Effect of Red Grape Cells, a Resveratrol Polyphenol Complex, on Glycemic Control and Clock Gene Expression on patients with Type 2 diabetes

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Short running title: Effect of Red Grape Cells on patients with Type 2 diabetes

Abstract

Introduction: Resveratrol, a naturally occurring polyphenol, exerts potent modulatory effects on clock gene expression thought to be linked with glycemic regulation. Effects of Red Grape Cells (RGC), a resveratrol polyphenol complex, on glycemic control and on clock genes (CGs) (Bmal1, Clock, Per 1&2, Cry1, Rev-Erbα and RORα) mRNA expression in white blood cell (WBC) have never been explored in patients with type 2 diabetes (T2D). The aim of the study was to explore the impact of RGC supplementation on CGs mRNA expression in WBC, glycated hemoglobin (HbA1c), plasma glucose, insulin and C-peptide in patients with T2D.

Material and methods: 33 participants with T2D aged 63.7±7.1 years, body mass index 30.3±4.6 kg/m^2 and HbA1c 7.76 ± 0.78% were randomized to receive a daily dose of either RGC or placebo (PLA).

Results*:* After 12 weeks, a greater reduction of HbA1c was observed in RGC patients: -0.55 ± 0.05% (from 7.85±1.01 to 7.30±0.75, p=0.0353) vs. PLA: -0.16±0.15% (from 7.67±0.55 to 7.51±0.52, p=0.2334).

The area under the curve (AUC) (0-240 min) of C-peptide concentration showed a steeper reduction of 27.2% for RGC vs. PLA (p=0.0409). Estimated insulin sensitivity calculated from fasting glucose and C-peptide was increased by 40.6% with RGC vs. PLA (p<0.0137). mRNA levels of most clock genes were reduced in the RGC group vs. PLA.

Conclusions: 12-week supplementation of RGC in T2D patients reduced HbA1c, improved insulin sensitivity and significantly influenced clock gene expression.

Additional studies are needed to determine the best dosage and whether RGC might be useful as adjuvant therapy in patients with T2D.

Keywords: Type 2 Diabetes; red grapes; polyphenol; resveratrol; clock genes; metabolic syndrome.

1. INTRODUCTION

Consumption of polyphenol-rich foods and beverages has been associated with various health benefits, including lower blood pressure and cholesterol, improved endothelial function, reduced cancer risk and neuroprotective effects. Polyphenol intake was shown to have an anti-obesity effect and to reduce the risk for developing type 2 diabetes (T2D) in animal and human studies [1-3]. Polyphenols of nutritional interest are commonly divided into three groups: phenolic acids, flavonoids and stilbens. The stilbene, resveratrol, has been investigated most often [4]. Due to its strong antioxidant activity, *in vitro* and *in vivo* studies in animal models found that resveratrol inhibited oxidation of low-density lipoproteins (LDL) and their accumulation in blood vessels; thus, preventing formation of atherosclerotic plaque [5]. Using human umbilical vein endothelial cells was also found to decrease systolic and diastolic blood pressure by increasing vasodilatation and inhibiting vasoconstriction [6]. Another positive effect was lower levels of pro-inflammatory markers in the blood in an *in vitro* study using human peripheral blood mononuclear cells [7]. Furthermore, it was demonstrated in a human clinical study that resveratrol activates sirtuins, known to be involved in energy metabolism and longevity. Specifically, it increases Sirtuin 1 (SIRT1) expression and its metabolic effect in several tissues, both directly and indirectly. For example, in an *in vitro* study using a human model, it was shown to improve insulin sensitivity in skeletal muscle, prevent weight gain [8,9], improve pancreatic beta-cell function and enhance insulin secretion in a human clinical trial [10], and glucose tolerance in mice [11,12]. Higher SIRT1 levels are also associated with increased lipolysis in white adipose tissue, decreased glycolysis and increased fatty acid oxidation in skeletal muscle, as

demonstrated in animal and human studies [8,13-15]. These observations suggest an important protective role for T2D.

Additional evidence from *in vitro* models suggested a sirtuin-mimetic effect of resveratrol on adenosine monophosphate (AMP)-activated protein kinase (AMPK) [17-19] and on SIRT1 activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1alpha (PGC-1α) [20,21]. Moreover, resveratrol was reported to act directly on clock genes and to induce an effect similar to calorie restriction [22]. control

The circadian clock regulates the activity of different systems participated in glucose metabolism. The BMAL1:CLOCK heterodimer involves in mediating the transcription of coactivators that regulate the circadian synthesis includes hormones and enzymes involved in glucose homeostasis.

CRYs inhibit gluconeogenic gene expression by regulating CREBP activity and their hepatic depletion increases circulating glucose. CRY over-expression reduces fasting blood glucose and improves whole-body insulin sensitivity in obese mice. The CLOCK:BMAL1 heterodimer regulates the expression of REV-ERBα and Rora. Depletion of REV-ERBα, the negative regulator of Bmal1 expression, leads to hyperglycemia. This regulator, is induced during normal adipogenesis and mediates a suppressive effect on gluconeogenesis and glucose output by regulating the expression of PEPCK and glucose-6-phosphatase (G6pase). In contrast, RORa, the positive regulator of Bmal1 expression, activates the gluconeogenic enzyme, G6pase and regulates lipogenesis and lipid storage in skeletal muscle. AMPK, the cellular energy sensor, involves in the clock mechanism by enhancing degradation of PERs and CRYs. Upregulation of AMPK signaling induced GLUT4 translocation and muscular glucose

uptake, affecting metabolic efficiency and improving postprandial glucose and insulin responses. Deficiencies in clock genes have been associated with reduced glucose-stimulated insulin secretion, insulin resistance, diminished b-cell proliferation, and apoptosis. Lower transcripts of Bmal1 and Cry2 are inversely correlated with HbA1c levels. In addition, higher risk of obesity, metabolic alterations, and type 2 diabetes have been observed in individuals and shift workers who underwent acute or chronic, forced circadian misalignment. This supports the idea that the clock gene plays an important role in preserving insulin sensitivity and b-cell function. Furthermore, it has recently been demonstrated, in rats and *in vivo* in humans, that secretion of glucagon-like peptide-1 (GLP-1), a key hormone that regulates glucose-dependent insulin secretion from intestinal L cells, shows a rhythmic pattern [31].

RGC powder manufactured by Bioharvest, Ltd., Israel, is a natural, patent-protected formulation of cells originating from the fruits of Vitis Vinifera L. cultivar. It consists of the whole matrix of polyphenols and other healthy ingredients naturally existing in RGC. Despite similar total polyphenolic content, the concentration of natural grape resveratrol in RGC is significantly higher than that found in fresh grapes [23,24]. Consumption of 0.4 g Red Grape Cells (RGC) powder is equivalent to 1000 red grapes and to 1 bottle of red wine without alcohol and sugar.

In light of the evidence demonstrating the beneficial effects of resveratrol, this study explored the effect of RGC in patients with T2D.

The aim of this study was to examine whether 12 weeks' supplementation with RGC powder affected mRNA levels and metabolic parameters in patients with T2D.

2. MATERIALS AND METHODS

2.1 The investigational product

RGC powder (Bioharvest, Ltd., Patent application US 2008/0166306A1) consists of dried red grape cells. Its nutritional values and composition were published previously [23].

2.2 Study Design

This randomized, double-blind, placebo-controlled trial was conducted for 12 weeks among patients with T2D. Eligible patients (appendix) were enrolled and randomized into 2 treatment arms: The RGC group received with daily supplementation with 1000 mg/day RGC powder and the placebo (PLA) group received 1000 mg of maltodextrin powder.

Potential candidates were evaluated during the screening visit (visit 1), which included blood chemistry (cholesterol, triglycerides, high density lipoprotein cholesterol (HDL-chol), insulin, C-peptide) and anthropometric measurements (weight, blood pressure and waist circumference). On visit 2, anthropometric measurements were recorded and fasting and post-breakfast bloods were drawn.

This study followed the tenets of the Declaration of Helsinki. Approval was obtained from the local Ethics Committee (number 0107-13-WOMC) and all participants provided written informed consent.

Treatments: The subjects visited the clinic on: Week -2 = Visit 1, Week 0= Visit 2, Week 6 = Visit 3 and Week 12 = Visit 4 (Figure 1). Telephone interviews were conducted during the weeks with no scheduled clinic visits.

Participants were asked to follow the anti-diabetic meal plan given to them by the dietitian. Eligible subjects entered a run-in period on the assigned diet only, without any supplementation from week -2 (Visit 1) to week 0 (Visit 2).

On visits 2 and 3, subjects received a package containing 48 daily doses of the investigational product or placebo according to their randomized treatment allocation. The investigational product or placebo was ingested once daily for 12 weeks, as instructed.

All patients were asked to follow the anti-diabetic plan given to them by the study dietitian. Food records were completed by subjects before study commencement to gain baseline intake data and on visits at weeks 0, 6, and 12 of the study.

Inclusion Criteria were T2D (HbA1C >7%), duration of diabetes 0.5 to 20 years, age ≥ 30 and ≤75 and BMI: 22 to 35 kg/m^2. All oral antidiabetic medications were allowed, with the exception of those specified in the exclusion criteria (see below). Normal liver, kidney and thyroid function. Agree to avoid the use of over-the-counter medications, herbs, or supplements, ingesting grapes, grape juice, red wine and any other foods that might contain polyphenols throughout the study. Stable physical activity pattern during the three months immediately preceding the study was required, as was a lifestyle of waking up between 06:00 and 07:00 and going to sleep between 22:00 and 24:00. Also, no shift work within 5 years preceding the study was allowed and no crossing time zones 1 month before the study.

Exclusion Criteria included Type 1 diabetes; clinically significant pulmonary, cardiac, renal, hepatic, neurologic, psychiatric, infectious, or malignant diseases. Pregnancy or lactation. Illicit drug abuse or alcoholism. Treatment with thiazolidinediones, insulin

and/or GLP-1 analogs, anti-hyperlipidemic treatment with fibrates, steroid treatment and post-bariatric surgery.

2.3 Efficacy Assessments

Participants' blood pressure, heart rate, weight, height, body mass index (BMI) and waist circumference were recorded at each visit.

HbA1C, glucose levels, and lipid profile were measured at each visit while fasting. On visits 2 and 4, postprandial glucose and lipids were taken, as well. HbA1C reflects chronic blood glucose values and presents the average amount of glucose attached to hemoglobin 3 months' prior the test.

Insulin and C-peptide levels were measured on visits 2 and 4, fasting and after breakfast.

Lipid peroxidase, inflammatory markers, Apo A-1S and Apo B: blood samples were collected during visits 2 and 4 while fasting.

GLP-1 (Glucagon-like-peptide 1) and ADMA (Asymmetric dimethylarginine)**:** blood samples were collected during visits 2 and 4 while fasting and post-prandial.

Clock Genes and Sirt1: mRNA expression of clock genes and Sirt1 was measured on visits 2 and 4, fasting and postprandial in peripheral blood cells (PBC).

2.4 Adverse Events

Serious adverse events (SAEs) included events reported by the subject, as well as clinically significant abnormal clinical examination or laboratory findings. Any new illness, symptom, sign or clinically significant clinical laboratory abnormality or worsening of a pre-existing condition or abnormality was considered an AE.

2.5 Statistical Analysis

AUC was calculated as sum of AUCs from time 0 to 240 minutes. Chi-square test was used to analyze the difference in frequency of responders, defined as decrease in HbA1c by 1% or 0.5% between the study groups. Paired T-test (paired observations) was used to analyze the changes from baseline in quantitative parameters within study groups. The two-sample T-test and non-parametric Wilcoxon-Mann-Whitney Rank sum test for independent samples were used to analyze the differences in the above changes between study groups.

Pearson correlations were used to analyze changes in gene expression versus other parameters: BMI, vital signs, Hba1c, glucose, insulin, C-peptide and lipids. Analysis of variance (ANOVA) used to analyze differences in changes in HbA1c and AUC from C-Peptide, with adjustment for other confounders (percent compliance and measurements). The mixed-effect model for repeated measures (MMRM) was used to analyze the difference between the groups in HbA1c and AUC C-peptide (measurements and changes from baseline) at any time, including the fixed effect time and adjusted for percent compliance.

BMAL1 mRNA expression levels was the primary endpoint. Statistical power calculations were performed based on a sample size of 15 having >90% power to detect a difference in means of 0.318 (e.g. a First condition mean, μ_1, of 1.0 and a Second condition mean, μ_2, of 0.682), assuming a standard deviation of differences of 0.1, using a paired t-test with a 0.05 two-sided significance level.

Gene expression results were expressed as means ±SE, using one-way ANOVA (time of day). Student's t-test was used for comparison between the control (Visit 2) and Visit 4 of each treatment.

All tests were two-tailed, and a p value of 5% or less was considered statistically significant. The data were analyzed using SAS®, version 9.3 (SAS Institute, Cary, North Carolina).

For gene expression analyses, the significance level was set at $P<0.05$. Statistical analysis was performed with JMP (version 7) software (SAS Institute, Inc., Cary, NC, USA).

3. RESULTS

3.1 Study population

A total of 45 patients were enrolled and randomized into the study; 22 in the treatment (RGC) arm and 23 patients in the placebo (control) arm. Of these, 7 patients dropped (5 patients from RGC and 3 from control) out and 3 patients were excluded from analysis (2 patients from RGC and 1 from control). One mistakenly discontinued the anti-diabetic medication during the first 6 weeks of the study, and two patients with BMI > 40 Kg **(** Inclusion criteria BMI: 22 to 35 kg/m^2).

Baseline characteristics are presented in Table 1. A total of 35 subjects completed the study; 25 males and 10 females. The mean age of the study population was 63.3±7.2 years (range 48–78 years), and the mean BMI was 30.3±4.3 kg/m^2.

Table 1: Baseline characteristics of the study ITT population

Parameter	RGC 1000 mg N=15	Placebo N=20	All N=35
Males, N (%)	11 (73)	15 (75)	26 (74)
Females, N (%)	4 (27)	5 (25)	9 (26)
Age, mean ± SD (years)	62.2±7.3	64.1±7.1	63.3±7.2
Age, range (years)	50 – 77	48 – 78	48 – 78
BMI, mean ± SD (kg/m^2)	30.1±4.6	30.5±4.3	30.3±4.3

3.2 Data sets analyzed

Intention to treat (ITT): 35 patients - 15 in the RGC arm and 20 in the placebo arm. ITT refers to all subjects who started the study on visit 1, and completed the study on visit 4, excluding the 3 subjects who dropped out.

Per protocol (PP): 33 patients - 14 in the RGC arm and 19 in the placebo arm. PP refers to ITT patients, with the exclusion of one subject from each group whose compliance level was lower than 85% and thus, were not included in the per-protocol (PP) group for efficacy analyses.

3.3 Effect of RGC on circadian genes and SIRT1 expression

Figure 2 shows a statistically significant reduction in mRNA expression of CLOCK, BMAL1, Per 2, Cry 1 and RevErb in WBC of patients receiving RGC, but not placebo. Per 1 expression was significantly reduced in both study groups, and hence, was considered to be a placebo effect. RORα expression was significantly reduced in the

control but not in the RGC group. Considering the time of blood collection, these results may reflect a phase advance in patients' circadian rhythm.

BMAL 1, PER1 T-test analysis results between interventions were found to be not significant (p=0.1506 , p=0.8163 accordingly). CLOCK, PER2, ROR, CRY1 and REVERB T-test analysis results between interventions was significant (P<0.0001). SIRT1 T-test analysis results between intervention was significant (P=0.0166).

3.4 Effect of RGC on HbA1c

Within PP patients, HbA1c levels decreased by 0.55% in the RGC group (p=0.0353) and by 0.16% in the control group (p=0.2334) (Figure 3A). Although the decrease was significant in the RGC population but not in the placebo group, when adjusted for percent compliance, the difference between the two study arms was not significant (p= 0.06; Figure 3B). When the changes from baseline were calculated for a sub-group of participants whose initial HbA1c values were particularly high (7.5-10.1), the decrease was 1.21% for the RGC group and 0.39% for the control (Figure 3C) (p=0.0247).

Within ITT patients, MMRM analysis adjusted for percent compliance showed a decrease in HbA1c from baseline by 0.24% (p=0.0139) in the RGC group compared to a decrease of 0.146% (p=0.0897) in the control group. HbA1c of RGC treatment and the placebo groups were compared at baseline, T-test revealed no significate differences between them (p=0.6525).

3.5 Effect of RGC on C-peptide and insulin levels

C-peptide values were decreased in the RGC group in contrast to an increase observed in the control group. The difference in C-peptide changes between groups was significant (p=0.0409 Wilcoxon test; p=0.0117 t-test (Figure 4A).

AUC values of insulin (concentration over 240 min) within the PP population demonstrated a slight elevation in RGC group and a considerable elevation in the control group. The difference between the two study arms was not significant. However, calculated Insulin Sensitivity Index, which is a function of C-peptide levels at fasting, showed a more substantial increase for the RGC group vs. placebo (Figure 4B).

HbA1c and insulin sensitivity of the treatment and the placebo groups were compared at baseline, T-test statistical analysis revealed. No significant differences were observed between groups.

3.6 Effect of RGC on glucose and lipid levels

Glucose measurements showed no significant differences between groups over time (p=0.6135 by Wilcoxon test, and 0.7970 by t-test). No significant differences were found for any of the various lipid types between groups. A significant increase in triglyceride levels was detected in both study groups, with no significant difference between arms.

Although the patients were given an anti-diabetic diet, they still were allowed to consume fat.

3.7 Effect of RGC on cardiovascular health – heart rate (anthropometric variables)

RGC consumption resulted in significantly increased mean heart rate (within the normal range) (p=0.0390 Wilcoxon test; p=0.0023 T-test), while it did not affect blood pressure.

3.8 Safety analysis and adverse events

Chemistry laboratory analysis demonstrated that none of the measures differed significantly from one another based on treatment assignment at any time point. All hematology results were within normal limits in both groups.

One mild SAE was reported and was not considered related to the study product. Two moderate SAEs were reported for two subjects prior to study product consumption. One severe SAE was reported for one subject and was considered unrelated to the study product.

3.9 Figures

Figure 1: Study scheme. RGC-Red Grape Cells; PLA- Placebo

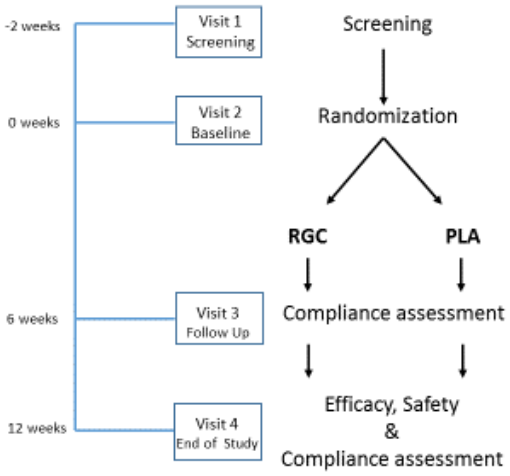


Figure 2: mRNA expression of CLOCK, BMAL1, Per 1, Per 2, Cry 1 and RevErb in white blood cells of patients receiving RGC vs. placebo. The results represent the normalized sum of gene expression at fasting and 120 and 240 minutes postprandial.

Results are expressed as mean ±SD.

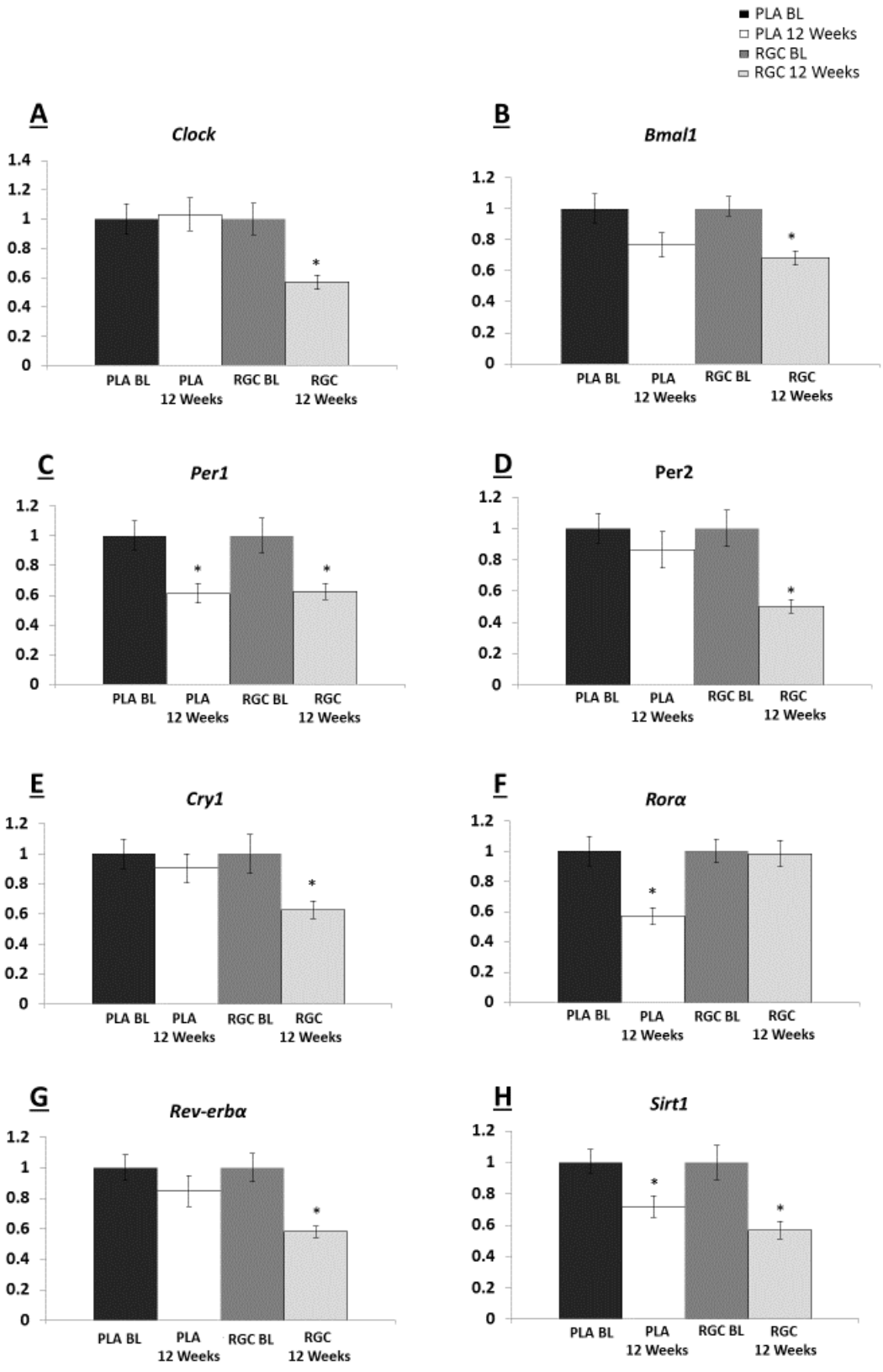

PLA BL
PLA 12 Weeks
RGC BL
RGC 12 Weeks
A
Clock
B
Bmal1
C
Per1
D
Per2
E
Cry1
F
Rorα
G
Rev-erbα
H
Sirt1
PLA BL
PLA 12 Weeks
RGC BL
RGC 12 Weeks

Figure 3: HbA1C levels at fasting

(A) HbA1c level in PLA and RGC groups. *p<0.05

(B) HbA1c after 12 weeks on RGC or PLA adjusted to % compliance. *p=0.05.

(C) HbA1c levels in subgroup of HbA1c 7.5-10.1. *p<0.05

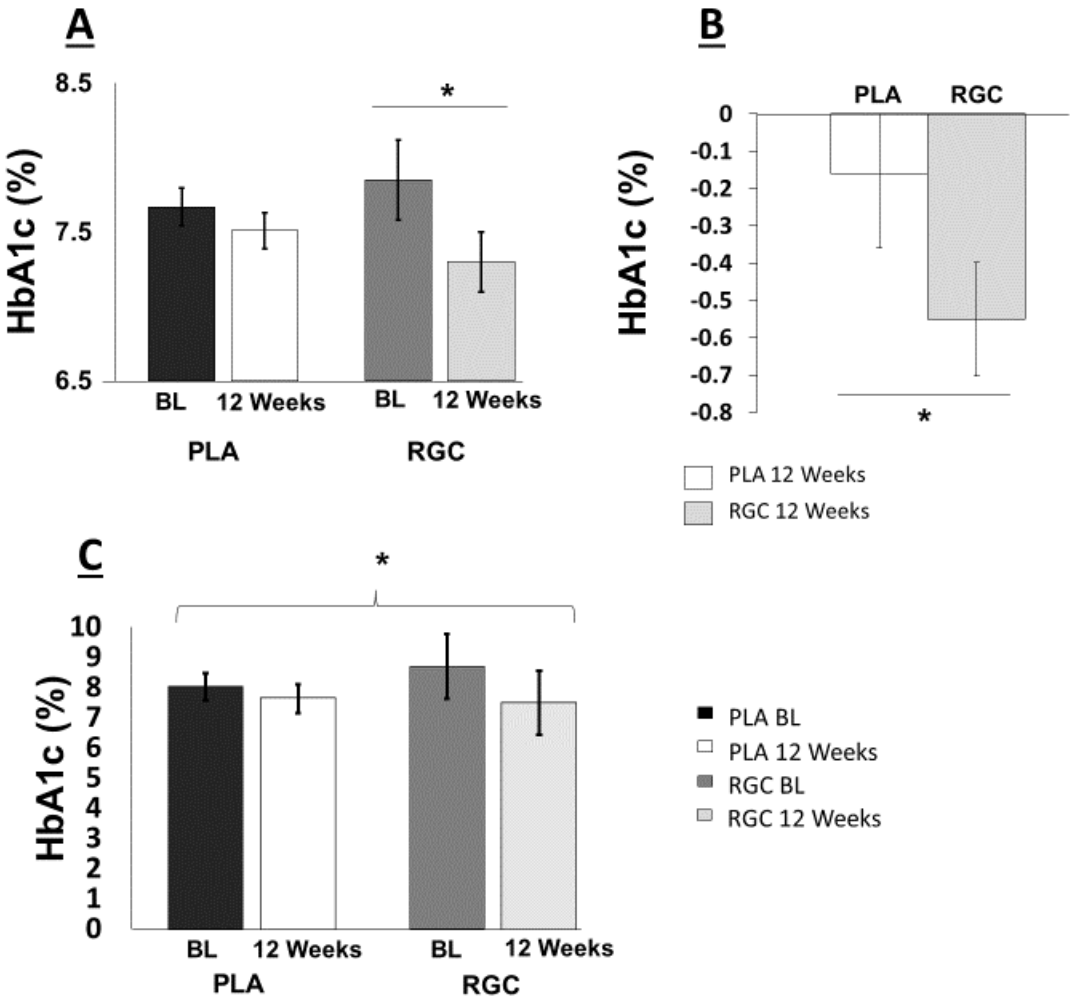
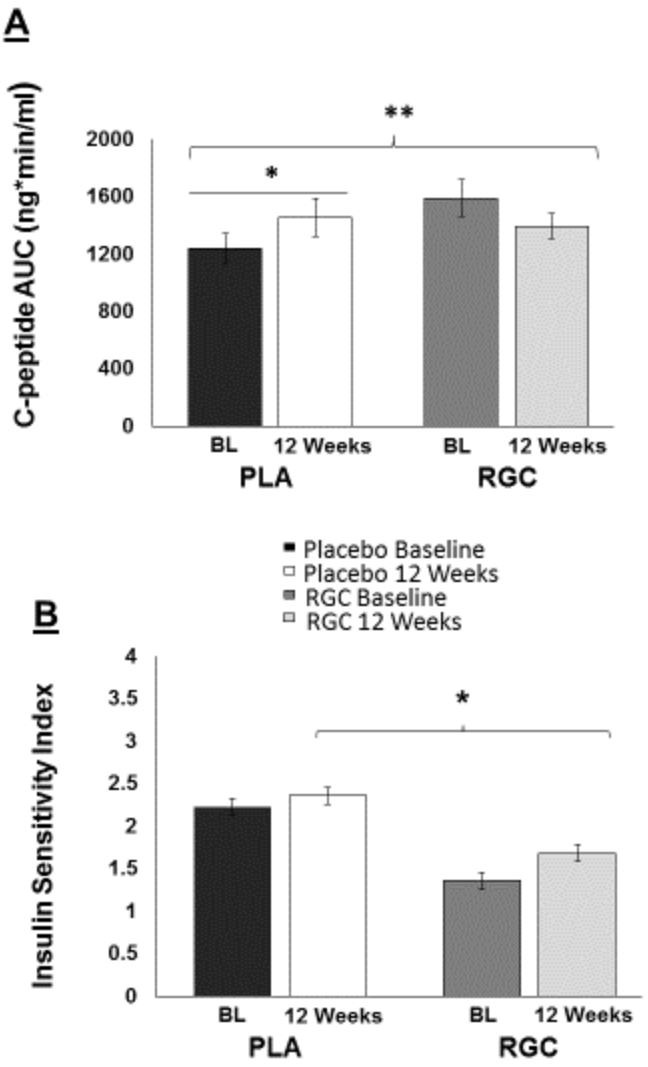


Figure 4:

(A) C-peptide AUC (at fasting and postprandial) in PLA and RGC group. *p<0.05. **p<0.05

(B) Insulin Sensitivity Index in PLA and RGC group. Calculated by 20/("fasting C−peptide × fasting plasma glucose") in PLA and RGC group. *p<0.05



4. DISCUSSION

A statistically significant decrease in mRNA expression of BMAL1 was detected in patients receiving RGC, but not in the placebo group. A similar outcome was also reported for CLOCK, PER 2, Cry 1 and Rev-Erbα, while RORα exhibited an opposite trend. This is in accord with the fact that Rev-Erbα and RORα exert opposite effects on

gene transcription [25]. The expression of Sirt1 was significantly reduced in both study groups, further research is needed to be performed to confirm these findings.

Considering the time of sample collection (9:00 AM and 4 hours later), the significant decrease in mRNA levels observed may reflect a phase advance in patients' circadian patterns. Importantly, this phase advance has been attributed to fasting and food restriction by various researchers. Barnea et al. showed that fasting leads to phase advance of Per2, Clock, and Bmal1 in mice livers, whereas a high-fat diet delays the clock gene Per1 [26,27]. Minami *et al.* demonstrated a phase advance of clock genes in murine hearts due to calorie restriction [28]. Phase advance is thought to occur through the activation of SIRT1 protein in yeast [29]. The following mechanism of action could explain the effect of RGC on clock gene expression: RGC activated SIRT1 protein and enhances its enzymatic activity, similarly to pure resveratrol and to calorie restriction and fasting. Activated SIRT1 deacetylates Bmal1, Per2 and histones, leading to relief of PER:CRY-mediated inhibition and promoting a phase advance. However, since serum samples were collected only during the morning and not throughout the day, further studies are needed to determine whether RGC consumption indeed induced a phase shift.

Effect of RGC on diabetic parameters was reflected by a significant decrease in glycated hemoglobin (HbA1c) levels, indicating a long-term effect of RGC on blood glucose concentration. The decrease in HbA1c was seen in PP patients, whose compliance was greater than 85%. Within the PP group, the most dramatic changes were observed for patients with initial HbA1c values >7.5. An additional effect was

observed with regards to C-peptide -- an indicator of insulin resistance. While the control group showed increased AUC values of C-peptide concentration, the RGC arm exhibited a clear decrease. Furthermore, calculated Insulin Sensitivity Index showed a more substantial increase for the RGC group vs. placebo. Moreover, as elevated C-peptide was associated with increased risk of cardiovascular diseases demonstrated in an in vitro model [30], the beneficial effect of RGC refers to cardiovascular health, as well. A similar randomized trial demonstrated that daily supplementation of RGC powder for 12 weeks improved endothelial function, diastolic BP and oxidative stress [4]. Furthermore, the effect of RGC on insulin resistance has been investigated in rats with metabolic-like syndrome [24]. The increase in plasma insulin was attenuated by RGC supplementation, suggesting an improvement in insulin resistance.

A minor increase of heart rate was demonstrated in RGC group patients, as compared to a slight decrease in control group. Both changes were within the normal range and were not accompanied with elevation in blood pressure.

5. **CONCLUSIONS**

Our results imply a possible effect of RGC on the expression of clock genes. This effect is demonstrated by reduced expression of BMAL1 and other clock genes, and may reflect a phase shift in circadian gene expression. Such shifts were shown to have beneficial effect on diabetic parameters. In addition, RGC was shown to have direct favorable effect on insulin resistance status, indicated by reduced HbA1c and C-peptide concentrations. Overall, this study demonstrates a potential beneficial effect of RGC on patients with diabetes. Further studies are needed to determine the best dosage and whether RGC might be useful as adjuvant therapy to achieve glycemic control in T2D.

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The formula RGC was manufactured and donated by Bioharvest, Ltd, Israel.

7. DECLARATION OF INTEREST

Bioharvest Ltd. supplied the study material and financial support.

JW, DJ, ST, TG, ZL, MM and YBD declare they have no conflict of interest in relation to this work.

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