ORIGINAL ARTICLE

Decoding Therapeutic Pathways of Micronutriomics: Interaction of Vitamin E-Tocotrienol Rich Fraction (TRF) in Insulin-Resistant Zebrafish Larvae Model (*Danio rerio*)

Nurliyana Najwa Md Razip[1,2] Huzwah Khaza'ai[1], Suzita Mohd Noor[3], Anwar Norazit[3], Norshariza Nordin[1,4]

[1] Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2] Pharmaceutical Services Programme, Lot 36, Ministry of Health Malaysia, Jalan Profesor Diraja Ungku Aziz, Pjs 13, 46200 Petaling Jaya, Selangor, Malaysia
[3] Department of Biomedical Science, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[4] Genetics & Regenerative Medicine (ReGEN) Research Group, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Introduction: This study investigates the intricate crosstalk between type 2 diabetes mellitus (T2DM) and neurodegenerative disorders, an interaction that remains poorly understood. By exploring micronutrient-based therapeutic strategies, particularly the tocotrienol and tocopherol isomers of vitamin E, we aim to evaluate the potential of a "micronutriomic" approach using tocotrienol-rich fraction (TRF)-vitamin E in modulating these interconnected disease pathways. **Materials and methods:** The oxidative stress status was evaluated by malondialdehyde (MDA) and glutathione (GSH) levels. Next Generation Sequencing (NGS) of transcriptome profiling analysis were performed to study the regulation of gene involved in insulin signaling and neurodegeneration. Insulin resistance-zebrafish larvae were treated with TRF-vitamin E (1 ug/mL) or metformin (50 uM) as a positive control. **Results:** Both TRF-vitamin E and metformin significantly decreased MDA levels ($p<0.05$) and increased GSH levels ($p<0.05$). Differential expression analysis in TRF-vitamin E group indicates 202 downregulated genes and 639 upregulated genes from a total of 841 dysregulated genes. Metformin, revealed a total of 1,500 dsyregulated genes with 1,300 were upregulated and 200 were downregulated. A gene ontology study discovered that the TRF-vitamin E group's dysregulated genes were considerably enriched in the calcium pathways. **Conclusion:** The findings suggest that MAPK-CA2+ signaling pathway may be an overlapping mechanism of action between TRF-vitamin E and metformin. Understanding these therapeutic pathwyas in TRF-vitamin E may provide into "micronutriomics" targeted strategies to restore this crosstalk diseases.
Malaysian Journal of Medicine and Health Sciences (2025) 21(6): 1-10. doi:10.47836/mjmhs.v21.i6.1353

Keywords: Type 2 *Diabetes mellitus*, Insulin resistance, Neurodegeneration, Tocotrienol rich fraction, Micronutrients, Vitamin E

Corresponding Author:
Nurliyana Najwa Md Razip, PhD
Email: nurliyananajwa@upm.edu.my
Tel : +603 - 9769 2303

INTRODUCTION

The increasing prevalence of non-communicable diseases, such as Type 2 Diabetes Mellitus (T2DM) and Alzheimer's disease (AD), presents a significant public health concern. In aging populations, the incidence of these metabolic and neurodegenerative disorders continues to rise, negatively impacting patients' quality of life. T2DM is characterized by impaired glucose metabolism, insulin resistance, and hyperglycemia, while AD is marked by beta-amyloid plaques and neurofibrillary tangles in the brain. Notably, individuals with T2DM have a significantly higher risk of developing AD compared to non-diabetic individuals (1). The underlying mechanisms linking these diseases remain unclear. Nevertheless, despite the complexity of their pathogenesis, oxidative stress and inflammation are believed to play pivotal roles in the connection between T2DM and AD.

Oxidative stress, caused by an imbalance between the formation of reactive oxygen species (ROS) has been linked to the development and progression of several diseases, including T2DM (2). ROS generation can induce cellular damage exacerbates insulin resistance and accelerates disease progression. Vitamin E, which is known for its antioxidant therapeutic effects, has been

studied for its potential benefits in reducing oxidative stress and inflammation in T2DM (3). Malaysia, a leading producer of palm oil, provides a natural source of vitamin E, predominantly in the form of tocotrienols. Tocotrienol-rich fractions of palm oil contain 78 to 82% tocotrienols and 18 to 22% tocopherols, making them one of the richest sources of vitamin E (4,5). Studies have suggested that vitamin E supplementation can reduce lipid peroxidation, increase antioxidant enzyme activity (6).

A supplementation of vitamin E at certain dose has been shown to decrease lipid peroxidation in the insulin resistance incidence with the by-product of malondialdehyde (MDA), and increase the activity of antioxidant enzymes, such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) (7). It has also been discovered that this supplementation increases the overall antioxidant capacity and glycemic control in persons with T2DM, potentially delaying the onset and progression (8). Vitamin E also stimulates the circulation of monocytes, which reduces C-reactive protein levels (9), interleukin-1 and -6 as well as tumour necrosis factor alpha (TNFalpha) formations (10,11). Additionally, T2DM has been linked to neurodegeneration; AD, and dementia. It is believed that the chronic inflammation caused by T2DM contributes to AD by causing neuronal damage and cognitive decline (12). The investigation of antioxidant therapy, such as vitamin E supplementation, holds promise for reducing oxidative stress and inflammatory processes in the progression of T2DM (13) associates AD in the context of this study. Yet, the information regarding vitamin E supplementation and T2DM progression is conflicting and inconsistent (14,15,16). The indicator such as sample size, socio-demographic, and socio-economic factors that influence the vitamin E status of study participants. Particularly, in-vivo research illuminates the targeted mechanisms for coping with oxidative stress and, as a result, disrupts the progression of the disease.

In biomedical research, bioinformatics tools have become increasingly valuable for analyzing a large amounts of gene expression data, thereby advancing our understanding of biological processes, molecular activities, and cellular components. These strategies contribute to the advancement of knowledge and have the potential to improve life expectancy and quality of life. In spite of the fact that articles about vitamin E contain a great deal of contradicting material, researchers are still trying to elucidate the targeted biochemical pathway.

The rising prevalence of chronic diseases, particularly T2DM and AD, presents a significant public health challenge. Targeting oxidative stress, inflammation, and gene expression through vitamin E supplementation, combined with bioinformatics analyses, offers a promising strategy to delay disease onset and progression. This approach could enhance the quality of life for affected individuals. In this study, transcriptome RNA sequencing was used to identify interaction genes and elucidate the molecular mechanisms of vitamin E supplementation in insulin resistance-induced zebrafish models.

MATERIALS AND METHODS

Fish husbandry and clearance of animal ethics approval

All experiments were conducted in compliance with the guidelines and regulations approved by the Institutional Animal Care and Use Committee (IACUC) protocol 2019-210404/BMS/SMN of the Faculty of Medicine, University of Malaya. Adult male and female wildtype zebrafish (Danio rerio) were maintained as a stock culture in automated water system in the Department of Biomedical Science, Faculty of Medicine, University of Malaya. In order to induce the fish's reproductive cycle, the fish were kept at temperatures between 25-27o C under a 14:10 light:dark cycle in a recirculating aquaculture system (Techniplast ZebTEC, Italy). Collected eggs were incubated at 28oC in egg buffer or known as E3 media (15 mM sodium chloride (NaCl, Sigma-Aldrich, St Louis, USA), 0.5 mM potassium chloride (KCl), 1 mM magnesium sulphate (MgSO4, Sigma-Aldrich, St Louis, USA), 0.15 mM potassium dihydrogen phosphate (KH2PO4, Sigma-Aldrich, St Louis, USA), 0.05 mM disodium phosphate (Na2HPO4, Sigma-Aldrich, St Louis, USA), 1 mM calcium chloride (CaCl2, Sigma-Aldrich, St Louis, USA), 0.7 mM sodium bicarbonate (NaHCO3, Sigma-Aldrich, St Louis, USA), pH was set at 7.0). At 2 hours post-fertilization (hpf), the eggs were observed under a conventional stereomicroscope with a 3X magnification (Leica, Microsystems, Germany). The normal fertilised embryos is characterised as those with intact chorion membranes and gastrulation (50% epiboly) (17). Any dead or unfertilized eggs were removed.

Preparation of insulin , human recombinant (E. coli)

Insulin (BioVision, USA) stock was reconstituted with 0.01 N HCl. Subsequently, the insulin desired concentration was diluted in E3 media to 100 nM and 250 nM insulins. These concentrations were selected to induce insulin resistance in zebrafish larvae aged between (72- 96 hpf).

Establishment of insulin resistant model in zebrafish larvae model

To establish an insulin resistance model, zebrafish embryos were placed into a 6-well plate, with each well containing 20 embryos per group. The development of this model in zebrafish larvae has been adopted by the same research group as Md Razip et al. (18).

Zebrafish larvae were exposed to 250 nM insulin at 72 hpf and re-challenge with an additional 100 nM insulin for the next 24 hours (at 96 hpf) to induce the insulin resistance. This exposure was carried out in a total

volume of 1 mL per well, based on the concentration established by Marin-Juex et al. (2014). The control group was maintained in 1 mL of E3 media without any induction or treatment. Fresh media were added to all wells daily, replenishing the solutions every 24 hours at room temperature.

Metformin preparation

The metformin-hydrochloride tablet (Glumet DC Tablet, 500 mg, Pharmaniaga™, Malaysia) was crunched and powdered and diluted in the water. Metformin has a molecular weight of 165.62 g/mol. The final working concentration to treat insulin-resistant zebrafish larvae was set at 50 µM based on the dose from a previous study (19). At 144 hpf, insulin resistance induced-zebrafish larvae were treated with metformin at the target concentration of 50 µM.

Treatment with tocotrienol rich fraction (TRF-vitamin E)

Vitamin E in the form of tocotrienol rich fraction (TRF) with 75% tocotrienol and 25% tocopherol, Gold E70 was purchased from Sime Darby Sdn. Bhd, Malaysia. The TRF-vitamin E stock solution was prepared in 100% ethanol. To achieve 0.1% volume vitamin E to the total volume of medium, vitamin E was prepared in microgram (µg) concentrations. The TRF-vitamin E was prepared in concentrations of 100 µg/mL. The treatment of TRF-vitamin E in insulin resistance induced-zebrafish larvae at concentration of 1 µg/mL were performed at 144 hpf based on previous study conducted by McDougall et al. with slight modifications (20).

RNA extraction and quality control for Next Generation Sequencing (NGS) analysis

Total RNA was extracted with PRIMEZOLTM phenol-chloroform reagent as described in the manufacturer's protocol. Genomic DNA was extracted from RNA samples using DNase I reagent (Canvax, Spain). The RNA quality and RNA integrity number (RIN) were assessed by spectrophotometer NanoDrop (Thermo Fisher, USA). Total RNA of larvae zebrafish was dissolved in 20 µL of RNAse-free water and submitted to the RNA sequencing service company GENEWIZ (Azenta Life Sciences, China) that provide a standard Illumina HiSeq and generated 300 bp pair-end reads of 2x150 base pair configuration with the HiSeq Control Software and GAPipeline-1.6 (Illumina). Fastq was obtained based on the quality of bases lower than 20 which passed filter data of its format using Cutadapt (V1.9.1). The raw RNA-seq data were deposited with NCBI BioProject ID under the accession number PRJNA743929.

Differential expression genes (DEGs) analysis

The DESeq2 Bioconductor and EdgeR (V3.4.6) tools were used for differentially expressed genes (DEG) analysis. Logarithmic fold change dispersion estimates were based on data-driven prior distributions with $p < 0.05$ and FDR < 1.

Gene Ontology (GO) and pathway enrichment analysis (PEA)

The Metascape open tool was used to reveal the interconnected networks in insulin resistance, metformin, and TRF-vitamin E. Gene ontology (GO) and pathway enrichment analysis (PEA) were conducted on the identified DEGs using Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis.

Lipid peroxidation and antioxidant assays

Biochemical assay kit of MDA analysis (Elabscience®, Houston, Texas, USA) evaluates the level of lipid peroxidation produced in the organism. Unsaturated fatty acids (polyunsaturated fatty acids), glycolipids, phospholipids and cholesterols, are easily exposed by the lipid peroxidation in biological system (21). Therefore, the degree of MDA that is produced from lipid peroxidation can reflect the progression of insulin resistance in experimental model.

Total glutathione (GSH) levels were also assessed to investigate the antioxidant effects of vitamin E in alleviating oxidative stress in insulin-resistant zebrafish .larvae In the current study, the OxiSelect™ Total Glutathione (GSSG/GSH) Assay Kit (Cell Biolabs, Houston, Texas, USA) was used to quantitatively measure total glutathione from both reduced GSH and oxidized glutathione (GSSG) in a sample. In principle, a reduced thiol group of GSH provides reducing equivalents for other unstable ROS, which in turn becomes unstable itself. This unstable GSH easily reacts with another unstable GSH to form a stable GSSG molecule. Therefore, the total glutathione was reflected from the GSH/GSSG.

Statistical analysis

Statistical analyzes were performed using a GraphPad Prism (version 9). Data from MDA and GSH analysis were expressed as the mean ± standard deviation (SD). Differences in statistical significance were determined using one-way ANOVA with Tukey's multiple comparison groups and unpaired t-test analysis in each of the parameters. The level of statistical significance was set at a p-value < 0.05. Meanwhile, the data expressed in transcriptomic profiles from NGS results were in accordance with the software generated in the bioinformatics analysis.

RESULTS

Differential Expressed Genes (DEGs)

To identify the differentially expressed genes (DEGs) associated with TRF-vitamin E supplementation in insulin resistance induced-zebrafish larvae, gene expression was quantified using fragments per kilobase of exon per million mapped (FPKM). A false discovery rate (FDR) threshold of 1 was applied for multiple testing

(q-value), and statistical significant was set at $p < 0.05$. Only transcripts with log fold-change (logFC) greater than $p < 0.05$ were considered DEGs compared to the untreated group.

A recent transcriptomic profiling study revealed 218 downregulated genes and 563 upregulated genes in the untreated insulin-resistant group. A previous study by the same research group identified dysregulated MAPK and Wnt/Ca2+-signaling pathways, along with lipid accumulation in the yolk sac of zebrafish larvae (18). Meanwhile, insulin-resistant zebrafish larvae treated with 50 µM metformin exhibited 200 downregulated genes and 1,300 upregulated genes. In contrast, insulin-resistant larvae treated with 1 µg/mL TRF-vitamin E showed 202 downregulated genes and 639 upregulated genes.

Effects of TRF-vitamin E and metformin on MDA levels in insulin resistance-induced zebrafish larvae

T2DM promotes insulin resistance, that can lead to oxidative stress generation. Lipid peroxidation is an endogenous chain reaction in which free radical species, such as reactive oxygen species (ROS) cause oxidative degradation of phospholipids, leading to the production of wide a variety of oxidation products. For example, free radicals can attack lipids containing carbon-carbon bonds such as polyunsaturated fatty acids (PUFAs) in the cellular membrane. The primary byproducts of lipid peroxidation are malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS), which are widely recognized as reliable of lipid peroxidation (22).

Our study demonstrated that MDA concentration was found to be greater in insulin resistance zebrafish larvae (2.725 ± 0.005) µmol/L compared to the control group (1.217 ± 0.005) µmol/L, with a significant difference ($p<0.0001$). Metformin and TRF-vitamin E groups, on the other hand, had significantly lower levels than the insulin-resistant group, with 1.705 ± 0.004 µmol/L and 1.572 ± 0.005 µmol/L, respectively (Figure 1).

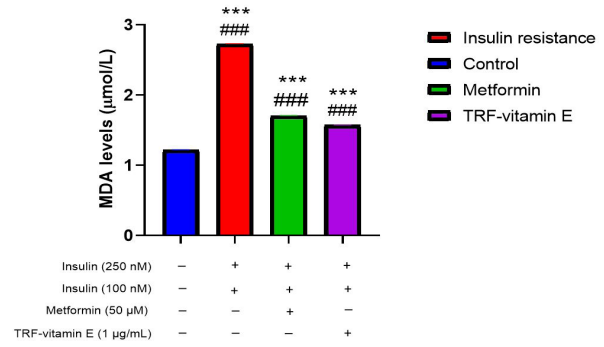

Figure 1: Effects of TRF-vitamin E on MDA levels in insulin resistance-induced zebrafish larvae. One-way ANOVA followed by Tukey's test (multiple comparison groups). The data are presented as mean ± standard deviation (SD) with 20 larvae aggregated from three independent replications for each group. The statistically significant analysis was reported as ####p < 0.001 relative to the control group. ***p<0.001 relative to the insulin-resistant group and treatment groups.

Effects of TRF-vitamin E and metformin on GSH production in insulin resistance-induced zebrafish larvae

The total GSH content was determined in the current study to assess the antioxidant potential of TRF-vitamin E in scavenging oxidative stress-induced free radicals. It was found that the total GSH levels in insulin-resistant group (0.174 ± 0.0038) µM was higher compared to control (0.0095 ± 0.0043) µM group. Metformin (0.1670 ± 0.0049) µM and TRF-vitamin E (0.0980 ± 0.0014) µM groups exhibited lower of total GSH content levels than insulin resistance group with significance different of $p< 0.0001$ (Figure 2).

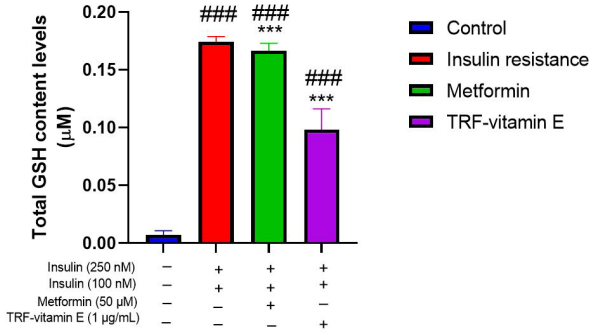

Figure 2: GSH concentration in insulin induced-zebrafish larvae. One-way ANOVA followed by Tukey's test (multiple comparison groups). The data are presented as mean ± standard deviation (SD) with 20 larvae aggregated from three independent replications for each group. The statistically significant analysis was reported as ####p < 0.001 relative to the control group. ***p<0.001 relative to the insulin-resistant group and treatment groups.

Gene ontology annotation from the DEGs analysis

To investigate the significance of the putative differentially expressed genes (DEGs) identified in relative to the control group, statistically significant differentially expressed transcripts with $p < 0.05$ were tested to a background set of all genes in DAVID v2023q1with Gene Ontology (GO) annotations. From the analysis, GO was found in this study was categorized into a cellular component (CC), molecular function (MF), and biological process (BP).

GO annotation of metformin

Figure 3 showed metformin treatment, enriched upregulated GO in CC (43%) was the highest compared to BP (31%) and MF (26%). For instance, in CC, the components highly expressed upregulated were plasma membrane (GO_CC:0005886, P=9.36E-12) with 139 uniquely upregulated genes, membrane (GO_CC:0016020, P=1.64E-06) with 231 upregulated genes, and integral component of plasma membrane (GO_CC:0005887, P=1.68E-04) with 41 upregulated genes. Transferase activity (GO_MF:0016740, P=0.03644), protein kinase activity (GO:0004672, P=0.00468) and kinase activity (GO_MF:0016301, P=0.01501) were among the most prevalent molecular functions identified in metformin treatment group. Protein phosphorylation (GO_BP:0006468, P=0.00513),

phosphorylation (GO_BP:0016310, P=0.01104) and ion transport (GO_BP:0006811, P=0.00134) were the most prominent biological processes metformin treated group. Meanwhile, Figure 4 showed downregulated genes of metformin treatment that enriched GO in CC which exhibited MF was the highest proportion with 50%, followed by BP (28%) and CC (22%). CC abundantly expressed in extracellular space (GO_CC: 0005615, P= 0.004), MF was an oxidoreductase activity (GO_MF: 0016491, P= 0.002) and BP in organic acid metabolic process (GO_BP: 0006082, P =8.19X10-7).

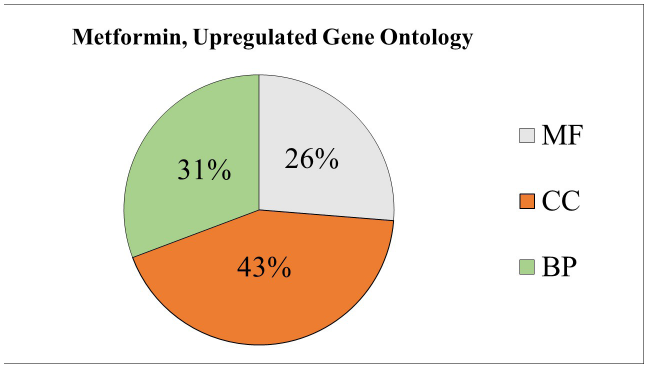

Figure 3: GO enrichment analysis of DEGs in insulin-resistant treated with 50 uM metformin at 120 dpf. The function of gene annotated represents three main levels of gene ontology categories: CC; cellular component, MF; molecular function and BP; biological process. The pie-chart displays the percentage (%) of total genes expressed in upregulated based on the GO categories.

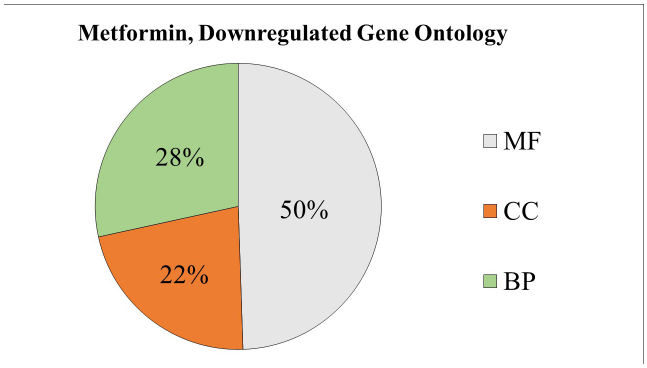

Figure 4: GO enrichment analysis of DEGs in insulin-resistant treated with 50 uM metformin at 120 dpf. The function of gene annotated represents three main levels of gene ontology categories: CC; cellular component, MF; molecular function and BP; biological process. The pie-chart displays the percentage (%) of total genes expressed in downregulated based on the GO categories.

GO annotation of TRF-vitamin E

The TRF-vitamin E upregulated GO cluster was widely distributed in CC (59%), followed by MF (23%), and BP (18%). Figure 5 shows significantly enriched CC in TRF-vitamin E treated group for membrane (GO_CC: 0016020, P<0.001), integral component of plasma membrane (GO_CC: 0005887, P<0.001), plasma membrane (GO_CC: 0005886, P<0.001), and voltage-gated calcium channel (GO_CC: 0005891, P=0.0016) and synapse (GO_CC: 0045202, P=0.04). Meanwhile, the MF for TRF-vitamin E group was upregulated in 3',5'-cyclic-nucleotide phosphodiesterase activity (GO_MF: GO:0004114, P<0.001, phosphoric diester hydrolase activity (GO_MF:0008081, P=0.0038), voltage-gated calcium channel activity (GO_MF:0005244, P=0.0043), metal ion binding (GO_MF: 0046872, P=0.009), voltage gated ion channel activity (GO_MF: 0005244, P=0.01), high voltage gated calcium channel activity (GO_MF:0008331, P=0.02) and cGMP-stimulated cyclic nucleotide phosphodiesterase activity (GO_MF:0004118, P=0.04). Furthermore, the most abundant gene expressed in TRF-vitamin E in the BP was homophilic cell adhesion via plasma membrane adhesion molecules (GO_BP:0007156, P=0.003), cell adhesion (GO_BP: 0007155, P=0.003), calcium ion transmembrane transport (GO_BP: 0070588, P=0.01), regulation of ion transmembrane transport (GO_BP: 0034765, P=0.01), signal transduction (GO_BP: 0007165, P=0.04), negative regulation of cGMP-mediated signaling (GO_BP: 0010754, P=0.04), and microtubule cytoskeleton organization (GO_BP: 0000226, P=0.05). On the other hand, the most abundance downregulated genes (Figure 6) involved in CC with 45% highly downregulated was extracellular space (GO_CC: 0005616, P= 0.00002), in MF (33%) was metal ion binding (GO_MF: 0046872, P=0.013) and in BP (22%) was extracellular matrix organization (GO_BP: 0030198, P= 0.001).

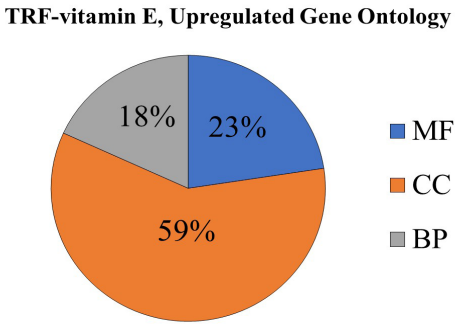

Figure 5: GO enrichment analysis of DEGs in insulin-resistant treated with TRF-vitamin E. The function of gene annotated represents three main levels of gene ontology categories: CC; cellular component, MF; molecular function and BP; biological process. The pie-chart displays the percentage (%) of total genes expressed in upregulated based on the gene ontology categories.

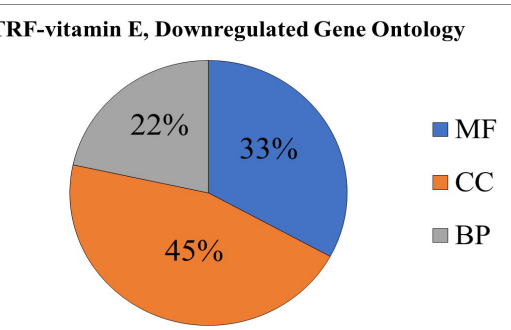

Figure 6: GO enrichment analysis of DEGs in insulin-resistant treated with TRF-vitamin E. The function of gene annotated represents three main levels of gene ontology categories: CC; cellular component, MF; molecular function and BP; biological process. The pie-chart displays the percentage (%) of total genes expressed in downregulated based on the gene ontology categories.

Metascape overview in the overlapping of crosstalk in micro-nutriomics signalling pathways

Protein-protein interaction (PPI) enrichment for each gene list was examined using a Metascape which derived from Search Tool for the Retrieval of Interacting Genes (STRING), BioGrid, OmniPath, and the InWeb IM databases. Gene Ontology, KEGG, Reactome, Canonical pathway and Hallmark Gene Sets are all integrated into Metascape (23). The resulting network contains a subset of proteins that physically interact with at least one from other members in the list. The Molecular Complex Detection (MCODE) technology was utilized to discover highly linked network components. The MCODE Pathway and Process Enrichment Analysis results are shown in Figure 7. The gene-gene interaction network is represented by the algorithms as a protein-protein interaction network with significant expression in insulin-resistant (red), metformin (blue), and TRF-vitamin E (green). MAPK signalling and calcium ion import across plasma membrane had shown the crosstalk TRF-vitamin E micro-nutriomics pathway.

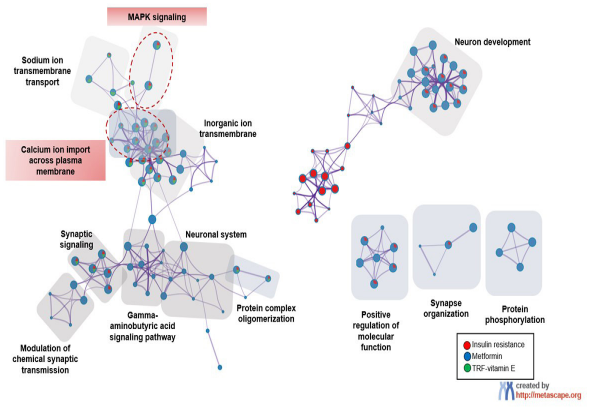

Figure 7: The network is displayed in Metascape with a "force-directed" structure with edge packaged. One term from each cluster is chosen to have its term description displayed as a label. Heatmap of upregulated DEGs displaying the top enrichment clusters with a distinct colour scale to denote statistical significance. The grey colour denotes an insignificant across group. The category dre04010 (MAPK signalling) is present in all metabolic processes. The enrichment network representation for the three treatment groups' outcomes, with nodes indicated by colour nodes. A data was resulted from the statistically significant enriched groups with the top 20 enriched GO terms (0.3 kappa score was applied as the threshold to cast the tree into term clusters).

DISCUSSION

This study explores the effects of TRF-vitamin E on insulin resistance and oxidative stress in zebrafish larvae, providing valuable insights into its potential as a therapeutic agent in T2DM which associating neurodegeneration. The promising results corroborates the significant effects of TRF-vitamin E can effectively reduce oxidative stress, lipid peroxidation, and promotes antioxidant defenses, focusing on a beneficial intervention for managing insulin resistance. The interplay of differential expression genes (DEGs) analysis in this model was confirmed by previous research using glucose dynamic study, lipid deposition at the area of yolk sac zebrafish larvae with Oil Red-O (ORO) staining, qPCR analysis, as well as gene expression analysis using transcriptome profilings (18).

The present study exhibits the long-term insulin resistance promotes ROS production in zebrafish. As a result of the implications of insulin resistance, enzyme antioxidants such as catalase, GPx, and SOD will diminish, resulting the capability of enzymes to quench free radicals (24). Therefore, we tested the effects of TRF-vitamin E at dosage of 1 µg/mL to elucidate the mechanistic pathways in modulating the insulin resistance that cause cholesterol trafficking leading to neurodegenerative disorders.

MDA levels were observed to be higher in insulin-resistant zebrafish larvae. In the occurrence of insulin-resistant, metformin and TRF-vitamin E levels declined considerably. The redox reaction imbalance developed as a result of an excessiveness of ROS generation and a decline in antioxidant defense systems (25). The increase in MDA levels found in the insulin-resistant zebrafish model suggests that insulin resistance may contribute to an increase in oxidative stress. In contrast, TRF-vitamin E does not work in isolation from other antioxidants rather than synergistically set of redox reaction. In this regards, TRF-vitamin E is able to scavenge peroxyl radicals essentially neutralizes them to form tocopheroxyl or tocotrienoxyl (26). Lipid peroxyl radicals are generated in lipid peroxidation. Lipid peroxidation consists of three reactivity stages: initiation, propagation and termination. Following an initiating event, ROS in insulin resistance of T2DM promotes the polyunsaturated fatty acids (PUFAs) to oxidize the double bond by reducing the hydrogen molecules. Consequently, the molecules turned to free radicals. The lipid is molecularly rearranged to a conjugated diene structure during this process, and O2 is added to the carbon-centered radical, giving continuously to a lipid peroxyl radical. At balance redox state, thioredoxin (txn) or glutathione endogenously will quench the free radicals to inhibit the chain reaction of lipid peroxidation. These objectives are dictated by the hypothesis that TRF-vitamin E is a good candidate for the free radical quencher at the chromanol ring of OH group (26). Therefore, it can inhibit lipid peroxidation to stop the chain-breaking lipid to become less reactive molecules (27). The lipid peroxidation reduction and free radicals accumulated in the cells is to avoid the cell damaging in the recurrent insulin resistant.

According to recent findings, TRF-vitamin E reduced MDA levels by 42% compared to metformin's 37%. Tocopherol functions acts as an oxygen radical scavenger and helps to maintain membrane structure (28). Tocotrienols, on the other hand, outperform tocopherols in terms of cholesterol-lowering, anti-proliferative, and anti-inflammatory effects (4). Clearly,

vitamin E inhibits lipid peroxidation by scavenging free radicals that attack PUFA on membrane phospholipids and plasma lipoprotein (29).

Metformin, a biguanide drug linked two molecules of guanide with lipophilic methyl side chain, incorporates with the similar results with TRF-vitamin E (30). It is a first-line drug to treat type 2 diabetic patients. Metformin treatment of diabetic patients was demonstrated to increase enzymatic antioxidants such as GPx, CAT, and SOD (31). The highest GSH levels were observed in insulin resistant larvae zebrafish compared to untreated and treated groups (TRF-vitamin E and metformin). GSH levels were increased in insulin resistance group to encounter the free radical attack . Goyal et al. (2011) reported that GPx levels were higher in non-obese diabetics compared to obese after post-insulin treatment corroborated that T2DM persistently promote the oxidative stress (32). Intriguingly, previous research has found that metformin combined with vitamin E can significantly lower the FBG, HbA1c, triglycerides and total cholesterol (33).

STRING and ClueGO is a solution to visualize enriched pathway when dealing with massive genomic data (34). Current study demonstrated a strong genomic dysregulation that leads to variation in gene expression. The metformin revealed enriched pathway genes with the biological process that signifies the mechanism targeted how insulin sensitizes the cells. For example, the MAPK signalling pathway revealed the relevance of metformin in studies that addressed these mechanisms (35,36). In our study, TRF-vitamin E also targeted the same mechanism as metformin. MAPK signalling was found to be dysregulated in insulin resistance, metformin, and TRF-vitamin E therapy groups.

In addition, the calcium signaling pathway indicates the role of beta-cell in insulin secretion. It is known that glucose homeostasis is regulated by Ca2+ ion, glycolysis and gluconeogenesis (37). We conclude that TRF-vitamin E works efficiently as a mediator of glucose homeostasis in facilitating the balance of glucose in the body. As previous studies reported that vitamin E improves the glycemic index and act as an insulin sensitizer (10). The high tendency of insulin binding to the insulin receptor on the cell surface leads to autophosphorylation of tyrosyl residues of the IR-beta subunit and phosphorylation of the insulin receptor substrates 1 and 2 (IRS1 and 2) could be postulated in TRF-vitamin E when employing the docking which has been demonstrated by Soleymani & his colleague (38). The subsequent cascade initiates the translocation of the insulin-responsive glucose transporter Glut4 into muscle cells and adipocytes (39). Furthermore, metformin acts as an insulin sensitizer (40) and its mechanistic pathway has been transcribed from the STRING platform.

Protein-protein network interaction curated five upregulated pathways in TRF-vitamin E such as MAPK signaling pathway, basal plasma membrane, intrinsic component of plasma membrane, sphingolipid metabolic process and voltage-gated calcium channel complex. TRF-vitamin E downregulated the extracellular matrix (adamts1, apoa1a, cmn, col14a1b, col1a1a, col1a1b, col1a2, col2a1b, col5a3b, lrig3, matn3a, and mmp2) as well as intracellular sequestering of iron-iron with the downregulation of fthl27, fthl29 and fthl31 expression. Furthermore, the downregulation of cardiac muscle contraction and ECM-receptor were observed in the TRF-vitamin E group.

A protein-protein network connection curated the metformin route, has been validated by current existing investigations. Particularly in voltage-gated calcium channel activity, MAP kinase activity and its cascade, JUN kinase activity, protein tyrosine kinase activity, MAPK signalling pathway, ErbB signalling pathway, kinase serine/threonine-protein kinase, ATP binding, calcium channel, stress-activated protein kinase signalling cascade, JNK cascade, regulation of voltage-gated calcium channel activity, and positive regulation of neuron apoptosis process. It appears that metformin acts not just as an insulin sensitizer but also attenuating the process of neurodegeneration. Prkcg gene is a hub of suppression of caspase-9 inflammation, which has been implicated in a neuroprotective function in neurological diseases such as ischemic stroke (41). Also, it is marked that in metformin there is upregulation of GTP-binding gene. It is well established that overexpressed of GTP-binding proteins reduce the amount of plaque in AD mouse models and contribute to the advancement of diabetic nephropathy through influencing cell shape, which is controlled by the tau microtubule-binding region (42).

In this current study we also screened for the gene that significantly expressed as neurodegenerative markers. Gene-disease association might over-representation when it comes to the single gene interest expression. Two genes, alkaline ceramidase (acer) and amyloid beta precursor protein family B (apbb) expressed in three groups of treatment. However, both genes do not exit the limit to be extended into enrichment pathway. Zebrafish expressed in acer1 abundantly expressed in skin, acer2 expressed in Golgi apparatus which highly specific and acer3 expressed in endoplasmic reticulum and golgi. Insulin resistance group indicated the acer is lower compared to TRF-vitamin E and metformin (Supplementary) . Acer is an enzyme that catalyze the hydrolysis of saturated long-chain ceramides (C18:1 and C20:1) into sphingosine. Wang et al. (2015) reported the acer3 knockout increased the ceramides to promote the Purkinje Cell disruption (43). Furthermore, Wang et al. suggest that the acer3 increased with the age relatively due to the compensation of the sphingosine and ceramides regulation (43). The treatment of TRF-vitamin E and metformin probably affect the quality of

ceramides level in the brain, yet further study need to be warranted. Apbb abundantly in cytoplasm binding to the intracellular domain of AD beta amyloid precursor protein (APP) (44). Apbb expression seems higher in metformin compared to TRF-vitamin E. On the other hand, insulin resistance group showed the lowest expression of apbb. Metformin is able to reach mouse brain as revealed by activation of AMPK demonstrating the ability of the drug to cross the blood brain barrier (45). Metformin can influence the integrity of brain function. Nevertheless, Picone et al. (2015) found that metformin has the same effect as H2O2 on APP metabolism which is counteracted by antioxidant molecules (46). Metformin causes mitochondrial membrane depolarization, whereas the presence of insulin results in mitochondrial membrane repolarization. This might be explained why metformin has a silent negative effect compared to TRF-vitamin E. Vitamin E bridge to the high binding energy to hydrophobic groove of insulin which demonstrated by Soleymani et al. (2019) by using a molecular docking (38). Vitamin E are bound to hydrophobic region between two alpha-helix structures in A and B-chain interface of insulin (38). Hence, the interaction of TRF-vitamin E with insulin activity is sustained despite T2DM progression.

CONCLUSION

This study reveals the key promising role of TRF-vitamin E micronutrients in combating T2DM by reducing inflammation, lipid peroxidation and oxidative stress. By utilizing zebrafish models, we provide a deeper understanding of dysregulated molecular pathways on how TRF-vitamin E targets the MAPK-Ca2+ signalling pathway to restore cellular balance. These findings not only highlight TRF-viatmin E's pontential to curb T2DM progression but also suggest its role in mitigating the onset of AD. Moving forward, integrating clinical trials and advanced bioinformatics will be essential to transforming these insights into viable therapeutic strategies, paving the way for innovative interventions in metabolic and neurodegenerative disorders.

ACKNOWLEDGEMENT

The authors would like to thank Nurul Munirah Manan and Shamala Devi Subramaniam for providing laboratory equipment.

REFERENCES

1. Barbagallo M, Dominguez LJ. Type 2 diabetes mellitus and Alzheimer's disease. World J Diabetes. 2014 Dec 12 [cited 2023 Jun 1];5(6):889. https://doi.org/10.4239/wjd.v5.i6.889
2. Chiroma AA, Khaza'ai H, Abd. Hamid R, Chang SK, Zakaria ZA, Zainal Z. Analysis of expression of vitamin E-binding proteins in H2O2 induced SK-N-SH neuronal cells supplemented with α-tocopherol and tocotrienol-rich fraction. Cao Y, editor. PLoS One [Internet]. 2020 Nov 24 [cited 2021 Feb 10];15(11):e0241112. https://doi.org/10.1371/journal.pone.0241112
3. Kiyose C. Absorption, transportation, and distribution of vitamin E homologs. Free Radic Biol Med. 2021 Dec 1;177:226–37. https://doi.org/10.1016/j.freeradbiomed.2021.10.016
4. Aggarwal BB, Sundaram C, Prasad S, Kannappan R. Tocotrienols, the vitamin E of the 21st century: Its potential against cancer and other chronic diseases. Vol. 80, Biochemical Pharmacology. NIH Public Access; 2010 [cited 2021 Apr 28]. p. 1613–31. https://doi.org/10.1016/j.bcp.2010.07.043
5. Zainal Z, Rahim AA, Khaza'ai H, Chang SK. Effects of palm oil tocotrienol-rich fraction (TRF) and carotenes in ovalbumin (OVA)-challenged asthmatic brown Norway rats. Int J Mol Sci. 2019 Apr 1;20(7). https://doi.org/10.3390/ijms20071764
6. Wong SK, Chin KY, Suhaimi FH, Ahmad F, Ima-Nirwana S. Vitamin E as a potential interventional treatment for metabolic syndrome: Evidence from animal and human studies. Front Pharmacol. 2017 Jul 5;8(JUL):444. https://doi.org/10.3389/fphar.2017.00444
7. Ryan MJ, Dudash HJ, Docherty M, Geronilla KB, Baker BA, Haff GG, et al. Vitamin E and C supplementation reduces oxidative stress, improves antioxidant enzymes and positive muscle work in chronically loaded muscles of aged rats. Exp Gerontol. 2010 Nov [cited 2023 Jun 1];45(11):882. https://doi.org/10.1016/j.exger.2010.08.002
8. Jain AB, Jain VA. Vitamin E, its beneficial role in diabetes mellitus (DM) and its complications. J Clin Diagnostic Res. 2012 Dec 15 [cited 2021 Jun 14];6(10):1624–8. https://doi.org/10.7860/JCDR/2012/4791.2625
9. Saboori S, Shab-Bidar S, Speakman JR, Yousefi Rad E, Djafarian K. Effect of vitamin E supplementation on serum C-reactive protein level: a meta-analysis of randomized controlled trials. Eur J Clin Nutr. 2015 Aug 8 [cited 2023 Jun 1];69(8):867–73. https://doi.org/10.1038/ejcn.2014.296
10. Asbaghi O, Sadeghian M, Nazarian B, Sarreshtedari M, Mozaffari-Khosravi H, Maleki V, et al. The effect of vitamin E supplementation on selected inflammatory biomarkers in adults: a systematic review and meta-analysis of randomized clinical trials. Sci Reports 2020 101. 2020 Oct 14 [cited 2023 Jun 1];10(1):1–17.https://doi.org/10.1038/s41598-020-73741-6
11. Sarir H, Emdadifard G, Farhangfar H, Taherichadorneshin H. Effect of vitamin E succinate on inflammatory cytokines induced by high-intensity interval training. J Res Med Sci [Internet]. 2015 Dec 1 [cited 2023 Jun 1];20(12):1177. https://doi.org/10.4103/1735-1995.172986
12. Kinney JW, Bemiller SM, Murtishaw AS, Leisgang AM, Salazar AM, Lamb BT. Inflammation as

a central mechanism in Alzheimer's disease. Alzheimer's Dement Transl Res Clin Interv. 2018 Jan 1 [cited 2023 Jun 1];4:575. https://doi.org/10.1016/j.trci.2018.06.014
13. Regner-Nelke L, Nelke C, Schroeter CB, Dziewas R, Warnecke T, Ruck T, et al. Enjoy Carefully: The Multifaceted Role of Vitamin E in Neuro-Nutrition. Int J Mol Sci 2021, Vol 22, Page 10087. 2021 Sep 18 [cited 2023 Jun 1];22(18):10087. https://doi.org/10.3390/ijms221810087
14. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. Cochrane Database Syst Rev. 2008;(2). https://doi.org/10.1002/14651858.CD007176.pub2
15. Cherubini A, Martin A, Andres-Lacueva C, Di Iorio A, Lamponi M, Mecocci P, et al. Vitamin E levels, cognitive impairment and dementia in older persons: The InCHIANTI study. Neurobiol Aging. 2005;26(7):987–94. https://doi.org/10.1016/j.neurobiolaging.2004.09.002
16. Farina N, Llewellyn D, Isaac MGEKN, Tabet N. Vitamin E for Alzheimer's dementia and mild cognitive impairment. Cochrane Database Syst Rev. 2017 Jan 27 [cited 2023 Jun 1];2017(1). https://doi.org/10.1002/14651858.CD002854.pub4
17. Diwan AD, Harke SN, Gopalkrishna SN, Panche AN. Cryobanking of Fish and Shellfish Egg, Embryos and Larvae: An Overview. Front Mar Sci. 2020 May 7;7:498013. https://doi.org/10.1016/j.aquaculture.2016.05.042
18. Md Razip NN, Mohd Noor S, Norazit A, Nordin N, Sakeh NM, Khaza'ai H. An Association between Insulin Resistance and Neurodegeneration in Zebrafish Larval Model (Danio rerio). Int J Mol Sci [Internet]. 2022 Aug 1 [cited 2023 Jun 1];23(15). https://doi.org/10.3390/ijms23158290
19. Wyett G, Gibert Y, Ellis M, Castillo HA, Kaslin J, Aston-mourney K. Metformin , beta-cell development , and novel processes following beta-cell ablation in zebrafish. 2017, doi: 10.1007/s12020-017-1502-3.
20. McDougall M, Choi J, Kim HK, Bobe G, Stevens JF, Cadenas E, et al. Lethal dysregulation of energy metabolism during embryonic vitamin E deficiency. Free Radic Biol Med. 2017 Mar 1;104:324–32. https://doi.org/10.1016/j.freeradbiomed.2017.01.020
21. Ayala A, Mucoz MF, Argьelles S. Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. Oxid Med Cell Longev [Internet]. 2014 [cited 2023 Jun 6];2014. https://doi.org/10.1155/2014/360438
22. Ito F, Sono Y, Ito T. Measurement and Clinical Significance of Lipid Peroxidation as a Biomarker of Oxidative Stress: Oxidative Stress in Diabetes, Atherosclerosis, and Chronic Inflammation. Antioxidants. 2019 Mar 1 [cited 2022 Nov 23];8(3).
23. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun 2019 101. 2019 Apr 3 [cited 2023 Jun 11];10(1):1–10. https://doi.org/10.1038/s41467-019-09234-6
24. Pasupuleti VR, Arigela CS, Gan SH, Salam SKN, Krishnan KT, Rahman NA, et al. A review on oxidative stress, diabetic complications, and the roles of honey polyphenols. Oxid Med Cell Longev. 2020;2020. https://doi.org/10.1155/2020/8878172
25. Tangvarasittichai S. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. World J Diabetes. 2015;6(3):456. https://doi.org/10.4239/wjd.v6.i3.456
26. Rimbach G, Moehring J, Huebbe P, Lodge JK. Gene-Regulatory Activity of α-Tocopherol. Mol 2010, Vol 15, Pages 1746-1761. 2010 Mar 12 [cited 2023 Jun 5];15(3):1746–61.. https://doi.org/10.3390/molecules15031746
27. Martemucci G., Costagliola c, Mariano M, L. D'andrea, P. Napolitano, and A. G. D'Alessandro, "Free Radical Properties, Source and Targets, Antioxidant Consumption and Health," Oxyg. 2022, Vol. 2, Pages 48-78, vol. 2, no. 2, pp. 48–78, Apr. 2022, doi: 10.3390/OXYGEN2020006.
28. Blokhina O, Virolainen E, Fagerstedt K V. Antioxidants, Oxidative Damage and Oxygen Deprivation Stress: a Review. Ann Bot. 2003 Jan 2 [cited 2023 May 28];91(2):179–94. https://doi.org/10.1093/aob/mcf118
29. Rizvi S, Raza ST, Ahmed F, Ahmad A, Abbas S, Mahdi F. The Role of Vitamin E in Human Health and Some Diseases. Sultan Qaboos Univ Med J. 2014 [cited 2023 Apr 15];14(2):e157.
30. Samson SL, Garber AJ. Metformin and other biguanides: pharmacology and therapeutic usage. Int Textb Diabetes Mellit. 2015 Mar 6 [cited 2023 May 28];641–56. https://doi.org/10.1002/9781118387658.ch43
31. Dogan Turacli I, Candar T, Yuksel EB, Kalay S, Oguz AK, Demirtas S. Potential effects of metformin in DNA BER system based on oxidative status in type 2 diabetes. Biochimie. 2018 Nov 1;154:62–8. https://doi.org/10.1016/j.biochi.2018.08.002
32. Goyal R, Singhai M, Faizy AF. Glutathione peroxidase activity in obese and nonobese diabetic patients and role of hyperglycemia in oxidative stress. J Midlife Health [Internet]. 2011 [cited 2023 May 28];2(2):72. https://doi.org/10.4103/0976-7800.92529
33. Dass AS, Narayana S, Venkatarathnamma PN. Effect of Vitamin E and omega 3 fatty acids in type 2 diabetes mellitus patients. J Adv Pharm Technol Res. 2018;9(1):32-36. doi:10.4103/japtr.JAPTR_309_17. https://doi.org/10.4103/japtr.JAPTR_309_17

34. Mlecnik B, Galon J, Bindea G. Comprehensive functional analysis of large lists of genes and proteins. J Proteomics. 2018 Jan 16;171:2–10. https://doi.org/10.1016/j.jprot.2017.03.016
35. Lu G, Wu Z, Shang J, Xie Z, Chen C, zhang C. The effects of metformin on autophagy. 2021 May 1;137:111286. https://doi.org/10.1016/j.biopha.2021.111286
36. Schultze SM, Hemmings BA, Niessen M, Tschopp O. PI3K/AKT, MAPK and AMPK signalling: Protein kinases in glucose homeostasis. Expert Rev Mol Med. 2012;14(January 2012).doi:10.1017/S1462399411002109
37. Klec C, Ziomek G, Pichler M, Malli R, Graier WF. Calcium Signaling in Я-cell Physiology and Pathology: A Revisit. Int J Mol Sci [Internet]. 2019 Dec 2 [cited 2023 Jun 20];20(24). Available from: /pmc/articles/PMC6940736/
38. Soleymani H, Ghorbani M, Allahverdi A, Shojaeilangari S, Naderi-manesh H. Activation of human insulin by vitamin E: A molecular dynamics simulation study. J Mol Graph Model [Internet]. 2019 S Boshtam M, Rafiei M, Golshadi ID, Ani M, Shirani Z, Rostamshirazi M. Long term effects of oral vitamin E supplement in type II diabetic patients. Int J Vitam Nutr Res. 2005 Sep [cited 2023 Jun 1];75(5):341–6. https://doi.org/10.1024/0300-9831.75.5.341ep 1 [cited 2023 Jun 1];91:194–203. doi:10.1016/j.jmgm.2019.06.006
39. Boshtam M, Rafiei M, Golshadi ID, Ani M, Shirani Z, Rostamshirazi M. Long term effects of oral vitamin E supplement in type II diabetic patients. Int J Vitam Nutr Res. 2005 Sep [cited 2023 Jun 1];75(5):341–6. https://doi.org/10.1024/0300-9831.75.5.341
40. Nasri H, Rafieian-Kopaei M. Metformin: Current knowledge. J Res Med Sci. 2014 [cited 2023 May 28];19(7):658. Available from: /pmc/articles/PMC4214027/.doi: 10.4103/JRMS.JRMS_62_24]
41. Wei H ping, Peng Z feng, Shao K mei, Zhang P hao, Chen L, Hu J an, et al. cPKCγ Inhibits Caspase-9-Initiated Neuronal Apoptosis in an Ischemia Reperfusion Model In Vitro Through p38 MAPK-p90RSK-Bad Pathway. Neurochem Res. 2023 Feb 1 [cited 2023 May 31];48(2):362–74. https://doi.org/10.1007/s11064-022-03747-1
42. Oyama F, Kotliarova S, Harada A, Ito M, Miyazaki H, Ueyama Y, et al. Gem GTPase and Tau: Morphological Changes Induced By GEM GTPase in CHO Cells are Antagonized by TAU. J Biol Chem. 2004 Jun 25;279(26):27272–7. https://doi.org/10.1074/jbc.M401634200
43. Wang K, Xu R, Schrandt J, Shah P, Gong YZ, Preston C, et al. Alkaline Ceramidase 3 Deficiency Results in Purkinje Cell Degeneration and Cerebellar Ataxia Due to Dyshomeostasis of Sphingolipids in the Brain. PLoS Genet. 2015 [cited 2023 Jun 1];11(10). doi: 10.1371/journal.pgen.1007190]
44. Haenig C, Atias N, Taylor AK, Mazza A, Schaefer MH, Russ J, et al. Interactome Mapping Provides a Network of Neurodegenerative Disease Proteins and Uncovers Widespread Protein Aggregation in Affected Brains. Cell Rep. 2020 Aug 18 [cited 2023 Jun 1];32(7). https://doi.org/10.1016/j.celrep.2020.108050
45. Chen Y, Zhou K, Wang R, Liu Y, Kwak YD, Ma T, et al. Antidiabetic drug metformin (GlucophageR) increases biogenesis of Alzheimer's amyloid peptides via up-regulating BACE1 transcription. Proc Natl Acad Sci U S A. 2009 Mar 10 [cited 2023 Jun 1];106(10):3907–12. doi:10.1073/pnas.0807991106
46. Picone P, Nuzzo D, Caruana L, Messina E, Barera A, Vasto S, et al. Metformin increases APP expression and processing via oxidative stress, mitochondrial dysfunction and NF-κB activation: Use of insulin to attenuate metformin's effect. Biochim Biophys Acta - Mol Cell Res. 2015 May 1;1853(5):1046–59. doi:10.1016/j.bbamcr.2015.01.017