Journal of Diabetes and Clinical Studies

RESEARCH ARTICLE

A Novel Mixture of δ-Tocotrienol, Vitamin D_3, Resveratrol (NS-3) Significantly Decreases Diabetes Biomarkers Including Inflammatory in People with Type 2 Diabetes

Asaf A Qureshi[1]*, Dilshad A. Khan[2], Wajiha Mahjabeen[2], Neerupma Silswal[1], and Nilofer Qureshi[1,3]

[1]Department of Biomedical Science, School of Medicine, University of Missouri-Kansas City, Holmes Street, Kansas City, USA. [2]Department of Chemical Pathology and Endocrinology, Armed Forces Institute of Pathology (AFIP), National University of Medical Sciences, Rawalpindi, Pakistan.: [3]Pharmacology/Toxicology, School of Pharmacy, University of Missouri-Kansas City, Charlotte Street, Kansas City, USA

Abstract

Aims: Diabetes mellitus is a metabolic disorder identified by hyperglycemia due to insulin resistance. Impaired serum/plasma fasting glucose, HbA1c, hs-CRP are biomarkers, normally used to determine onset of diabetes. δ-Tocotrienol, vitamin D_3, and resveratrol (nutritional supplement-NS-3) are potent anti-cholesterolemic, anti-oxidative and anti-inflammatory agents. We hypothesized that a mixture of δ-tocotrienol, vitamin D_3, resveratrol (NS-3) will be more effective treatment for reducing diabetes biomarkers as compared to its individual components, in people with type 2 diabetes mellitus (T2DM).

Methods: To test our hypothesis, evaluation of NS-3 mixture and its individual components was carried out on diabetes inflammatory biomarkers using peripheral blood mononuclear cells (PBMC) obtained from healthy, normal and people with T2DM. A randomized placebo controlled double-blinded prospective trial of individual components (n = 30/component), and NS-3 trial of people with T2DM (n = 56/group), were given two capsules/d of cellulose/olive oil as placebo, individual components, or NS-3 mixture for 24-weeks.

Results: Significant down-regulation (15 - 74; $P < 0.002$) of gene expression was observed with individual components and NS-3 on diabetes biomarkers (IRS-1, SOD-2, GCKR, ICAM-1, VCAM- 1, IL-6, IL-8) in PBMCs of T2DM, and in serum values of fasting glucose (11%), HbA1c (10%), hs-CRP (23%), fasting insulin (9%), HOMA-IR (20%), MDA (20%) of NS-3 treated people with T2DM after 24-weeks. Treatment with individual components showed significant decrease but were less effective than the mixture. RT-PCR analysis of blood RNA obtained from NS-3 treated people with T2DM for 24-weeks resulted in significant ($p < 0.01$) down-regulation of gene expression in diabetes biomarkers (IRS-1, SOD-2, GCKR, IGFBP-2) compared to pre-dose values.

Conclusions: Present results of *in vitro* and *in vivo* studies support our hypothesis that NS-3 mixture is more effective in lowering serum levels of several diabetes biomarkers including inflammatory gene expression markers compared to its individual components in people with T2DM.

Keywords: T2DM, PBMC, δ-tocotrienol, vitamin D_3, Resveratrol, diabetes biomarkers, glucose, HbA1c, HOMA-IR, hs-CRP, MDA. ICAM-1, VCAM-1, IL-6, IL-8.

Introduction

Diabetes mellitus is a complex, chronic metabolic disorder due to chronic inflammation, infection and oxidative stress, are important factors for development of insulin resistance (IR) during the progression of type 2 diabetes mellitus (T2DM) [1]. It is a global health problem, and its prevalence is continuously increasing worldwide. The detection of T2DM often occurs late, although damage to organs or tissue starts early, even before the disease become clinical manifested, and chances of development of complications are more, and will also increase rate of morbidity, mortality, retinopathy with potential blindness, nephropathy that may lead to renal failure, and risk of foot, amputation. Therefore, a timely diagnosis can reduce the development of complications and augment the efficiency of treatment given to these individuals.

Hyperglycemia induced oxidative stress and inflammation, are associated with complications, such as diabetic nephropathy, retinopathy and others [2]. The levels of glucose control and occurrence of diabetes associated complications occurred in different phases of disease, and strongly associated with enhanced oxidative stress [3]. Malondialdehyde (MDA) is considered to be the most commonly assessed biomarker during diabetic complications, and chronic inflammation is thought to be

Correspondence to: Asaf A Qureshi, Department of Biomedical Science, School of Medicine, University of Missouri, USA. Email: qureshia [AT] umkc [DOT] edu

Received: May 28, 2021; **Accepted:** June 03, 2021; **Published:** June 07, 2021

an important factor for development of insulin resistance during the progression of T2DM [3]. Inflammation causes the release of different inflammatory markers such as hs-C-reactive protein (hs-CRP) and different cytokines such as interleukin (IL)-1α, IL-6, IL-8, ICAM-1, VCAM-1, and tumor necrosis factor-α (TNF-α). The increase in systemic markers of inflammation (hs-CRP, IL-6 and TNF-α) are associated with complications such as diabetic nephropathy and weakly associated with development of diabetic retinopathy [3,4].

Evaluation of other biomarkers and IL-1α in initiation and development of T2DM have been reported [5,6]. IL-1α plays its role in diabetes by enhancing β-cell failure, and it is a potential marker for onset and progression of T2DM *in vitro* studies [6]. Plasma monocytes chemoattractant protein-1 (MCP-1), a chemokine is responsible for inducing insulin resistance and its levels are elevated in people with diabetes. Likewise, IL-8, an inducer of insulin resistance in diabetes has also been reported [7]. The vascular endothelial growth factor (VEGF) and IL-10 are also considered important factors in pathogenesis of diabetic nephropathy [8]. Excessive levels of these markers result in increased activation of nuclear factor kappa B (NF-κB). This causes the increase transcription of cytokine and chemokine genes responsible for development of insulin resistance, and impaired insulin action and ultimately results in production of hyperglycemia. Hyperglycemia induces altered protein, lipid, nucleic acid function, and gene expression causes cellular dysfunction thus leads to various complications associated with diabetes [3 - 9]. The risk factors like impaired fasting glucose, glucose tolerance and glycosylated hemoglobin (HbA1c) are used nowadays to diagnose the onset of diabetes mellitus, which are not sensitive enough and thus do not have the definite predictive values [10]. Beside these biomarkers, hs-CRP, adipokines and cytokines considered as potential novel biomarkers of metabolic diseases including T2DM [11].

The dietary supplements like δ-tocotrienol, vitamin D_3, and resveratrol have been established as potent anti-oxidative and anti-inflammatory agents, which are continuously re-evaluated for a long time in humans, animals, and in tissues separately [12 - 25]. Data showing combined effects of these nutritional supplements in T2DM is still lacking, and due to these diverse results and limited number of studies, there is a need of large trials *in vivo* to determine impact of effects of nutritional supplements of naturally occurring compounds in people with T2DM. Thus, we have used a number of relatively inexpensive, commercially available naturally occurring, and FDA approved compounds with inhibitory properties of diabetes biomarkers including inflammation, such as levels of fasting glucose, HbA1c, hs-CRP, IRS-1, SOD-2, IGFBP-2, GCKR, PTPRN, IL-6, IL-8, ICAM-1, VCAM-1, TNF-α. The functions of IRS-1 are to lower blood glucose level and alleviates insulin resistance, SOD-2 is a free radical scavenging enzyme, PTPRN plays role in maintaining normal level of insulin, and GCKR for the accumulation of normal levels of insulin-containing vesicles in T2DM (based on Google search). Previously, we conducted several studies to evaluate the treatment response of combination of different nutritional supplements (δ-tocotrienol, resveratrol, quercetin, pterostilbene, nicotinic acid) in hypercholesterolemic subjects. All these studies showed significant post treatment changes in different inflammatory markers, cytokines, oxidative stress, lipid profile and different miRNAs in these individuals [26 - 28]. The oral hypoglycemic treatments, change of lifestyle (increasing physical activity), and dietary habits (consumption of fruits and vegetables) have been found most effective in preventing progression of diabetes and associated complications [29]. We have recently demonstrated that a mixture of δ-tocotrienol, resveratrol, quercetin, pterostilbene and nicotinic acid (NS-5 mixture) lowers serum total cholesterol levels and pro-inflammatory cytokines in hypercholesterolemic subjects more effectively than their individual components [26]. The hypothesis of present study is that a combination therapy would be more effective in inhibiting several diabetic biomarkers and those anti-inflammatory properties of naturally occurring compounds, δ-tocotrienol, vitamin D_3 resveratrol (Figure 1), and their mixture (NS-3) would decrease the serum/plasma levels of fasting glucose, HbA1c, hs-CRP, MAD, other diabetic biomarkers, and levels of cytokines in people with T2DM more effectively than its individual components. To test our hypothesis, first comparative evaluation of NS-3 mixture versus its individual components was tested *in vitro* using peripheral blood mononuclear cells (PBMCs) obtained from healthy normal, and those with T2DM. This was followed by clinical investigation of effects of a mixture of NS-3, and its individual components on oxidative stress, inflammation, different cytokines, and diabetic biochemical profile in people with T2DM to improve the quality of life of these individuals. (Figure 1)

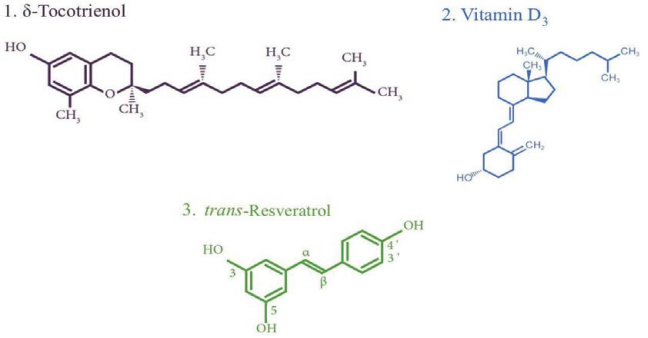


Figure 1. Chemical structures of δ-tocotrienol, vitamin D_3, and resveratrol.

Materials And Methods

Materials

The 70% tocotrienol mixture (typical composition 90% δ-tocotrienol and 10% γ-tocotrienol) purified from annatto seeds was purchased from American River Nutrition, Inc. (Hadley, MA, USA); trans-Resveratrol from "Mega Resveratrol" (60 Newton Road # 32 Danbury CT, USA) and

Vitamin D_3 purchased from Piping Rock, NY. The peripheral blood mononuclear cells (PBMCs) from healthy normal subjects and T2DM subjects were purchased from Stem Cell Technologies, Vancouver, BC, Canada.

The 70 % δ-tocotrienol was purified to 98% as described earlier [30]. The capsulation of mixture of δ-tocotrienol (125 mg) + resveratrol (125 mg) + vitamin D_3 (62.5 µg [2500 IU]) = 250.062 mg/capsule; 90 capsules/bottle), and placebo capsule (125.062 mg cellulose + 125 mg olive oil, after removing micro-components of olive oil by washing with ethanol) = 250.062 mg/capsule. Capsules were prepared at Kabco Inc. New Jersey, USA. The bottles were blindly labeled as AMR-1 and AMR-2 by the capsulation company. RNeasy mini kits were obtained from QIAGEN Sciences (Germantown, MD, USA).

Experimental

Evaluation of a mixture of NS-3 and its components *in vitro* on various diabetes biomarkers and inflammatory cytokines in peripheral blood mononuclear cells (PBMCs) obtained from healthy normal and people with T2DM.

The stock solutions of each compound were prepared (1 mg/mL) in 95% ethanol and stored at -20˚C. The working solutions (10 µM) of each compound and their mixture (NS-3) were prepared by diluting the appropriate volume of each stock solution in culture medium to give a final ethanol concentration 0.1% (v/v). The peripheral blood mononuclear cells (PBMCs) obtained from healthy normal or people with T2DM were added in each well (500,000 cells/well) in 96-well tissue culture plates and treated individually each compound as well as mixture of these compounds (triplicate of each compound and mixture), incubated at 37 ˚C under 5% CO2 for 1h. After 1 h, total RNAs were isolated from cell lysates using Qiagen RNeasy Mini kit according to manufacturer's instructions for RT-PCR analyses of various biomarkers associated with diabetes. Quality of RNAs were assessed by spectrophotometric measurements. The purity of total RNA was carried out by measuring the absorption at several wavelengths using a Thermo Scientific NanoDrop 1000 Spectrophotometer. The purity of total RNA was determined by using the ratio of 260/280 (2.02 -2.08).

Real-time PCR of purified NS-3 treated RNAs

Real-time PCR (RT-PCR) was performed on total mRNAs isolated from untreated (control) and NS-3 treated PBMCs by using One-Step qRT-PCR kit (Life Technologies, Foster City, California). All reactions were performed in triplicate using equal amount of mRNA per reaction in 96-well (500000 cells/well) PCR plates. Gene expression from cell cultures was normalized ($2^{-\Delta\Delta C}$ t analysis) to GAPDH.

Impact of a mixture NS-3 and its components on autophagy using peripheral blood mononuclear cells (PBMCs) obtained from healthy normal and people with T2DM.

The PBMC (200,000 cells/well in medium + 0.2% DMSO) were first differentiated with PMA (10ng/mL) in media of 96-tissue culture white plate for 4 h at 37 ˚C under 5% CO2 in an incubator, then washed with fresh media, followed by incubation with vehicle (medium + 2% DMSO, blank control), rapamycin, chloroquine, and mixture of rapamycin + chloroquine (as positive control) or δ-tocotrienol + vitamin D_3 + resveratrol or mixture of these three compounds (NS-3) using a dose response of 0.0 µM, 10 µM, 20 µM, 40 µM, 80 µM of each compound or mixture for 4 h at 37 ˚C under CO2. The cells were washed with assay buffer followed by incubation with microscopy dual detection reagent (100 µL) for 30 min at 37 ˚C in dark. The microscopy dual detection reagent was prepared according the protocol of the manufacturer. After incubation the PBMC were finally washed with assay buffer three times. The Fluorescence measurements were performed under Cytation-3-fluorometric reader using DAPI and FITC filer sets.

Impact of a mixture NS-3 and its components on diabetes biomarkers in healthy normal and

People with T2DM.

Study Design

The present studies were double-blind, randomized, placebo-controlled trial (RCT). A non-probability convenience sampling technique was used. The study protocol was registered with WHO regional office in Asia (World Health Organization Sri Lanka Clinical Trials Registry, Sri Lanka Center; srilankactr @ gmail.com), after ethical approval by the Institutional Review Board of Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. The registry number was SLCTR/2018/019, dated 6.21.2018. The study was carried out according to the guidelines provided by the United States Food and Drug Administration (FDA, 2003) at (AFIP), Rawalpindi, and University of Health Sciences (UHS), Lahore. Pakistan. All participants of study # 1 and study # 2 have signed an informed consent form before start of the study. The purified total RNA samples were delivered at UMKC, School of Medicine after getting approval by the members "Compliance Officer (Christopher Winders) and "Chemical Biological Safety Officer (Timothy Sturgis, RBP) of Institution Board of UMKC School of Medicine, Kansas City, MO. USA.

Study Population

People with T2DM (n = 232) aged >30 years were selected over a period of 8- weeks for study # 1 and study # 2. The inclusion criteria for each study group were male and female participants diagnosed with T2DM, and exclusion criteria were acute illness, liver, renal, thyroid disorders or malignancy or history of taking anti-inflammatory drugs or vitamins (A, B, C, D, E, K) regularly or in the last 2-weeks. For the diagnosis of diabetes, their serum fasting glucose, random glucose, oral glucose tolerance (OGT) and HbA1c levels were measured. Individuals were labeled as diabetic if their fasting glucose

were ≥ 126 mg/dL (7.0 mmol/L) or random blood glucose levels were ≥ 200 mg/dl (11.1 mmol/L), OGT ≥ 200 mg/dl (11.1 mmol/L) and HbA1c ≥ 6.5%.

All participants with T2DM in this project were screened at Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. Their clinical history and general physical examination were carried out. The clinical history and general physical examination of each participant was recorded. A questionnaire was given to them. Questionnaire data was comprised of participant's height, weight, BMI, systolic and diastolic blood pressure at rest, history of smoking, physical activity, medicine intake. Drug histories of participants were taken in detail (oral hypoglycaemic drugs [Metformin, Glimepiride, Vildagliptin, Gliclazide, Glibenclamide], insulin, nitrates, oral aspirin, calcium antagonist, ACE inhibitors and diuretics). Height and weight were measured without shoes. Systolic

and diastolic blood pressures were measured at rest. Body mass index (BMI, kg/m2) was used as a measure of relative body weight. All relevant investigations have been carried out in the AFIP, Rawalpindi. The liver function tests, TSH and serum urea were analyzed to exclude liver, thyroid and renal disorders respectively.

In Study # 1, baseline venous blood samples (12 h fast, 7:00 - 9:00 am) were drawn after 8-weeks (phase I). Then participants were randomly divided into four groups. The participants of

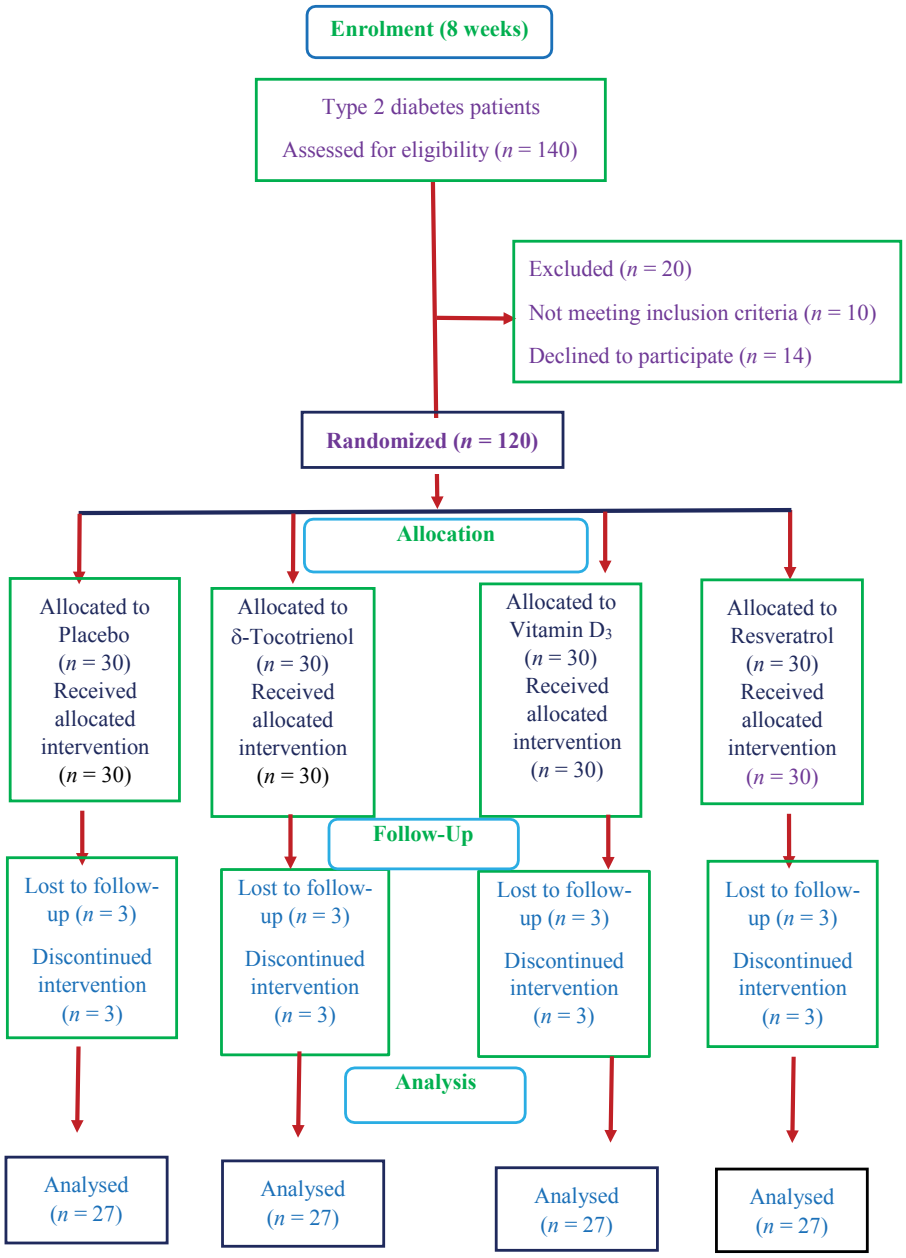


Figure 2. Study # 1 protocol of effect of placebo vs. δ-tocotrienol, vitamin D_3 and resveratrol on diabetes biomarkers people with T2DM.

Group A were provided capsules of AMR-1 (placebo group, n = 30 participants; 2 capsules/d of 250.062 mg/capsule of cellulose + olive oil (125.062 mg + 125.00 mg); group B (n = 30) two capsules/d of δ-tocotrienol (250 mg/capsule); group C (n = 30) two capsules/d of vitamin D_3 (5000 IU/capsule); and group D (n = 30) capsules/d of resveratrol (250 mg/capsule) were given (one capsule after breakfast and second after dinner) for 24-weeks (phase II) as outlined in Figure 2.

In study # 2, baseline venous blood samples (12 h fast, 7:00 - 9:00 am) were also drawn after 8- weeks (phase I), then T2DM participants of placebo group (n = 56) were given two capsules/d of cellulose + olive oil (150.062 mg +150 mg/capsule) and NS-3 group (n = 56) was given two capsules/d of a mixture of NS-3 (125 mg + 5000 IU + 125 mg/capsule) for 24-weeks (phase II) as outlined in Figure 3. The capsules in each group were administered one capsule after breakfast and second after dinner throughout the study. In order to ascertain full compliance of dietary recommendations and intake of nutritional supplements, participants were contacted by telephone. Two tubes (6 mL/tube) fasting venous blood sample were collected at the end of each phase, one sample into EDTA tubes for plasma and second set for serum. The blood tubes were centrifuged at 1200 x g for 10 minutes, followed by careful separation of plasma samples into three aliquots. One aliquot (2.0 ml) was immediately processed for total mRNAs purification. Plasma and serum (1.0ml/tube) and purified total RNAs were stored at –80 ºC for further analyses.

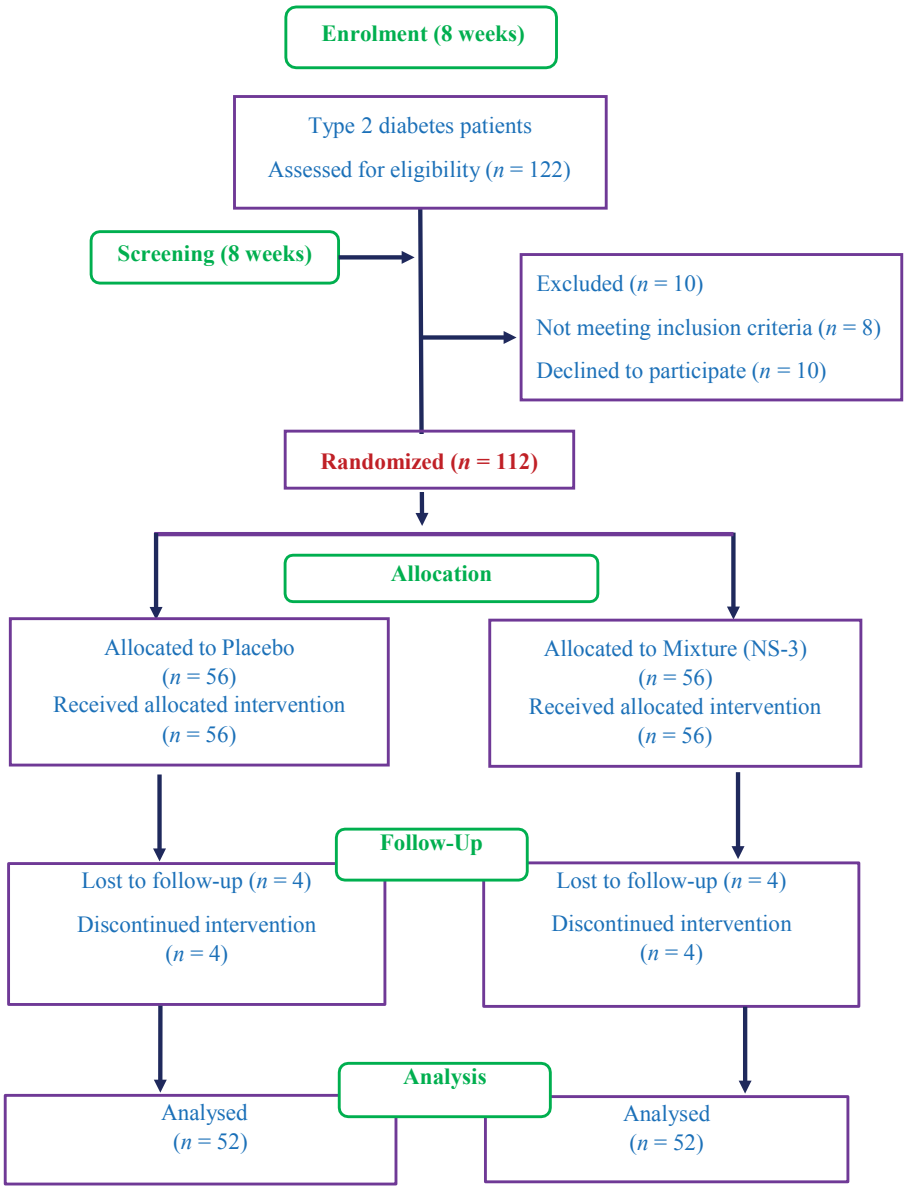


Figure 3. Study # 2 protocol of effect of placebo vs. mixture of δ-tocotrienol, vitamin D_3, resveratrol (NS-3) on diabetes biomarkers people with T2DM.

Evaluation of a mixture of NS-3 on diabetes biomarkers and cytokines in RT-PCR of purified RNAs of NS-3 treated people with T2DM.

The total mRNAs were isolated from people with T2DM treated with a mixture of NS-3 for 24- weeks by using One-Step qRT-PCR kit (Life Technologies, Foster City, California). The Real-time PCR assays of total mRNA per reaction (200,000 RNAs/treatment) of pre-dose and post-dose were carried out as described above.

Biochemical Analysis

Several biochemical assays were performed in all groups at the end of Phases I and II. The serum fasting glucose was determined by glucose oxidase method. Serum hs-CRP was analyzed by 2-site sequential chemiluminescent immune-metric assay kit (Seimen, LA) on Immulite 1000 (Immulite; Diagnostic Product Corporation). Serum hemoglobinA1c (HbA1c), bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, urea, uric acid, the estimation of these parameters was carried out in Department of Pathology, Rawalpindi, Pakistan. The serum/plasma levels of insulin, insulin resistance, oxidative stress, inflammatory biomarkers in people with T2DM were also determined.

Sample Size Calculation in NS-3 Group

The sample size was carried out by G Power 3.1 software (Experimental Psychology institute, Dusseldorf, North Rhine-Westphalia, Germany) available online using 80% power and 95% confidence level. The levels of post-treatment of HbA1c, Hs-CRP and MDA in NS-3 treatment and placebo groups (Table 5) were analyzed based on previous studies [31,32].

Statistical analyses

The Statistical Package for Social Sciences (SPSS) software version 21.0 (IBM Corporation, New York, USA) was used for statistical analyses. Kolmogorov–Smirnov test was applied on data to assess distribution for all the variables, mean, SD, median, and interquartile range (IQR) calculate for descriptive statistics. Serum cytokine levels, oxidative markers and glycosylated HbA1c among the people with T2DM in both groups were compared using Mann–Whitney U test. Pearson correlation was also applied between HbA1c, MDA, hs-CRP, cytokines (Interleukin-6, IL-8, ICAM-1, VCAM-1, TNF-α, and IFN-γ). Analysis of covariance (ANOVA) was used to compare means of pre-treatment versus post-treatment. Data reported as mean ± SD (Standard Deviation) in Tables. The statistical significance level was set at 5% ($P < 0.05$).

Results

Evaluation of a mixture of NS-3 and its individual components on biomarkers of type 2 diabetes *in vitro* using PBMCs from normal and people with T2DM.

The results of RT-PCR tested in PBMCs of people with T2DM indicated significant ($p > 0.05 – 0.02$) down regulation in gene expression of important diabetes biomarkers (IRA-1, SOD-2, IGFBP-2, PTPRN, GCKR, resistin) with δ-tocotrienol (20 - 74), vitamin D_3 (37 - 73), resveratrol (58 - 74), and NS-3 mixture (15 - 56) as compared to their respective controls (Table 1). Similarly, significant down-regulation was also observed with these compounds and NS-3 mixture in ICAM- 1, VCAM-1, IL-6, IL-8 (23 – 70), respectively compared to their respective controls (Table 1). These results clearly supported our original hypothesis that the NS-3 mixture will effectively better down-regulate gene expression of T2DM biomarkers compared to its individual components, except for δ-tocotrienol showed significant up-regulation in IFN-γ (138 and with 80 µM 287 [data is not shown]), and IL-8 (108). The summary of these results was presented as percentage down- or up-regulation in Figures 4A and 4B. The above reported biomarkers were also tested in PBMCs obtained from healthy normal subjects under same conditions showed non-significant changes in these biomarkers compared to their respective controls (data not shown).

Toxicity of a mixture of NS-3 and its components on PBMCs obtained from people with T2DM by MTT or autophagy.

The toxicity of a mixture of NS-3 and its components were treated PBMC from people with T2DM with different concentrations (10 µM – 80 µM) of these compounds for 4 h.

Table 1: RT-PCR analysis of RNAs obtained from PBMCs of people with T2DM after treatment with a mixture of NS-3 and its components *in vitro* on Diabetes biomarkers including inflammatory.

Biomarkers	Control	δ-Tocotrienol	Vitamin D_3	Resveratrol	Mixture (NS-3)
1. Insulin Receptor Substrate-1 (IRS-1)	0.99 ± 0.04*	0.33 ± 0.06	0.84 ± 0.07*	0.74 ± 0.06	0.31 ± 0.06
2. Superoxide Dismutase (SOD-2)		0.75 ± 0.06	0.80 ± 0.08*	0.89 ± 0.07*	0.56 ± 0.08
3. Insulin Llike FactorBinding Protein-2 (IGFBP-2)		0.20 ± 0.06	0.73 ± 0.07	0.70 ± 0.08	0.39 ± 0.06
4. Protein Tyrosine Phosphtase Receptor Type (PTPRN)		0.81 ± 0.08*	0.96 ± 0.07*	0.60 ± 0.07	0.30 ± 0.11
5.Glucokines Regulator (GCKR)	1.00 ± 0.04*	0.45 ± 0.09	0.37 ± 0.10	0.58 ± 0.09	0.29 ± 0.06
6. Resistin		0.50 ± 0.06	0.57 ± 0.08	0.69 ± 0.08	0.50 ± 0.13
7. Intercellular Adhesion Molecule-1 (ICAM-1)		0.45 ± 0.09	0.67 ± 0.08	0.89 ± 0.08*	0.58 ± 0.07
8. Vascular Adhesion Molecule-1 (VCAM-1)		0.23 ± 0.06	0.49 ± 0.10	0.54 ± 0.09	0.15 ± 0.06
9. Interleukin-6 (IL-6)	1.00 ± 0.04*	0.69 ± 0.09	0.85 ± 0.13*	0.81 ± 0.19*	0.26 ± 0.08
10. Interleukin-8 (IL-8)		1.08 ± 0.12*	0.82 ± 0.12*	0.84 ± 0.09*	0.61 ± 0.08
11. Tumor Necrosis Factor-α(TNF-α)		0.98 ± 0.11*	0.70 ± 0.10	0.98 ± 0.09*	0.66 ± 0.10
12. Interferon-γ (IFN-γ)		1.38 ± 0.15	0.65 ± 0.08	0.75 ± 0.15*	0.51 ± 0.12

ANOVA test is applied for analyses; *Values in a row sharing a common asterisk are non significant; all other are significant as compared with control.

The MTT and LDH cell death assays indicated that cells were more than 95% viable with these concentrations. Moreover, the effect of these compounds, and mixture (NS-3) was also examined on autophagy assays using PBMCs obtained from people with T2DM. None of the concentrations of these compounds or their mixture (NS-3) used induced autophagy in these cells after 4 h incubation (Figure 5, reported only for 20 µM). However, rapamycin + chloroquine treatment (positive control) resulted in increased green fluorescence, as evidence of autophagy vacuole accumulation (Figure 5). These results clearly demonstrated that these compounds can be safely be used for human studies. Both of these findings prompted us to investigate the comparative effect of a mixture of NS-3 as well as its individual components on various diabetes biomarkers, and later gene expression of miRNAs and mRNAs associated with diabetes *in vivo* in people with T2DM.

Evaluation of δ-tocotrienol, vitamin D_3 and resveratrol in people with T2DM.

The first study of people with T2DM (n = 120) was carried out of four groups: of placebo, δ- tocotrienol, vitamin D3 and resveratrol (n = 30/group) fed individual capsules for 24-weeks as outlined in (Figure 2). Three subjects in each group were not able to complete the treatment. The physical characteristics of all the participants of pre-dose values are reported in Table 2. There were no changes in the body weight, height, body mass

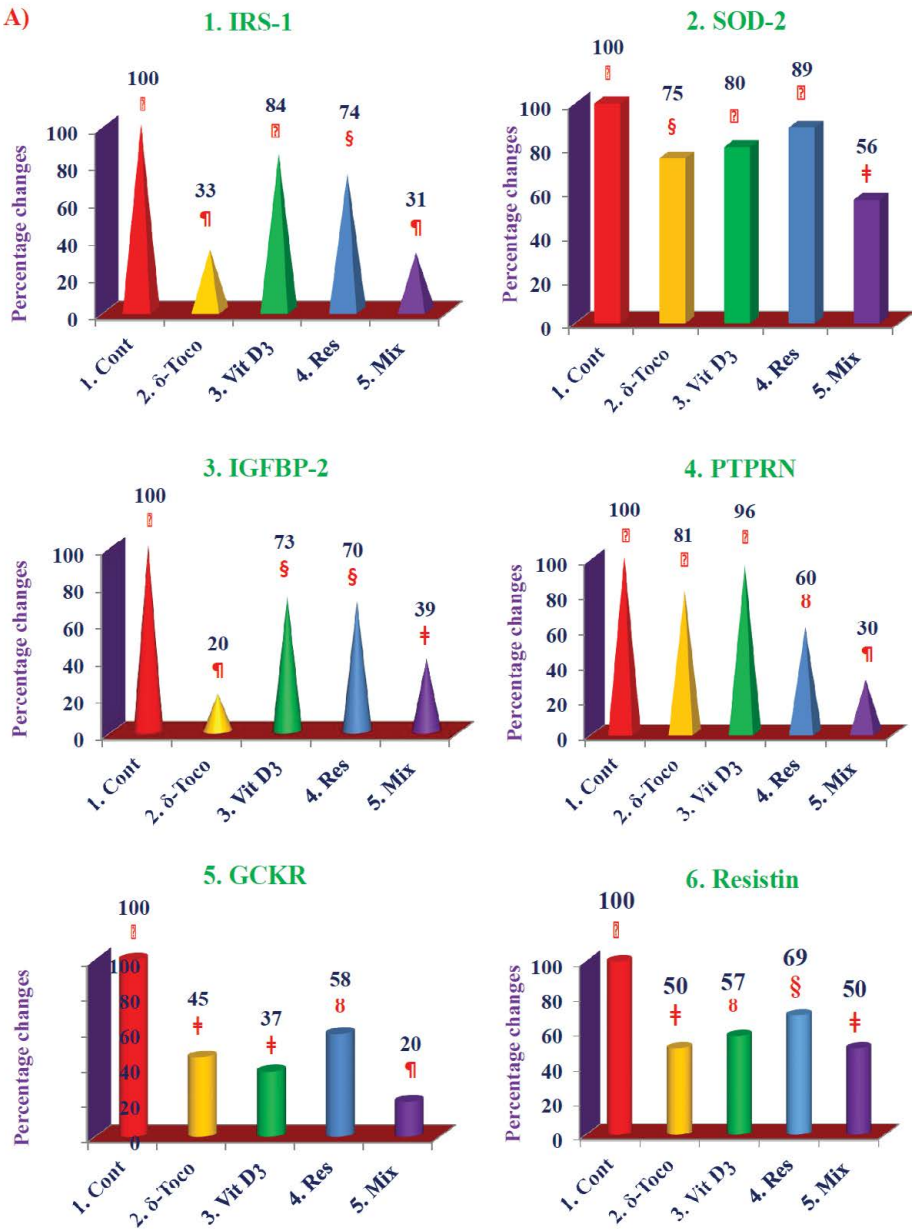


Figure 4A. Effect of a mixture of NS-3 and its components *in vitro* on diabetes biomarkers and cytokines in PBMCs obtained from people with T2DM.
The assay procedure of estimating effect of NS-3 mixture on diabetes biomarkers and cytokines in peripheral blood mononuclear cells (PBMCs) of people with T2DM has been described in detail in method section. Data are means ± SD. Values in a column sharing a common symbol are significantly different at compared to †control, **§*P*** < 0.05, **ϑ*P*** <0.02, **≠*P*** <0.01, **¶*P*** <0.001.

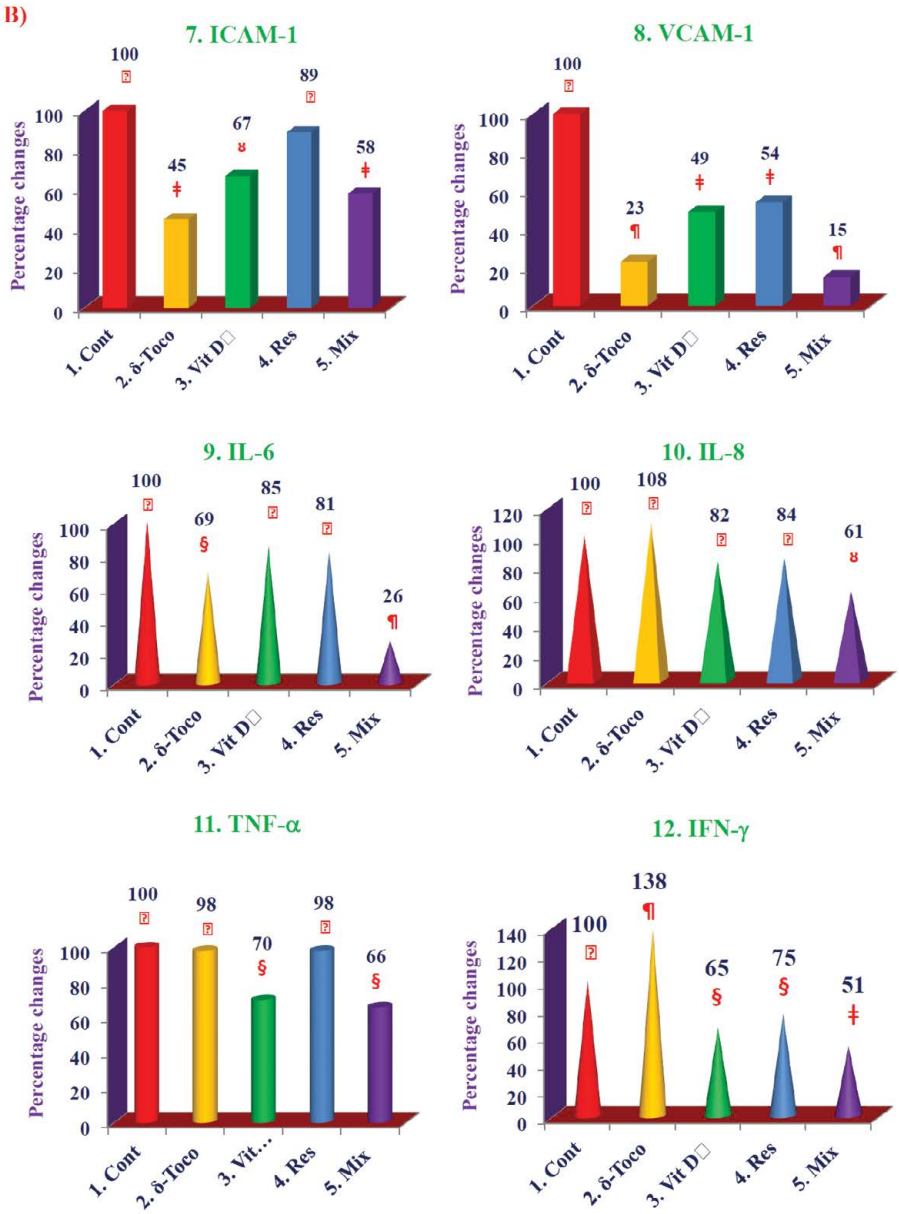
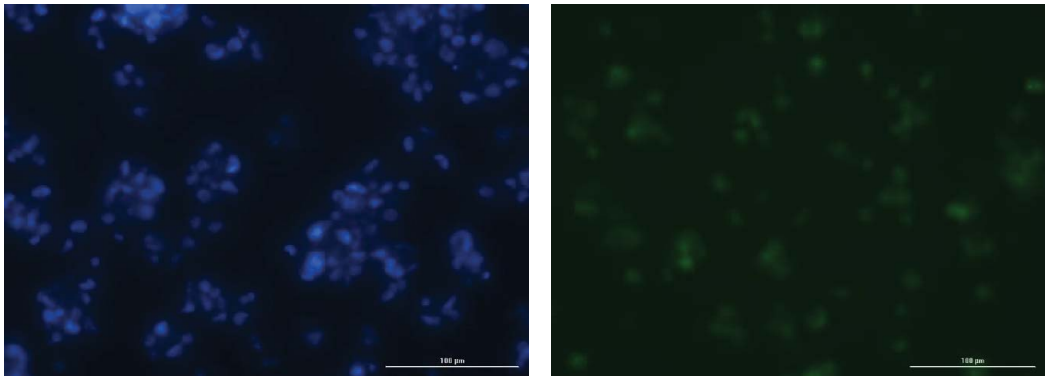


Figure 4B. Effect of a mixture of NS-3 and its components *in vitro* on diabetes biomarkers and cytokines in PBMCs obtained from people with T2DM.

The assay procedure of estimating effect of NS-3 mixture on diabetes biomarkers and cytokines in peripheral blood mononuclear cells (PBMCs) of people with T2DM has been described in detail in method section. Data are means ± SD. Values in a column sharing a common symbol are significantly different at compared to †control, **§*P*** < 0.05, ***ɷP*** <0.02, ***≠P*** <0.01, ***¶P*** <0.001.

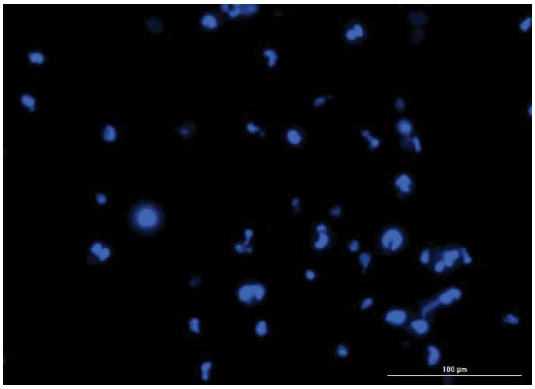
index, waist circumference, systolic blood pressure, diastolic blood pressure at the end of post-dose treatment (Table 2). There were also insignificant changes in placebo group in serum/plasma values in various biomarkers of post-dose as compared to pre-dose values after the treatment (Table 3A). However, there were significant (p <0.001 – 0 .05) decreases in serum/plasma values of fasting glucose (7%), HbA1c (8%), hs-CRP (12%), fasting insulin (9%), HOMA-IR (14), MDA (11%), TNF-α (14%), IL-6 (9%), total cholesterol (9%), LDL-Chol (4%), triglycerides (8%) of post-dose values as compared their respective pre-dose values at the end of δ-tocotrienol treatment (Table 2A). Similar significant (p <0.001 – 0.032) percentages decreases in all these parameters were also observed in serum/plasma levels of post-dose versus pre-dose values at the end of treatments with vitamin D_3 fasting glucose (5%), HbA1c (7%), hs-CRP (6%), fasting insulin (8%), HOMA (IR-11%), TNF-α (12%), IL-6 (14%), and with resveratrol, fasting glucose (5%), HbA1c (8%), hs-CRP (11%), fasting insulin (6%), HOMA-IR (7%), TNF-α (13%), IL-6 (15%) as compared to their respective pre-dose values (Table 3B).

In the second study, people with T2DM (n = 112) were selected for placebo group (n = 56) and for a mixture NS-3 (δ-tocotrienol + vitamin D_3 + resveratrol, (n = 56) group.

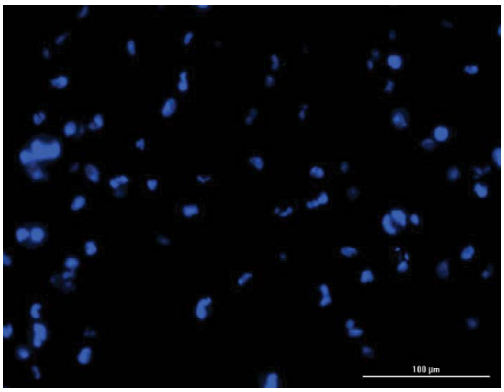


A: Vehicle (Medium-Blank)

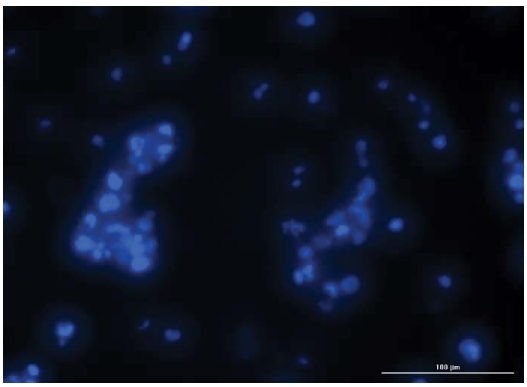
B: Rapamycin + Chloroquin (Positive Conl)



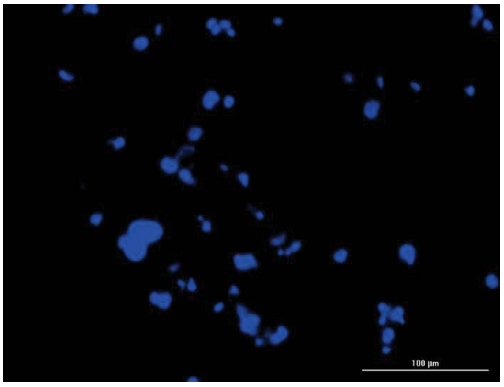
C: δ-Tocotrienol (20 µM)



D: Vitamin D_3 (20 µM)



E: Resveratrol (20 µM)



F: Mixture (NS-3; 20 µM)

Figure 5. Effect of a mixture of NS-3 and its component on autophagy assay in PBMCs of people with T2DM.
The PBMC (200,000 cells/well in medium + 0.2% DMSO) were first differentiated with PMA (10ng/mL) in media of 96-tissue culture white plate for 4 h at 37 ˚C under 5% CO2 in an incubator, then washed with fresh media, followed by incubation with vehicle (medium + 2% DMSO, blank control), a mixture of rapamycin + chloroquine (as positive control), δ-tocotrienol, vitamin D3, resveratrol, or NS-3 using a dose of 20 µM of each compound or mixture for 4 h at 37 ˚C under CO2. The cells were washed with assay buffer followed by incubation with microscopy dual detection reagent (100 µL) for 30 min at 37 ˚C in dark. After incubation the PBMC were finally washed with assay buffer three times. The Fluorescence measurements were performed under Cytation-3- fluorometric reader using DAPI and FITC filer sets.

They were fed placebo or NS-3 capsules for 24-weeks. Four subjects in each group did not complete at the end of the study. The physical characteristics of all the participants (n = 52) of pre-dose values are reported in Table 4. There were no changes in the body weight, height, body mass index, waist circumference, systolic blood pressure, diastolic blood pressure at the end of post-dose treatment (Table 4). There was also no change in all the parameters values of serum/plasma of post-dose compared to pre-dose levels of the placebo after 24-week treatment (Table 4). However, there were significant ($p < 0.001 – 0.04$) decreases in serum/plasma values of fasting glucose (11%), HbA1c (10%), hs-CRP (23%), fasting insulin (9%), HOMA-IR (20%), MDA (20%), microalbuminuria (5%), creatinine (8%), total cholesterol (8%), LDL-cholesterol

Table 2: Impact of placebo, δ-tocotrienol, vitmin D_3, and resveratrol on demographic charateristics of people with T2DM (n = 108).

#	Biomarkers	[a]Placebo (n = 27)	[a]δ-Tocotrienol (n = 27)	[a]Vitamin D_3 (n = 27)	[a]Resveratrol (n = 27)
1	Sex:				
	Male	15	15	15	15
	Female	12	12	12	12
2	Age (years)	53.67 ± 11.71	58.33 ± 9.92	58.41 ± 10.35	51.67 ± 8.02
3	Weight (kg)	89.59 ± 13.59	89.30.33 ± 12.21	86.37 ± 7.52	85.96 ± 13.13
4	Height (m^2)	3.34 ± 0.32	3.21 ± 0.38	2.89 ± 0.30	2.96 ± 0.28
5	Body mass index (kg/m^2)	27.62 ± 3.62	27.93 ± 3.62	30.06 ± 3.28	29.13 ± 4.55
6	Waist Circumference (cm)	39.70 ± 4.05	39.15 ± 5.14	39.07 ± 4.63	37.19 ± 2.20
7	Duration of diabetes (years)	9.07 ± 5.18	9.04 ± 5.21	9.48 ± 2.93	7.15 ± 2.73
8	Systolic blood pressure (mmHg)	137.04 ± 7.50	137.22 ± 9.54	142.22 ± 12.89	139.81 ± 6.12
9	Diastolic blood pressure (mmHg)	89.20 ± 7.50	90.00 ± 6.93	91.67 ± 8.20	96.30 ± 5.47
10	Fasting blood sugar (mmol/L)	7.65 ± 1.83	7.62 ± 1.84	7.55 ± 1.22	7.59 ± 1.31
11	Hemoglobin A1c (%)	8.46 ± 2.46	8.42 ± 1.24	8.86 ± 0.77	8.58 ± 1.30
12	hs-C reactive protein (mg/L)	3.48 ± 1.68	3.59 ± 1.64	3.37 ± 0.72	3.69 ± 0.73
13	Total cholesterol (mmol/L)	5.42 ± 0.54	5.42 ± 0.66	5.44 ± 0.60	5.67 ± 71
14	Triglycerides (mmol/L)	2.27 ± 1.56	2.22 ± 1.44	2.20 ± 0.56	2.19 ± 0.57

[a]Two capsules of cellulose/olive oil (250 mg/capsule; placebo) or two capsules of δ-tocotrienol or resveratrol (250 mg/capsule) or vitamin D_3 (5000.00 IU/capsule) were administered to people with T2DM for 24-weeks.

Table 3A: Impact of placebo vs δ-tocotrienol on various biomarkers of diabetes in people with T2DM (n = 27/group).

Biomarkers	[a]Placebo (n = 27)	Placebo	P-value	[a]δ-tocotrienol (n = 27)	-Tocotrienol	P-values
	Pre-dose	Post-dose		Pre-dose	Post-dose	
1. Fasting blood sugar (mmol/L)	7.62 ± 1.84 (100)[b]	7.57 ± 1.66 (99)	0.303	7.35 ± 1.98 (100)[b]	6.85 ± 2.00 (93)	0.001
2. Hemoglobin A1c (%)	8.42 ± 1.24 (100)	8.42 ± 1.23 (100)	0.619	8.44 ± 1.09 (100)	7.79 ± 1.70 (92)	0.001
3. hs-C reactive protein (mg/L)	3.59 ± 1.64 (100)	3.47 ± 1.81 (100)	0.751	3.53 ± 2.05 (100)	3.10 ± 1.93 (88)	0.019
4. Fasting Insulin (IU/L)	15.79 ± 4.83 (100)	15.79 ± 4.64 (100)	0.208	15.46 ± 5.40 (100)	14.10 ± 5.18 (91)	0.000
5. HOMA-IR	5.51 ± 2.62 (100)	5.44 ± 2.40 (99)	0.791	5.23 ± 2.60 (100)	4.51 ± 2.53 (86)	0.001
6. Malondialdehyde (µmol/L)	3.57 ± 0.61 (100)	3.58 ± 0.59 (100)	0.731	3.63 ± 0.72 (100)	3.22 ± 0.68 (89)	0.001
7. Tumor Necrosis Factor-a (pg/L)	8.22 ± 2.64 (100)	8.04 ± 2.48 (98)	0.032	9.61 ± 4.11 (100)	8.25 ± 0.25 (86)	0.001
8. Interleukin-6 (pg/L)	14.33 ± 3.89 (100)	14.21 ± 3.22 (99)	0.570	15.22 ± 3.64 (100)	13.86 ± 3.46 (91)	0.001
9. Total cholesterol (mmol/L)	5.42 ± 0.68 (100)	5.25 ± 0.74 (99)	0.910	5.73 ± 0.77 (100)	5.20 ± 0.74 (91)	0.000
10. HDL-C (mmol/L)	0.89 ± 0.34 (100)	0.91 ± 0.35 (102)	0.355	0.81 ± 0.14 (100)	0.82 ± 0.12 (101)	0.315
11. LDL-C (mmol/L)	3.50 ± 0.76 (100)	3.43 ± 0.94 (98)	0.767	3.22 ± 0.67 (100)	3.10 ± 0.54 (96)	0.001
12. Triglycerides (mmol/L)	2.25 ± 1.44 (100)	2.21 ± 0.75 (98)	0.387	2.61 ± 1.55 (100)	2.40 ± 1.47 (92)	0.752

[a]Two capsules of cellulose/olive oil (250 mg/capsule; placebo) or two capsules of δ-tocotrienol (250 mg/capsule) were administered to people with T2DM for 24-weeks.

[b]Percentage of control values are in parentheses.

Table 3B: Impact of vitamin D_3 & resveratrol on various diabetes biomarkers in people with T2DM (n = 27/group).

Biomarkers	[a]Vitamin D_3 (n = 27)	Vitamin D_3	P-value	[a]Resveratrol (n = 27)	Resveratrol	P-value
	Pre-dose	Post-dose		Pre-dose	Post-dose	
1. Fasting blood sugar (mmol/L)	7.55 ± 1.22 (100)	7.16 ± 1.11 (95)	0.039	7.39 ± 1.31 (100)[b]	6.98 ± 1.58 (94)	0.026
2. Hemoglobin A1c (%)	8.81 ± 0.77 (100)	8.19 ± 0.90 (93)	0.001	8.58 ± 1.30 (100)	7.86 ± 1.36 (92)	0.001
3. hs-C reactive protein (mg/L)	3.37 ± 0.72 (100)	3.09 ± 0.72 (94)	0.023	3.69 ± 0.73 (100)	3.28 ± 0.70 (89)	0.001
4. Fasting Insulin (IU/L)	15.84 ± 2.32 (100)	14.97 ± 2.27 (92)	0.001	15.59 ± 2.91 (100)	14.65 ± 2.88 (94)	0.001
5. HOMA-IR	5.32 ± 1.05 (100)	4.74 ± 0.91 (89)	0.001	5.37 ± 1.38 (100)	4.98 ± 1.33 (93)	0.001
6. Malondialdehyde (µmol/L)	3.59 ± 0.78 (100)	3.48 ± 0.62 (97)	0.089	3.82 ± 0.94 (100)	3.49 ± 0.94 (91)	0.001
7. Tumor Necrosis Factor-a (pg/L)	9.71 ± 1.33 (100)	8.55 ± 1.23 (88)	0.001	10.37 ± 2.06 (100)	9.02 ± 2.49 (87)	0.001
8. Interleukin-6 (pg/L)	14.92 ± 2.23 (100)	12.90 ± 2.53 (86)	0.001	16.19 ± 2.22 (100)	13.69 ± 3.83 (85)	0.001
9. Total cholesterol (mmol/L)	5.44 ± 0.60 (100)	5.36 ± 0.73 (99)	0.309	5.69 ± 0.71 (100)	5.50 ± 0.81 (97)	0.654
10. HDL-C (mmol/L)	0.85 ± 0.20 (100)	0.88 ± 0.20 (104)	0.506	0.83 ± 0.20 (100)	0.83 ± 0.19 (100)	0.912
11. LDL-C (mmol/L)	3.59 ± 0.69 (100)	3.52 ± 0.94 (98)	0.878	3.84 ± 0.80 (100)	3.69 ± 0.80 (96)	0.451
12. Triglycerides (mmol/L)	2.20 ± 0.56 (100)	2.14 ± 0.58 (97)	0.361	2.19 ± 0.57 (100)	2.14 ± 0.63 (98)	0.713

[a]Two capsules of vitamin D_3 (5000/capsule) or two capsules of resveratrol (250 mg/capsules were administered to people with T2DM for 24 weeks.

[b]Percentage of control values are in parentheses.

(10%), triglycerides (8%) TNF-α (25%), and IL-6 (25%) of post-dose values compared their respective

pre-dose values at the end of NS-3 mixture treatment (Table 5). The serum levels of HDL- cholesterol were not changed in both sets of study (Tables 4). These results clearly indicate that treatment by a mixture of NS-3 to people with T2DM resulted significant reductions in the levels of fasting glucose, hs-CRP, HOMA-IR, MDA, TNF-α and IL-6 (Table 5).

The results of comparison of between NS-3 and placebo groups analysis revealed that supplementation of NS-3 significantly reduced [mean difference at 95% confidence level of fasting glucose -1.076-(-0.534), HbA1c -1.240-(-0.714), hs-CRP-1.105-(-0.379), fasting insulin -2.218-(-0.707), HOMA-IR -1.422-(-0.667) and MAD -0.341-(0.451) were significantly ($P < 0.05$) decreased as compared to placebo group (Table 6).

In summary, the results of the impact of NS-3 mixture and its components on some important biomarkers associated with diabetes are shown in Table 7. The mixture (NS-3) displayed maximum reduction in serum/plasma values of fasting glucose, HbA1c, hs-CRP, HOMA-IR, MDA as compared to individual components (Table 7). None of the subjects reported any side-effects with the NS-3 mixture during six months of trial period, and these results supported our original hypothesis that a mixture of natural products (δ-tocotrienol, vitamin D3, resveratrol) is more effective in lowering serum/plasma levels of diabetes biomarkers and pro-inflammatory cytokines than their individual compounds (Tables 4, 5).

Table 4: Impact of a mixture of NS-3 on demographic characteristics of people with T2DM (*n* = 104)

Various biomarkers	[a]Placebo (*n* = 52)	[b]Mixture (*n* = 52)	*p* -value
Sex			
Male	26 (50)	26 (50)	0.082
Female	26 (50)	26 (50)	0.081
Age (Years)	50.48 ± 12.50[c]	49.73 ± 12.08	0.951
Weight (kg)	86.77 ± 12.56	82.63 ± 11.71	0.207
Height (m^2)	3.25 ± 0.37	3.10 ± 0.35	0.125
Body mass index (kg/m^2)	26.78 ± 3.26	26.82 ± 4.05	0.999
Waist Circumference (inches)	38.67 ± 4.2	36.98 ± 4.41	0.116
Systolic BP (mmHg)	137.60 ± 8.55	137.94 ± 14.38	0.987
Diastolic BP (mmHg)	88.27 ± 6.63	88.92 ± 7.49	0.886
Duration of Diabetes (years)	8.42 ± 4.72	8.14 ± 5.13	0.953
Fasting blood glucose (mmol/L)	7.65 ± 1.66	7.39 ± 1.71	0.703
HbA1c (%)	8.53 ± 1.16	8.20 ± 1.30	0.301
hs-CRP (mg/L)	3.46 ± 1.51	3.65 ± 1.31	0.802
Fasting Insulin (mIU/L)	15.96 ± 4.37	15.90 ± 5.78	0.950
HOMA-IR	5.58 ± 2.44	5.44 ± 2.85	0.792
Malondialdehyde (MDA; µmol/L)	3.75 ± 0.65	3.81 ± 0.52	0.720
Microalbuminuria (mg/mmol)	11.32 ± 0.96	12.56 ± 1.95	0.821
Creatinine (µmol/L)	89.79 ± 12.30	89.75 ± 18.10	0.990
Total Cholesterol (mmol/L)	5.37 ± 0.71	5.36 ± 0.72	0.997
HDL-C (mmol/L)	0.92 ± 0.29	0.92 ± 0.34	0.987
LDL-C (mmol/L)	3.49 ± 0.83	3.36 ± 0.79	0.734
Triglycerides (mmol/L)	2.13 ± 1.27	2.36 ± 1.00	0.562
TNF-α (pg/mL)	8.98 ± 4.37	9.65 ± 5.60	0.500
IL-6 (pg/mL)	14.97 ± 7.82	14.86 ± 8.01	0.944

[a]Two capsules of cellulose/olive oil (250 mg/capsule) were administered to people with T2DM for 24-weeks.

[b]Two capsules of NS-3 mixture (250 mg/capsule) were administered to people with T2DMs for 24-weeks.

[c]Mean ± SD (Standard Deviation).

Table 5: Impact of a Mixture of NS-3 on various biomarkers of diabetes in serum/plasma of people with T2DM (n = 104).

#	Various biomarkers	[a]Placebo (n = 52) Pre-dose	Placebo (n = 52) Post-dose	[c]P-values	[b]Mixture (n = 52) Pre-dose	Mixture (n = 52) Post-dose	[c]P-values
1	Fasting glucose (mmol/L)	7.65 ± 1.66	7.59 ± 1.49 (99)[d]	0.098	7.39 ± 1.71	6.56 ± 1.66 (89)[d]	0.000
2	Fasting HbA1c (%)	8.53 ± 1.16	8.50 ± 1.17 (98)	0.764	8.20 ± 1.30	7.40 ± 0.93 (90)	0.000
3	hs-CRP (mg/L)	3.46 ± 1.51	3.42 ± 1.68 (99)	0.690	3.65 ±1.31	2.82 ± 1.07 (77)	0.000
4	Fasting Insulin (mIU/L)	15.96 ± 4.37	15.94 ± 4.26 (100)	0.601	15.90 ± 5.78	14.42 ± 5.56 (91)	0.000
5	HOMA-IR	5.58 ± 2.44	5.50 ± 2.25 (99)	0.060	5.44 ± 2.85	4.35 ± 2.25 (80)	0.000
6	Malondialdehyde (MDA; µmol/L)	3.75 ± 0.65	3.76 ± 0.63 (100)	0.960	3.81 ± 0.65	3.04 ± 0.47 (80)	0.030
7	Microalbuminuria (mg/mmol)	11.32 ± 0.96	11.03 ± 9.45 (97)	0.345	12.56 ± 1.19	11.90 ± 1.21(95)	0.015
8	Creatinine (µmol/L)	89.79 ± 12.30	88.77 ± 12.69 (99)	0.109	89.75 ± 18.10	82.40 ± 16.97 (92)	0.000
9	Total cholesterol (mmol/L)	5.37 ± 0.71	5.34 ± 0.99 (99)	0.844	5.36 ± 0.72	4.95 ± 0.72 (92)	0.000
11	HDL-C (mmol/L)	0.92 ± 0.29	0.92 ± 0.30 (100.00)	0.255	0.92 ± 0.34	0.94 ± 0.28 (102)	0.498
12	LDL-C (mmol/L)	3.49 ± 0.83	3.49 ± 1.21 (100.00)	0.956	3.36 ± 0.79	3.02 ± 0.78 (90)	0.000
13	Triglycerides (mmol/L)	2.12 ± 1.27	2.03 ± 0.89 (96)	0.621	2.36 ± 0.00	2.18 ± 0.98 (92)	0.038
14	TNF-α (pg/mL)	8.98 ± 4.37	8.77 ± 4.12 (98)	0.154	9.65 ± 5.60	7.28 ± 4.41 (75)	0.000
15	IL-6 (pg/mL)	14.97 ±7.82	14.82 ± 7.22 (99)	0.591	14.86 ± 8.01	11.13 ± 6.96 (75)	0.003

[a]Two capsules of cellulose/olive oil (250 mg/capsule; placebo) were administered to people with T2DM for 24-weeks.

[b]Two capsules of a mixture of NS-3 (250.062 mg/capsule) were administered to people with T2DM for 24-weeks.

[c]The calculation of post treatment variables is based on an analysis of covariance (ANCOVA), adjusted for one covariates: baseline (pre-treatment) variables.

[d]Percentage of control values are in parentheses.

Table 6: Comparison of between-groups of serum/plasma biovhemical levels in placebo and mixture groups in people with T2DM (n = 104).

#	Various biomarkers	[a]Placebo (n = 52) Pre-dose	[b]Mixture (n = 52) Pre-dose	[c]Mean difference (95% CI)	P-values	Placebo (n = 52) Post-dose	Mixture (n = 52) Post-dose	Mean difference (95% CI)	P-values
1	Fasting glucose (mmol/L)	7.65 ± 1.66	7.39 ± 1.71	-0.92 - 0.39	0.432	7.59 ± 1.49 (99)[d]	6.56 ± 1.66 (89)[d]	-1.076 - (-0.534)	0.000
2	Fasting HbA1c (%)	8.53 ± 1.16	8.20 ± 1.30	-0.96 - 0.14	0.168	8.50 ± 1.17 (98)	7.40 ± 0.93 (90)	-1.240-(-0.714)	0.000
3	hs-CRP (mg/L)	3.46 ± 1.51	3.65 ±1.31	-0.36 -0.74	0.503	3.42 ± 1.68 (99)	2.82 ± 1.07 (77)	-1.105-(-0.379)	0.000
4	Fasting Insulin (mIU/L)	15.96 ± 4.37	15.90 ± 5.78	-2.06-1.93	0.950	15.94 ± 4.26 (100)	14.42 ± 5.56 (91)	-2.218-(-0.707)	0.000
5	HOMA-IR	5.58 ± 2.44	5.44 ± 2.85	-1.17-0.89	0.792	5.50 ± 2.25 (99)	4.35 ± 2.25 (80)	-1.422-(-0.667)	0.000
6	Malondialdehyde (MDA; µmol/L)	3.75 ± 0.65	3.81 ± 0.65	-0.38-0.66	0.515	3.76 ± 0.63 (100)	3.04 ± 0.47 (80)	-0.341-(0-0.451)	0.000
7	Microalbuminuria (mg/mmol)	11.32 ± 0.96	12.56 ± 1.19	-2.97-5.45	0.561	11.03 ± 9.45 (97)	11.90 ± 1.21(95)	-1.142-(-462)	0.403
8	Creatinine (µmol/L)	89.79 ± 12.30	89.75 ± 18.10	-6.06-5.98	0.990	88.77 ± 12.69 (99)	82.40 ± 16.97 (92)	-1.021-(-4.42)	0.000
9	Total cholesterol (mmol/L)	5.37 ± 0.71	5.36 ± 0.72	-0.29-0.27	0.939	5.34 ± 0.99 (99)	4.95 ± 0.72 (92)	-0.654-(-0.125)	0.004
11	HDL-C (mmol/L)	0.92 ± 0.29	0.92 ± 0.34	-0.11-0.13	0.895	0.92 ± 0.30 (100)	0.94 ± 0.28 (102)	-0.035-(-0.045)	0.796
12	LDL-C (mmol/L)	3.49 ± 0.83	3.36 ± 0.79	-.044-0.19	0.432	3.49 ± 1.21 (100)	3.02 ± 0.78 (90)	-0.72-(-0.050)	0.023
13	Triglycerides (mmol/L)	2.12 ± 1.27	2.36 ± 0.00	-0.21-0.68	0.301	2.03 ± 0.89 (96)	2.18 ± 0.98 (92)	-0.24-(-0.351)	0.790
14	TNF-α (pg/mL)	8.98 ± 4.37	9.65 ± 5.60	-1.29-2.62	0.500	8.77 ± 4.12 (98)	7.28 ± 4.41 (75)	-2.77-(-1.201)	0.000
15	IL-6 (pg/mL)	14.97 ±7.82	14.86 ± 8.01	-3.19-2.97	0.944	14.82 ± 7.22 (99)	11.13 ± 6.96 (75)	-5.69-(-1.570)	0.001

[a]Two capsules of cellulose/olive oil (250 mg/capsule; placebo) were administered to people with T2DM for 24-weeks.

[b]Two capsules of a mixture of NS-3 (250.062 mg/capsule) were administered to people with T2DM for 24-weeks.

[c]The calculation of post treatment variables is based on an analysis of covariance (ANCOVA), adjusted for one covariates: baseline (pre-treatment) variables.

[d]Percentage of control values are in parentheses.

Impact of a mixture of NS-3 on diabetes associated biomarkers and cytokines in real-time PCR of purified treated RNAs obtained from people with T2DM.

In order to confirm *in vitro* results of RT-PCR effects of NS-3 and its individual components on diabetes biomarkers using RNAs purified from PBMC isolated from T2DM subjects, the RT-PCR of pre-dose RNAs versus post-dose RNAs after NS-3 administered to persons with T2DM for 24-weeks was also carried out with several T2DM biomarkers, which indicated significant down- regulated gene expression of IRS-1 (79%), SOD-2 (90%), GCKR (87%), IGFBP-2 (88%), IL-4 (82%), IL-6 (76%) and iNOS (78%) as compared to pre-dose values (Figure 6). These *in vivo* results of down-regulated gene expression in diabetes biomarkers were more significant than *in vitro* results as reported in Figures 4A,4B. These results prompted us to carry out mRNA-sequencing and miRNA-sequencing of these RNAs. This will be the subject of the next publication.

Discussion

The hypothesis of our study is that synergistic effect of a mixture (NS-3) of natural compounds on biological functions will be more effective than its individual components in lowering the diabetes biomarkers. The results of a mixture of NS-3 when tested *in vitro* in PBMCs of people with T2DM indicated more effective down-regulation on gene expression of several diabetes biomarkers and cytokines as compared to its individual components (Table 1, Figures 4A, 4B), except δ-tocotrienol treatment showed up-regulation in interferon-d (138%, 10 µM; 287%, 80 µM) only in PBMC of diabetes and not in normal healthy PBMC. It is well known IFN-γ is a cytokine associated with both innate and adaptive immunity against viral infection, and functions as primary activator of macrophages and stimulate natural killer cells and neutrophils, thus δ-tocotrienol might be a very good candidate to induce immunity in humans.

Table 7: Summary of impact of placebo supplement or a mixture of (NS-3) or its components after treatment for 24-weeks on various biomarkers of diabetes in serum of people with T2DM.

	Biomarkers	Fasting Glucose Pre-dose	Fasting Glucose Post-dose	Fasting HbA1c Pre-dose	Fasting HbA1c Post-dose	hs-CRP Pre-dose	hs-CRP Post-dose	HOMA-IR Pre-dose	HOMA-IR Post-dose	MDA Pre-dose	MDA Post-dose
	Values in---------->	mmol/L	mmol/L	%	%	mg/L	mg/L			µmol/L	µmol/L
1	Control[a] (placebo)	7.62 (100)[b]	7.57 (99)	8.42 (100)	8.42 (100)	3.59 (100)	3.47 (100)	5.51 (100)	5.44 (99)	3.57 (100)	3.58 (100)
2	δ-tocotrienol[a]	7.35 (100)	6.85 (93)	8.44 (100)	7.79 (92)	3.53 (100)	3.10 (88)	5.23 (100)	4.51 (86)	3.63 (100)	3.22 (89)
3	Vitamin D_3[a]	7.55 (100)	7.16 (95)	8.81 (100)	8.19 (93)	3.37 (100)	3.09 (92)	5.32 (100)	4.74 (89)	3.59 (100)	3.48 (97)
4	Resveratrol[a]	7.39 (100)	6.98 (94)	8.58 (100)	7.86 (92)	3.69 (100)	3.28 (89)	5.37 (100)	4.98 (93)	3.82 (100)	3.49 (91)
5	Mixture (2 + 3 + 4)[a]	7.39 (100)	**6.56 (89)**	8.20 (100)	**7.40 (90)**	3.65 (100)	**2.82 (77)**	5.44 (100)	**4.35 (80)**	3.81 (100)	**3.04 (80)**

[a]Two capsules of placebo (cellulose/olive oil; 250 mg/capsule) or two capsules of δ-tocotrienol (250 mg/capsule), or vitamin D_3 (5000 IU = 0.062/capsule) or resveratrol (250 mg/capsule) were administered to people with T2DM for 24-weeks.

[b]Percentage of control values are in perentheses.

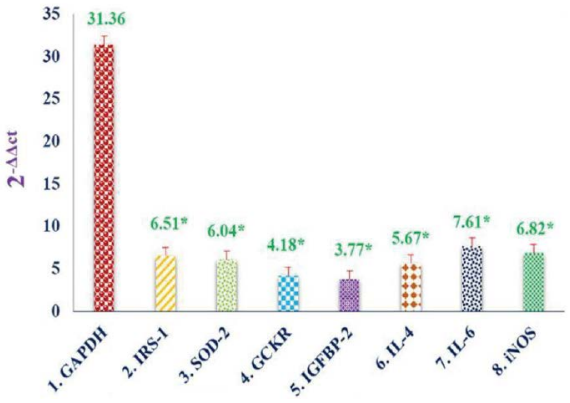


Figure 6. Effect of a mixture of NS-3 on diabetes biomarkers and cytokines by RT-PCR of total RNAs obtained from NS-3 administered people with T2DM after 24-weeks.

Real-time PCR (RT-PCR) was performed on total RNAs isolated from people with T2DM treated with a mixture of NS-3 for 24-weeks. All reactions were performed in triplicate using equal amount of mRNA per reaction (200,000 RNAs/treatment) of pre-dose as well as post-dose. Real-time PCR assays were completed by a Step-one plus RT-PCR system as described in Figures 4A, 4B. Data are means = SD. Values in a column sharing a common asterisk are significantly different at *P <0.001 compared to control.

Next, the toxicity of NS-3 mixture and its components was carried out by MTT test with concentration (10 µM – 80 µM) in diabetic PBMC showed the cells were > 95 % viable with these concentrations, and under same conditions as of MTT test, the NS-3 mixture and its components did not induce autophagy in these PBMC (Figure 5). The results clearly demonstrate that NS-3 mixture safely can be used for human studies. A mixture of NS-3 showed much more significant decreases in the levels of serum/plasma of fasting glucose, HbA1c, hs-CRP, fasting insulin, HOMA-IR, MDA as compared to its individual components (Table 5). There were no side-effects reported by any of the subjects. These *in vitro* (PBMC) and *in vivo* results supported our hypothesis that a mixture NS-3 of δ-tocotrienol, vitamin D_3 resveratrol is more effective in lowering the diabetes biomarkers and pro-inflammatory cytokines compared to its individual components.

The RT-PCR estimation of NS-3 treated *in vivo* of pure RNAs of pre-dose vs. post-dose on several Key biomarkers (IRS-1, SOD-2, IGFBP-2, GCKR), and IL-4, IL-6, iNOS of diabetes showed significant down-regulation in gene expression (>70%) in these biomarkers and cytokines compared to pre-dose values (Figure 6).

The number of pre-diabetes and people with T2DM will ultimately increase to 482 million people by year 2040. Therefore, more effective methods and new biomarkers will be required to diagnose pre-diabetes and diabetes people and their complications. The current parameters, hs-CRP, HbA1c, and HOMA-IR as well as some new biomarkers will be required to diagnose pre-diabetes and diabetes and its complications. Recently, a comprehensive review of novel biomarkers for prediabetes, diabetes, and associated complications has been reported [33]. They have discussed the functions of nineteen novel general (such as HbA1c, GA, OGT, adiponectin, LP(a), THBS1, GPLD1, miRNAs) and seven inflammatory (hs-CRP, IL-6, WBCs, fibrinogen, PAI-1, IL-18, IL- IRA) biomarkers in detail [33]. The present results clearly indicate that six biomarkers (IRS-1, SOD-2, IGFBP-2, PTPRN, GCKR, resistin), and cytokines (ICAM-1, VCAM-1, IL-6, IL-8, TNF-α, IFN- γ) may be used in future for diagnosing early onset of people of pre-diabetes and with T2DM.

The current-well-designed, long-term population studies that show association between plasma glucose levels, HbA1c and hs-CRP, but has some limitations, such as moderate sensibility and specificity are inaccurate in certain clinical conditions. Therefore, these novel biomarkers (gene expression of IRS-1, SOD-2, GCKR, IGFBP-2, resistin) might be useful for diagnosing and identifying early onset of people with T2DM. IRS-1 regulates body growth and peripheral insulin action. Phosphorylation in IRS-1, increases body weight, lowers blood glucose level, and alleviates insulin resistance (IR) in T2DM. SOD-2 is a free radical scavenging enzyme that forms a major component in guarding against oxygen radical species produced during cellular metabolism. It functions as a homodimer that binds copper and zinc. IGFBP-2 is

a pleiotropic polypeptide that functions as an autocrine and/or paracrine growth factor. PTPRN plays a role in vesicle-mediated secretory processes and is required for the accumulation of normal levels of insulin-containing vesicles and preventing their degradation. It is also required for normal accumulation of the neurotransmitter norepinephrine, dopamine, and serotine in the brain. Glucokinase regulator (GCKR) is associated with elevated T2DM risk, and acts as an allosteric switch for glucokinase in blood glucose control by the liver and resistin is involved in insulin resistance (IR) in T2DM (based on Google Search). In the future, the present suggested biomarkers may predict individual risk of diabetes complications after long-term population studies, and RT-PCR technique will be used routinely for diagnostic purposes in clinical laboratories in the near future. Recently, function of miRNA as regulatory marker of glucose and lipid metabolism has been reported in studies of different tissues and animal [34, 35]. This will be the subject of our next publication.

Conclusions

The present study confirms the important roles played by IRS-1, SOD-2, IGFBP-2, PTPRN, GCKR as novel biomarkers, and cytokines (ICAM-1, VCAM-1, IL-6, IL-8, IFN-γ) in diagnosing T2DM. The *in vitro* (PBMC) and *in vivo* (clinical study) results supported our hypothesis that a mixture NS-3 of δ-tocotrienol, vitamin D_3, resveratrol will be more effective in lowering the diabetes biomarkers and pro-inflammatory cytokines as compared to its individual components. Although, present mixture of NS-3 effectively lowers the elevated levels of all the tested diabetes and inflammatory biomarkers, but there are some limitations of present study. The elevated plasma level of HbA1c decreased only 10% in T2DM and not below its normal value of < 7%. There are many commercial products of synthetic compounds (Jardiance, Farxiga, Trulicity, Ozempic) available in the market, which are very effective in lowering the elevated plasma level of HbA1c in people with T2DM, but most of them have severe side-effects (nausea, diarrhea, vomiting, abdominal pain, discomfort, frequent bowel movements, indigestion, decreased appetite, and fatigue). Therefore, there is still a need to find out a mixture of natural products, which should be able to lower elevated plasma level of HbA1c below normal value of < 7.0%. This might be achieved by carrying out a dose-response (400 mg/d, 800 mg/d, 1200 mg/d) study by changing composition of present mixture or by adding some other potent natural compounds in the present mixture and then carrying out a study of (NS-4) in people with T2DM for 24-weeks. The other limitation is that in our first pilot preliminary study, few T2DM participants (n = 30 for each component) were enrolled for individual components compared to (NS-3) mixture (n = 56). However, Dr. Wajiha Mahjabeen (Ph.D. student) of Dr. Dilshad A Khan has completed the clinical trial of "Effects of δ-tocotrienol, vitamin D3, resveratrol (n = 55 for each compound) feeding on glycemic control, oxidative stress, inflammatory biomarkers, and miRNA expression in type 2 diabetes mellitus" for her Ph.D. thesis, and obtained results for δ-tocotrienol-fasting glucose 93%, HbA1c 94%, hs-CRP 90%, HOMA-IR 87%, MDA 91%). The decrease in T2DM biomarkers with NS-3 mixture is more significant and better (Table 7) than these values (personal communication).

In summary, present study using a mixture of natural products (NS-3) has demonstrated an effective product which can effectively decrease several current well-established diabetes biomarkers and some new ones in people with T2DM, without any side-effects.

Acknowledgements

We thank Ms. Julia C Reis, and Subhan Burale for their technical help. We also thank Dr. Betty M. Drees (Department of Internal Medicine and Department of Biomedical and Health Informatics, University of Missouri-Kansas City, School of Medicine, 2411 Holmes, Kansas City, MO 64108) for valuable suggestions and editing the manuscript.

Additional Information And Declarations

Funding

The study was funded by Advanced Medical Research (AMR), Madison, Wisconsin, 53719.

Competing interest

All authors declare that they have no competing interests.

Author's contributions

AAQ and DAK conceived and NQ, NS, and BMD planned the study. DAK and WM carried out human study and prepared total mRNAs. WM collected the data and carried out most of estimation of all the parameters/biomarkers. DAK and AAQ carried out analysis of the data. NS carried out RT-PCR analyses of PBMC and autophagy assays. AAQ wrote the manuscript. NQ and BMD edited and proof-read the manuscript. All authors have read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this article is available in USB.

Consent of publication

All contributing authors agree to the publication of this article.

Abbreviations: T2DM = type 2 diabetes mellitus, PBMC = peripheral blood mononuclear cells, IRS-1 = insulin receptor substrate-1, SOD-2 = superoxide dismutase-2, IGFBP-2 = insulin like factor binding protein-2, PTPRN = protein tyrosine phosphatase receptor type N, GCKR = glucokinase regulators, ICAM-1 = intercellular adhesion molecule-1, VCAM-1 = vascular cell adhesion molecule-1, IL-6 = interleukin-6, IL-8 = interleukin-8, TNF-α = tumor necrosis factor-α, IFN-γ = interferon-γ, NF-κB = nuclear factor kappaB.

References

1. Gardner DG, Shoback D (2011) Greenspan's basic & clinical endocrinology. 9th ed. New York:
2. Bandeira SM, Fonseca LJS, Guedes GS, Rabelo LA, et al. (2013) Oxidative stress as an underlying contributor in the development of chronic complications in Diabetes Mellitus. *Int J Mol Sci* 14: 3265-3284. [View Article]
3. King LG (2008) The role of inflammatory cytokines in diabetes and its complications. *Periodontal* 79: 1527-1534. [View Article]
4. Navarro-Gonzalez JF, Mora-Fernandez C (2008) The role of inflammatory cytokines in diabetic nephropathy. *J M Soc Nephrol* 19: 433-442. [View Article]
5. Benerjee M, Saxena M (2012) Interleukin-1 (IL-1) family of cytokines: role in type 2 diabetes. *Clinica Chimica Acta* 413: 1163-1170. [View Article]
6. Zhao G, Dharmadhikari G, Maedler K, Meyer-Hermann M (2014) Possible role of interleukin-1β in type 2 diabetes onset and implications for 22 anti-inflammatory therapy strategies. *PloS Comput Biol* 10: e1003798. [View Article]
7. Lontchi-Yimagou E, Sobngwi E, Matsha TE, Kengne AP (2013) Diabetes mellitus and inflammation. *Curr Diab Rep* 13: 435-444. [View Article]
8. Rangasamy S, McGuire PG, Das A (2012) Diabetic retinopathy and inflammation: novel therapeutic targets. *Middle East Afr J Ophthalmol* 19: 52-59. [View Article]
9. Popov D (2019) Endothelial cell dysfunction in hyperglycemia: phenotypic change, intracellular signaling modification, ultrastructural alteration, and potential clinical out-comes. *Int J Diabetes Mellit* 2: 189-195. [View Article]
10. American Diabetes association (ADA). Standards of medical care in diabetes. Diabetes care 34: 11-61. [View Article]
11. Muller G (2012) Microvesicles/exosomes as potential novel biomarkers of metabolic diseases. *Diabetes Metab Syndr obes* 5: 247-282. [View Article]
12. Budin SB, Othman F, Louis SR, Bakar MA, Srijit Das et al. (2009) The effects of palm oil tocotrienol- rich fraction supplementation on biochemical parameters, oxidative stress, and the vascular wall of streptozotocin-induced diabetic rats. *Clinics (Sao Paulo)* 64: 235-244. [View Article]
13. FangF, Kang Z, Wong C (2010) Vitamin E tocotrienols improve insulin sensitivity through activating peroxisome proliferator-activated receptors. *Mol Nutr Food Res* 54: 345-352. [View Article]
14. Kuhad A, Bishnoi M, Tiwari V, Chopra K (2009) Suppression of NF-kappa B signaling pathway by tocotrienol can prevent diabetes associated cognitive deficits. *Pharmacol Biochem Behav* 92: 251-259. [View Article]
15. Tiwari V, Kuhad A, Chopra K (2013) Neuroprotective effect of vitamin E isoforms against chronic alcohol-induced peripheral neurotoxicity: possible involvement of oxidative-nitroadative stress. *Phytother Res* 26:1738–1745. [View Article]
16. Siddiqui S, Ahsan H, Khan MR, Siddiqui WA (2014) Protective effects of tocotrienols againstlipid-induced nephropathy in experimental type-2 diabetic rats by modulation in TGF-beta expression. *Toxicol Appl Pharmacol* 273: 314–324. [View Article]
17. Ahsan H, Ahad A, Iqbal J, Siddiqui WA (2014) Pharmacological potential of tocotrienols: a review. *Nutrition metabol* 11: 52. [View Article]
18. Bhatt JK, Thomas S, Nanjan MJ (2012) Resveratrol supplementation improves glycemic control in type 2 diabetes mellitus. *Nutr Res* 32: 537-41. [View Article]
19. Tome-carneiro J, Larrosa M, Yanez-Gascon MJ, Davalos A, et al. (2013) One-year supplementation with a grape extract containing resveratrol modulates inflammatory- related microRNAs and cytokines expression in peripheral blood mononuclear cells of type 2 diabetes and hypertensive patients with coronary artery disease. *Pharmacology Research* 69-82. [View Article]
20. Botella-Carretero JI, Alvarez-Blasco F, Villafruela JJ, Balsa JA, et al. (2007) Vitamin D deficiency is associated with the metabolic syndrome in morbid obesity. *Clin Nutr* 26: 573-580. [View Article]
21. Hirai M, Suzuki S, Hinokio Y, Hirai A, et al. (2000) Variations in vitamin D-binding protein (group-specific component protein) are associated with fasting plasma insulin levels in Japanese with normal glucose tolerance. *J Clin Endocrinol Metabolism* 85: 1951-1953. [View Article]
22. Orwoll E, Riddle M, Prince M (1994) Effects of vitamin D on insulin and glucagon secretion in non-insulin dependent diabetes mellitus. *Am J Clin Nutrition* 59: 1083-1087. [View Article]
23. Green A (2013) Effects of vitamin E, C and D supplementation on inflammation and oxidative stress in streptozotocin-induced diabetic mice. *Int J Vitamin Nutrition Research* 83: 168-175. [View Article]
24. Li YC, Chen Y, Liu W, Thadhani R (2014) MicroRNA-mediated mechanism of vitamin D regulation of innate immune response. *J Steroid Biochem Mol Biol* 144: 81-86. [View Article]
25. Zitman-Gal T, Green J, Pasmanik-Chor M, Golan E, et al. (2014) Vitamin D manipulates miR-181c, miR-20b and miR-15a in human umbilical vein endothelial cells exposed to a diabetic-like environment. *Cardiovascular diabetology* 13: 8. [View Article]
26. Qureshi AA, Khan DA, Mahjabeen W, Silswal N, et al. (2015) Comparative evaluation of NS-5 mixture and its components on superoxide production in HUVEC and inflammatory biomarkers in humans. *Journal Clinical & Experimental Cardiology* 6:7. [View Article]
27. Qureshi AA, Khan DA, Mahjabeen W, Papasian CJ, et al. (2012) Suppression of nitric oxide and cardiovascular risk factors in healthy seniors and hypercholesterolemic subjects by a combination of polyphenols and vitamins. *J Clin Exp Cardiolog* 5: 8. [View Article]
28. Qureshi AA, Khan DA, Mahjabeen W, Qureshi N (2015) Dose-dependent modulation of lipid parameters, cytokines and RNA by δ-tocotrienol in hypercholesterolemic subjects restricted to AHA Step-1 diet. *Br J Med Res* 6: 351-366. [View Article]
29. Bandeira SM, Fonseca LJS, Guedes GS, Rabelo LA, et al. (2013) Oxidative stress as an underlying contributor in the development of Chronic complications in diabetes mellitus. *Inter J Mol Sci* 14: 3265-3284. [View Article]
30. Qureshi AA, Mo H, Packer L, Peterson DM (2000) Isolation and structural identification of novel tocotrienols from rice bran with hypocholesterolemic, antioxidant and antitumor properties. *J Agr Food Chem* 48: 3130-3140. [View Article]

31. Tan SMQ, Chiew Y, Ahmad B, Kadir KA (2018) Tocotrienol-rich vitamin E from palm oil (tocovid) and its effects in diabetes and diabetic nephropathy: a pilot phase II clinical trial. *Nutrients* 10: 1315. [View Article]

32. Haghighat N, Vafa M, Eghtesadi S, Huidari I, et al. (2014) The effects of tocotrienols added to canola oil on microalbuminuria, inflammation, and nitrosative stress in patients with type 2 diabetes: a randomized, double-blind, placebo-controlled trial. *International Journal of preventive Medicine* 5: 617- 623. [View Article]

33. Dorcely B, Katz K, Chiang SS, Oluwadare B, et al. (2017) Novel biomarkers for prediabetes, diabetes, and associated complications. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy* 10: 345-361. [View Article]

34. Claudiane Guay, Romano Regazzi (2013) Circulating microRNAs as novel biomarkers for diabetes mellitus. *Nat Rev Endocrinol* 9: 513-521. [View Article]

35. Baldeon RL, Weigelt K, Wit HD, Ozcan B, et al. (2015) Type 2 diabetes monocytes MicroRNA and mRNA increased differentiation-related genes but not inflammatory activation. [View Article]

Citation: Qureshi AA, Khan DA, Mahjabeen W, Silswal N, Qureshi N (2021) A Novel Mixture of δ-Tocotrienol, Vitamin D_3, Resveratrol (NS-3) Significantly Decreases Diabetes Biomarkers Including Inflammatory in People with Type 2 Diabetes. J Diab Clin Stud 5(1): 001-016.