Effects of dapagliflozin against streptozotocin and isoproterenol-induced heart failure via investigating NLRP3 and PPAR-γ signaling

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Abstract: Heart failure is a condition in which the heart's one or both ventricles are unable to either receive an adequate amount of blood or eject an adequate amount of blood. Diabetes is considered one of the major risk factors for cardiovascular diseases. The current research is designed to evaluate the cardioprotective effects of dapagliflozin in streptozotocin and isoproterenol-induced comorbid rats. The COX-2, TNF-α, NF-KB, NLRP3, PPAR-γ, CKMB, TROP-I, AR, GP and SGLT were docked against dapagliflozin, propranolol and metformin. Dapagliflozin restored adequate blood flow and halted myofibril damage. Moreover, it's evident from this study that dapagliflozin significantly decreased serum concentration of various blood markers, decreased relative growth rate and QT interval prolongation, as compared to the negative control group. However, it improved the ventricular ejection fraction in rats of the treatment group. The GST, GSH and CAT levels were increased, as compared to normal. On the contrary, a decrease in LPO concentrations was observed. Evaluation of the coronal section of heart tissues showed the anti-inflammatory expressions evaluated through H & E staining and immunohistochemical techniques and with ELISA and PCR. In a nutshell, dapagliflozin reverses myocardial necrosis and apoptosis.

Keywords: Cardioprotective, docking, dapagliflozin, ELISA, PCR.

INTRODUCTION

The heart is among the vital organs that serves the function of blood supply to the body (Buckberg *et al.*, 2018). Heart failure is a condition in which the heart's one or both ventricles are unable to either receive an adequate amount of blood from the venous system or eject an adequate amount of blood to the arteriolar system (Berry *et al.*, 2001). Heart failure is manifested by ventricular hypertrophy, decreased heart ejection fraction, and cardiac output (Garg *et al.*, 2019) Diabetes is metabolic disorder, directly linked with cardiovascular functioning, and its control can mitigate the risk of hospitalization among cardiac patients. Type-2 Diabetes Mellitus prevalence has been increasing with every passing year, which is obvious from the literature and claims that 439 million people will be diabetic by 2030, which is asignificantly higher number (Olokoba *et al.*, 2012). Approximately 41% population in Pakistan is obese and prone to attacks of diabetes, heart, kidney and liver diseases. The impaired blood flow, deficient with oxygen and nutrients, if persists for a longer duration, it can lead to necrosis due to ischemia (Rodius *et al.*, 2020).

Different animal studies have been performed on streptozotocin (STZ) and isoproterenol (ISO) alone, to evaluate the cardioprotective activity of SGLT-2 (Sodium-glucose Cotransporter-2) antagonist, dapagliflozin (Afroz *et al.*, 2016) . However, the current study aimed to evaluate the underlying mechanism of the

cardio-protective effect of the SGLT-2 antagonist, dapagliflozin, in STZ and isoproterenol (Agrawal *et al.*, 2014)-induced comorbid rats, which were previously kept on high fatty diet for four to five weeks before disease induction. Multiple entities have been tested to cope with oxidative stress, mitochondrial changes, Ca^{+2} influx, free radical and inflammatory mediators' production to estimate the cardio-protective effects (Li *et al.*, 2021)

Computational analysis from the best pose dock showed E-values (Kcal/mol), hydrogen bond and bonding residues formed by 3D and 2D-interaction among the ligands and protein structures of cyclooxygenase-2 (COX-2), tumor necrotic factor alpha (TNF-α), nuclear factor kappa beta (NFκB), Janus kinase (JNK), nod-like receptors pyrin domain-3 (NLRP-3), peroxisomes proliferated-activated receptor-γ (PPAR-γ), creatine kinase-MB (CK-MB), cardiac troponin-I (Trop-I), aldose reductase (AR), glycogen phosphorylase (GP) and SGLT, docked against dapagliflozin, propranolol and metformin. Dapagliflozin improves blood flow and prevents myofibril structural loss. We further performed some tests on the serum concentration of CKMB, trop-I, fasting blood glucose (FBS), hemoglobin-A1c (HbA1c), alanine aminotransferase (Ullah *et al*., 2022), and aspartate transaminase (Agrawal *et al.*, 2014) levels in the blood. Moreover, we examined the relative growth rate, QT interval prolongation and left ventricular ejection fraction (LVEF) of rats. Ejection fraction (EF) is the total percentage of blood that the left ventricle pumps out with each contraction. Its normal value is between 50-70%;

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sometimes, heart failure may present with preserved ejection fraction (p-EF) from 41-49% in the case of humans, and slightly less in the case of rats (i.e., ±5-10%). It does not always indicate that an individual is having heart failure, rather it may indicate the damage from the preceding ischemic attack. The effect of dapagliflozin on the ejection fraction has been estimated by using echocardiogram (ECHO) testing (Paik *et al.*, 2015). However, various cellular signals induce the activation of NLRP3, which include Ca^{+2} signaling, K^+ efflux, lysosomal rupture, and mitochondrial dysfunction. Toll-like Receptors (TLRs) are activated by cytokines, like TNF-α, their toxins lead to downstream activation of NLRP3 and NF-ƙβ (Hoseini *et al*., 2018). The effective therapeutic target for ameliorating myocardial injury is PPAR-γ, a ligand-inducible nuclear receptor that depressingly regulates NLRP3 (Luo *et al.*, 2018) .

This FDA-approved drug for the treatment of heart failure has been studied to reveal the underlying molecular mechanisms that how dapagliflozin (fig. 1) works on heart tissues and blood flow (Garg *et al.*, 2019). Although, there are a number of therapies to manage heart failure (Inamdar and Inamdar, 2016), we were lacking potent candidates for type-2 diabetes mellitus associated with heart failure with reduced ejection fraction (HF r-EF). This research will determine how the potent drug dapagliflozin reverses heart failure. The selected different inflammatory markers have rebutted the results via effects of dapagliflozin on myocardial necrosis, and apoptosis associated with over expression of PPAR-γ and attenuated activation of NLRP3-complexes by using different established techniques.

MATERIALS AND METHODS

Materials
Chemicals
STZ was imported from Japan;, propranolol was from ICI Pakistan Ltd; normal saline 0.9%, ISO-hydrochloride were from Sigma-Aldrich CO.LLC, USA; metformin from Merk Pvt Ltd) and dapagliflozin propanediol monohydrate from AmBeed. Mouse monoclonal antibodies of COX-2, TNF-α, NF-kB, NLRP-3, PPAR-γ and ABC Elisa kit were from Santa Cruz Biotechnology, USA.

Animals
Thirty-five mature Sprague Dawley rats in good health, weighing between 180g and 250g, were purchased from Riphah International University, Islamabad. All animal studies were conducted in accordance with the recommendations of Riphah International University, Islamabad's Experimental Animal Ethics Committee (Ref.NO.REC/RIPS/2022/05). Animals were kept in a setting with a constant temperature of 25°C±0.5, 55±5% humidity and a 12-hour cycle of light and darkness, each.

Before the onset of the condition, a high-fat diet was administered for four to five weeks.
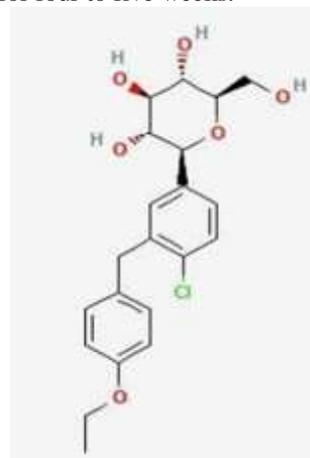
Fig. 1: Structure of Dapagliflozin
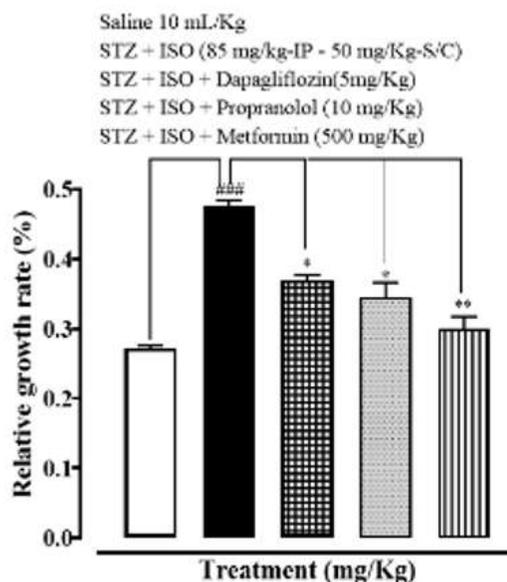
Fig. 2: Effects of dapagliflozin on relative growth rate in streptozotocin (STZ) and isoproterenol (ISO)-induced comorbid rats. Values expressed as mean ± SEM (n=5). One-way ANOVA with post hoc Tukey's test. $^{###}P< 0.001$ vs. saline group, $^*P<0.05$, $^{**}P<0.01$ vs. STZ+ISO group.

Induction of diabetes
Diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (STZ) (50 mg/kg body weight), dissolved in 0.1M cold citrate buffer (pH 4.5), after a 24-h fasting (Agrawal *et al.*, 2014).

Induction of experimental myocardial infarction
A standardized dose of ISO (85 mg/kg, subcutaneously (SC), for two consecutive days at 24h intervals) was used for the induction of myocardial infarction (Agrawal *et al.*, 2014).

Study design

Rats were randomly divided into seven groups of five rats each, after assuring the onset of diabetes (Agrawal *et al.*, 2014).

1. *Diabetic Control group:* A 0.5% hydroxyethyl cellulose and 2% DMSO was administered orally and intraperitoneally (I.P.) for 14 days; saline was also administered subcutaneously (SC) on days 13 and 14.
2. Diabetic ISO group: A0.5% hydroxyethyl cellulose, and 2% DMSO solution was administered to rats, as described above and isoproterenol (ISO) was also administered on days 13 and 14 (85 mg/kg, SC at 24-h intervals).
3. Diabetic ISO + Dapagliflozin (5mg/kg) group: Rats were administered dapagliflozin (5mg/kg) orally for a period of 14 days with a concurrent injection of ISO (85 mg/kg, SC at 24-h intervals) on days 13 and 14.
4. Diabetic ISO + Dapagliflozin (10mg/kg) group: Rats were administered dapagliflozin orally (10 mg/kg) for a period of 14 days with concurrent injection of ISO (85mg/kg, SC at 24-h intervals) on days 13 and 14.
5. Diabetic ISO + Dapagliflozin (15mg/kg) group: Rats were administered dapagliflozin (15mg/kg) orally for a period of 14 days with a concurrent injection of ISO (85mg/kg, SC at 24-h intervals) on days 13 and 14.
6. Diabetic ISO + Propranolol group: Rats were administered propranolol (10mg/kg) orally for a period of 14 days with a concurrent injection of ISO (85mg/kg, SC at 24-h intervals) on days 13 and 14.
7. Diabetic ISO + Metformin (15mg/kg) group: Rats were administered metformin orally (500mg/kg) for a period of 14 days with a concurrent injection of ISO (85mg/kg, SC at 24-h intervals) on days 13 and 14.

Throughout the study, the experimental animals were observed for any changes in body weight and food and water intake. All of the rats were anesthetized after the treatment period and then sacrificed. To obtain serum for the assessment of cardiac biomarkers, blood samples were obtained by cardiac puncture. For the biochemical study, isolated hearts were snap-frozen after being isolated. For histological and ultra structural investigations, the hearts were additionally put in the appropriate fixatives.

Computational studies

The selected protein targets, Cox-2 (4e1g), NF-KB (1SVC), TNF-α (5WUX), p-JNK (5AWM), NLRP3 (7PZD) PPAR-γ (6KOT) CKMB (1lOE), TROP-I (2M2P), AR (5IR3), GP(1A81) and SGLT (2XQ2), were docked against dapagliflozin, propranolol and metformin by using Discovery Studio Visualizer (Irshad *et al.*, 2021).

Cardioprotective effect estimation

Biochemical testing

Different biochemical markers, using the serum, have been examined to scrutinize the effects of dapagliflozin on blood parameters. Biochemical assays were performed to estimate CK-MB, trop-I and HbA1c, from the CMH-RWP pathology lab. Furthermore, FBS was determined by using on-call EZ-II and alanine aminotransferase and aspartate transaminase (Agrawal *et al.*) levels in the blood were examined by using a biochemistry analyzer i.e. Beckman Coulter AU480, USA (Shimizu and Ichihara, 2019).
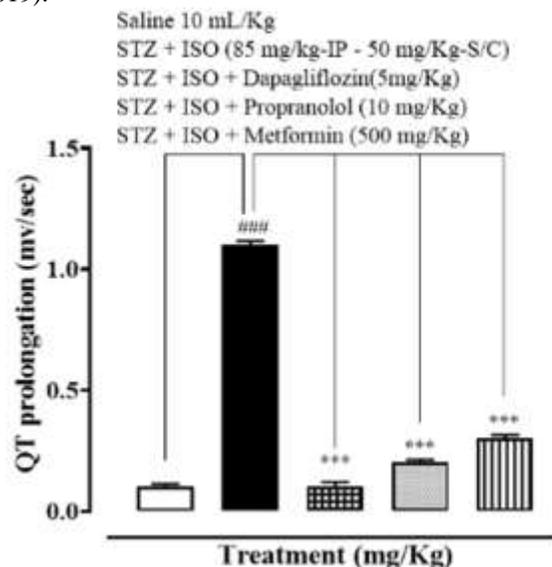
Fig. 3: Effect of dapagliflozin on QT interval prolongation of streptozotocin (STZ) and isoproterenol (ISO)-induced comorbid rats. Values expressed as mean ± SEM (n=5). One-way ANOVA with post hoc Tukey's test. $^{###}P<0.001$ vs. saline group, $^{***}P<0.001$ vs. STZ+ISO group.

Relative growth rate

Effects of dapagliflozin on relative growth rate are calculated by measuring heart weight to body weight ratio in STZ and ISO-induced comorbid rats using the following formula:

Relative growth rate (%) = heart weight / body weight

QT-intervals prolongation

Electrocardiogram (ECG) testing, for assessment of the effects of dapagliflozin on QT interval prolongation, was traced by using a power lab (ADI instruments). Results are compiled by applying snip and sketch tools and standard guidelines (Kim *et al.*, 2019).

Left ventricular ejection fraction (LVEF)

The effect of dapagliflozin on the LVEF of the rats was examined through the echocardiogram (ECHO) technique. The measuring device was a Mindray Ultrasound Machine, attached to the smallest voltage probe (Mcleod *et al.*, 2018).
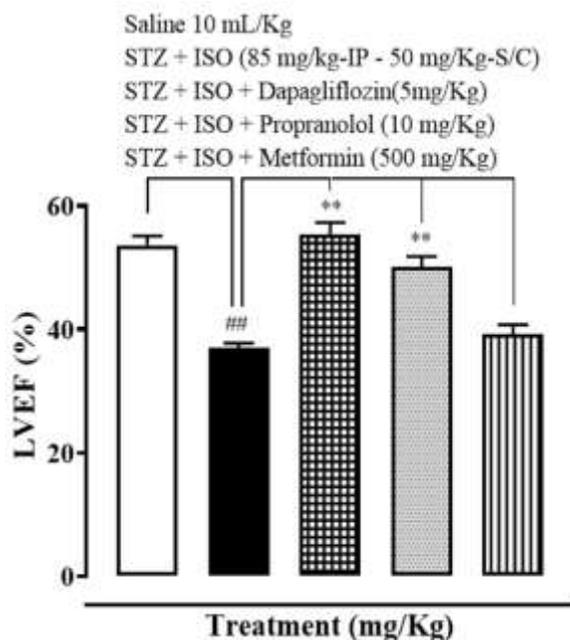
Fig. 4: Effect of dapagliflozin on left ventricular ejection fraction (LVEF) of streptozotocin (STZ) and isoproterenol (ISO)-induced comorbid rats. Values expressed as mean ± SEM (n=5). One-way ANOVA with post hoc Tukey's test. $^{##}P<0.01$ vs. saline group, $^{**}P<0.01$ vs. STZ+ISO group.

Antioxidant assay
The effects of dapagliflozin on oxidative stress and decreased levels of glutathione-s-transferases (GSH), glutathione (GST) and catalase concentration have been measured for evaluating the destructive mechanism in cells as a result of increased oxidation. Therefore, lipid peroxidase (LPO) was estimated by measuring thiobarbituric acid reactive substances (TBARS). The assay mixture containing ascorbic acid and potassium buffer supernatant was allowed to incubate in a water bath for 60 min at 37°C, afterward, incubated for at least 1 hour followed by the addition of trichloro acetic acid (TCA) to tests. Ice cold water bath was used to cool the solution after the centrifugation. The absorbance wavelength was kept near 535 nm to scrutinize the convergence of protein (Zaidi *et al.*, 2005, Kaynar *et al.*, 2005).

Hematoxylin and eosin (H&E)
The glass slides were first coated with different sections of cardiac tissues of rats, their wax coating was removed by using xylene (100%) for deparaffinization and forwarded by rehydration with graded ethanol series (100%, 90%, 80% & 70%). After rinsing with distilled water, nuclei were stained with hematoxylin and eosin. The dye was kept for at least 10 to 15 minutes (Guntawang *et al.*, 2021). The stained section was washed with running tap water. After drying, these slides were dehydrated with graded ethanol series (70%, 95% and 100%), cleared by using xylene and mounted with

mounting media, so they could be observed under a light microscope to take slide images; their analyses were done by Image J software (Ali *et al.*, 2020).
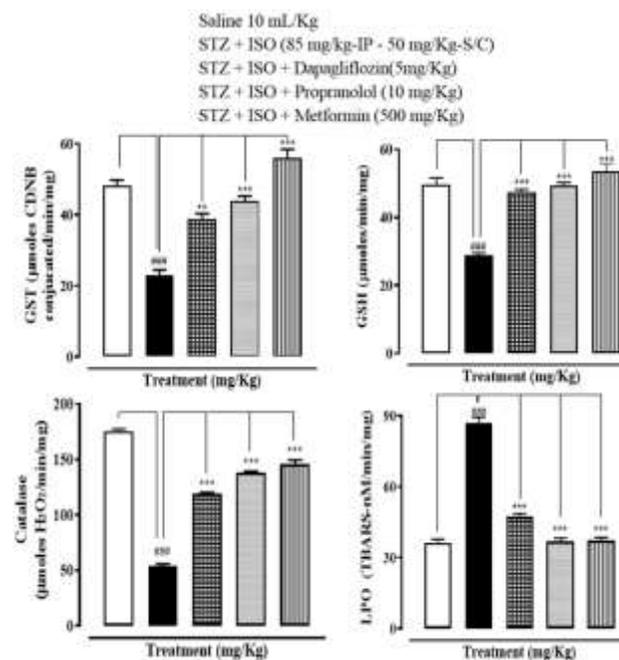
Fig. 5: Effect of dapagliflozin on glutathione sulfotransferases (GST), reduced glutathione sulfotransferases (GSH), catalase (CAT) and lipid hydroperoxide (LPO) concentrations in streptozotocin (STZ) and isoproterenol (ISO)-induced comorbid rats heart tissues. Data expressed as mean ± SEM (n=5), one-way ANOVA with post hoc Tukey's test. $^{###}P<0.001$ vs. saline group and $^{**}P<0.01$, $^{***}P<0.001$, vs. STZ + ISO group

Immunohistochemical analysis (IHC)
The above-mentioned slides, with some modifications, were treated with protein kinase, followed by application of 3% hydrogen peroxide H_2O_2 and blocked by 5% normal goat serum containing 0.1% Triton X 100 to incubate overnight with mouse anti-Cox-2, mouse anti-TNF-α, mouse anti-NF-Κβ and mouse anti-IL-8 antibodies (dilution 1:100, Santa Cruz Biotechnology). The next day, the slides were washed with PBS, and then treated with secondary antibodies and ABC Elisa kit (Santa Cruz Biotechnology) for one hour. Slides were washed with PBS and then stained with DAB solution. The next step was air drying of slides and mounted with DPX mounting medium. In the last step, the slides were optimized and analyzed under a light microscope. To take slide images, their analysis were done by Image J software to determine their expression by optimizing the images according to the threshold intensity for each group and showing relative integrated density of the samples in contrast to the normal saline group (Ullah *et al.*, 2022). The analyses of the slides were done by Image J software, the images were optimized as per the threshold intensity

of each group and shown as the relative integrated density of the samples in contrast to the normal saline group.
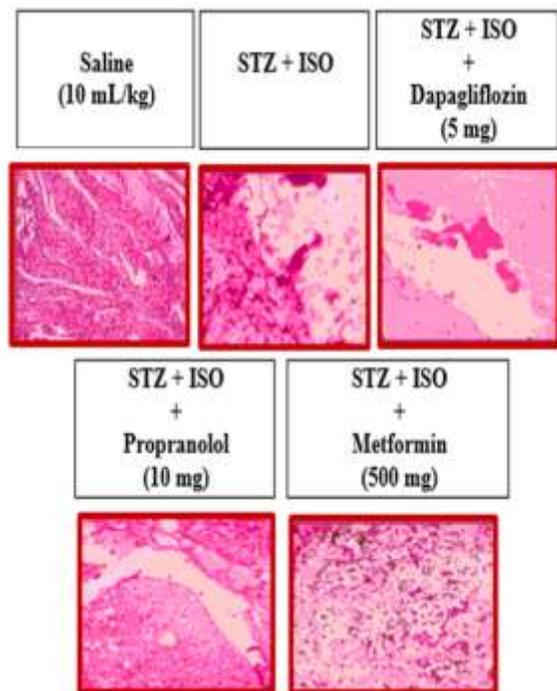
Fig. 6: Effect of dapagliflozin on coronal section of ischemic heart tissues, characterized by area of necrosis, collagen deposition, edematous region and cellular infiltration in streptozotocin (STZ) and (ISO) induced comorbid rats, by using hematoxylin and eosin (H and E) staining and visualized under a microscope with bar 50 μm, magnification 40x.

Enzyme-linked immunosorbent assay (ELISA)
To scrutinize the effects of dapagliflozin against COX-2, NFκB, NLRP3 and PPAR-γ, quantitatively, a suitable quantity of extracted tissues from the cardiac cortical section was homogenized, followed by collecting the supernatant after centrifugation (at 4000rpm for 20 min), and deparaffinized slides were processed for antigen retrieval step, followed by PBS buffer solution. The kits of COX-2, NFκB, PPAR-γ, NLRP-3 antibodies, against which the samples were tested in a 96-well plate, the absorbance w as measured by microscope reader and concentrations in picogram per liter (pg/mL) were normalized to total protein content in pg/mg. All these steps were performed in the triplicate (Ullah *et al.*, 2022) .

Real-time polymerase chain reaction (Rt-PCR)
The heart tissues were homogenized for m-RNA expression analysis. The ratio of NLRP-3-m-RNA copy number to the β-actin m-RNA copy number was determined. After that, RNA was extracted by using 1 mL of TRI-reagent by blending in heart tissue and was homogenized using ultrasound. 200 μL of chloroform was added to the solution, and then mixed vigorously and

centrifuged for 15 minutes. Then, the RNA was subjected to reverse transcription using the cold Strand cDNA Synthesis Kit (Palikša *et al.*, 2018) for 60 minutes at 42ºC, and for 10 minutes at 70ºC. Then, fast start Taq DNA polymerase was applied and 3mM $MgCl_2$ was added. We amplified the 0.4μM primer in the Light Cycler Instrument. Furthermore, pre-incubation, denaturation and annealing for specific time and temperature were recorded. After the completion of PCR, a melting curve analysis was performed by increasing the temperature gradually from 65ºC to 95ºC (0.2ºC /s) to validate the authenticity of the PCR products. Data were compiled using Graph Pad Prism. NLRP-3, and mRNA, in the presence of beta-actin housekeeping gene concentrations between five groups, were analyzed. The mRNA levels were corrected using the transcription level of the β-actin, as an internal standard.

Acute toxicity test
A dose of up to 5-10mg/kg was estimated as a safe dose, with no significant side effects. Five rats in each group were fasted overnight and dapagliflozin (1mg/kg, 5 mg/kg, 15mg/kg and 30mg/kg) was administered IP. All animals were kept under keen observation for 24 hours, and toxicity reactions were observed and the doses were documented to determine the safety profile of the drug. All the treated rats survived with no significant physiological changes within 24 hours of dapagliflozin administration. However, a dapagliflozin dose of 30 mg/kg caused the sudden death of three rats.

STATISTICAL ANALYSIS

All the values were expressed as mean ± SD (n=5). All morphological data were analyzed using Image J software. One-way ANOVA was performed, followed by post hoc Tukey's test, by using graph-pad prism-5. GraphPad prism-5 was also used for graphs. For PCR, the Delta $2^{\cdot}Ct$ method was used for the estimation of gene expression.

RESULTS

Computational Studies
Best pose dock analysis' E-values (Kcal/mol), hydrogen bond formation by the ligands, and protein structures are presented in table 1. The dapagliflozin binding against Cox-2, NFκB, TNF-α, p-JNK, NLRP3, PPAR-γ, CK-MB, Trop-I, AR, GP and SGLT exhibited E-values (Kcal/mol) of -6.9, -6.7, -6.9, -6.7, -6.9, -6.7, -5.8, -5.9, -6.7, -6.5 and -7.2, respectively. This shows that dapagliflozin showed striking interaction with inflammatory, cardiac biomarkers and antioxidant parhways, that play important role in ISO and STZ inducecd heart failure. The dapagliflozin binding against Cox-2, NFKB, TNF-α, p-JNK, NLRP3, PPAR-γ, CK-MB, Trop-I, AR, GP and SGLT exhibited H-bonding values of 2, 2, 1, 2, 1, 0, 1, 2,

2, 1 and 3, respectively. The Standard drug (Propranolol) binding against Cox-2, NFκB, TNF-α, p-JNK, NLRP3, PPAR-γ, CK-MB and Trop-I showed E-values (Kcal/mol) of -6.8, -5.7, -6.7, -6.8, -6.7, -6.9, -6.7 and -6.9, respectively. The Standard drug (Propranolol) binding against Cox-2, NFκB, TNF-α, p-JNK, NLRP3, PPAR-γ, CK-MB, and Trop-I showed H-bond values of 3,2,3,4,2,2,2 and 1, respectively. The standard drug metformin binding against AR, GP and SGLT exhibited E- values (Kcal/mol) of -6.8, -6.7 and -8.6, respectively. The standard drug metformin binding against AR, GP, and SGLT exhibited H-bond values of 2, 1 and 3, respectively.
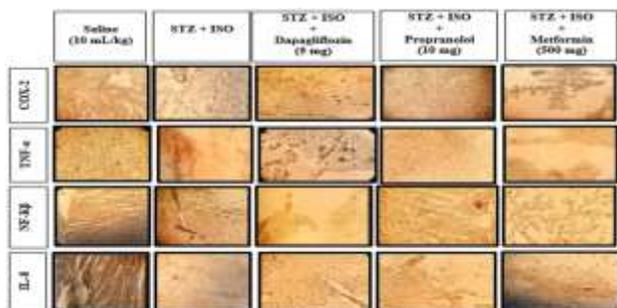
Fig. 7: Effects of dapagliflozin, against cyclooxygenase-2 (COX-2), nuclear factor kappa beta (NF-KB), tumor necrotic factor alpha (TNF-α) and interleukin-8 (IL-8) expression in streptozotocin (STZ) and isoproterenol (ISO)- induced comorbid rats heart tissues, using the immunohistochemical technique with bar 50 μm, magnification 40x and IMGJ software to analyze images.

Relative growth rate
The STZ+ISO group showed a marked increase in relative growth rate when compared with the normal saline group (10 ml/kg). The dapagliflozin (5mg/kg), Propranolol (10mg/kg) and Metformin (500mg/kg) groups showed a reduction in relative growth rate when compared with the STZ+ISO group. Among the dapagliflozin (5mg/kg), propranolol (10mg/kg), and metformin (500mg/kg) groups, the metformin (500mg/kg) showed the highest reduction in relative growth rate when compared with STZ+ISO group (fig. 2).

Cardiac biomarkers
Table 2 shows the effect of dapagliflozin on cardiac biomarkers, HbA1c and FBS, in different experimental groups. In the diabetic ISO group, the serum level of cardiac biomarkers, HbA1c and FBS, were markedly increased (P<0.001), as compared to the normal group. Dapagliflozin treatment in diabetic ISO rats significantly (P<0.001; P<0.01) restored the serum level of cardiac biomarkers, HbA1c and FBS, when compared with the respective normal group.
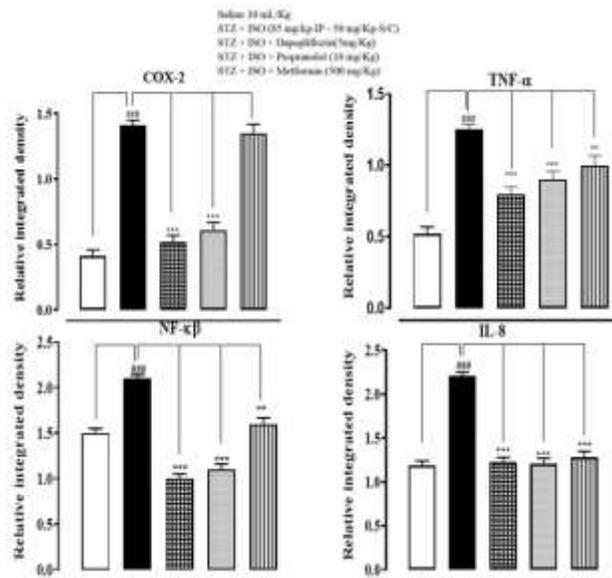
Fig. 8: Effect of dapagliflozin against cyclooxygenase-2 (COX-2), tumor necrotic factor-α (TNF-α), nuclear factor kappa beta (NFKβ), interleukin-8 (IL-8) expression in streptozotocin (STZ) and isoproterenol (ISO)-induced comorbid rats heart tissues, using immunohistochemical technique. Values expressed as mean ± SEM (n=5). One-way ANOVA with post-hoc Tukey's test. ###P<0.001 vs. saline group, **P<0.01,***P<0.001 vs. STZ+ISO group
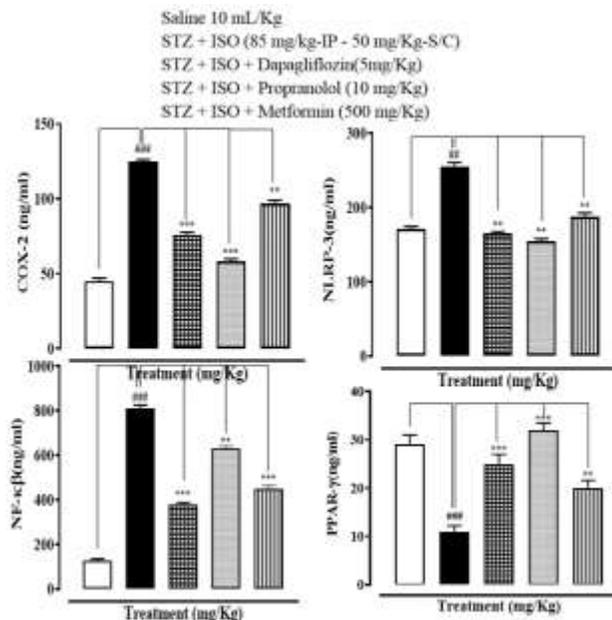
Fig. 9: Effect of dapagliflozin against cyclooxygenase-2 (COX-2), nod-like receptor pyrin domain 3 (NLRP-3), nuclear factor kappa-b (NFKB), peroxisomes proliferated-activated receptor-γ (PPAR-γ) concentration in streptozotocin (STZ) and isoproterenol (ISO) induced comorbid rats coronal heart tissues, by using enzyme-linked immunosorbent assay technique (ELISA). Values expressed as mean ± SEM (n=5). One-way ANOVA with

post hoc Tukey's test. ##P<0.01, ###P<0.001 vs. saline group, **P<0.01, ***P<0.001 vs. STZ+ISO group

QT-intervals prolongation
The STZ+ISO group showed a significant increase in the QT interval when compared with the normal saline (10 ml/kg) group. The dapagliflozin (5mg/kg) group showed relatively less QT prolongation compared to propranolol (10mg/kg) and metformin (500mg/kg) groups, showing values of 0.1, 0.2 and 0.3 (mv/sec), respectively (fig. 3).
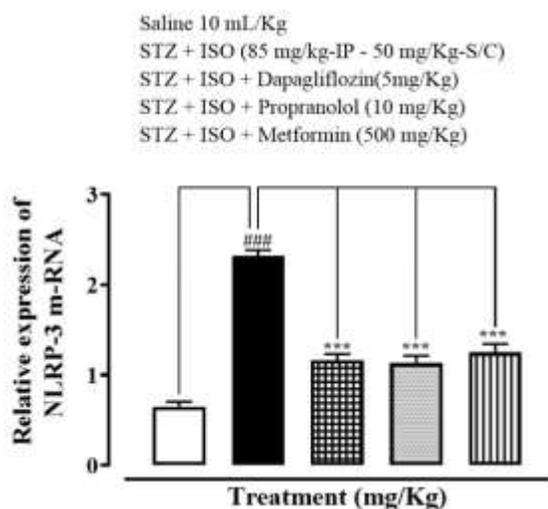
Fig. 10: Effect of dapagliflozin against the messenger ribose nucleic acid (m-RNA) expression of nod-like receptor pyrin domain-3 (NLRP3) in streptozotocin (STZ) and isoproterenol (ISO)-induced comorbid rats coronal heart tissues, by using reverse transcriptase polymerase chain reaction (Rt-PCR). Values expressed as mean± SEM (n=5). One-way ANOVA with post hoc Tukey's test. ###P<0.001 vs. saline group, ***P<0.001 vs. STZ+ISO group.

LVEF
The LVEF (%) in dapagliflozin (5mg/kg), propranolol (10mg/kg) and metformin (500mg/kg) groups were 57, 55, and 40, respectively (fig. 4).

Antioxidant assay
The STZ+ISO group showed significantly reduced levels of GST, GSH and CAT, as compared to the normal saline group. The levels of GST, GSH and CAT were significantly increased in dapagliflozin (5mg/kg), propranolol (10mg/kg) and metformin (500mg/kg) when compared with the STZ+ISO group, and metformin (500mg/kg) showed more prominent results, as compared to dapagliflozin (5mg/kg) and propranolol (10mg/kg). Whereas, the level of LPO increased significantly in the STZ+ISO group when compared with the normal saline group. Dapagliflozin (5mg/kg), propranolol (10mg/kg), and metformin (500mg/kg) decreased the level of LPO significantly when compared with the STZ+ISO group (fig. 5).

Hematoxylin and eosin (H&E) staining
The effect of dapagliflozin on the coronal section of ischemic hearts tissues, which is characterized by an area of necrosis, collagen deposition, edematous region and cellular infiltration in STZ and ISO induced comorbid rats, is shown in fig. 3.5. by using H and E staining and visualized under the microscope with bar 50 µm (fig. 6).

Immunohistochemical analysis (IHC)
In the saline group, no change was observed in the regulation of COX-2, NFκB, TNF-α and IL-8 expression. The dapagliflozin (5mg/kg) group showed a marked decrease in the COX-2, NFκB, TNF-α and IL-8 expression with relative integrated densities of 0.5, 0.75, 1.0 and 1.2, respectively. The propranolol (10mg/kg) group showed relative integrated density against COX-2, NFκB, TNF-α and IL-8 expression of 0.6, 0.8, 1.1 and 1.1, respectively. The metformin (500mg/kg) group showed relative integrated density against COX-2, NFκB, TNF-α and IL-8 expression of 1.4, 1.0, 1.5 and 1.1, respectively (figs. 7 & 8).

Enzyme-linked immunosorbent assay (ELISA)
In the coronal heart tissues of the saline group, COX-2, NLRP-3, NFκB and PPAR-γ concentrations (µg/ml) were 48, 175, 120 and 29, respectively. In the dapagliflozin (5mg/kg) group, COX-2, NLRP-3, NFκB and PPAR-γ concentrations (µg/ml) were 70, 175, 390 and 25, respectively. In the propranolol (10mg/kg) group, COX-2, NLRP-3, NFκB and PPAR-γ concentrations (µg/ml) were 60, 170, 620 and 32, respectively. In the metformin (500mg/kg) group, COX-2, NLRP-3, NFκB and PPAR-γ concentrations (µg/ml), were 100, 180, 440 and 20, respectively (fig. 9).

Real-time polymerase chain reaction (Rt-PCR)
In dapagliflozin (5mg/kg) and propranolol (10mg/kg), NLRP-mRNA expression was decreased to almost a similar extent with relative values of 1.1 and 1, respectively, whereas, metformin (500mg/kg) group showed a slightly less decrease in the NLRP-mRNA expression, comparatively (fig. 10).

Acute toxicity test
All the treated rats survived with no significant physiological changes within 24 hours of dapagliflozin 5-10mg administration, but on dapagliflozin administration of 30mg/kg, sudden death of three rats occurred with the mortality rate being about 8.5%.

DISCUSSION

Cardiovascular diseases are among the life-threatening diseases and several therapies have been designed to deal with these fatal diseases.

Table 1: Best pose dock analysis showing E-values (Kcal/mol), hydrogen bond and bonding residues formed by interaction of dapagliflozin and standard drugs with targets: cyclooxegenase-2 (COX-2), tumor necrotic factor alpha (TNF-α), nuclear factor kappa B, phosphorylated c-Jun N-terminal kinase (p-JNK), nod-like receptor pyrin domain containing-3 (NLRP3), peroxisomes proliferator-activated receptor-γ (PPAR-γ), creatine kinase-MB (CKMB), cardiac troponin-I (TROP-I), aldose reductase (AR) and glycogen phosphorylase (GP).

Target protein	PDB ID	Dapagliflozin				Standard drugs				
-	-	E-value (Kcal/mol)	H-bond	H-Bonding residues	Bonding Residues Forming Other bonds	Drug Name	E-value (Kcal/mol)	H-bond	H-Bonding residues	Bonding residues forming other bonds
Cox-2	4e1g	-6.9	2	GLU 106, ASN 102	THR 93, CYS 113, ARG 196	Propranolol	-6.8	3	PHE 371, LYS 369, GLN 370	GLU 30 GLY 35 VAL 34
NF-KB	1SVC	-6.7	2	ALA 36, VAL 164	VAL 154, MET 91, SER 34	Propranolol	-5.7	2	SER 108, THR 149	LEU 139, LYS 153, VAL 141
TNF-α	5WUX	-6.9	1	ASN 267	ASN 260, GLU 88	Propranolol	-6.7	3	LEU 108, SER 190, LYS 358	ASN 200, ALA 36, GLN 37
p-JNK	5AWM	-6.7	2	SER 21 ARG 34	SER 169 LEU 163 VAL 134	Propranolol	-6.8	4	GLY 91, SER 158 ASN 112	VAL 98 LEU 166 ALA 51
NLRP3	7PZD	-6.9	1	ARG 62	THR 83 SER 21 CYS 22	Propranolol	-6.7	2	GLN 39, VAL 48	CYS 91 THR 19, SER 79
PPAR-γ	6KOT	-6.7	0	-	ASN 402 ARG 234	Propranolol	-6.9	2	SER 221, GLU 298	LYS 216, GLU 427, SER 382
CKMB	1lOE	-5.8	1	MET 191 GLY 95	LYS 608, GLY 132	Propranolol	-6.7	2	SER 190, ASN 200	PRO 542, ASP 29, LEU 108
TROP-I	2M2P	-5.9	2	ARG 189, ASP	TYR 90, ALA 129	Propranolol	-6.9	1	GLU 107	ASP 169 VAL 156 MET 108
AR	5IR3	-6.7	2	LYS 48, PRO 62	GLN 39, SER 79	Metformin	-6.8	2	ALA 36, GLN 37	PRO 192, ILE 32, ASN 156,
GP	1A81	-6.5	1	ARG 234	GLU 298, SER 382, ASN 402	Metformin	-6.7	1	ILE 84	GLU427 SER 221 LYS 216
SGLT	2XQ2	-7.2	3	PRO 64, GLU 142, ASP 156	ASP 437, MET 102	Metformin	-8.6	3	GLN 428, ASN 64, SER 91	ASN 142, ILE 427, GLN 69

Amino acids are: alanine (Ali et al.), arginine (V. Garg et al.), asparagine (ASN), aspartic acid (ASP), cysteine (CYS), glutamine (GLN), glutamic acid (GLU), glycine (GLY), histidine (Shimizu et al.), isoleucine (ILE), lysine (LYS), methionine (Guntawang et al.), phenylalanine (PHE), proline (PRO), serine (SER), threonine (THR), tryptophan (TRP), tyrosine (TYR) and valine (VAL).

Dapagliflozin, an SGLT-2 receptor antagonist being used for the treatment of type-2 diabetes, has been a Pandora box for the researchers for the last five years. Formally, many combination therapies of this drug have been designed to mitigate the likelihood of diabetes-related complications. Recently, it has been approved by the FDA for the treatment of heart failure. Several studies have been conducted successfully which are in favor of the cardioprotective effect of dapagliflozin in cardiotoxicity induced by STZ and ISO, separately (Afroz *et al.*, 2016). However, this study is the extended form that reveals the cardioprotective effect of the selected compound in STZ+ISP comorbid rats that were on obesity-inducing

high-fat diet for four to five weeks before the disease induction and additional analyses like computational studies, to confirm various targets for the drug, RT-PCR, acute toxicity studies and ECG, have also been included.

In this study, the underlying molecular mechanism of selected drugs on cardiovascular and anti-inflammatory pathways, through the utilization of computational, molecular and clinical testing on rats and their extracted heart tissues, was revealed. This compound is an addition to the lifesaving interventions for heart failure with reduced and preserved LVEF. The use of a high-fat diet before disease induction was intended to explore the

Table 2: Effect of dapagliflozin on serum creatine kinase-MB (CKMB), troponin-I, fasting blood glucose (FBS), hemoglobin-A1c (HBA1C), alanine amino transferase (Ullah et al.) and aspartate transaminase (Agrawal et al.) levels in blood, in streptozotocin (STZ) and isoproterenol (ISO)-induced comorbid rats.

Blood markers	Saline	STZ+ISO	STZ+ISO + Dapagliflozin (5 mg/Kg)	STZ+ISO + Propranolol (10 mg)	STZ+ISO + Metformin (500 mg/Kg)
CKMB (U/L)	638.75 ± 33.25	$1273.39 \pm 97.36^{\#\#\#}$	$379.33 \pm 9.59^{***}$	$446.09 \pm 16.19^{***}$	$453.09 \pm 12.17^{***}$
Trop-I (ng/ml)	5.23 ± 0.12	$14.87 \pm 1.57^{\#\#\#}$	$4.96 \pm 0.07^{***}$	$4.67 \pm 0.05^{***}$	$6.72 \pm 0.17^{***}$
FBG (mg/dl)	105.14 ± 18.26	$302.35 \pm 26.54^{\#\#\#}$	$129.75 \pm 7.73^{***}$	$198.47 \pm 15.36^{**}$	$141.39 \pm 9.75^{***}$
HbA1C (ng/dl)	4.4 ± 0.02	$8.7 \pm 0.17^{\#\#\#}$	$4.19 \pm 0.02^{***}$	7.17 ± 0.12	$4.35 \pm 0.03^{***}$
ALT (u/mL)	73.83 ± 11.37	$477.07 \pm 113.28^{\#\#\#}$	$245.6 \pm 18.38^{**}$	$238.31 \pm 15.54^{**}$	$305.22 \pm 41.48^{*}$
AST (u/mL)	100.13 ± 17.31	$293.67 \pm 27.83^{\#\#}$	$187.46 \pm 12.36^{**}$	$174.28 \pm 10.92^{**}$	$193.69 \pm 17.62^{*}$

Values expressed as mean $\pm$ SEM (n=5). One-way ANOVA with post hoc Tukey's test. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. saline group, $^{*}P< 0.5$, $^{**}P<0.01$, $^{***}P<0.001$ vs. STZ+ISO group, using graph

natural physiological mechanism of the disease. STZ 50mg/kg administered intraperitoneally, induced diabetes in rats. After 24 hours, ISO 85mg/kg was administered subcutaneously to induce heart failure in diabetic rats, in order to validate its potential to save a life and the protective mechanism of dapagliflozin 5mg/kg in the diseased group for two to three weeks. Simply, the implemented model mimics the same computational, biochemical, electrocardiogram, echocardiogram, antioxidant, immunohistochemical and histopathological changes as the human cardiac tissues undergo with a significantly low mortality rate (Zaibi *et al.*, 2021). Docking analysis performed for dapagliflozin, propranolol and metformin against their respective targets, their corresponding binding affinities, the stabilities, the binding energies and the hydrogen bond formation were determined. The relative growth rate of the animals depicted the effect of the drug on the lessening of the disease intensity. The results indicated increased levels of cardiac markers i.e., CK-MB and Trop-I in blood promptly after the cardiac injury being more pronounced in the diseased group, whilst an almost normal range in the case of the treated group. Results plotted for diabetic blood markers i.e. FBS and HbA1c also suggested its efficacy. The LFT plotted data suggested the tissue recovered status in the treated group was far better than the diseased group i.e. increased AST and ALT levels in the diseased group compared to the treatment group. The ECG changes, after induction of comorbidity and its treatment by the selected drug, were recorded. The significant effect on QT interval prolongation and its reversal had been observed, and the LVEF reflected impaired functioning of blood vessels. The normal pattern of ECG and the normal percentage of ECHO proved the cardio protective effects of dapagliflozin in STZ-induced diabetic plus ISO-induced heart failure in rats. In cardiovascular dysfunction, the potent phenomenon of oxidative damage is the major concern of discussion. The cascades induced by HFD, STZ, and ISO cause oxidative changes which have been examined by using GSH, GST, CAT and LPO assessment. The oxidized glutathione ratio in the heart,

under high oxidative stress, acts as an indicator of cardiac insufficiencies. This experimental approach of administering dapagliflozin to treat diseased rats suggests that there is an improvement of CAT and glutathione levels. LPO is a major redox reaction, which makes structural dysfunction in the heart cells and dapagliflozin reverses the disruptions caused. Multiple changes like calcium uptake by the mitochondrial cells and less ATP production have been observed, and the oxidative stress in case of cardiovascular disease caused several damages to DNA, and this has been witnessed as the major reason for histopathological changes (Vanessa Fiorentino *et al.*, 2013). The counteracting roles of PPAR-γ and NLRP-3, in cardiac muscles' inflammation pathways, are mainly described in the study. To quantify these contrast molecules, ELISA kits were used. Previous studies highlighted that NLRP3 is responsible for inducing inflammation and PPAR-γ counteracts it. A ligand-stimulated cardio protective transcription factor, PPAR-γ identifies the inhibition of inflammatory processes in myofibrils through transcription factors. PPAR-γ's over expression reduced the expression of different inflammatory mediators, majorly TNF-α (Garg *et al.*, 2020).

The diseased group showed significant changes in morphology, as well as changes in metabolic order that caused the heart to function improperly and worsen its coronary blood supply. Moreover, changes in extra cellular matrices were also observed, that is, the typical decrease in phosphate level, as described previously. Dapagliflozin in combination with many oral antidiabetic drugs has been studied, whereas, its potential as a cardio protective agent needs exploration. The current study was designed to avert myocardial injuries by restoring the blood flow and ruling out necrosis by enhancing the level of antioxidant and anti-inflammatory activity. The pores created and damaged in the blood vessels were also recovered with the use of the drug. The drug showed promising action for longer period of time on the heart cells and a protective agent against NLRP-3, and a modulator of PPAR-γ. The antagonist of PPAR-γ

significantly worked against atherosclerosis, necrosis, apoptosis and other cardiovascular changes, which is suggestive of its antioxidant, as well as anti-inflammatory and cardio protective effects (Mahmoud *et al.*, 2019). The effects of dapagliflozin against multiple anti-inflammatory cardiac markers, COX-2, TNF-α, NFκB, JNK, NLRP-3, PPAR-γ, Trop-I, CK-MB, AR, GP and SGLT, have been established for the first time.

The effects of dapagliflozin on various histochemical changes against COX-2, TNF-α, NFκB, NLRP-3 and PPAR-γ have been described in the current study. Furthermore, the activation of NFκB and p- JNK resulted in the release of NLRP-3 which is forwarded by suppression mediated by over expression of PPAR-γ. The IL-8 has a potential role in the ischemic perfusion of cardiac cells. Dapagliflozin against IL-8 has a significant effect to prove its protective mechanism. Multiple approaches to the underlying inflammatory mechanisms of cardiovascular injuries have been studied, but in the current study, the specially selected dapagliflozin worked as a protective agent and the NLRP-3, as a contributor to ischemic inflammation (Ran *et al.*, 2021). It has been previously reported that the activation of NLRP-3 inflammasome by the initiation of caspases causes mitochondrial disruption. This study is aimed to prove dapagliflozin is a revival of the therapeutic strategies used to treat heart failure. The target was inhibition of the NLRP-3 in the heart by dapagliflozin. This targeted therapy down regulates the NLRP-3 by reducing and reversing cardiovascular injuries and must preserve cardiac physiological functioning.

Several studies have proved that PPAR-γ shows a protective mechanism on heart tissues, it prevents ischemic instabilities by the regulation of different inflammatory responses such as apoptosis, as well as lipid metabolism (Chong *et al.*, 2012). According to a report, the dysfunction of mitochondria is in fact due to oxidative stress that is the major target of PPAR-γ, which is a dominant stimulator of mitochondrial respiration, and biogenesis due to ATP levels alteration (Picard *et al.*, 2012). Furthermore, PPAR-γ is mainly expressed in adipose tissues of the heart, having high oxidative activity, which plays an important role in increasing the mitochondrial biogenesis and antioxidant pathways that slowdown or reverse the mitochondrial damage. In cardiac oxidative injury, PPAR-γ is over expressed, works as an anti-inflammatory agent, and delays myocardial injuries via NLRP-3 suppression and NFκB transgression (Doenst *et al.*, 2013). Acute toxicity testing of dapagliflozin was performed to evaluate the potential adverse effects, when administered in a singlehigh dose (Chinedu *et al.*, 2013). The results indicated that dapagliflozin showed toxicity at 30 mg/kg as three rats were died. However there was no symptoms of toxicity were seen below 30 mg/kg. So he proposed therapeutic

doses of 5 and 10 mg/kg was considered to be safe in the context of acute toxicity study.

CONCLUSION

It is sufficient to say that dapagliflozin overexpressed the PPAR-γ, and is significantly involved in the suppression of NLRP-3, oxidative stress and cardiac cell inflammation by modulating the COX-2, TNF-α, JNK and NFκB. Henceforth, it is proposed that dapagliflozin has been approved by the FDA previously for the treatment of heart failure, however, the underlying mechanism was not revealed properly. The current study claims dapagliflozin is the best therapeutic choice to protect the heart and to reverse cardiovascular changes. A detailed pharmacological profile of dapagliflozin has been described which needs further exploration.

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