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Ameliorative effect of lixisenatide on diabetic cardiovascular damage and its enhancement via ticagrelor co-administration in rats: possible role of eNOS and NrF_2/HO-1 signaling

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In this study, we hypothesized that lixisenatide (LIX) and ticagrelor (TIC) could have a protective effect against type 2 diabetes mellitus (T2DM)-induced vascular damage. Furthermore, we explored the possible additional protective effect of co-administering LIX and TIC in the treatment regimen. *Methods:* 50 male rats were divided into five groups, each comprising 10 rats: C (control), D (T2DM rats), D + LIX (T2DM rats treated with LIX for 4 weeks), D + TIC (T2DM rats treated with TIC for 4 weeks), and D + LIX + TIC (T2DM rats treated with LIX + TIC for 4 weeks). *Results*: The D group showed an increase in body weight, blood glucose, hemostatic model assessment for insulin resistance (HOMA-IR), aorta reactive oxygen species (ROS), and nuclear factor kappa B (NF-κB), along with a reduction in serum insulin, aorta superoxide dismutase (SOD), glutathione reduced (GSH), nuclear factor erythroid-2 (NrF_2), hemeoxygenase-1 (HO-1), and endothelial nitric oxide synthase (eNOS). Deterioration in the aorta histopathological condition, coupled with a noticeable impairment in vascular reactivity compared to the C group, was observed. A single administration of LIX showed a reduction in body weight, blood glucose, HOMA-IR, aorta ROS, and NF-κB, accompanied by an increase in serum insulin, aorta SOD, GSH, NrF_2, HO-1, and eNOS. Amelioration in the aorta histopathological condition and improved vascular reactivity compared to the D group were reported. Similarly, a single administration of TIC showed a reduction in aorta ROS and NF-κB, along with an increase in aorta SOD, GSH, NrF_2, HO-1, and eNOS. A slight amelioration was detected in the aorta histopathological condition, with improved vascular reactivity compared to the D group. The combined administration of LIX and TIC showed a reduction in aorta ROS and NF-κB, along with an increase in aorta GSH, SOD, HO-1, and eNOS. This was combined with evident amelioration in the aorta histopathological condition and noticeable improvement in vascular reactivity compared to the single treatment with either LIX or TIC group. *Conclusion*: The present study introduces clear evidence that the administration of LIX and TIC can improve metabolic and vascular complications of T2DM through modulating eNOS and NrF_2/HO-1 signaling. The combined administration of LIX and TIC produced more significant effects than a single treatment.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a long-term and ongoing destructive disease characterized by alterations in glucose metabolism (Lisco et al. 2023). The consequences of associated chronic hyperglycemia may worsen the function of organs and tissues, such as the cardiovascular system and kidneys (Giri et al. 2018). Among the most severe manifestations of diabetes mellitus (DM) are vascular complications, with atherosclerosis and endothelial dysfunction being the main reasons for impaired life expectancy (Dubsky et al. 2023). Persistently elevated glucose levels are a significant cause of vascular dysfunction in DM (Wu et al. 2016), initiating metabolic alterations such as increased levels of advanced glycation end products and reactive oxygen species (ROS) (Sharma et al. 2023). Advanced glycation end products can decrease the availability of nitric oxide (NO), negatively affecting endothelium-dependent vasodilation in DM. Elevated ROS and nuclear factor-kappa B (NF-κB) activation promote atherosclerosis and inflammation (Liu et al. 2017). NF-κB is among a family of inducible transcription factors that regulate various genes responsible for different processes of the immune system and inflammatory reactions (Zhao et al. 2021). Additionally, it regulates the functions of inflammatory T cells (Lawrence 2009).

The nuclear factor erythroid 2 (NrF_2) regulates oxidative stress damage in different cells. Under conditions of elevated oxidative stress, the transcription process of NrF_2 is activated, leading to the introduction of the downstream production of a series of activated antioxidant enzymes, such as superoxide dismutase (SOD), glutathione reduced (GSH), and heme oxygenase-1 (HO-1). These enzymes mediate the removal of ROS and facilitate other cell protection mechanisms (Cui et al. 2019). HO-1 levels are low in most tissues. The activities of HO-1 are associated with protective effects during cellular stress, such as inflammation. Additionally, HO-1 is believed to be critical in preventing vascular injury (Gall et al. 2019). The signaling pathway related to NrF_2 and HO-1 is considered an essential pathway in oxidative stress generation and is involved in decreasing inflammation, programmed cell death, and other processes. Consequently, it is considered an essential objective for managing vascular complications in DM (Fiorelli et al. 2019).

Glucagon-like peptide-1 (GLP-1) has recently attracted attention as a therapeutic strategy for diabetes, heart disease, and obesity (Sivakumar et al. 2021). The GLP-1 receptor agonist, lixisenatide (LIX), is approved as an additional therapy to diet and exercise to improve glycemic control in patients with T2DM. The adminis-

tration of LIX improves glycemic control through its effects on glucose-triggered insulin secretion, glucagon secretion, gastric emptying time, and appetite-limiting effects (Kuwata et al. 2021). Previous studies have reported that LIX possesses anti-inflammatory and anti-ROS properties, protecting endothelial cells against vascular injury (Abdel-Latif et al. 2018). Furthermore, LIX has been shown to inhibit free fatty acid-induced NF-κB activation (Zhao et al. 2019). Elevated levels of free fatty acids are known to cause vascular inflammation and enhance ROS production, potentially deteriorating endothelial cell function (Inoguchi et al. 2000). Ticagrelor (TIC) is a direct P_2Y_{12} receptor antagonist of adenosine diphosphate (ADP), which is highly expressed in thrombocytes and plays an essential role in thrombosis (Gachet 2012). P_2Y_{12} receptor antagonists, including TIC, effectively block platelet aggregation and activation. In addition, TIC has been reported to exert a reduction in inflammatory markers, leading to anti-inflammatory effects in patients with T2DM (Triska et al. 2022). Evidence from previous studies revealed the pronounced anti-inflammatory and anti-fibrotic effects of TIC (Ye et al. 2015). A study showed that TIC diminished apoptosis and protected against oxidative damage (El-Mokadem et al. 2021). Moreover, an *in vitro* study identified that TIC activates endothelial nitric oxide synthase (eNOS) (Ariotti et al. 2018).
Therefore, the present study investigated the possible ameliorative effects of LIX or TIC and their combination in managing T2DM-induced vascular injury in rats. Furthermore, this study aimed to explore the role of eNOS and NrF_2/HO-1 pathways in these effects.

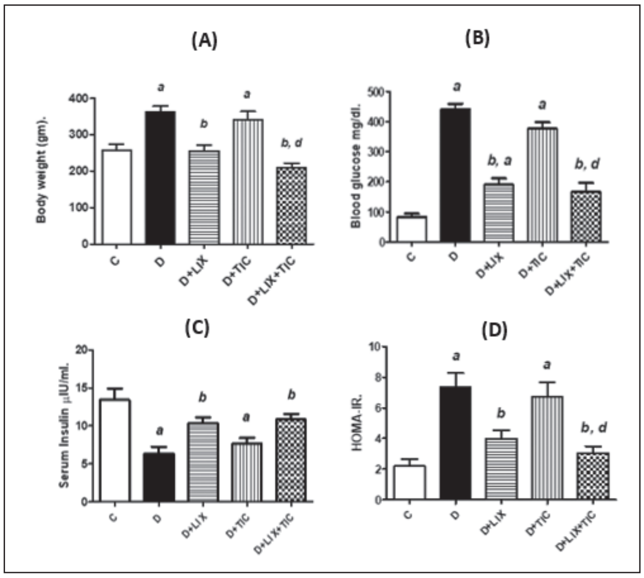

Fig. 1: Effects of single and combined administration of LIX and TIC on body weight (A), blood glucose (B), serum insulin (C), and HOMA-IR (D). Data represent the mean±SEM (n=6), a significant from C, b significant from D, and d significant from D+TIC at p <0.05.
C: Control; D: Diabetic; LIX: Lixisenatide; TIC: Ticagrelor.

2. Investigations and results

2.1. Effects of single and combined administration of LIX and TIC on body weight, blood glucose, serum insulin, and HOMA-IR

Figure 1 (A-D) shows the changes in body weight, blood glucose, serum insulin, and HOMA-IR in T2DM rats, n = 6, for each treatment group following single and combined administration of LIX and TIC. Body weight, blood glucose, and HOMA-IR were significantly elevated while serum insulin was significantly reduced in the D group compared to the C group. Interestingly, the D+LIX and D+LIX+TIC groups significantly reduced body weight, blood glucose, and HOMA-IR while significantly increased serum insulin compared to the D group, with almost no significant effect found in the D+TIC group. Combined administration of LIX and TIC to diabetic rats showed significant reduction on body weight, blood glucose, and HOMA-IR relative to D+TIC.

2.2. Effects of single and combined administration of LIX and TIC on aortic ROS, GSH, and SOD

Figure 2 (A-C) illustrates the changes in aortic ROS, GSH, and SOD in T2DM rats following single and combined administration of LIX and TIC. The D group exhibited a significant increase in aortic ROS and a significant reduction in aortic GSH and SOD compared to the C group. Meanwhile, the D+LIX, D+TIC, and D+LIX+TIC groups showed a significant reduction in aortic ROS and significant elevations in aortic GSH and SOD compared to the D group. In addition, the D+LIX+TIC group significantly reduced aortic ROS while significantly elevated aortic GSH and SOD compared to the D+LIX and D+TIC groups.

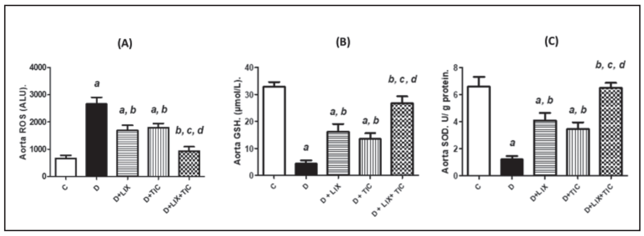

Fig. 2: Effects of single and combined administration of LIX and TIC on aortic ROS (A), GSH (B), and SOD (C). Data represent the mean±SEM (n=6), a significant from C, b significant from D, c significant from D+LIX, and d significant from D+TIC at p <0.05.
C: Control; D: Diabetic; LIX: Lixisenatide; TIC: Ticagrelor.

2.3. Effects of single and combined administration of LIX and TIC on aortic NrF_2, HO-1, eNOS, and NF-κB expression

Changes in the aorta NrF_2, HO-1, eNOS, and NF-κB in T2DM rats following single and combined administration of LIX and TIC are shown in Fig. 3 (A-E). The results showed a significant reduction in aorta NrF_2, HO-1, and eNOS while showed a significant elevation in NF-κB in the D group compared to the C group. Conversely, the D+LIX, D+TIC, and D+LIX+TIC groups showed significant NrF_2, HO-1, and eNOS elevations while showed a significant reduction in NF-κB compared to the D group. Additionally, the D+LIX+TIC group demonstrated significant increases in aortic HO-1 and eNOS while exhibited a significant reduction in NF-κB relative to the D+LIX and D+TIC groups.

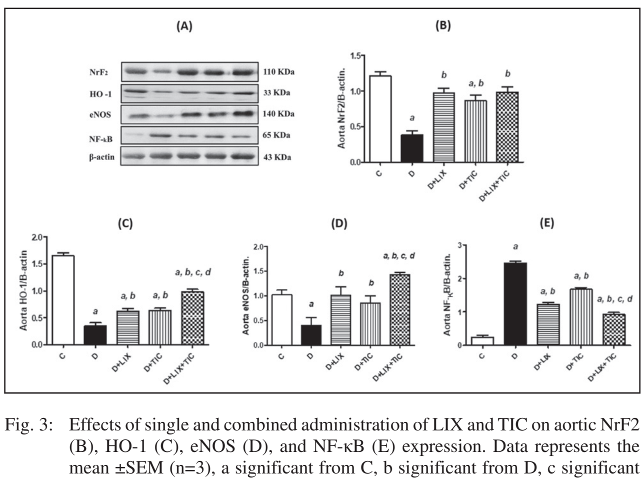

Fig. 3: Effects of single and combined administration of LIX and TIC on aortic NrF2 (B), HO-1 (C), eNOS (D), and NF-κB (E) expression. Data represents the mean ±SEM (n=3), a significant from C, b significant from D, c significant from D+LIX, and d significant from D+TIC at p <0.05. C: Control; D: Diabetic; LIX: Lixisenatide; TIC: Ticagrelor

2.4. Effects of single and combined administration of LIX and TIC on aorta TGF-β

Figures 4 (A-B) showed the changes in aortic expression of TGF-β in T2DM rats following single and combined administration of LIX and TIC. Immunohistochemical analysis revealed that the C group showed weak cytoplasmic expression of TGF-β in endothelial cells. In contrast, the diabetic rats in D group showed significantly elevated cytoplasmic expression of TGF-β in endothelial cells relative to C group. Single and combined administration of LIX and TIC to Diabetic rats showed significant attenuation of cytoplasmic expression of TGF-β in endothelial cells relative to D. Interestingly, combined administration of LIX and TIC to diabetic rats showed significant reduction of cytoplasmic expression of TGF-β in endothelial cells relative to D+TIC.

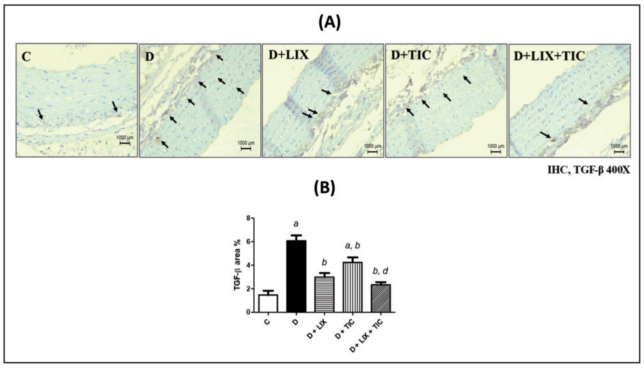

Fig. 4: Effects of single and combined administration of LIX and TIC on aorta TGF-β. (A) Immunohistochemical analysis revealed that the C group showed weak cytoplasmic expression of TGF-β in endothelial cells (black arrows). In contrast, the D group showed significantly elevated cytoplasmic expression of TGF-β in endothelial cells (black arrows) relative to control. The D+LIX, D+TIC, and D+LIX+TIC groups showed weak cytoplasmic expression of TGF-β in endothelial cells (black arrows). D+LIX+TIC group showed significantly reduced weak cytoplasmic expression of TGF-β in endothelial cells relative to D+TIC. (B) Data represents the mean ±SEM (n=4), a significant from C, b significant from D, and d significant from D + TIC at p <0.05. C: Control; D: Diabetic; LIX: Lixisenatide; TIC: Ticagrelor.

2.5. Effects of single and combined administration of LIX and TIC on vascular reactivity

Figure 5 illustrates changes in endothelial-dependent vascular reactivity in the studied groups. The results showed a significant reduction in aortic endothelial-dependent relaxation in the D group compared to the C group. Conversely, the D+LIX, D+TIC, and D+LIX+TIC groups showed significant elevations in endothelial-dependent relaxation compared to the D group. Additionally, the D+LIX+TIC group showed a significant elevation in aortic endothelial-dependent relaxation compared to the D+LIX and D+TIC groups.

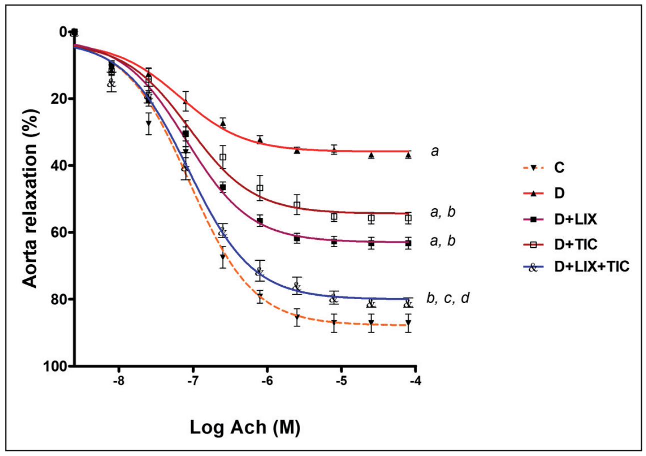

Fig. 5: Effects of single and combined administration of LIX and TIC on endothelial-dependent vascular reactivity. Data represents the mean ±SEM (n=4), a significant from C, b significant from D, c significant from D+LIX, and d significant from D+TIC at p <0.05.
C: Control; D: Diabetic; LIX: Lixisenatide; TIC: Ticagrelor.

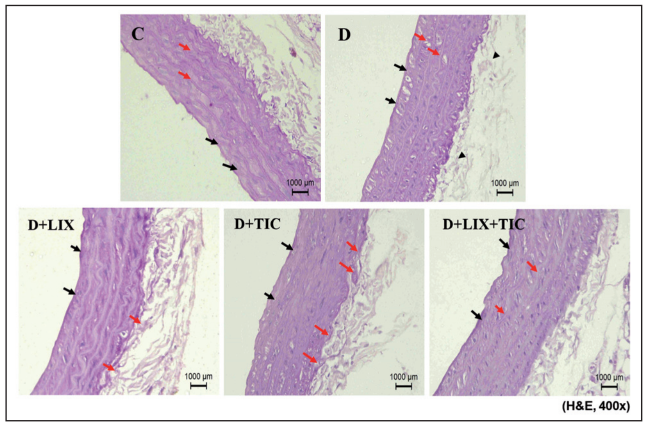

Fig. 6: Effects of single and combined administration of LIX and TIC on diabetes-induced vascular histopathological injury. C displayed uniform aortic walls with regular outlines, a uniform intima endothelial lining (black arrows), uniform tunica medium, and regular smooth muscle cells (red arrows). D exhibited focal swelling in intima endothelial cells (black arrows), vacuolization in tunica media cells (red arrows), mild edema in the adventitia (black arrowheads). D+LIX showed a uniform aortic wall with regular outlines, a uniform intima endothelial lining (black arrows), and uniform tunica medium. Edema in tunica adventitia (red arrows). D+TIC displayed uniform aortic walls with regular outlines, a uniform intima endothelial lining (black arrows), and a uniform tunica medium. Edema in tunica adventitia (red arrows). D+LIX+TIC demonstrated a uniform aortic wall with regular outlines, a uniform intima endothelial lining (black arrows), and regular tunica medium smooth muscle cells in tunica medium (red arrows).
C: Control; D: Diabetic; LIX: lixisenatide; TIC: ticagrelor.

2.6. Effects of single and combined administration of LIX and TIC on diabetes-induced vascular histopathological injury

Diabetes-induced vascular histopathological injury in the aorta of the studied groups is illustrated in Fig. 6 and Table 1. In the present study, hematoxylin-eosin staining revealed deterioration in the aortic tissues of the D group. The aortic wall showed focal swelling in the endothelial cells of the intima and extensive vacuolization in the tunica media cell, adventitia showed mild edema compared to the C group. single administration of LIX and TIC resulted in a uniform aortic wall with regular outlines. The intima exhibited a uniform endothelial lining, and the tunica medium showed uniformity. Tunica adventitia displayed areas of edema. Notably, histopathological injury ameliorated significantly compared to the D group. In contrast, the combined administration of LIX and TIC in the D+LIX+TIC group showed a uniform aortic wall with regular outlines. Intima exhibited a uniform endothelial lining, and the tunica medium was uniform and displayed regular smooth muscle cells. There was a more significant effect on histopathological regular outlines compared to the D+LIX and D+TIC groups.

2.7. Effects of single and combined administration of LIX and TIC on diabetes induced fibrosis and collagen deposition

Histopathological changes in the aorta in C, D, and different treated groups are illustrated in Fig. 7 (A-B). In the present study, Masson trichome staining revealed deterioration in the aortic tissues of the D group as evidenced by significant collagen deposition as compared to the C group. The administration of LIX resulted in significant reduction on fibrosis or collagen deposition. Also, TIC administration resulted in significant reduction on fibrosis and collagen deposition. In contrast, the combined administration of LIX and TIC in the D+LIX+TIC group showed no evidence of fibrosis or collagen deposition compared to the D group.

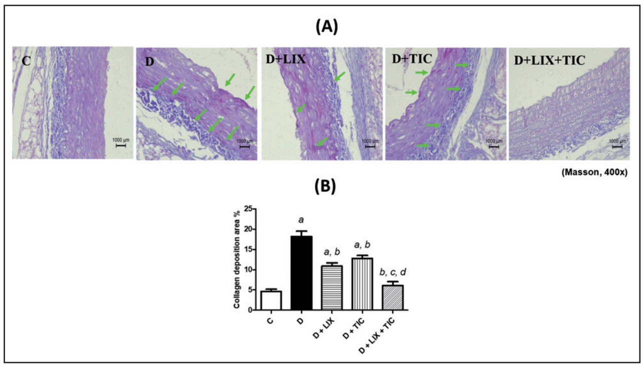

Fig. 7: Effects of single and combined administration of LIX and TIC on diabetes-induced vascular fibrosis and collagen deposition. (A) The C group has uniform aortic walls, regular outlines, and no fibrosis or collagen deposition. The D group showed adventitial fibrosis and collagen deposition in the aortic wall (green arrows). The D+LIX group exhibited uniform aortic walls and evidence of fibrosis or collagen deposition. The D+TIC group displayed uniform aortic walls with regular outlines and evidence of fibrosis or collagen deposition. The D+LIX+TIC group demonstrated a uniform aortic wall with regular outlines and no fibrosis or collagen deposition. (B) Data represent the mea ±SEM (n=4), a significant from C, b significant from D, c Significant from D+LIX, d Significant from D+TIC group at $p < 0.05$.
C: Control; D: Diabetic; LIX: Lixisenatide; TIC: Ticagrelor.

Results obtained from treatment with drugs under investigation also agreed with previous reports that LIX can cause a notable weight reduction attributed to its appetite-suppressing effects and delayed gastric emptying time (Becker et al. 2015). Also, LIX, a member of the GLP-1 agonist family, is reported to exert a significant increase in insulin levels in diabetic rats (Roca-Rodriguez et al. 2017). On the other hand, previous reports indicated that TIC has no significant effect on insulin release or glycemic control in diabetic nephropathy in mice with T2DM. (Birnbaum et al. 2022), along with reports documenting that TIC did not appear to have any effect on altering animals' body weight in a carcinogenicity study in male and female rats (Brott et al. 2014).

Oxidative stress is an imbalance between ROS and cellular antioxidant defense systems that may result from alterations in glucose metabolism or from dysregulation of several enzymes not directly involved in glucose metabolism (Giugliano et al. 1996). Oxidative stress in rats was found to cause cellular dysfunction by promoting the formation of advanced glycation end products (Schleicher and Friess 2007). Also, it was discovered that endothelial dysfunction is strongly enhanced by the generation of ROS in diabetic patients (Shi and Vanhoutte 2017).

In the present study, a marked elevation of ROS along with a significant reduction in SOD and GSH in the aorta of diabetic rats was observed relative to control. These results agree with the previous investigation, which reported that hyperglycemia in T2DM patients increases the production of ROS and reduction in the actions of antioxidant elements such as SOD and GSH, causing damage to various tissues (Song et al. 2007).

Table 1: Summary of the histological changes to the H&E-stained aorta tissue

Aorta histopathology scoring H&E staining					
Histopathological alterations	**C**	**D**	**D + LIX**	**D + TIC**	**D + LIX + TIC**
Swelling in endothelial cells	—	+	—	—	—
Vacuolations in tunica media	—	++	—	—	—
Edema in the tunica adventitia	—	++	+	++	—

C: Control; D: Diabetic; LIX: Lixisenatide; TIC: Ticagrelor. +: mild injury, ++: moderate injury

3. Discussion

The present study examined the effects of GLP-1 agonist, LIX, and P_2Y_{12} inhibitor, TIC, on attenuating and reversing STZ-HFD-induced T2DM metabolic and vascular changes contributing to the progression of endothelial dysfunction in T2DM. Previous studies demonstrated that animals that were fed HFD to induce peripheral insulin resistance, followed by a low dose of STZ to target pancreatic beta cells, would closely resemble the pathogenesis of T2DM (Reed et al. 2000).

In this study, our results showed that the STZ-HFD-induced T2DM model resulted in a significant increase in animals' body weight, blood glucose levels, and HOMA-IR, together with a significant reduction in blood insulin levels compared to control.

These results are in accordance with previous reports where administration of HFD was claimed to be responsible for hyperglycemia, obesity, and insulin resistance (Flanagan et al. 2008), while STZ administration can result in decreased blood insulin levels in rats (Zhang et al. 2021). Besides, HOMA-IR is a frequently used indicator for assessing insulin resistance in the diabetic population (Mekseepralard et al. 2015). Also, another study showed that the STZ-HFD-induced T2DM model revealed a significant increase in animals' body weight (Gheibi et al. 2017), elevated blood glucose levels and HOMA-IR (Jiao et al. 2017), together with a significant reduction in blood insulin levels compared to control (Srinivasan et al. 2005). Hyperglycemia, obesity, and insulin resistance are among the primary metabolic abnormalities considered accountable for long-term vascular alterations in T2DM (Maranta et al. 2021).

In the current study, treatment with drugs under investigation showed that single and combined administration of LIX with TIC resulted in a notable reduction in animals' weight, blood glucose, and HOMA-IR, with a remarkable enhancement in insulin release compared to diabetic rats. On the other hand, single administration of TIC did not appear to have any significant effect on altering animals' body weight, blood glucose, HOMA-IR, and insulin release compared to diabetic rats.

Our study results for the drugs under investigation revealed that single administration of LIX or TIC showed a significant reduction in aortic ROS and a significant increase in aortic GSH and SOD relative to diabetic rats. Interestingly, the combined administration of LIX and TIC showed a significant reduction in aortic ROS and a significant elevation in both aortic GSH and SOD compared to the single administration of either LIX or TIC.

These results are strongly in agreement with other reports which indicated that a low dose of LIX demonstrated antioxidant effects in diabetic nephropathy in T2DM rats (Abdel-Latif et al. 2020). A different investigation on rats with doxorubicin-induced renal fibrosis showed that administering LIX significantly restored the antioxidant capacity in renal fibrotic tissues and showed a significant increase in GSH and SOD compared to the untreated group (Guo et al. 2019). Additionally, a study found that TIC reduced ROS production in high glucose-incubated cardiomyocytes and can postpone apoptosis by reducing oxidative and endoplasmic reticulum stress (Bitirim et al. 2022).

HO-1 is thought to play a key role in the prevention of vascular injury. Activities of HO-1 involve protective effects during times of cellular stress, such as inflammation (Gall et al. 2019). A recent study on humans revealed that activation and transcription of NrF_2 exert a main role in HO-1 expression (Fiorelli et al. 2019). Also, activation of HO-1 through the action of NrF_2 can enhance an antioxidant protective effect, further delaying the progression of endothelial dysfunction (Zhang et al. 2021). Also, oxidative stress in rats was found to cause dysfunction of eNOS and activate other stress-activated pathways (Wang et al. 2019). Additionally, reducing eNOS levels in vascular endothelial cells reduces NO synthesis and may contribute to the potentiation of endothelial dysfunction in rats (El-Mokadem et al. 2021).

Results in our study highly confirmed previous reports where NrF_2, HO-1, and eNOS levels were significantly reduced in T2DM rats. These results are in agreement with a study that demonstrated a significant reduction in aortic and renal HO-1, NrF_2, and eNOS in diabetic mice (Wang et al. 2020). Another study examined the effects of sulfasalazine on endothelial dysfunction induced by high glucose showed significant reduction in aortic HO-1, NrF_2, and eNOS in diabetic albino mice (Sonmez et al. 2022). Moreover, same results were reported in a study examined inflammation on STZ-induced aortic injury on rats (Alzahrani et al. 2021).

Drugs under investigation showed notable effects where treatments with LIX or TIC showed significant elevations in NrF_2, HO-1, and eNOS levels compared to diabetic rats. This could be confirmed by results from a separate research study of ischemia-induced endothelial injury model where LIX pretreatment increased NrF_2 and HO-1 and enhanced the phosphorylation of eNOS and intracellular NO production (Xiao et al. 2020). Also, clinical studies recorded that TIC improved endothelial function by activating eNOS in the vascular endothelium (Vieceli Dalla Sega et al. 2018). Additionally, TIC enhanced NrF_2/HO-1 axis in a renal ischemia reperfusion rat model (El-Mokadem et al. 2021)

From another perspective, NrF_2 and HO-1 have been shown to inhibit the inflammatory transcription factor NF-κB in animal models (Ahmed et al. 2017). Transcription factor NF-κB is responsible for releasing different inflammatory molecules, contributing to vascular complications associated with T2DM in animal models (Patel and Santani 2009). As previously reported, cardiac and renal fibrosis and collagen deposition were evident in STZ-induced T2DM in rats (Miric et al. 2001). Also, NF-κB presence is enhanced in hyperglycemic patients, leading to endothelial cell apoptosis and inflammatory reactions (Evans and Goldfine 2016). Moreover, the increased activity of NF-κB also leads to altered production of TGF-β, resulting in vascular cell damage and angiogenesis (Kitada et al. 2010). Besides, T2DM-induced damage to the aortic endothelium and vascular smooth muscle was previously investigated in HFD-STZ-induced rats (Alzamil et al. 2020).

Results in our study are in line with these previous reports where NF-κB and TGF-β together with fibrosis and collagen deposition were significantly elevated in the aorta of diabetic rats compared to controls. These effects were markedly confirmed by the significant deterioration in aorta histopathological picture and significantly reduced vascular reactivity.

Interestingly, treatment with LIX and TIC revealed a significant reduction in NF-κB, TGF-β, fibrosis, and collagen deposition. Also, histopathological status and vascular reactivity were significantly improved in the aorta of treated rats compared to untreated T2DM rats.

These results could be augmented by other reports indicating that LIX alleviated doxorubicin-induced renal fibrosis in rats by inhibiting NF-κB and TGF-β signaling (Guo et al. 2023). On the other side, TIC has been reported to reduce apoptosis, inflammation, and fibrosis in a rat model of ischemia reperfusion (Birnbaum et al. 2019). Another report revealed that TIC ameliorates pulmonary fibrosis in rats by inhibiting TGF-β (Wanas et al. 2022). Also, TIC alleviated cellular dysfunction through suppressing the NF-κB signaling in human umbilical vein endothelial cell culture (Jia et al. 2019).

Moreover, as previously stated, LIX can improve endothelial function by restoring NO bioavailability in T2DM rats (Zhao et al. 2019). Also, a study on patients with stable coronary artery disease revealed that vascular reactivity was improved with treatment with TIC compared with baseline (Waksman et al. 2015). TIC effects on rats were reported to reduce endothelial dysfunction, ROS generation, and eNOS phosphorylation, leading to increased production of NO (Wang et al. 2018). Moreover, an in vitro study on rats stated that TIC may provoke adenosine-related effects, such as coronary vasodilatation and anti-inflammatory effects (Armstrong et al. 2014). From a clinical perspective, TIC markedly reduces cardiovascular events in patients with T2DM and stable coronary artery disease (Steg et al. 2022).

Thus, the diabetic rat model showed an evident deterioration in endothelial-dependent vascular reactivity and histopathological condition compared to healthy nondiabetic rats. These observations were partially ameliorated following a single treatment with either LIX or TIC, and markedly ameliorative effects were observed with the combined administration of LIX and TIC compared to the single drug treatment. These effects could be related to modulating eNOS, NrF_2/HO-1, and NF-κB signaling. Further studies are required to investigate the clinical efficacy of the combination of LIX and TIC.

4. Experimental

4.1. Chemicals

Drug LIX, Cat. No. SML2836, Sigma Aldrich, Germany. Drug TIC, Cat. No. 5857, RAMEDA, Egypt. Streptozotocin (STZ), Cat. No. S8050, Solar Bio® Life Science, Beijing. Rat Insulin ELISA Kit, Fine Test, Cat#: ER1113 China. Assay kits for SOD Cat#: SD 25 21 and GSH Cat. No. GR 25 11, Bio Diagnostic, Egypt. Lucigenin, Cat. No. M8010. NADPH, Cat#: 481973. Casein Cat#: C3400, d-l-methionine Cat#: M9500. Acetylcholine, Cat#: 101264957. Phenylephrine, Cat#: SL 5495, Sigma Aldrich, Germany. Isoflurane, Cat#: SM 1036, Sunny Medical, Egypt. Sodium nitroprusside, tween 80, normal-pellet diet (N.P.D.), Mineral mix, and vitamin mix were supplied by the South Valley University (SVU) lab.

Western blot analysis antibodies used include polyclonal anti-NrF_2 Cat#: YPA1865, polyclonal HO-1 antibody Cat#: YPA1919 (BIOSPES, Chongqing), anti-NF-κBp65 antibody, Cat#: abx012874 (Abbexa, UK), anti-eNOS (B-5) Cat#: sc-136977 (SANTA CRUZ BIOTECHNOLOGY, INC. USA), and anti-β-actin monoclonal antibody, Cat#: E-AB-20031 (ELABSCIENCE, USA). Anti- TGF-β antibody Cat#: EPR21143(ABCAM, USA) was used for immunohistochemical analysis. Antibodies were used in dilutions of 1:2000 for the primary antibody, 1:3000 for the anti-β-actin antibody, and 1:5000 for the alkaline phosphatase-conjugated (Biospes, China) secondary antibody.

4.2. Animals

Animals were handled, and procedures were conducted following South Valley University (SVU) ethical approval committee with approval number: P.S.V.U. 011/22. Male Sprague Dawley rats weighing between 120 and 170 g (50 rats in total) were supplied by the National Research Center in Giza, Egypt. The animals were kept under constant environmental conditions, with a dark/light cycle of 14:10 hours and free access to food and water. However, the animals were deprived of food only for 12 hours before taking blood samples. Anesthesia was induced using isoflurane inhalation (Bhatia et al. 2022).

4.3. Experimental design

Rats were divided into five groups (n=10) as follows:

Group 1 (C): Control rats received a basal diet for 12 weeks, followed by a single intraperitoneal (i.p.) injection of citrate buffer and daily water for injection subcutaneously (S.C) and tween 80, orally for 4 weeks. *Group 2* (D): Diabetic rats received a high-fat diet (HFD) for 12 weeks, followed by a single i.p. injection of streptozotocin (STZ) (35 mg/kg) dissolved in citrate buffer. *Group 3* (D+LIX): Diabetic rats treated with LIX (10 μg/kg/day, S.C. dissolved in water for injection for 4 weeks (Wohlfart et al. 2013). *Group 4* (D+TIC): Diabetic rats treated with TIC (25 mg/kg/day, orally) dissolved in tween 80 for 4 weeks (Moustafa Ahmed et al. 2019). *Group 5* (D+LIX+TIC): Diabetic rats treated with LIX (10 μg/kg/day, S.C.) + TIC (25 mg/kg/day, orally) for 4 weeks.

4.4. Preparation of HFD and induction of T2DM

Preparation of HFD and induction of T2DM were performed using the following ingredients: Powdered normal pellet diet (N.P.D.) (1 kg), butter (531 g), casein (125 g), d-l methionine (3 g), vitamin mix (7 g), and mineral mix (42 g) (Gheibi et al. 2017). Diabetic rats were fed HFD (over 40% of calories from fat) for 12 weeks. Subsequently, a single injection of STZ (35 mg/kg) dissolved in citrate buffer was administered. After three days, rats were selected and examined to identify animals with fasting blood glucose greater than 200 mg/dL labeled as diabetic (Li et al. 2023). After induction of T2DM, drug treatment was continued for an additional 4 weeks (Jeric et al. 2015).

4.5. Serum and tissue sampling

At the end of the experiment, animals were fasted for 12 hours, weighed, and had their blood glucose levels measured using a glucometer. Animals' blood was collected from the retro-orbital vein, and serum was separated by centrifugation at 1000 × g for 10 minutes, then used to measure serum insulin levels (Huang and Chen 2022).

Aorta tissues were dissected from each animal, washed with a physiological solution, and divided into parts. One part was homogenized in a cold Krebs-HEPES buffer that had 10 mmol/l glucose, 0.02 mmol/l Ca-Tri triplex, 25 mmol/l $NaHCO_3$, 1.2 mmol/l KH_2PO_4, 120 mmol/l NaCl, 1.6 mmol/l $CaCl_2.2H_2O$, 1.2 mmol/l $MgSO_4.7H_2O$, 5 mmol/l KCl, and pH was 7.4. Tissue homogenates were used to evaluate aortic SOD, GSH, and ROS (Ertik et al. 2023). Another part was used to perform western blot analysis. The second part was preserved in a 10% buffered-formalin solution for histopathological examination and immunostaining procedures. The third part was used to examine vascular reactivity.

4.6. Assessment of biochemical markers

4.6.1. Body weight

Animals' body weight was measured using the conventional laboratory animal weight balance, model: PS 2100, R2 RADWAG, UK.

4.6.2. Blood glucose levels

Blood obtained from the tails of the experimental rats and glucose levels were analyzed by the Tango® TD-4235 glucometer, Taiwan.

4.6.3. Serum insulin levels

Following manufacturer instructions, the assay was conducted using an enzyme-linked immune-sorbent assay (ELISA) kit. The density of the yellow color at 450 nm is proportional to the concentration of rat insulin in the sample (Kato et al. 1977) .

4.6.4. Hemostatic model assessment for insulin resistance (HOMA-IR)

The following formula was used to determine HOMA-IR:
HOMA-IR = fasting glucose (mmol/L) × fasting insulin (μU/mL)/ 22.5 (Bian et al. 2023).

4.6.5. Determination of aorta SOD activity

SOD activity in aorta tissue homogenates was estimated based on the inhibition of pyrogallol autoxidation. The inhibition is directly proportional to the activity of SOD in the tested sample (Flohe and Otting 1984).

4.6.6. Aorta GSH content

The reduced GSH content in aorta tissue homogenates was determined based on the reduction of Ellman's reagent [5, 5'-dithio-bis (2-nitrobenzoic acid)] by SH groups. The yellow color of the nitro-mercaptobenzoic acid can be determined spectrophotometrically at 412 nm (Ellman 1959).

4.6.7. Aorta ROS production

Superoxide production was detected in the presence of 5 μmol/l lucigenin and incubated for 20 min. The reaction was initiated with 100 μmol/l NADPH, and the relative light units of chemiluminescence were measured over 30 min. Results were expressed per minute and normalized to the protein content in each sample (Brehm et al. 1996).

4.6.8. Western blot analysis for aorta NrF_2, HO-1, eNOS, and NF-κB expression

Aorta tissues were homogenized in Tris lysis buffer (400 mM NaCl, 0.5% Triton X-100, and 50 mM Tris at pH 7.4) along with a protease inhibitor cocktail (BIOSPES, China) at 4 °C for 30 min, according to a previously described method (Henry et al. 1986). Tissue remnants were isolated by centrifugation at 10000×g for 10 min at 4 °C. Protein concentrations were measured by the Biuret method (Jayle et al. 1951; Wang et al. 1996). Equal amounts of protein (30 μg of total protein in each lane) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Merk, USA) using semi-dry transfer methods (Towbin et al. 1979). The membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween (TBST) buffer at room temperature for one hour. Following this, they were incubated overnight with primary antibodies at 4 °C. Afterwards, the membranes were incubated for one hour with an alkaline phosphatase-conjugated secondary antibody from BIOSPES, China. Bands were visualized using the BCIP/NBT substrate detection kit (GENEMED, Biotechnologies, USA). The produced bands were analyzed using imageJ® software (National Institutes of Health, Bethesda, USA).

4.6.9. Vascular reactivity and endothelial-dependent relaxation

Aorta tissues were isolated, handled carefully, and regularly transferred to a Krebs-Henseleit buffer solution. The buffer solution is composed of 115 mmol/l NaCl, 5.9 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 1.2 mmol/l NaH_2PO_4, 1.2 mmol/l Na_2SO_4, 2.5 mmol/l $NaHCO_3$, and 10 mmol/l glucose, with a pH of 7.4, a temperature maintained at 37±0.5 °C, and was aerated continuously (Wahba et al. 2019). Segments of the aorta, each 1mm long, were hung in 10 ml of Krebs buffer at a constant temperature of 37 °C in organ baths, continuously aerated, and placed between two metallic pins of 200 μm in diameter. Changes in tissue force tension were recorded in gram (g) tension units. A starting tension of 1.5 g was gradually applied to the tissue rings throughout the experiment using calibrated isometric force displacement transducers (MLT0201/D) and (MLT0201/A), connected to a Power-lab/15T, PL 15T02 data acquisition system, and Lab-Chart® software from AD Instruments, Australia.
Aortic rings were left to gain equilibrium for 40–60 min with physiological solution changed every 15 min. Cumulative concentration-response curves were generated for the endothelium-dependent relaxing factor, acetylcholine, after submaximal contraction induced by phenylephrine.

4.6.10. Histopathological examination and immunohistochemical analysis

Aorta sections from different animal groups were fixed in 10% buffered-formalin for 24 hours. Then, the sections were cleared in xylene and embedded in paraffin at 56 °C in a hot air oven for a day. The paraffin-beeswax blocks were sliced using a microtome (Slaoui and Fiette 2011).
Hematoxylin and eosin (H&E) staining were used in sections of 3 μm thickness from each block, Masson trichrome staining was performed on sections of 3 μm thickness from each block (Goodman and Bernstein 1977). For immunohistochemical analysis, sections of 3 μm thickness were mounted to the slide, incubated with primary anti-TGF-β antibody, and lightly counterstained with hematoxylin for 30 s before dehydration and mounting (Taylor and Burns 1974). An independent pathologist examined all slides, and imaging was performed using the LEICA Microsystems DM750 ICC50 W electronic microscope, Germany. A digital imaging software program Image J® software (version 1.32j, NIH, Bethesda, USA) was used to measure the percentage area occupied by the immunopositive cells and Mason trichrome staining in six separate microscopic fields in each slide.

4.7. Statistical analysis

The results are expressed as mean±standard error of the mean (SEM) and were analyzed for statistically significant differences using one-way analysis of variance (ANOVA), followed by the Tukey-Kramer post-analysis test to compare all groups. A significance level of $p < 0.05$ was considered significant. Statistical calculations were performed using GraphPad Prism® (Version 5.00 for Windows, Graph Pad Software, San Diego, California).

Availability of data and materials: All data in this study are available upon request by contact with the corresponding author.

Ethics approval and consent to participate: Animals were handled, and procedures were conducted following SVU's ethical approval committee number: P.S.V.U. 011/22.

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Author contributions: AT, BA and WR designed the research study; EM and MS performed the research; AT provided help and advice in vascular reactivity experiment; MS and EM analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

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