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Zinc, Selenium, and Vitamin E Combination Ameliorate Carbohydrate Metabolic Enzymes and Liver Antioxidant Activities in Diabetic Rats through Regulation of Glycogen Synthase Kinase 3 Beta and Pyruvate Kinase M2 Genes Expression

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Abstract:
The ameliorative effects of micronutrients and multi-vitamins in decreasing diabetes complications along with their role in regulating carbohydrate metabolic enzymes and antioxidant status of the liver have become the focus of several studies. Consequently, the current study was conducted to evaluate the efficiency of zinc, selenium and vitamin E supplementation either separately or in combinations in the regulation of carbohydrate metabolism, antioxidant status and oxidative stress in streptozotocin-induced diabetes in rats. For this purpose, a total of 48 male Wister albino rats (200-250 gm each) were divided equally into six groups: (Group I) controlled rat with a normal diet, (Group II) diabetic rats, (Group III) diabetic rats supplemented with vitamin E, (Group IV) diabetic rats supplemented with zinc, (Group V) diabetic rats supplemented with selenium and (Group VI) diabetic rats supplemented with a combination of vitamin E, zinc and selenium. After one month of diabetic induction, blood samples were collected to measure insulin level while liver and leg tissue samples were collected to evaluate oxidative stress markers (malondialdehyde and nitric oxide), antioxidant markers (superoxide dismutase and glutathione peroxidase), as well as SYBR Green real-time PCR for pyruvate kinase m2 (Pkm2), and glycogen synthase kinase 3 beta (GSk 3beta) genes expression. The results revealed that supplementation of vitamin E, zinc, and selenium in combination significantly decreased MDA level and PKm2 expression with a marked increase in insulin level, antioxidant markers (SOD and GPx), and GSk3 beta gene expression when compared with diabetic group. These findings highlighted the potential hypoglycemic effects of vitamin E, zinc, and selenium in combination and their role in regulating carbohydrate metabolism through regulation of Pkm2, GSk3 beta gene expression, and in ameliorating the antioxidant status of liver tissues.

Keywords: Diabetes, vitamin E, zinc, selenium, carbohydrate metabolism, oxidative stress.

INTRODUCTION

Diabetes mellitus is a metabolic disease characterized by an increase in blood glucose level, disorders of carbohydrates, proteins, and fats metabolism and is caused by the lack of insulin secretion or a decreased sensitivity to insulin (Lin and Sun, 2010). World Health Organization (WHO) estimated that 3.4 million people worldwide died from diabetes-related complications. The prevalence of DM continues to increase and is predicted to be the seventh main cause of death in 2030 (International Diabetes Association, 2010). Diabetic complications are common in type 1 or type 2 diabetic patients and are divided into microvascular and macrovascular complications (Deshpande *et al.*, 2008). Microvascular complications include neuropathy and retinopathy, while macrovascular complications include cardiovascular disease, stroke, peripheral artery disease (PAD), and increased oxidative stress which has a role in the development and progression of diabetes and its complications (Ceriello, 2000). Moreover, diabetes is associated with increased production of free radicals or impaired antioxidant defenses (Bajaj and Khan, 2012). Oxidative stress can be reduced through the administration of nutritional supplementation of antioxidants which can help protect tissues from ROS damage (Ramkumar *et al.*, 2007). This supplementation includes micronutrient which is a vital component of antioxidant enzymes as (copper, zinc) in superoxide dismutase (Ighodaro and Akinloye, 2018) and the selenium in case of glutathione peroxidase (Nazıroğlu, 2009). Another micronutrient that is considered as an antioxidant is vitamin E which has a role in the progression of diabetes (Balbi *et al.*, 2018). This supplementation may have a protective role in decreasing the incidence of various degenerative diseases, such as diabetes and its complications (Ramkumar *et al.*, 2007). In addition to diabetes-related oxidative stress, DM is also associated with a disorder in carbohydrate metabolic enzymes. GSk3β and pkm2 are two important enzymes involved in the maintenance of a normal plasma glucose concentration. GSk3beta is a phosphorylating and an inactivating agent for the glycogen synthase, with a resultant increase in glycogen synthesis (Ilouz *et al.*, 2002). In diabetic animals, the activity of GSK3 β was increased in peripheral tissues (Nikoulina *et al.*, 2000) . and GSK3beta inhibitor can be used in treating diabetes in obese diabetic mice (Rao *et al.*, 2006). Moreover, transgenic overexpression of GSK-3β in the skeletal muscles of mice resulted in impaired glucose tolerance, elevated plasma insulin levels, and reduced glycogen content (Pearce *et al.*, 2004). Conversely, GSK-3 inhibitors can mimic insulin action in cell lines and tissues (MacAulay *et al.*, 2003)**.** Pyruvate kinase catalyzes the last step of glycolysis (conversion of phosphoenolpyruvate (PEP) to pyruvate) which yields ATP (Gupta and Bamezai, 2010). Diabetes and high glucose levels induced mitochondrial dysfunction which was reversible with PKM2 activator (Sharma 2015; Susztak, 2013). In general talking, several micronutrients have an important role in regulating carbohydrate metabolic enzymes. Zinc, an essential nutrient, has insulin coadjuvant properties and can enhance carbohydrate metabolism through increasing glucose uptake and transport in several tissues, inhibiting pancreatic insulin secretion and increasing pancreatic insulin content (Siddiqui *et al.*, 2014). On the other hand, Selenium has cytoprotective properties because it can arrange antioxidant selenoenzymes. Se supplementation could prevent the onset of metabolic diseases, such as type 2 diabetes (T2D), by counteracting the oxidative stress. It was found to act as insulin-mimetic, exhibiting anti-diabetic effects (Stapleton, 2000). Vitamin E is non-enzymatic antioxidant which protects cell membrane especially against lipid peroxidation (Sumien *et al.*, 2003). Vitamin E supplementation can improve glucose metabolism in muscle cells and the circulation to the islets of Langerhans and other tissues (Soliman, 2013). Therefore, the current study was conducted to evaluate the potential protective effects of vitamin E, zinc and selenium in decreasing the severity of diabetes

in rats through regulation of glucose uptake and carbohydrate metabolism by controlling PKm2 and GSk3beta gene expression and ameliorating the antioxidant status of liver tissues during the pathogenesis of diabetes.

MATERIALS AND METHODS

Experimental animals

A total of forty-eight male waster albino rats weighting (250 ±50 gm) were obtained from the Medical Experimental Research Center (MERC) at the faculty of medicine, Mansoura University, Egypt. The rats were conditioned in standard polypropylene cages (five rats/cage) with 12-h light/dark cycles at room temperature of 25 ± 2 °C. All animals were fed with pellet diet prepared according to the NRC (1995) and obtained from MERC, Faculty of Medicine, Mansoura University, Egypt.

Animal Ethical Approval Permission

All Institutional and National Guidelines for the care and use of animals were followed according to the Egyptian Medical Research Ethics Committee (no. 14–126).

Chemicals

Vitamin E was purchased from Pharco Company, Cairo, Egypt in the form of capsules containing 1000 mg per capsule. Zinc chloride and Sodium selenite were obtained from sigma company, Cairo, Egypt.

Experimental induction of STZ Diabetes Model

For induction of diabetes, a single intraperitoneal injection of streptozotocin (STZ) was injected to the rats at a low dose of 65 mg/kg body weight (STZ was freshly dissolved in 0.05 M citrate buffer, PH 4.5) (Vats *et al.*, 2004). The development of hyperglycemia was confirmed by the increased fasting blood sugar (FBS). The blood was taken from the tail vein, and glucose level was determined at 72 h and then on day seven after injection. Overnight fasting rats with a threshold value of FBS level >250 mg/dl by one week following injection were considered diabetic (Andrade Cetto *et al.*, 2000).

Experimental design and Animal Grouping

Forty-eight male albino rats were divided into 6 groups (8 rats in each group) Group I: control group (normal healthy rats) which received normal diet, Group II: diabetic group which was injected with a single intraperitoneal dose of STZ (65mg/kg body weight), Group III: diabetic group which administered vitamin E by stomach tube (1000mg/kg body weight) for 30 days (Lemoyne *et al.*, 1987). Group IV: Diabetic group-administered zinc in the form of zinc chloride ($ZnCl_2$) by stomach tube (180 mg/kg body weight) for 30 days (Blalock *et al.*, 1988). Group V: diabetic group which administered selenium in form of sodium selenite by stomach tube (1.5 mg/kg body weight) for 30 days (Kahya *et al.*, 2015; Yazici *et al.*, 2014) and Group 6: diabetic group which administered a combination of vitamin E (1000mg/ kg body weight) + zinc chloride (100 mg /kg body weight) + sodium selenite (1.5 mg /kg body weight) by stomach tube for 30 days.

Determination of insulin level

After 30 days of micronutrient supplementation, blood samples were collected through cardiac puncture and placed into a sterile, dry, capped tube then left in a vertical position at room temperature. Centrifugation was performed at 3000 rpm for 15 minutes and then the clear, straw-colored serum sample was aspirated using an automatic pipette and kept in the deep freezer at -20°C. Serum samples were used for determination of insulin level by immulite 1000 according to Chevenne *et al.* (1998) using kits from sigma company, Cairo, Egypt.

Determination of liver tissue antioxidant activities and oxidative stress markers

The right lobe of the liver from each animal was dissected and perfused with phosphate-buffered saline (PBS) solution (pH 7.4), then homogenized with 5 -10 ml cold buffer (i.e., 50 mM potassium phosphate, pH 7.5) per gram tissue. Samples were centrifuged at 4000 r.p.m for 15 min. The supernatant was removed and used for estimation of nitric oxide level according to Montgomery and Dymock (1961), malondialdehyde level according to Satoh (1978) and Ohkawa *et al.* (1979), Superoxide dismutase activity according to Nishikimi *et al.* (1972) and glutathione peroxidase activity according to Paglia and Valentine (1967). All chemical kits were obtained from Sigma Company, Cairo, Egypt.

SYBR Green real-time PCR

A small section of the left lobe of the liver and left leg muscle from each rat were collected and preserved in RNA later solution to determine Glycogen Synthase Kinase 3 beta gene (GSK3 β) as well as pyruvate kinase m2 gene (PKm2), respectively. Total RNA was extracted from collected tissues (RNeasy Mini Kit) (QIAGEN Company) according to the manufacturer's instructions. Quantification of total RNA was performed spectrophotometrically. The expression of liver GSK3β gene and muscle PKm2 were determined by RT-PCR of total RNA. Six oligonucleotide PCR primers specific for GSK3 β, PKm2, and rat β-actin were used in this study as shown in Table 1. The 25 µl reaction mixture contained 12.5 µl2x QuantiTect SYBR Green PCR Master Mix, 0.25 Reverse transcriptase, 0.5µl of forward and reverse primer (20 pmol), RNase Free water 8.25µl and 3µl of RNA template. The Reverse transcription was done at 50˚C for 30 min followed by amplification conditions included 40 cycles with primary denaturation at 94˚for 15 min, secondary denaturation at 94˚C for 15 sec, primer annealing for GSK3beta at 60˚C for 30 sec and then extension at 72˚C for 30 sec. Amplification conditions for PKm2 gene included 40 cycles with Primary denaturation at 94˚for 15 min, secondary denaturation at 94˚C for 15 sec, primer annealing at 59˚C for 30 sec and then extension at 72˚C for 30 sec.

Table 1. The sequence of forward and reverse primers used in the determination of the gene expression of studied genes.

Gene	Primer sequence (5'-3')	Reference
Rat ß-actin	TCCTCCTGAGCGCAAGTACTCT	Banni *et al.,* 2010
	GCTCAGTAACAGTCCGCCTAGAA	
GSK3 ß	TCG CCA CTC GAG TAG AAG AAA	Sklepkiewicz *et al.,* 2011
	ACT TTG TGA CTC AGG AGA ACT	
PKM2	AATGGGATCAGATGCAAAGC	Sajic *et al.,* 2013
	CGATCTTGGAGATGCTGAA	

Statistical Analysis

The obtained data were analyzed by GraphPad Prism (Windows version 5.0; GraphPad Software, Inc., San Diego, CA, USA) using the Kruskal-Wallis test with post hoc Dunn multiple comparison tests. $P < 0.05$ was considered statistically significant in all tests.

RESULTS

Effect of vitamin E, zinc and selenium separately or in combination on the insulin level

A combination of Vitamin E, zinc and selenium resulted in a significant increase in serum insulin concentration ($P < 0.05$) and return to a normal level as in control group with a slight increase in insulin level in other groups administered vitamin E, zinc and selenium separately when compared with diabetic group (Table 2).

Table 2. Effect of micronutrients supplementation on serum insulin concentration

Groups	Insulin (µIU/ml)
Group I	5.6 ± 0.28^{a}
Group II	2.1 ± 0.42^{c}
Group III	3.85 ± 0.35^{b}
Group IV	3.85 ± 0.07^{b}
Group V	3.4 ± 0.42^{b}
Group VI	5.3 ± 0.28^{a}

The mean with different letter revealed a significant change ($P<0.05$). Values represent (mean ± SD).

Effect of vitamin E, zinc and selenium separately or in combination on antioxidant activities and oxidative stress markers

A combination of Vitamin E, zinc, and selenium resulted in a more significant increase in GPx and SOD activities ($P < 0.05$) with a rate near to normal level, while other groups showed a slight increase in GPx and SOD activities when compared with diabetic group. Besides, the induction of diabetes increased MDA and decreased NO levels as shown in the diabetic group and micronutrient combination exhibited a significant effect in reducing MDA and increasing NO levels when compared with diabetic group Table 3.

Expression of GSK3 beta and PKm2

Supplementation with Vitamin E + zinc and selenium in combination significantly down-regulated GSK3 beta gene expression while resulted in up-regulation of expression of PKm2 when compared to the diabetic group, with level near to normal levels in control group Figs.1 and 2 respectively.

Table 3. The activity of oxidative stress and antioxidant in micronutrients supplemented diabetic rats

Groups	Glutathione peroxidase activity (µmoles /mg tissue)	Superoxide dismutase activity (µ/ml)	Malondialdehyde concentration (nmol/g.tissue)	Nitric Oxide concentration (mol/g.tissue)
Group I	31.73 ± 3.55^{a}	331.76 ± 20.98^{ab}	28 ± 1.91^{c}	1.46 ± 0.2^{a}
Group II	17.16 ± 2.05^{b}	227.53 ± 16.43^{d}	71.13 ± 3.05^{a}	0.63 ± 0.15^{c}
Group III	30.93 ± 1.98^{a}	288.13 ± 28.5^{bc}	47.46 ± 4.3^{b}	1 ± 1.1^{b}
Group IV	30.83 ± 5.07^{a}	274.2 ± 12.29^{c}	52.16 ± 11.35^{b}	1.1 ± 0.1^{b}
Group V	32.4 ± 4.15^{a}	269.13 ± 44.14^{cd}	45.86 ± 7.96^{b}	1.03 ± 0.15^{b}
Group VI	22.5 ± 3.35^{b}	335.73 ± 18.55^{a}	38.1 ± 3.7^{bc}	1 ± 1.4^{ab}

The mean with different letter revealed a significant change ($P<0.05$).

Values represent (mean ± SD).

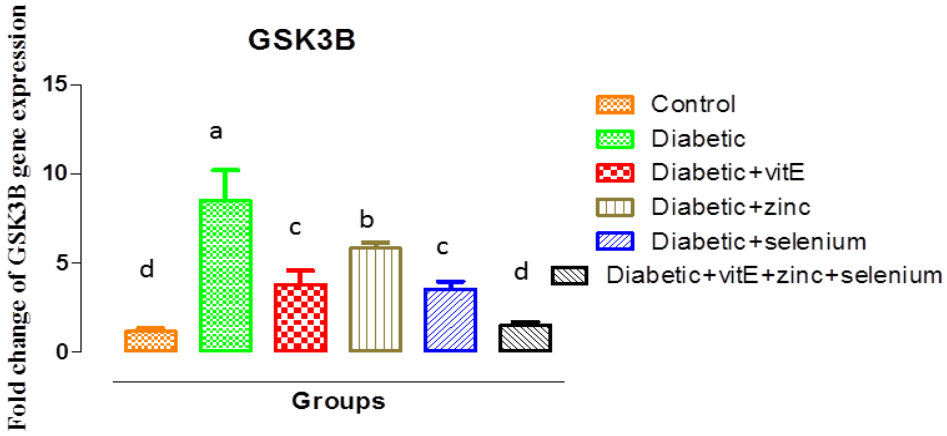

Fig. 1. Effect of vitamin E, zinc, selenium, and their combination on GSK3B gene expression.

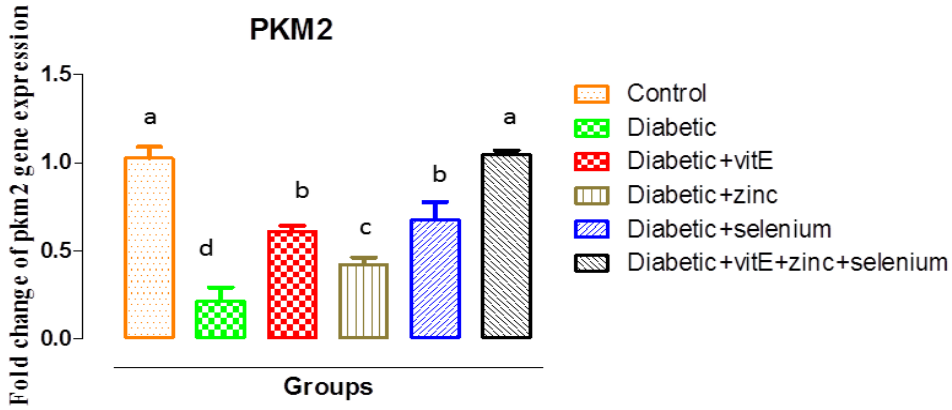

Fig. 2. Effect of vitamin E, zinc, selenium, and their combination on PKm2 gene expression.

DISCUSSION

Complications and pathogenesis of diabetes can result from oxidative stress due to an imbalance between reactive oxygen species (free radicals) production and the ability of antioxidant defense (Ceriello and Motz, 2004). For clarification, under normal physiological conditions, different antioxidant enzymes including glutathione peroxidase-1 (GPx-1), catalase, and superoxide dismutase (SOD) can detoxify ROS and when these intermediates are not efficiently detoxified, they accumulate and interact with different components in the cell including proteins, lipids or DNA resulting in cellular damage or death (Tsukahara, 2007). Antioxidant therapy such as vitamins and minerals is a valuable approach to decrease

diabetic complications (Fadupin *et al.*, 2007). By using streptozotocin to induce diabetes, it results in cytotoxic effects on beta-cells of the pancreas, via a mechanism associated with the generation of ROS causing insulin deficiency (Punitha *et al.*, 2005). In the current study, the improved insulin levels among all groups, in comparison with diabetic groups after treatment with micronutrients combination, may be attributed to the ability of vitamin E to reduce the glycaemic index in diabetes (Bonnefont-Rousselot, 2004), whereas the marked increase in insulin level after zinc chloride administration was due to the correlation between zinc and insulin action which was approved by many studies that reported the role of zinc in prevention of insulin hexamers degradation (Taylor, 2005). Additionally, the selenium normalized serum glucose, insulin, and antioxidant enzyme activity in STZ-induced diabetic mice and suppressed *α*-amylase and *α*-glucosidase activities in vitro gastric and intestinal models (Liu *et al.*, 2015).

According to our study, after the injection of rats with STZ (65mg/kg), we noticed an elevation in MDA as one of the oxidative stress markers. Such increase was due to the production of high levels of oxygen free radicals as a result of glucose autoxidation, protein glycation, and interaction of advanced glycation end products with their receptors in macrophage (Singh *et al.*, 2014). Chu and Bohlen (2004) reported that NO decrease was due to that high level of glucose could suppress the activity of endothelial nitric oxide synthase in glomeruli via protein kinase c associated mechanism. Moreover, the current study stated that STZ caused a reduction in levels of glutathione peroxidase and superoxide dismutase due to glycation. Ahmed (2005) suggested that the decrease in Gpx activity in diabetic rats' liver might be due to oxidative stress which causes exhaustion or inactivation due to insulin deficiency which promotes beta-oxidation of fatty acids resulting in H_2O_2formation. In the setting of decreased GPx activity, H_2O_2 accumulates in the liver and causes Cu, Zn-SOD inactivity. Besides, Cu, Zn-SOD glycation occurs in diabetes causing inactivation of SOD. The integration of all these reasons causes inactivation of GPx (Sharma *et al.*, 2013). Micronutrients and vitamins administered to diabetic rats can strengthen endogenous antioxidant defense against ROS. This study reflects the ability to use zinc, selenium, and vitamin E separately or in combination to reduce MDA levels and increase SOD, GPX, NO, and insulin levels in STZ diabetic rats. Our data are in agreement with Taylor (2005) who reported the role of zinc in the prevention of the degradation of insulin hexamers, improvement of the binding of insulin to liver receptors, and inhibition of the degradation by liver plasma membranes. Moreover, zinc act as a protective agent against free radicals and can decrease lipid peroxidation in several tissues protecting them against increased MDA levels through its ability to bind and stabilize cellular membranes against MDA and degeneration, this is linked to the competition of zinc with other elements such as iron and copper at binding sites (Wang *et al.*, 2012). According to our study, Zinc Chloride controlled hyperglycemia, preserved the liver architecture, ameliorated NO, superoxide dismutase (SOD) and GSH levels and prevented the renal oxidative damage via up-regulation of nuclear factor-erythroid 2-related factor (Nrf2) (Li *et al.*, 2014).

Treatment of diabetic rats with selenium was able to improve the results as reported by Liu *et al.,* (2015). Selenium can normalize serum glucose, insulin, and antioxidant enzyme activity in STZ-induced diabetic mice. Besides, Se has a biological function similar to insulin. Furthermore, the antioxidant role of selenium can delay diabetes development through decreasing oxidative stress markers such as MDA as revealed in the present study (Steinbrenner and Sies, 2009). Also, Kahya *et al.,* (2015) study reported a negative correlation between MDA and serum selenium level. On the other hand, there is an increase in nitric oxide as selenium contains sugar which can enhance antioxidant activity in aortae and prevent hyperglycemia-induced endothelial dysfunction

(Ng *et al.*, 2017). Also, the elevation of antioxidant SOD and GPX is associated with overexpression of PTP1B and decrease of glutathionylation (Mueller *et al.*, 2009). It was found that vitamin E supplementation resulted in an elevation of GPX and SOD (Varvařovská *et al.*, 2004) due to its role as antioxidant making it able to reduce glycemic index in diabetes leading to an increase in insulin level (Bonnefont-Rousselot, 2004). Whereas the increased Nitric oxide is due to the ability of vitamin E to eliminate free radicals and decrease the possibility of interaction between radicals and NO giving peroxide nitrite and thus elevating nitric oxide availability to tissue (Tronchini *et al.*, 2010).In this study, we studied pyruvate kinase M2 and Glycogen synthase kinase 3 beta gene expressions and their role in diabetes and carbohydrate metabolism. GSK-3 was noticed to have an important role in glycolysis. Both hexokinase II (HK II) and GLUT4 have a necessary role in phosphorylation and transport of glucose to glucose 6-phosphate and GSK-3 can regulate HK II via a negative action (Pastorino *et al.*, 2005). In our study, the induction of diabetes increased the expression of GSK-3B. This may be attributed to reducing its phosphorylation and enhancing phosphorylation of Akt (Montanari *et al.*, 2005). Rayasam *et al.* (2009) claimed that the treatment of diabetes with competitive inhibitors of GSK-3 was able to increase glucose tolerance in diabetic mice (Jope *et al.*, 2006). Zinc has an inhibitory effect on GSK-3β through its activity on PI3K/AKT (Ohsaka *et al.*, 2014). The inhibition of Glycogen synthase kinase-3 (GSK-3β), which is a phosphorylating and an inactivating agent of glycogen synthase, increased glycogen synthesis and reduction of the effect of accumulated toxic glucose (Ilouz *et al.*, 2002) .

Gupta and Bamezai (2010) clarified the role of PKm2 in catalyzed last step of glycolysis to give pyruvate and ATP. Moreover, Sharma (2015) explained that diabetes was able to induce mitochondrial dysfunction and result in toxic effects related to hyperglycemia and accumulated glucose which are reversible with PKM2 activator. Supplementation of Vitamin E, Zinc and Selenium can activate PKM2 as Zinc increases the activity of glycolytic enzymes, phosphofructokinase (PFK) and pyruvate kinase (PK) in a concentration- and time-dependent manner (Canesi *et al.*, 2001) and this activation as well as apoptosis in *PKm*-knockdown podocytes mimic the effects of diabetes (Sharma *et al.*, 2013) and is associated with increased metabolic flux through glycolysis which neutralizes the toxic effects related to hyperglycemia.

CONCLUSION

The current study found that the synergistic supplementation with vitamin E, zinc and Se in combination yielded better results than being used separately due to their potent effects in controlling hyperglycemia, serving as antioxidant agents, scavenging free radical, and their role in treatment and improvement of liver damage at the cellular level.

AUTHOR CONTRIBUTIONS

MEA, SAE conceived and designed the experiments. MEA performed the experiments: EEE, SAE analyzed the data. All authors contributed reagents/materials/analysis tools. MEA, SAE wrote the paper. All authors reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in the current research work.

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