also showed that tadalafil possesses anti-secretory and mucogenic properties as part of its anti-ulcer mechanism and that these properties are modulated by the presence of sex hormones and thyroxine [4]. The current study aimed to ascertain firstly the involvement, if any, of oestrogen and thyroxine on gastric blood flow in

tadalafil-treated ulcerated rats and secondly to examine the influence of these same hormones on local vascular mediators prostaglandin and nitric oxide production in same rats.

MATERIALS AND METHODS:

Chemicals and Kits: Rat chow (Ladokun feeds, Ibadan), Dissecting set, Glass wares, Small gauge cannula, Olympus optical microscope (Olympus, Japan), Magnifying lens, Langerdoff apparatus (Harvard apparatus), Spectrophotomer (Molecular devices, USA), Homogeniser (Potter-Elvehjem model, USA), T206 Dual channel blood flowmeter (Transonic, USA), PGE_2 immunoassay kit (Elabscience, China).

Indomethacin (Sigma Aldrich, Germany), testosterone, thyroxine, oestrogen, histamine (Kermel, China), formalin, Tris HCl, potassium chloride (Qualikems). Tadalafil (Evans Pharmaceutical), Ketamine Hydrochloride from Rotex medica (Germany).

Animals: Adult male and female Wistar rats (20 weeks old) weighing between 140-160g were procured from the Central Animal House, College of Medicine, University of Ibadan (Ibadan, Nigeria). They were housed in clean, well ventilated polypropylene cages with comfortable ambient temperature. They were acclimatized for at least a period of two weeks before any experimental work was done and maintained under standard condition of 12 h of alternating light and dark cycle. The animals were fed with standard rat chow (admixture of cornstarch, sucrose, vegetable oil, cellulose and mineral supplements).

Water was provided *ad libitum*. The animals were treated humanely under globally accepted guidelines for good laboratory practice and the principles of laboratory animal care.

EXPERIMENTAL DESIGN:

Ulcer Induction: The indomethacin-induced ulceration model was adapted for this study [5]. Animals were fasted for 24 h prior to experimentation but had free access to clean tap water *ad libitum.* The Control group received Indomethacin (40 mg/kg bw, p.o).

All other groups received Indomethacin (40 mg/kg bw, p.o) 30 min after receiving their respective pre treatments. All animals were euthanized 4 h later with ether overdose in order to obtain clear ulceration patterns; the stomachs were removed and assessed for ulcer lesions by planimetry [6, 7]. The following investigations were carried out following ulcer induction.

- Measurement of blood flow to the gastric region.
- Prostaglandin and nitrite concentration evaluation from stomach tissue homogenate by ELISA and spectrophotometry techniques respectively.

Animals were Divided According to the Following Experimental Groups:

- Group I (Control) – distilled water 0.2ml/100g bw p.o
- Group II- Thyroidectomised
- Group III - Tadalafil 10mg/kg bw p.o
- Group IV- Thyroidectomised + Tadalafil 10mg/kg bw p.o
- Group V– Hormonal replacement (Thyroxine i.p.) + Tadalafil 10mg/kg bw p.o.
- Group VI - was repeated for the ovariectomised animals.

Experimental Procedures:

Direct Blood Flow Measurement: Total blood flow to the gastric region was estimated by direct flow measurement using the T206 dual channel blood flow meter from Transonic, USA [9, 10, 11]. The rats' coeliac trunk was exposed by a midline section just below the sternum and the vessel was hooked to a PS series flow probe to monitor blood flow through the artery, which was converted to a digital output on the flow meter (mL/min). Flow was measured at 15 min interval over a 90 min period starting 1 h post-treatment.

Homogenization of Stomach Tissues: The excised stomachs were first blotted on filter papers in order to remove blood and other extraneous tissues that may compromise the assays. The tissues were washed in ice cold 1.15% potassium chloride solution, weighed and chopped into bits

before homogenizing in four volumes of the homogenizing buffer (50mM Tris HCl, 1.15% KCl, pH 7.4) using a Potter-Elvehjem homogenizer. The resulting homogenate was centrifuged at 10,000g and at 4 °C for 10 min. The supernatant was collected and then used for both the prostaglandin and nitrite assays [12].

Protein Determination: Protein concentration of the homogenate was determined by means of the Biuret reaction as described by [13] with some modifications. It is based on the principle that copper sulphate in alkaline solution turns from blue to violet/blue in the presence of proteins. Potassium iodide was added to the Biuret reagent to prevent the precipitation of Cu^{2+} ions as cuprous oxide.

PGE_2 Elisa Assay: Prostaglandin E_2 (PGE_2) has been widely studied due to its role in inflammation and is of great interest as a therapeutic target because its synthesis can be modulated by the COX inhibitors (NSAIDS).

PGE_2 immunoassay kit, procured from Elabscience, China uses the principle of specific antigen-antibody interaction which is the standard method of immobilizing reactants to the bottom of a 96 well plate and then conjugated to an antibody that is linked to an enzyme. Detection is accomplished by estimating the amount of end product resulting from the conjugated enzyme activity. This method is also called the Sandwich ELISA assay.

Nitric Oxide Determination (Cadmium Reduction Method): This assay is based on the principle of reduction of nitrate by copper cadmium alloy, followed by colorimetric assessment with griess reagent (sulfanilamide and N naphthyl ethylenediamine) in acidic medium.

The assay is sensitive and suitable for different biological fluids, including body fluids with a high lipid concentration [14]. The present method of copper–cadmium substrate is of choice because it is easy to reproduce and reaction time for reducing nitrate to nitrite can be achieved within an hour. After 10 min incubation, the absorbance was read at 545nm as by ELISA method using a micro-plate reader (Biobase Biodustry Co. Ltd, Shandong, China). Nitrite concentrations were assessed using sodium nitrite as standard [15].

Statistical Analysis: Data were expressed as Mean ± SEM. Statistical difference between test groups and control group was calculated using one way ANOVA. Newman Keul's post-Hoc test was done. $p<0.05$ was considered as significant.

Ethical Approval and Consent to Participate: Ethical approval for this study was obtained from the University of Ibadan Animal Care and Use Research Ethics Committee (UI-ACUREC). Approval number is Ui-ACUREC/17/0077.

RESULT:

Prostaglandin E_2 (PGE_2) Production Pattern in Thyroidectomised, Tadalafil, Thyroxine - Treated Rats: The pattern of PGE_2 production in response to thyroidectomy, thyroxine replacement and tadalafil treatment in indomethacin-induced gastric ulceration is shown in **Fig. 1.** PGE_2 production is significantly reduced in thyroidectomised rats compared to control (637.38 ± 10.41 *vs.* 768.48 ± 6.80; $p<0.05$). Tadalafil significantly increased PGE_2 production when compared to control (1124.52 ± 32.22). This PGE_2 secretory effect was diminished in thyroidectomised rats treated with tadalafil (879.50 ± 16.88). Thyroxine replacement in combination with tadalafil treatment in thyroidectomised rats significantly elevated PGE_2 production when compared to control (999.63 ± 41.11).

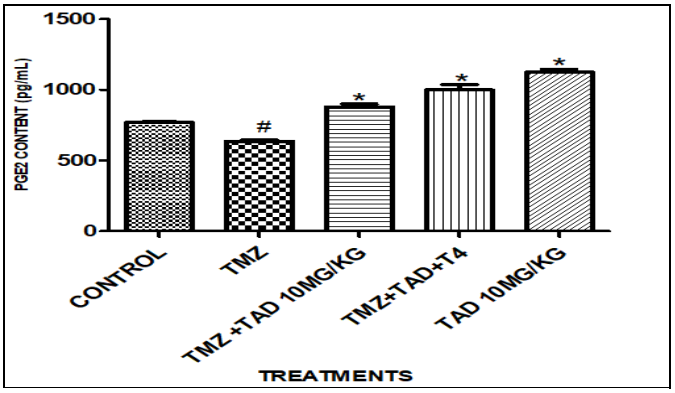

FIG. 1: PROSTAGLANDIN E_2 PRODUCTION PATTERN IN THYROIDECTOMISED, TADALAFIL AND THYROXINE-TREATED RATS. * - significant at $p<0.05$ when compared with Control # - significant at $p<0.05$ when compared with Control.

Prostaglandin E_2 Production Pattern in Ovariectomised, Tadalafil and Oestrogen-Treated Rats: The pattern of PGE_2 production in response to ovariectomy, oestrogen replacement and tadalafil treatment in indomethacin - induced

gastric ulceration is shown in **Fig. 2.** PGE_2 production is significantly reduced in ovariectomised rats compared to control (457.74 ± 24.87 *vs.* 788.88 ± 16.96; $p<0.05$). Tadalafil significantly increased PGE_2 production when compared to control (1230.86 ± 18.57). This PGE_2 secretory effect was diminished in ovariectomised rats treated with tadalafil (953.94 ± 20.63). Oestrogen replacement in combination with tadalafil treatment in thyroidectomised rats significantly elevated PGE_2 production when compared to control 1108.41 ± 67.11).

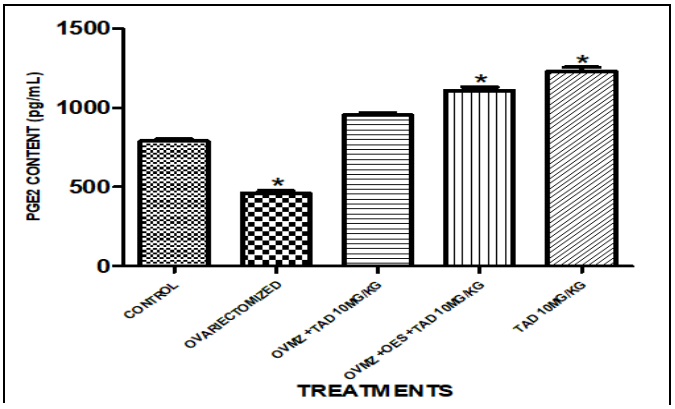

FIG. 2: PROSTAGLANDIN E_2 PRODUCTION PATTERN IN OVARIECTOMISED, TADALAFIL AND OESTROGEN-TREATED RATS. * - significant at $p<0.05$ when compared with Control OVMZ- ovariectomized; TAD- Tadalafil; OES- oestrogen.

Effects of Graded Doses of Tadalafil and Cimetidine on Gastric Blood Flow in Indomethacin-Induced Ulceration: Effect of graded doses of tadalafil on gastric blood flow is shown in **Fig. 3.**

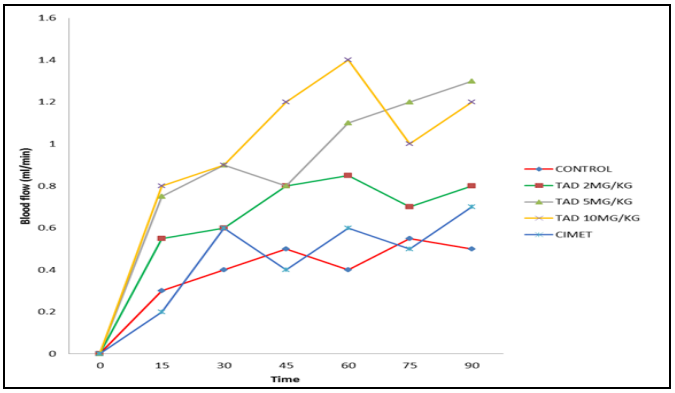

FIG. 3: EFFECTS OF GRADED DOSES OF TADALAFIL AND CIMETIDINE 100 mg/kg ON GASTRIC BLOOD FLOW IN INDOMETHACIN-INDUCED ULCERATION. TAD- Tadalafil; CIMET- Cimetidine

A known mechanism of action of tadalafil is *via* increase in blood flow. There was a significant dose-dependent increase in the blood flow to the gastric region by tadalafil by the tested doses of 5mg/kg bw (1.00 ± 0.06 mL/min) and 10 mg/kg bw (1.08 ± 0.04 mL/min) compared to control (0.44 ± 0.03 mL/min; $p<0.05$). The reference drug, cimetidine, did not produce any significant increase in blood flow (0.50 ± 0.04 mL/min).

Effects of Thyroidectomy, Ovariectomy, Castration and Tadalafil on Gastric Blood Flow in Indomethacin-Induced Ulceration: Changes in blood flow to the various surgical procedures and to TAD (10 mg/kg) is shown in **Fig. 4.** Ovariectomy (0.53 ± 0.04 mL/min) and castration (0.62 ± 0.05 mL/min) did not produce significant changes to the blood flow pattern when compared to control (0.44 ± 0.03 mL/min; $p<0.05$). There was a significant decrease in the blood flow to the gastric region in thyroidectomised rats compared to control (0.38 ± 0.04 mL/min) while TAD 10 mg/kg BW group had a significant increase in gastric blood flow (1.08 ± 0.03 mL/min).

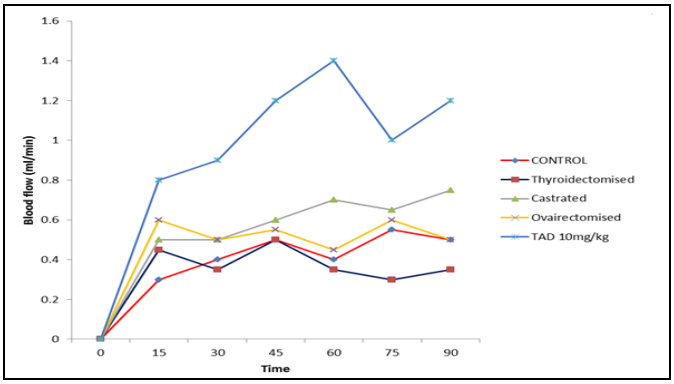

FIG. 4: EFFECTS OF THYROIDECTOMY, OVARIECTOMY, CASTRATION AND TAD 10 mg/kg ON GASTRIC BLOOD FLOW IN INDOMETHACIN-INDUCED ULCER *, # - significant at $p<0.05$ when compared with Control.

Effects of Co-Administration of Tadalafil with Either of Thyroxine, Oestrogen or Testosterone on Gastric Blood Flow in Indomethacin-Induced Ulcer: Changes in blood flow following the re introduction of tadalafil and the corresponding hormone (thyroxine, oestrogen and testosterone) after surgical removal of the corresponding gland is shown in **Fig. 5.** TAD 10 mg/kg BW had a significant increase in gastric blood flow (1.08 ± 0.03 mL/min). Administration of tadalafil to thyroidectomised and castrated rats did not significantly change the blood flow pattern. However, ovariectomised rats administered with

tadalafil had a significant increase in blood flow (0.80 ± 0.02 mL/min) when compared to control (0.45 ± 0.03 mL/min; $p<0.05$). Co-administration of tadalafil and testosterone to castrated rats failed to improve the blood flow (0.64 ± 0.04 mL/min). Co-administration of tadalafil with either of oestrogen (1.24 ± 0.03 mL/min) or thyroxine (1.07 ± 0.03 mL/min) significantly increased the blood flow pattern when compared to control.

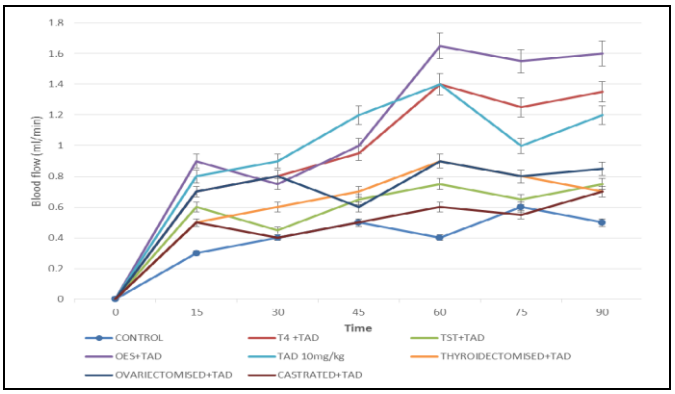

FIG. 5: EFFECTS OF THYROXINE, OESTROGEN AND TESTOSTERONE SUPPLEMENTATION ON GASTRIC BLOOD FLOW IN INDOMETHACIN-INDUCED ULCERATION. * - significant at $p<0.05$ when compared with Control TST- Testosterone; TAD- Tadalafil; OES- Oestrogen.

Effect of Thyroidectomy and Thyroxine Replacement on Nitric Oxide Production in Indomethacin-Induced Gastric Ulcer: Nitric oxide level in response to thyroidectomy and thyroxine replacement is shown in **Fig. 6.**

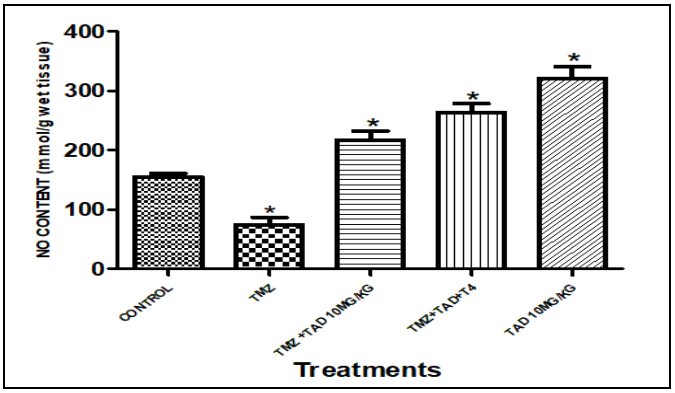

FIG. 6: EFFECT OF THYROIDECTOMY AND THYROXINE REPLACEMENTS ON NO PRODUCTION IN INDOMETHACIN-INDUCED ULCERATION. TAD- Tadalafil; TMZ- Thyroidectomised; T_4- Thyroxine * - significant at $p<0.05$ when compared with Control.

Nitric oxide level is reduced in thyroidectomised rats when compared to control (154.88 ± 14.20 nmol/g wet tissue, $p<0.05$). There is a significant increase in nitric oxide level in normal rats treated with TAD 10 mg/kg (217.00 ± 27.28 nmol/g wet tissue) and in thyroidectomised rats treated with thyroxine and TAD 10 mg/kg (263.76 ± 30.71 nmol/g wet tissue)

Effect of Ovariectomy and Oestrogen Replacements on Nitric Oxide Production in Indomethacin-Induced Gastric Ulceration: Nitric oxide level in response to ovariectomy and oestrogen replacement is shown in **Fig. 7.** Nitric oxide is significantly elevated across all treated groups when compared to control (160.74 ± 10.51 nmol/g wet tissue, $p<0.05$). The TAD only group (369.78 ± 11.87 nmol/g wet tissue), the oestrogen replacement group (247.01 ± 6.33 nmol/g wet tissue), the ovariectomised + TAD group (283.99 ± 28.64 nmol/g wet tissue) and the ovariectomised group (192.30 ± 31.15 nmol/g wet tissue) all had a significantly elevated NO content when compared to control.

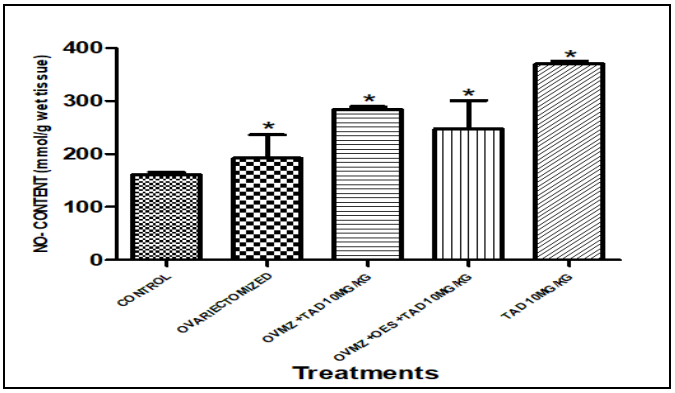

FIG. 7: EFFECT OF OVARIECTOMY AND OESTROGEN REPLACEMENT ON NO PRODUCTION IN INDOMETHACIN-INDUCED ULCER * - significant at $p<0.05$ when compared with Control. OVMZ- ovariectomised; TAD- tadalafil; OES- oestrogen.

DISCUSSION: A more recent approach directed at tackling the gastric ulcer problem focused on improving microcirculation around the ulcerated gastrum. A great deal of focus is still being directed at agents that improves blood flow to tissues. One of such agents is nitric oxide and its donors [1]. We have previously established that tadalafil at high doses, ameliorated the effects of indomethacin induced ulcerations in rats [3]. We also showed that tadalafil possesses anti-secretory and mucogenic properties as part of its anti-ulcer mechanism and that these properties are modulated by the presence of sex hormones and thyroxine [3]. The current study was aimed at investigating the influence of the sex hormones and thyroxine on gastric blood flow in tadalafil-treated ulcerated rats and the possible

involvement of local mediators such as prostaglandin and nitric oxide in the mechanistic pathway.

Mucus production has been linked to the presence or absence of prostaglandin. Prostaglandin E_2 and I_2 (PGE_2 and PGI_2) of the gastric and duodenal mucosae have been shown to be responsible for both the production of mucus and the maintenance of cellular integrity of the gastric mucosa. The PGE_2 is central to mucosal defence; it stimulates mucus and bicarbonate (HCO_3^-) production, acid reduction, increases blood flow, epithelial resistance and angiogenesis [16]. Result showed significant levels of expression of PGE_2 in the gastric homogenate in both the tadalafil-treated rats and the thyroxine and the oestrogen administered rats **Fig. 1** and **Fig. 2**.

Thyroidectomised and ovariectomised rats had significantly lower levels of PGE_2 expression when compared to control. This shows that both oestrogen and thyroxine presence enhances PGE_2 production [17]. Demonstrated a relationship between PDE5 inhibitors and HCO_3^- secretion and also between PDE5 inhibitors and PGE_2 when reporting that PDE5 inhibitors increases intracellular levels of cGMP and then stimulate gastric HCO_3^- secretion in two ways; either directly *via* cGMP or indirectly by PGE_2/EP_1 receptors and also that NO promotes the secretion of HCO_3^- *via* endogenous PGE_2.

HCO_3^- is a vital component of the 'mucus barrier' which is produced by PGE_2. This result provides plausible pathway for the ulcer mitigating effect of tadalafil in the presence of either of oestrogen or thyroxine in rats. The major mechanism of the mucogenic activity of PGE_2 has been shown to be via glycoprotein synthesis mediated by cAMP as a secondary messenger [18].

Furthermore, the short term protective effects of some gut hormones and local mediators such as nitric oxide, growth factors, leptin, ghrelin, calcitonin-gene related peptide (CGRP) *etc*. has been attributed to the release of prostaglandin or activation of sensory nerves [19]. The NSAID-induced ulcer has been shown to occur via depletion of cyto-protective prostaglandin leading to inhibition of the cyclooxygenase (COX) pathway [20]. Other isoforms of prostaglandin, especially PGI_2 which increases mucus production in the superficial epithelial cells, have also been reported to be significantly inhibited and depleted by NSAID use [21]. Therefore, we propose that one mechanism by which gastric mucosal damage induced by oral indomethacin is attenuated in rats treated with tadalafil in combination with either of thyroxine or oestrogen is *via* up-regulation of PGE_2 synthesis resulting in increased mucus production in the gastrum.

This study was based on the concept that tadalafil, being a nitric oxide (NO) donor, will ultimately increase blood flow in a non-erectile tissue such as the gastric mucosa.

Earlier studies have demonstrated tadalafil use in erectile dysfunction [22]. However, there is limited data on tadalafil effect in non-erectile tissues such as the stomach, or whether the no released by tadalafil will be adequate enough to modulate gastric functions [23] proposed a pathway for the treatment of NSAID-induced gastric ulceration that involves modulation of no synthesis. In order to confirm the production of NO by tadalafil in the stomach, total nitric oxide content in the gastric mucosal homogenate was assayed for by estimating the reduction of nitrate to nitrite using cadmium reduction method. NO levels were significantly elevated in the tadalafil-treated rats. This increase in NO content was also observed in thyroidectomised rats treated with tadalafil **Fig. 6**. However, thyroidectomy produced reduced NO concentration in rats. Thyroxine replacement in thyroidectomised rats resulted in significant increases in NO concentration. Increases in NO content have been reported in hyperthyroid rats [24] and also that the capacity for vascular NO formation is decreased in hypothyroidism and increased in hyperthyroidism [25] buttressing the findings in this study and confirming the involvement of the NO pathway in modulating gastric ulceration by NSAID irritants. The observed protective effect of oestrogen during vascular injury has been attributed to be partially mediated by an enhancement in nitric oxide production [26]. Increased NO and nitrite concentration in saliva have been demonstrated to increase blood flow and mucus production.

This is in consonance with the finding of this study in which there was significant increase in the NO content of the gastric homogenate of the oestrogen-treated rats.

Enhanced blood flow is a major premise upon which this study was predicated. Earlier studies on PDE V inhibitors have been on erectile and smooth muscle invested tissues especially the carvernosum and the lung tissue [22]. Blood flow to the gastric region was evaluated in response to the various doses of Tadalafil used in the preliminary study (2, 5, 10 mg/Kg bw) **Fig. 3** and in response to oestrogen and thyroxine withdrawal and supplementation. From the results, it was observed that blood flow was significantly increased in the 5 mg/kg and 10 mg/kg doses of tadalafil when compared to control. This buttresses the conclusion from the study that, at high doses, tadalafil ameliorates ulcerogenesis.

Thyroidectomy significantly reduced blood flow to the gastric region while ovariectomy and castration produced no significant changes in blood flow pattern. The combination of oestrogen and tadalafil had a higher blood flow than the tadalafil treated only and is significant when compared to control. Similar results were obtained with the combination of tadalafil and thyroxine although the increment in blood flow is not as high as that of tadalafil combined with oestrogen. Decreased mucosal blood flow has been suggested as part of the mechanisms responsible for mucosal damage and delayed ulcer healing [27]. Prostaglandin, which has been linked to tadalafil action and the influence of oestrogen and thyroxine on tadalafil action, has been shown to markedly prevent the reduction in blood flow due to indomethacin administration [29]. It is therefore evident from this study that increased mucosa blood flow theory is a definite mechanism of action for tadalafil action in NSAID-induced gastric ulceration and it is influenced by the availability or otherwise of oestrogen and thyroxine on tadalafil.

CONCLUSION: This study was designed to ascertain the involvement of oestrogen and thyroxine on gastric blood flow in tadalafil-treated ulcerated rats while also looking at the influence of these same hormones on local vascular mediators, prostaglandin and nitric oxide production in same tadalafil-treated Wistar rats. This was done via measurement of blood flow to the gastric region. Tissue homogenates were used to assay for the molecular anti-ulcer mechanisms by estimating prostaglandin and nitrite concentration.

Blood flow, gastric PGE_2 and nitric oxide contents were significantly elevated by tadalafil, and by co administration of tadalafil and oestrogen or thyroxine. Result showed that the gastro-protective effect of tadalafil is mediated by increased PGE_2 and NO generation. This study was able to uniquely show that thyroxine and oestrogen enhanced this gastro-protective effect in Wistar rats by boosting the production of local vascular mediators, prostaglandin and nitric oxide.

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: The authors declare that they have no competing interests or conflicts that may have influenced study outcome.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' Contributions: Both authors are responsible for the concept development and execution of the research. Dr. Kolawole Ajiboye conceptualized, carried out the hands-on experiments and wrote the initial manuscript while Dr. Francis Oluwole revised the methodology, donated the lab space used, reviewed and edited the raw manuscript.

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How to cite this article:
Kolawole IA and Francis SO: Oestrogen and thyroxine modulate blood flow mechanisms in tadalafil-treated Wistar rats. Int J Life Sci & Rev 2019; 5(12): 172-79. doi: 10.13040/IJPSR.0975-8232.IJLSR.5(12).172-79.