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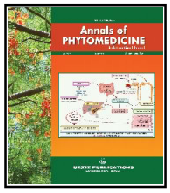

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Multicentric assessment of empagliflozin, sitagliptin and cholestyramine on n-2 streptozotocin diabetic rats

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Abstract

Diabetes mellitus stands as a major global epidemic, rapidly spreading across the world. WHO reports a continuous and concerning upsurge in both the number of cases and prevalence. In India alone, diabetes prevalence escalated from 7.1% in 2009 to 8.9% in 2019. This research endeavor aimed to investigate the combined effects of three distinct classes of medications; empagliflozin, sitagliptin, and cholestyramine on countering type 2 diabetes induced by streptozotocin (STZ) in neonatal rat pups. The assessment encompassed individual drug therapies as well as their triple combination and evaluated *in vitro* measurements of α-amylase and α-glucosidase activities. Encouraging results were observed with the triple drug combination in diabetic treatment. The study employed neonatal albino rat pups of both genders, administering a single intraperitonial dose of 90 mg/kg of the potent diabetes-inducing agent streptozotocin. This treatment regimen spanned 4 weeks, after which the animals were anesthetized, and blood, liver, pancreas and kidney samples were collected for antioxidants, histopathological and biochemical analysis. The findings indicated that the triple drug combination exhibited the most favorable outcomes, displaying significant levels (p<0.05), (p<0.01), (p<0.001) against STZ induced type 2 diabetes in neonatal rat pups. Consequently, the study concludes that triple therapy demonstrates synergistic effects in mitigating type 2 diabetes in neonatal rat pups.

1. Introduction

Diabetes mellitus is an abating degenerative, multi-systemic, metabolic disorder characterized by elevated levels of glucose in fasting as well as post-prandial conditions. Diabetes is categorized majorly into 2 sub types, type 1 diabetes often termed IDDM which relates to lack of stimulation of insulin from pancreatic β cells resulting in high levels of blood glucose concentration, and secondly, type 2 diabetes which is also termed NIDDM which constitutes the presence of insulin but the inability of cells to respond towards insulin produced (Wilding, 2014). NIDDM holds 90% of the carriers which makes it comparatively more prevalent than IDDM all over the globe. The main symptoms of NIDDM were obesity and hyperglycemia (Sruthy and Balasubramaniam, 2023). Excessive fat results in insulin resistivity in muscles; which the pancreas prevents by releasing more insulin but lastly step-by-step it results in NIDDM (Sendraya perumal *et al.*, 2014).

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This sweet killer ailment is unstopping and its prevalence is gradually increasing. In 2015, it affected 415 million people worldwide and is expected to achieve the figure of 642 million by 2040. India leads the globe and is titled the diabetic capital of the world by having the largest number of diabetes patients all over the world (Prasanna Kumar, 2018). According to the data published in diabetes atlas 2015 (seventh edition) by IDF, India, currently has approximately 69.2 million diabetics, and this number is expected to increase to 123.5 million by the year 2040 (Cavan *et al.*, 2015).

Various animal models were present to correlate diabetes with human diabetes but none fits to its best. The maximum possible outcomes were observed from the neonatal pups induced with diabetes. As stated by Arulmozhi *et al.* (2004), there were several models to induce type 2 diabetes like genetic and spontaneous models constitutes sand rats *(Psammomyms obesus),* the fatty zucker rats (Zucker fatty diabetic-ZDF rats), the JCR rat (LA-Cp rat), the OLETF rats (Outsuka-Long-Evans-Tokoshima-Fatty rats) and experimentally induced models like neonatal rat pups induced with type 2 diabetes by injecting chemical streptozotocin (STZ) intraperitoneally termed as neonatal STZ induced rat (n-STZ model of type 2 diabetes) with different doses and at diverse time duration like induction just after birth (n0-STZ model), 2nd day after birth (n2-STZ model), 5th day after birth (n5-STZ model) (Arulmozhi *et al.,* 2004). Comparing the

three neonatal models n0-STZ and n2-STZ results profoundly over the n5-STZ model as it results in unaltered glucose intolerance, elevated HbA1c, and insulin hyperglycemia. So, the n5 STZ model was pulled out of the chain.

Fixed dosage combinations (FDC) are effective healthcare interventions that combine various active components in a single dose, making it easier to treat symptoms or diseases while also increasing efficacy (Arya *et al.,* 2019). A new approach towards the therapeutic activity for the treatment of patients who were suffering from NIDDM came into force as SGLT2 inhibitors, which act by restraining the re-absorption of renal glucose (Devineni *et al.,* 2014). Empagliflozin, a selective potent active orally SGLT2 inhibitor was recently approved for NIDDM. As per studies, it is observed that single and multiple doses results in the elimination of glucose from blood and urine and a reduction in fasting serum glucose and glycated hemoglobin (HbA1c) levels, respectively (Haring *et al.*, 2014; Kern *et al.,* 2016).

Another novel class for treating NIDDM is DPP-4 inhibitors. Incretin helps the body to produce extra insulin as needed and diminishes glucose levels by the liver when not needed. These hormones were released round the clock and elevated levels are observed during meal times. Now DPP-4 is an enzyme used to destroy incretin hormone, for this DPP-4 inhibitors were used to treat this condition (Santwana *et al.,* 2023). Sitagliptin is a novel, oral hypoglycemic therapeutic agent preferred for NIDDM. It could be used either as a mono drug or in combination which provides an improvement in glycemic control (Karabulut *et al.,* 2015).

Cholestyramine is a BAS observed to produce a productive decrease in HbA1c and glucose levels and is said to be a fruitful target drug for NIDDM (Staels *et al.,* 2007). It is observed with a patient suffering from NIDDM, hyperlipidemia and cardiovascular diseases are among the serious symptoms, BAS can be used either as monotherapy or in combination with HMG CoA reductase inhibitors and nicotinic acid to produce cholesterol lowering effects (Ast and Frishman, 2013).

STZ is used to induce diabetes with a toxic effect on pancreatic β-cells in experimental models (Vikas and Mohan, 2022). STZ is chemically 2-deoxy -2-[(methylnitrosoamino) carbonyl] amino-D-glucose, used to induce both types of diabetes, i.e., IDDM and NIDDM as per the dose dependent model used (Venkatachalam *et al.,* 2021). STZ analogs to glucose moiety get incorporated with DNA and produce toxicity by DNA fragmentation and as a result, DNA strands get broken which ultimately leads to cell injury (Arulmozhi *et al.,* 2004).

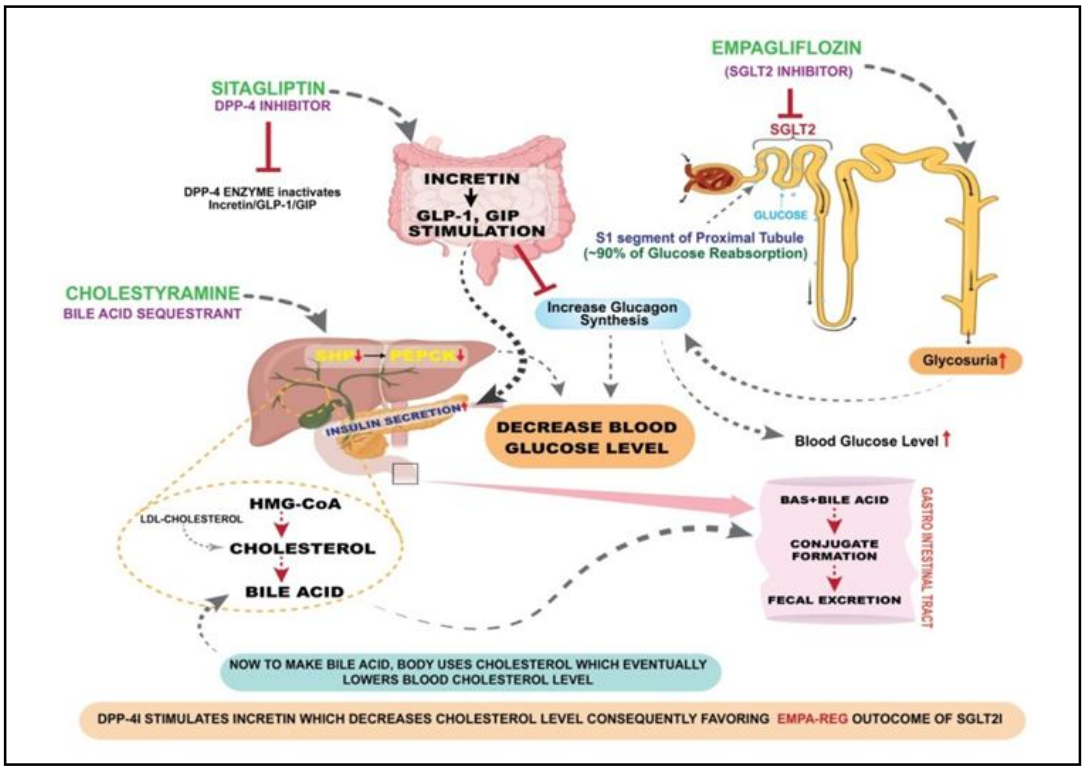

Figure 1: Schematic representation of synergistic effect of triple combination of test drugs.

Our research endeavor aimed to assess the synergistic mechanism (Figure 1) of empagliflozin, sitagliptin, and cholestyramine representing different classes of drugs and their combined anti-diabetic effect on neonatal rat pups with STZ induced type 2 diabetes.

2. Materials and Methods

2.1 Selection of drugs

A plausible synergistic and supportive mechanism of triple therapy is that the three variant categories of drugs with diverse mechanisms of action have been used in this study. First and foremost the selection of the drug was based on the combination of our hypothesis that was never been researched before and there is no data available till date related to this novel combination. Secondly, the advantages of selected the drug over the other drugs of the same category, *i.e.,* we considered the major adverse effects associated. Further, we also hypothesized that the mechanism of action of one drug improves the functioning of other drug synergistically.

2.2 *in vitro* study

In vitro assessment of α-amylase and α-glucosidase was conducted to confirm the efficacy of medication monotherapy and triple combination. This combination's inhibitory efficacy against α-amylase and α-glucosidase has not before been described. To establish the anti diabetic potential of this new combination, *in vitro* and *in vivo* investigations were conducted.

2.2.1 α-amylase inhibition assay

The α-amylase inhibition test was done as per Ahmed *et al.* (2013), 500 µl amount of the reported volume of samples were taken likewise empagliflozin (10 mg/kg/l), sitagliptin (10 mg/kg/l), cholestyramine (5 mg/kg/l), triple combination of all 3 test drug, metformin (25 mg/kg/l) drug used as standard and 500 µl of incubate 0.5 mg/ml of á-amylase solution in sodium phosphate buffer (pH 6.9) for 10 min. at 25°C. After pre-incubation, add 1% of 500 µl of starch solution in 20 mM of sodium phosphate buffer (pH 6.9) to each test tube. This mixture was incubated for 10 min at 25°C before being further clogged with 1 ml of DNS (3,5 dinitro salicylic acid), a coloring agent. Now the tubes were placed in a boiling water bath for 5 min before being cooled to room temperature. Lastly, mixtures were diluted with 10 ml distilled water and results were measured at 540 nm. As a positive control, acarbose was used.

Inhibition activity was estimated using the following formula;

$$\text{Inhibition \%} = \frac{(\text{Absorbance control} - \text{Absorbance test})}{\text{Absorbance control}} \times 100$$

2.2.2 α-glucosidase inhibition assay

Ahmed *et al.* (2013), investigated the inhibitory action of α-glucosidase. In 96 well plates, 60 µl of test sample volume containing empagliflozin (10 mg/kg/l), sitagliptin (10 mg/kg/l), cholestyramine (5 mg/kg/l), triple combination of all three test drugs, metformin (25 mg/kg/l) of methanol, drug used as standard, and 50 µl of phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U/ml) were incubated at 37°C for 10 min. After pre-incubation, add 50 µl 5 mM PNPG (pnitrophenyl-a-D-gluco-pyranoside) solutions in 0.1 M phosphate buffer (pH 6.8) to each well and incubate at 37°C for 30 min. After incubation, samples were occluded with 160 µl of 0.2 M sodium carbonate, and the absorbance was measured at 405 nm.

α-glucosidase inhibitory activity was estimated as follows:

$$\text{Inhibition \%} = \frac{(\text{Absorbance control} - \text{Absorbance test})}{\text{Absorbance control}} \times 100$$

2.3 Animal procurement

Albino neonatal rat pups weighing 5-10 g of either sex were acquired along with their mothers. For 90 days, all of the pups were raised in separate polypropylene cages with their mother at the animal home. Animals were separated into 7 groups of 6 animals each and kept under ideal conditions of 20°C - 25°C with 12 h of light and dark cycles, relative humidity 50% - 55%, and free access to a standard pellet food (Lipton rat feed, Ltd., Pune) and water ad libitum. The Sam Higginbottom University of Agriculture Technology and Science's IAEC approved the whole experimental procedure under permission number IAEC/SHIATS/PA16III/SMIKDA003.

2.4 Drugs and chemicals

Empagliflozin API- catalogue number: 864070-44-0 was a generous gift from Mylan (Hyderabad, India). Sitagliptin- catalogue number: 486460-32-6 was obtained from MSD (Mumbai, India). Cholestyramine- catalogue number: 11041-12-6 was purchased from Sandoz (Mumbai, India). STZ- catalogue number: 18883-66-4 was purchased from SRL. Various diagnostic kits were gifts from Vanguard Diagnostics (New Delhi, India).

All other compounds used were analytical grade.

2.5 Diabetes induction

Type 2 diabetes was developed in 2 day old pups weighing 5-10 g. STZ was prepared in citrate buffer with pH 4.5 (Kamble and Bodhankar, 2013) and administered intraperitoneally with a single dose of 90 mg/kg (Hafizur *et al.* 2015), and the pups were left with their respective mothers for 90 days. On the 91st day, blood glucose levels (BGL) were evaluated, and rats with BGLs more than 200 mg/dl were chosen. The selected numbers of rats were further classified into the group specified in (Table 1), and the treatment procedure was followed for four weeks. Blood samples were obtained by retro orbital, and the rats were euthanized, with the liver, pancreas, and kidney removed for additional biochemical analysis.

Table 1: Treatment schedule

Group No.	Groups	Dose and route of administration
I.	Normal control	3 ml/kg, N.S. for 4 weeks, p.o.
II.	Toxic control [STZ]	90 mg/kg once, i.p.
III.	STZ + empagliflozin [E]	90 mg/kg, i.p. + 10 mg/kg/day for 4 weeks, p.o.
IV.	STZ + sitagliptin [S]	90 mg/kg, i.p. + 10 mg/kg/day for 4 weeks, p.o.
V.	STZ + cholestyramine [C]	90 mg/kg, i.p. + 5 mg/kg/day for 4 weeks, p.o.
VI.	STZ + [E] + [S] + [C]	90 mg/kg, i.p. + 10 mg/kg/day for 4 weeks + 10 mg/kg/day for 4 weeks + 5 mg/kg/day for 4 weeks.
VII.	STZ + SM	90 mg/kg, i.p. + 25 mg/kg/day for 4 weeks, p.o.

NS = normal saline; STZ = streptozotocin; SM = standard drug (metformin); i.p. = intraperitonial; p.o. = per oral

2.6 Biochemical estimation

2.6.1 Blood glucose estimation

Animals were made to fast for last 8-10 h before the estimation. At the time of estimation the blood is obtained through retro-orbital puncture using thin capillary. On the 31st day following medication therapy from serum, blood glucose levels were measured using Dr. Morepen's glucometer.

2.6.2 Oral glucose tolerance test (OGTT)

Adult rats were fasted for 8-10 h before the test. Neither the pellet feed nor the water given to them. The time slot is divided into six categories and OGTT was performed by oral ingestion of 2 g/kg of glucose (Chaimum-aom *et al.,* 2017). Six time slot to measure blood glucose level were as -60, 0, 30, 45, 60 and 120 min, respectively, after glucose was loaded using oral gavage and blood glucose was measured using Dr. Morepen glucometer.

2.6.3 Measurement of serum insulin

With the help of Vanguard's insulin kit, India, test for insulin estimation was performed.

2.6.4 Measurement of glycogen

Liver glycogen test was performed as per Anthrone method. Formula used to determine the amount of glycogen is:

$$\mu g \text{ of glycogen aliquot} = \frac{100 \times U}{1.11 \times S}$$

U = Optical density of the unknown test solution;

S = Optical density of 100 µg glucose standard.

2.6.5 Measurement of lipid profile

The lipid profile, which included total cholesterol (TC), triglycerides (TG), and high density lipoprotein (HDL) (Laxmipriya *et al.,* 2021), was determined using diagnostic kits provided by Vanguard diagnostics. Friedewald's formula is used to calculate low density lipoprotein (LDL) cholesterol as shown below (Ahmed *et al.,* 2017).

$$\text{LDL Cholestreol} = \frac{\text{Total cholesterol} - \text{Triglyceride}}{5 - \text{HDL cholesterol}}$$

The other two formulas were used for the calculation of very low density lipoprotein (VLDL) and atherogenic Index (AI) (Ahmed *et al.,* 2017).

$$\text{VLDL} = \frac{\text{Triglyceride}}{5}$$

$$\text{AI} = \frac{\text{TC} - \text{HDL}}{\text{HDL}}$$

2.6.6 Measurement of change in body weight

The animal's body weight change was assessed by comparing its initial and final weights at the beginning and conclusion of the therapy.

2.6.7 Measurement of antioxidant parameters

10% tissue homogenate was prepared in ice cold potassium chloride (0.15) and centrifuged at 10,000 rpm at 40°C for 15 min. The tissue supernatant was collected with a micropipette and stored at -20°C for further investigation of numerous antioxidant markers, including TBARS, glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD), as previously reported (Ahmed *et al.,* 2017).

2.6.8 Histopathological assessment

After collecting blood samples, the animals were euthanized and their organs, specifically the pancreas and kidney, were promptly removed. Organs were washed with DNS immediately and then preserved in a 10% formalin solution at -20°C until processing. The tissues were fixed in paraffin, and 4 mm thick slices were cut on a semi-automated rotator microtome (Ahmed *et al.,* 2015).

2.7 Statistical analysis

Data was collected as Mean ± SEM and analyzed using one-way ANOVA and the bonferroni test to identify significant differences between groups. ***p<0.001, **p<0.01 and *p<0.05 were deemed statistically significant. The statistical analysis was carried out using Graph Pad Prism (5.0) (San Diego, California).

3. Results

3.1 Effect of monotherapy as well as triple therapy on α-amylase and α-glucosidase

The percentage inhibition activity of α-amylase is produced as 57.2%, 63.2%, 64.8% and 96.2% for empagliflozin (10 mg/kg), sitagliptin (10 mg/kg), cholestyramine (5 mg/kg) and triple combination of all three drugs respectively. Concluding the monotherapy of three different drugs as well as the triple combination shows specific á-amylase inhibitory activity but the triple combinatorial therapy showed the highest inhibitory effect (96.2% ± 1.5%) (Figure 2A). In the same manner, the triple combinatorial therapy exerts best result (82.7% ± 1.16%) with highest α-glucosidase inhibition activity. The other monotherapy drugs namely empagliflozin (10 mg/kg), sitagliptin (10 mg/kg) and cholestyramine (5 mg/kg) results as 71.6%, 63.6% and 60.9%, respectively. Therefore, the triple combinatorial therapy favors the best α-amylase and α-glucosidase inhibitory outcome in STZ treated diabetic rats (Figure 2B).

3.2 Effect of monotherapy as well as triple therapy on serum glycemic control and serum insulin

The results obtained from (Figure 3 A) show a significant increase in blood glucose levels in the diabetic treated group versus to the normal control group. Although, the declined levels of blood glucose is observed in monotherapy drug, *i.e.*, empagliflozin (10 mg/kg), sitagliptin (10 mg/kg), cholestyramine (5 mg/kg) but the best blood glucose lowering results produced from the group receiving triple combinatorial therapy to a significant level (p<0.001) compared to toxic diabetic group and other individual drug therapy. Furthermore, (Figure 3.B) confirms the low level of insulin in diabetic untreated group compared to control treated rats. Significant elevation of plasma insulin observed in different monotherapy drug groups, however, the maximum enhancement with significant level (p<0.01) was reported from triple combinatorial therapy group with addition to standard group receiving metformin.

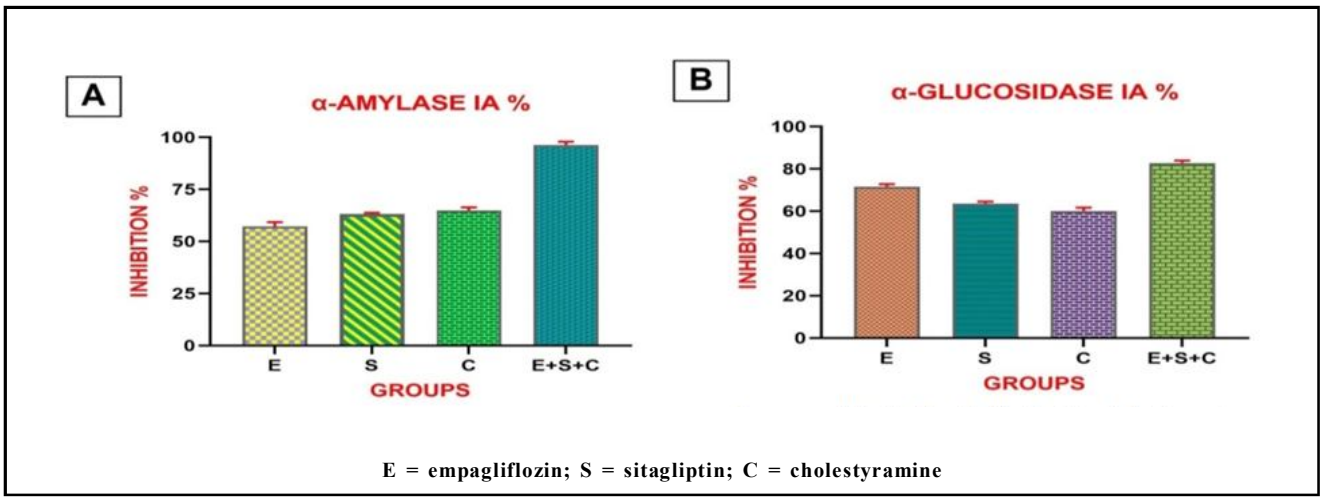

Figure 2: ***In vitro*** **inhibitory affect (IA) of monotherapy and triple combination of test drugs, A-α-Amylase Inhibitory activity; B-α-glucosidase inhibitory activity.**

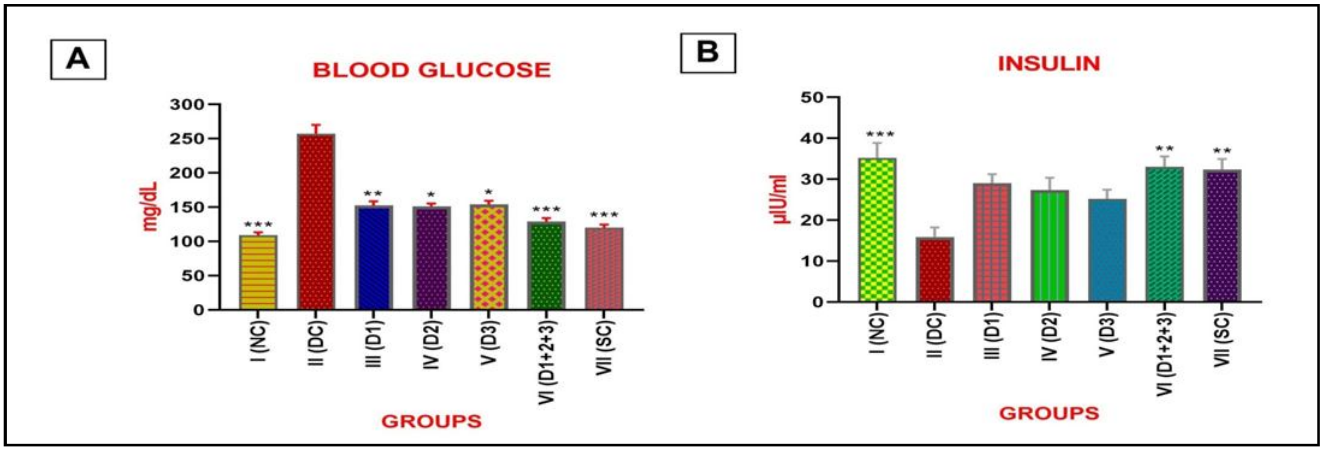

Figure 3: Effect of monotherapy and triple combination of test drug on A= Blood glucose levels and B= Insulin levels. NC=Normal control; DC=Diabetic control (STZ); D1= empagliflozin (10 mg/kg); D2= sitagliptin (10 mg/kg); D3= cholestyramine (5 mg/kg); SC=Standard control (metformin; 25 mg/kg). (Values are Mean ± SEM), n=6. Comparisons were made on the basis of the one-way ANOVA, followed by bonferroni test. (*p<0.05, **p<0.01, ***p<0.001).

3.3 Effect of monotherapy as well as triple therapy on oral glucose tolerance test (OGTT)

The OGTT output (Table 2) of the research clearly indicates that the triple drug combinatorial therapy [empagliflozin (10 mg/kg) + sitagliptin (10 mg/kg) + cholestyramine (5 mg/kg)] as well as standard metformin remarks a significant level (p<0.01) after 2 h (120 min) of oral administration of glucose load of 2 mg/kg compared to diabetic control.

Table 2: Effect of monotherapy and triple combination of test drug on plasma oral glucose tolerance test (OGTT) of the STZ treated diabetic rat

Time (in min)	NC	DC	D 1	D 2	D 3	D (1+2+3)	S C
-60	106.4*	244.8	153.6*	155	169.6*	121***	123.4**
0	101.8*	234.8	147.6*	150.2*	161.6*	111.4**	118.6**
45	144*	288.6	175.8*	186.2*	216*	156.6**	161.2**
60	127.8	265	163.6*	168*	202	138.6**	140**
120	111*	242.6	156.2*	157.8	179.4*	126.4**	127.6 **

NC = Normal Control; DC = Diabetic Control; D1 = empagliflozin [E]; D2 = sitagliptin [S]; D3 = cholestyramine [C] ; SC = Standard MetforminValues are Mean ± SEM. n=6. Comparisons were made on the basis of one way ANOVA, followed by bonferroni Test. * p<0.05 ; ** p<0.01 ; *** p<0.001.

3.4 Effect of monotherapy as well as triple therapy on lipid profile

Diabetic group deteriorates the lipid profile (227.8 ± 10.36), whereas it is observed that the group receiving treatment either monotherapy or triple combination, there is a significant decrease in blood cholesterol. However, the triple combination therapy shows the most approving results (127.45.33) with significant level (p<0.001) in STZ.

STZ treated diabetic group worsen the triglyceride level in blood. The advantageous results were observed with monotherapy and triple combination drug therapy but the best significant level (p<0.05) of triglyceride was reported through triple drug combination.

Serum HDL levels was observed to get decreased whereas LDL, VLDL and AI gets increased in toxicant streptozotocin treated diabetic rats. The treatment group both monotherapy and triple combinatorial therapy shows progressive results in increasing HDL level and decreased LDL, VLDL and AI levels. However, the best promising results were evaluated through triple combination therapy with HDL (25.10.37), LDL (76.75.16), VLDL (25.50.72) and AI (4.10.28) with significant levels (p<0.01), (p<0.001), (p<0.05) and (p<0.001), respectively (Table 3).

Table 3: Lipid profile of monotherapy and triple combination of test drug and standard metformin of the STZ treated diabetic rat

Groups	Treatment	Total Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	AI (mg/dl)
I	**NC**	118 ± 3.89**	110.3 ± 2.48***	25.9 ± 0.18**	70.1 ± 4.07***	22.1 ± 0.49**	3.6 ± 0.15***
II	**DC**	227.8 ± 10.36	197.5 ± 7.65	16.6 ± 0.87	171.7 ± 10.4	39.5 ± 1.52	12.9 ± 0.85
III	**D1**	168.8 ± 6.47**	174.6 ± 6.14	23.1 ± 1.5	110.7 ± 6.68	34.9 ± 1.22	6.4 ± 0.45*
IV	**D2**	163.6 ± 6.32**	175.3 ± 11.4	23.6 ± 0.24*	104.9 ± 7.84	35.1 ± 2.27	5.9 ± 0.27*
V	**D3**	184.8 ± 5.51**	189.8 ± 6.23	22.5 ± 1.13	124.3 ± 5.47	37.9 ± 1.24	7.3 ± 0.46
VI	**D (1+2+3)**	127.4 ± 5.33***	127.7 ± 3.63 **	25.1 ± 0.37**	76.7 ± 5.16***	25.5 ± 0.72**	4.1 ± 0.28***
VII	**SC**	141.4 ± 3.59***	173.8 ± 3.93*	24.9 ± 0.35**	81.6 ± 3.07**	34.8 ± 0.78*	4.6 ± 0.16***

NC = Normal Control; DC = Diabetic Control; D1 = empagliflozin[E]; D2 = sitagliptin [S]; D3 = cholestyramine [C] ; SC = Standard MetforminValues are Mean ± SEM. n=6. Comparisons were made on the basis of one way ANOVA, followed by bonferroni Test. *p<0.05 ; **p<0.01 ; *** p<0.001

3.5 Effect of monotherapy as well as triple therapy on antioxidants

Increased value of TBARS (353.46 ± 34.28 Nm of MDA/mg of protein) and down regulation of SOD (0.69 ± 0.3 unit of SOD/mg of protein), CAT (2.18 ± 0.58 nM of H_2O_2/min/mg of protein) and GSH (1.61 ± 0.35 µg %) was observed in STZ treated diabetic group. However, the levels get restored with the treatment of individual drugs but 2-3 folds productive restoration were observed by triple combinatorial therapy with value of restored TBARS (122.3120.89) with significant level (p<0.001), SOD (1.540.00) with significant level (p<0.05), CAT (3.960.34) and GSH (2.810.33) (Table 4).

Table 4: Antioxidant effect, level of glycogen and weight variation of monotherapy and triple combination of test drug and standard metformin of the STZ treated diabetic rat

Parameters/Groups	I	II	III	IV	V	VI	VII
Treatment	**NC**	**DC**	**D1**	**D2**	**D3**	**D (1+2+3)**	**SC**
TBARS (Nm of MDA /µg of protein)	100.96 ± 14.92***	353.46 ± 34.28	163.46 ± 11.42*	186.73 ± 8.69*	261.34 ± 25.22*	122.31 ± 20.89***	135.77 ± 9.27***
GSH*10^{-4} (µg%)	3.06 ± 0.43*	1.61 ± 0.35	2.16 ± 0.33	2.3 ± 0.31	2 ± 0.58	2.81 ± 0.33*	2.52 ± 0.39*
SOD (unit of SOD/ µg of protein)	1.65 ± 0.14*	0.69 ± 0.3	1.22 ± 0.12	1.14 ± 0.13	0.92 ± 0.12	1.54 ± 0.00*	1.41 ± 0.00*
Catalase (nM of H_2O_2 disappeared /min/µg of protein)	4.21 ± 0.29	2.18 ± 0.58	3.49 ± 0.75	3.44 ± 0.82	3.25 ± 0.56	3.96 ± 0.34	3.64 ± 1.57
Liver glycogen (mg/gm)	72.57 ± 2.19***	30.13 ± 1.63	55.25 ± 1.95*	48.74 ± 1.97	45.33 ± 2.65	61.67 ± 2.13**	65.99 ± 1.69**
			Initial weight				
Weight variation (in gm)	214.8 ± 9.58	201 ± 11.6	216.6 ± 12.02	168.8 ± 8.92	171.4 ± 5.72	232.8 ± 7.91	199.4 ± 7.44
			Final weight				
	217.4 ± 9.32	155.4 ± 6.15	207.4 ± 11.65	163 ± 8.41	167 ± 4.7	214.8 ± 7.77	214.8 ± 7.14

NC = Normal Control; DC = Diabetic Control; D1 = empagliflozin[E]; D2 = sitagliptin [S];D3 = cholestyramine [C] ; SC = Standard MetforminValues are Mean ± SEM. n=6. Comparisons were made on the basis of one way ANOVA, followed by bonferroni test. *p<0.05 ; **p<0.01 ; ***p<0.001.

3.6 Effect of monotherapy as well as triple therapy on liver glycogen and body weight

Liver glycogen gets deteriorated with STZ induced animals and the same results were restored when given with monotherapy as well as triple combinatorial therapy. Same as other parameters, triple combination therapy proves itself superior to other test treatments (61.67 ± 2.13) with significant level (p<0.01) (Table 4). With respect to body weight, individual weight of rats from each group was taken at the start and end of the treatment schedule. As it is clearly noticeable from (Table 4) there is a decrease in weight in rat within diabetic group whereas triple combinatorial therapy proved notable outcome.

3.7 Effect of monotherapy as well as triple therapy on tissue histology

Pancreas: Normal control group reveals usual histopathological structure. Pancreatic acini were surrounded by several rounded islets of Langerhans as well as islets surrounded with prominent nuclei with well-arranged lobules as a normal pattern. Toxic group receiving STZ disclosed damaged pancreatic acini and islets resulting in damaged pancreatic β cells. Treatment either with monotherapy or triple combinatorial therapy produces positive impact towards damage but the triple combinatorial therapy marked a remarkable reduction to damaged β cell (Figure 4).

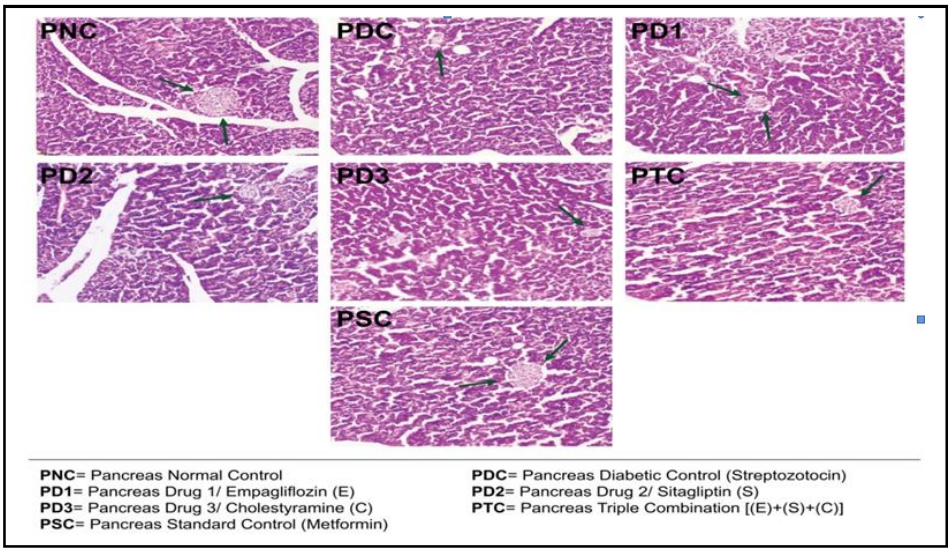

Figure 4: Effect of monotherapy and triple combination of test drug on pancreatic tissue histology following H&E stains under 20X magnification.

Kidney: Prominent glomeruli, ascending and descending loop of henle, collecting ducts and other morphological features of kidney observed in normal control group. Diabetic untreated group indicates the presence of damaged glomeruli, glomeruli deposited with crystals and infiltration of RBC. With triple combinatorial therapy, optimum removal of crystal deposition, restoration of damaged glomeruli and other correlated features of kidney were observed (Figure 5).

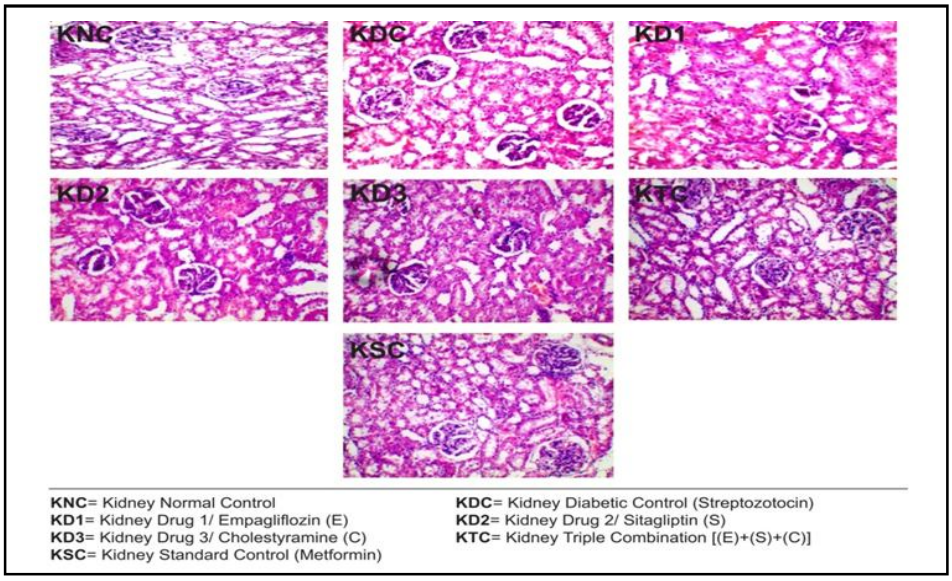

Figure 5: Effect of monotherapy and triple combination of test drug on kidney tissue histology following H&E stains under 20X magnification.

4. Discussion

Diabetes is an intricate, multifaceted, polygenic, endocrinal disease. Key aspect of type 2 diabetes is body's irresponsiveness/resistance towards insulin which concludes in lacking action potential of insulin in body. Henceforth, preference of animal model which mimics human type 2 diabetes should be cautionary considered. Although, the animal model could not be identical to human syndrome but n-STZ models mimics best as an experimental model to type 2 diabetes (Arulmozhi *et al.*, 2004).

Drug is an essential element of healthcare system. In case of ailments, either single or multiple, one or more medicines often used for therapeutic efficacy. When more than one drug is pooled in a single dose in a fixed amount/concentration it is termed as fixed dose combination (FDCs). FDCs are accepted in case they proof themselves beneficial in conditions like (a) synergistic effect among drugs, (b) reducing adverse effect, (c) combating pill burden, (d) improved pharmacokinetics, (e) economical benefits (Kumar and Ramchandra, 2016). FDCs should be carefully justified and clinically relevant.

A hypothesis regarding the synergistic impact of the triple combination mentioned is illustrated in Figure 1. Empagliflozin, acting as SGLT2 inhibitor, hinders glucose reabsorption at the S1 segment of the proximal tubule, leading to increased glycosuria (Nguyen *et al.*, 2024). This process boosts glucagon synthesis, potentially raising blood glucose levels, but this effect is counteracted by sitagliptin, a DPP-4 inhibitor. Sitagliptin primarily blocks DPP-4 enzyme activity, which otherwise deactivates incretin, responsible for insulin secretion (Vella, 2012). Additionally, cholestyramine, a bile acid sequestrant, aids in removing cholesterol (Lent-Schochet and Jialal, 2024), while Sitagliptin's incretin stimulation contributes to reducing cholesterol levels, aligning with the favorable EMPA-REG outcomes observed with empagliflozin.

An *in vitro* outcome of this present research exertion confirms the best lucrative anti diabetic property of triple dose combinatorial therapy over other monotherapy drug treatment. Any individual drug or a combination of drug inhibits the activity of α-amylase and α-glucosidase is stated among major mechanism possessing anti diabetic efficacy. Herewith, although monotherapy treatment drug possess inhibiting α-amylase and α-glucosidase but the most leading inhibition was reported by triple dose combinatorial therapy. Therefore, triple dose treatment proved to be more virtuous in managing type 2 diabetes mellitus (Ali *et al.*, 2013).

STZ is a crucial chemical used for inducing diabetes in rats. Variance in dose concentration used to induce different type of diabetes, *i.e.,* a single high dose of STZ used to induce type 1 diabetes whereas type 2 induced either by multiple low dose of STZ with high fat diet (Zhang *et al.,* 2008) or STZ in addition with chemical like Nicotinamide (Ahmed *et al.,* 2017). In this research exertion, the neonatal rat model (n2-STZ) used which is reported to imitate human diabetes to its best Arulmozhi *et al.* (2004). During this study it is observed that 90 mg/kg dose (Hafizur *et al.* 2015) (Barragán-Bonilla *et al.,* 2019), do induced glucose intolerance as well as other key characteristics of diabetes in test animals. The correct i.p. dosage to neonatal pups was a crucial task of this research and the practical implementation with maximum possible precaution was effectively done resulting successful matured diabetic rats with negligible mortality (5%).

The present study indicates that the STZ treated neonatal pups after attaining adulthood shows an increased blood glucose concentration. Drug given as a monotherapy lowers the glucose concentration but with triple drug FDCs shows a remarkable blood glucose lowering concentration with a significant level (p<0.001). As per outcome received, our result supports that when empagliflozin, sitagliptin and cholestyramine combined as a single FDC drug reduces the blood glucose level effectively than the monotherapy of same individual drugs.

The OGTT is a widely used test for evaluation of evident insulin release/resistance. In this study OGTT was estimated with 2 g/kg loading dose of glucose (Chaimum-aom *et al.* 2017) and the blood samples were further analyzed at -60, 0, 30, 45, 60 and 120 minutes, respectively. The single FDC of triple drug therapy produced significant effect in STZ treated diabetic rats. It was compared with Metformin which has been used as standard to treat diabetes and enhance insulin release.

Deteriorated lipid profile was also observed parallel to type 2 as well as increased worsening of coronary artery disease (CAD). Diabetic group deteriorates the lipid profile (227.8 ± 10.36), whereas it is observed that the group receiving treatment either monotherapy or triple combination, there is a significant decrease in blood cholesterol. However, the triple combination therapy shows the most approving results (127.45.33) with significant level (p<0.001) in STZ.

STZ treated diabetic group worsen the triglyceride level in blood. The advantageous results were observed with monotherapy and triple combination drug therapy but the best significant level (p<0.05) of triglyceride was reported through triple drug combination.

Elevation in the reactive oxygen species (ROS) is widely recognized in the development as well as progression of diabetes mellitus. As per Brownlee's concept free radical production brought on by hyperglycemia at the mitochondrial level is believed to be the primary cause of the diabetes oxidative stress cycle. Increased TBARS levels in STZ induced group, furthermore diminished SOD, CAT, and GSH levels signify increased free radical production. Lipid hydro peroxidase and hydrogen peroxide levels can be decreased by the additional antioxidant glutathione peroxidase (GSH). By altering these proteins and causing induced oxidative stress, STZ induced diabetic rats with higher blood glucose levels can inactivate the SOD, CAT, and GSH levels, which in turn can lead to lipid peroxidation. The level of SOD, CAT, and GSH activities in STZ induced diabetic rats treated with various combinations of empagliflozin, sitagliptin, and cholestyramine were nearly restored to normal levels in the control group of rats, demonstrating the effectiveness of the combinational drug in reducing oxidative stress in diabetic rat.

Liver glycogen levels were markedly diminished in the STZ treated group compared with the control treated group. As it was clearly stated by Ahmad *et al.* (2018) that elevated insulin levels increases intracellular glycogen deposition by inhibiting glycogen phosphorylase and stimulating glycogen synthase activity. However, it was clearly observed that with significant level of (p<0.01) triple combinatorial therapy regimens proves itself best in glycogen correlating effect with other single monotherapy drugs.

With respect to body weight, the considerable effect of triple combination in reducing body weight was not necessarily connected to lower food consumption, but the calorie loss associated with this

increased glycosuria is probable to be a primary source of the weight reduction reported in the STZ treated animals.

STZ's diabetogenic effect is a direct result of permanent damage to the pancreatic β cells, resulting in degranulation and loss of insulin secretion (Alaofi, 2020; Zafar and Naqvi, 2010). From this presented study, it is observed that the toxic group receiving STZ displayed damaged pancreatic acini and islets resulting in damaged pancreatic β-cells while treatment with monotherapy or triple combinatorial therapy produces positive impact towards damage, but the triple combinatorial therapy proved to be a remarkable reduction to damaged β-cell (Figure 4).

STZ induced diabetes is typically associated with a substantial kidney hypertrophy index because to hypoinsulinemia, hyperglycemia and muscle protein loss (Alaofi, 2020) Diabetic untreated group indicates the presence of damaged glomeruli, glomeruli deposited with crystals and infiltration of RBC. With triple combinatorial therapy, optimum removal of crystal deposition, restoration of damaged glomeruli and other correlated features of kidney were observed (Figure 5).

5. Conclusion

Concludingly, authors wish to assert that the evidence presented in this study validates our hypothesis that the administration of a triple dose combination involving empagliflozin, sitagliptin, and cholestyramine resulted in enhanced glycemic control, improved lipid management, and a reduction in oxidative stress.

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Abbreviations

API	-	Active Pharmaceutical Ingredient
BAS	-	Bile Acid Sequestrants
DPP-4	-	Dipeptidyl-Peptidase 4
GIP	-	Gastric Inhibitory Polypeptide
GLP-1	-	Glucagon like peptide-1
H&E	-	Hematoxylin & Eosin
HbA1C	-	Glycated Hemoglobin
HMG-Co-A	-	Hydroxymethylglutaryl-Co-A
IAEC	-	Institutional Animal Ethical Committee
IDDM	-	Insulin Dependent Diabetes Mellitus
IDF	-	International Diabetes Federation
NIDDM	-	Non- Insulin Dependent Diabetes Mellitus
PEPCK	-	Phosphoenolpyruvate carboxykinase
SGLT2	-	Sodium glucose co transporter
SHP	-	Small Heterodimer Partner
STZ	-	Streptozotocin
WB	-	Western Blotting

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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