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RESEARCH ARTICLE

A novel red grape cells complex: health effects and bioavailability of natural resveratrol

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Abstract

In this study, we present a novel product consisted of red grape cells (RGC) grown in culture and evaluated its effect on human LDL oxidation (*in vitro*) and inflammatory stress (in an *in vivo* rat model). We analyzed RGC for its polyphenols content and characterized RGC-derived resveratrol (RES) and its properties; and finally, we characterized the pharmacokinetic profile of RGC-RES in human plasma. RGC has demonstrated a strong inhibitory effect on LDL oxidation with IC_{50} as low as 8.0 μg/ml. RGC significantly reduced rats inflamed paw size induced by carrageenan injection. LC/MS analysis has shown that the main polyphenol in RGC was RES with one hexose moiety. The human pharmacokinetic analysis (clinicaltrials.gov NCT01747252) revealed relatively high bioavailability and two distinctly separated plasma concentration peaks at 1 and 5 h. The present study demonstrates antioxidant and anti-inflammatory traits of RGC that warrants further research in both pre-clinical and clinical settings.

Keywords

Piceid, polyphenols, red grapes, resveratrol

History

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Introduction

Polyphenols are secondary metabolites synthesized by plants, encompassing a broad variety of compounds, such as hydroxybenzoic acids, flavonoids, anthocyanins, proanthocyanidines, catechins, and stilbenes, including resveratrol (RES) (Manach et al., 2005; Williamson & Manach, 2005). There is a growing body of evidence demonstrating that polyphenols possess antioxidant, anti-inflammatory and anti-atherosclerotic activity, as well as cardiovascular protective activity (Pandey & Rizvi, 2009). Among the known polyphenols, the phytoalexin RES (*trans*-3,5,4′-trihydroxystilbene), found in red grapes, red wine, and other foods such as different kind of berries and peanuts (Goldberg et al., 1996) has drawn most of the attention. It is believed to be responsible for the ''French paradox'', a phenomenon associated with low incidence of cardiovascular diseases despite high-fat diet as a result of moderate red wine consumption (Opie & Lecour, 2007).

The aromatic groups in RES structure enable it to function as an antioxidant and prevent important reactions in diseases processes such as LDL oxidation occurring in atherosclerosis (Catalgol et al., 2012). RES was also shown to modulate the inflammatory responses underlying chronic diseases such as cancer and diabetes (Vang et al., 2011). Animal studies have shown RES involvement in attenuation of pain as well as acute inflammation (Bazzo et al., 2013; Bralley et al., 2008).

RES bioavailability is compromised by its physicochemical properties such as low water solubility and also its high hepatic uptake. Moreover, oral bioavailability of RES is extremely low due to rapid and extensive metabolism with the consequent formation of various metabolites such as RES-glucuronides and RES-sulfates (Walle, 2011).

Studies investigating RES activity and effects rely mainly on three sources of RES, namely pure synthetic RES, natural plant-derived RES (e.g. *Poligonum cupcidatum* extracts) products, or to lesser extent whole red grapes or their products (red wine, grape juice, and grape extracts). Herein, we introduce a novel red grape cells (RGC) product, a natural patent-protected formulation of cultured cells originated from the fruits of *Vitis Vinifera L.* cultivar comprising the whole matrix of polyphenols, and other ingredients naturally existing in red grapes.

In the present work, our aims were to investigate whether RGC demonstrates the well-documented anti-oxidative and ant-inflammatory properties associated with polyphenols that may be attributed to the complete red grape polyphenolic composition found in RGC; to characterize the structure of RES found in RGC and its solubility. Finally, we examined RES-RGC pharmacokinetic profile in human plasma.

Materials and methods

The investigational product

RGC powder (Fruitura Bioscience Ltd. Patent application US 2008/0166306A1) consists of dried red grape cells grown in culture. Nutritional values of RGC and of agricultural grown red grapes mixture of different varieties were analyzed by an external analytical laboratory (Aminolab Ltd., Ness-Ziona, Israel).

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Five different batches of RGC powder and agricultural red grapes cultivars were prepared for HPLC analysis by dissolving RGC powder or a lyophilized, frozen-crushed preparation of agricultural red grapes in 80% methanol. The solution was sonicated for 10 min at 30 °C, centrifuged at 16 900 × *g* to remove non-soluble matter and filtered through 0.45 μm filter, and the filtrated supernatant was used for analysis. Polyphenols and *trans*-RES in RGC powder were quantified by HPLC using JASCO PU-2089 HPLC system and the operation software ChromNAV (Jasco Inc, Easton, MD). Total phenolic compounds content was evaluated at 280 nm and expressed as epicatechin equivalent. Anthocyanins content was evaluated at 520 nm and expressed as delphinidine equivalent. *Trans*-RES content was evaluated at 306 nm, based on its characteristic absorption profile. Total tannins content was evaluated using a colorimetric method as previously described (Giner-Chavez et al., 1997).

In vitro LDL oxidation assay

Measurements of LDL susceptibility to oxidation are commonly used in evaluating the effects of dietary or pharmacological antioxidants on LDL oxidation (Aviram & Vaya, 2001). LDL was prepared from human blood as described elsewhere (Aviram, 1983). The LDL protein concentration was determined with the Folin–phenol reagent (Lowry et al., 1951) prior to oxidation, LDL was dialyzed against EDTA-free, phosphate-buffered saline (PBS) solution, pH 7.4, and at 4 °C.

Copper ion-induced LDL oxidation: LDL (100 mg of protein/ml) was incubated for 10 min at room temperature with increasing concentrations (up to 50 μg/ml) of RGC ethanolic extract in each experiment. Then, 5 mmol/l of $CuSO_4$ was added and the tubes were incubated for 2 h at 37 °C. At the end of the incubation, the extent of LDL oxidation was determined by measuring directly in the medium the generated amount of thiobarbituric acid reactive substances (TBARS) at 532 nm, using malondialdehyde (MDA) for the standard curve and of lipid peroxides.

Lipoprotein oxidation was also determined by the lipid peroxide test which analyzes lipid peroxide formation by its capacity to convert iodide to iodine, as measured spectrophotometrically at 365 nm (Aviram & Vaya, 2001)

Anti-inflammatory effect

For the purpose of assessing the anti-inflammatory effect of RGC, the acute carrageenan-induced oedema and thermal stimulus models were used (Morris, 2003) according to National Institute of Health (NIH) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines and in compliance with ''The Israeli Animal Welfare Act''.

Sprague–Dawley male rats ($n = 8$ per group) were administered RGC (400 mg/kg body weight daily), or the non-steroidal anti-inflammatory drug Indomethacin (2 mg/kg body weight) or drinking water (vehicle) daily for 7 d via oral gavage. The number of animals per group was based on previous studies demonstrating that this was the minimum number of animals per group sufficient to obtain indicative/significant information (Falchi et al., 2010). RGC dose was chosen based on previous dose range study that demonstrated RGC efficacy in rat model (Leibowitz et al., 2014). Indomethacin dose was chosen based on other studies that showed inhibition of carrageenan induced paw edema (e.g. Amann & Schuligoi, 2000). On day 8, rats were injected with 100 μL of 1% solution of λ-carrageenan (Sigma, St. Louis, MO) in saline into the planter pad of the paw. Carrageenan injected paw volumes were measured just before carrageenan injection defined as time 0, 1, 2, and 4 h after injection using a caliper. The paw dimensions were measured in two axes and the apparent paw volume was calculated by multiplication of the axes measurements. About 1.5 h before and 2 and 4 h post-carrageenan injection the rats were also tested for thermal stimulus. Rats were placed on a hot plate platform maintained at a temperature of 50 °C and the time to paw lifting or jump (the response) was measured. In the absence of response, a 60 s cut-off was used to prevent tissue damage.

LC/MS analysis

Detection of RES in RGC powder was performed by LC-MS using Accela LC system coupled with the LTQ Orbitrap Discovery hybrid FT mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with a electrospray ionization source. A mass spectrometer was operated in a negative ionization mode. The LC-MS system was controlled and data were analyzed using Xcalibur software (Thermo Fisher Scientific Inc., Waltham, MA). Chromatographic separation was achieved on Kinetex Hexyl-Phenyl column (2.6 μm, 150 × 2.1 mm, Phenomenex, Torrance, CA)

Solubility assay

RGC, plant derived RES (plant RES) and synthetic RES (S-RES) were prepared for HPLC analysis as described above. These preparations were used as the 100% reference for each source of RES dissolution in water at pH 2 and pH 7. RES was monitored at 306 nm, based on its characteristic absorption profile, and its concentration was determined by a calibration curve of Trans-RES analytical standard.

Bioavailability study design

The study was a randomized three-way crossover pharmacokinetic study. Fifteen adult healthy fasting male subjects received, in randomized order, the investigational product RGC (oral doses equivalent to 50 mg or 150 mg of trans-RES), or plant-RES (150 mg) on three occasions separated by 7 d washout periods. The sample size was determined based on previous studies using similar population of subjects and/or investigational product (Burkon & Somoza, 2008; Nunes et al., 2009; Ortuno et al., 2010). A standard meal was served at 4 h post-dosing. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Soroka University Medical Center IRB and registered at clinicaltrials.gov (NCT01747252). All the subjects signed an informed consent form prior to the beginning of the study.

Fifteen healthy non-smoking male volunteers were recruited into the study. Volunteer eligibility criteria included ages of 18–55 years; BMI ≥ 19 and ≤ 30, non-smoking and good health judged by medical history and routine laboratory tests. Subjects were asked to refrain from RES-containing food, nutritional supplements or drinks and from all drugs including over the counter medications from 7 d before the first dosing, and throughout the entire study period. The use of xanthine containing beverages such as coffee, tea, cola, and chocolate or alcoholic beverages was prohibited for 12 h prior to dosing and throughout the following 12 h.

Sample collection and management

Venous blood samples were collected into K_2EDTA containing tubes before dosing (t_0) and at 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, and 12 h post dosing. Blood was kept in an ice bath and immediately handled under yellow light. Blood was spun (3000 × *g*, 10 min, 4 °C) and transferred into amber-colored polypropylene tubes and deep frozen (−75 °C) until analysis of free and conjugated RES concentrations.

Analysis of RES content in plasma

Sample preparation and LC-MS analysis for free and total RES in plasma samples were performed in an external lab (PRAC Institute, Canada) using completely validated methods. Plasma sample were liquid-liquid extracted and used for LC-MS/MS analysis. The lower limits of quantification (LLOQ) were 0.5 and 20 ng/ml for free and total RES, respectively. For the analysis of total RES, enzyme hydrolysis and protein precipitation extraction were performed prior to LC-MS/MS analysis.

The liquid chromatography system used a Thermo Hypersil Gold column, 100 mm × 2.1 mm (5 μm particle size) set at 40 °C with an isocratic flow of 70:30 (v/v) deionized water:acetonitrile at a flow rate of 0.35 ml/min. The analyte and internal standard were detected using an API 4000 LC-MS/MS system equipped with Turbo Ion Spray ionization source operated in the negative ion mode (AB Sciex, Framingham, MA). The following MRM transitions of the respective $[M–H]^-$ ions were used to monitor $m/z = 227.1$ (RES) and $m/z = 233.1$ (the internal standard).

Pharmacokinetic analysis

The following pharmacokinetic variables were calculated for free and for total RGC-RES (free and conjugated) using a noncompartmental pharmacokinetic approach: maximal plasma concentration (C_{max}), time to reach maximal plasma concentration (T_{max}) and area under the plasma concentration versus time curve (AUC) from time (0) to the last quantifiable concentration (C_{last}, above LOQ), calculated by the trapezoidal method.

Statistical analysis

Summary statistics of all data for each treatment and scheduled sampling time were reported, as appropriate, using the arithmetic mean, CV, SEM, and median. Statistical analysis was carried out for the solubility assay using a one-way ANOVA. For the anti-inflammatory test, a two-way ANOVA for repeated measures, followed by the Bonferroni *post hoc* test have been used. p Values <0.05 were considered statistically significant. The statistical package SAS (Version 9.1, SAS Institute, Cary, NC) was used.

Results

RGC composition

RGC composition indicates that the levels of carbohydrates and the resulting caloric value are much lower in RGC as compared with agricultural grown red grapes (Table 1). Polyphenols content of RGC is similar to agricultural red grapes except for the RES content, which in some cases was higher by two orders of magnitude in RGC as compared with red grapes (Table 2).

In vitro LDL oxidation

Addition of increasing concentrations of ethanolic RGC extract inhibited copper ion-induced LDL oxidation in a dose-dependent manner (Figure 1). The IC_{50} (inhibition of LDL oxidation by 50%) of RGC was 12.0 μg/ml for the TBARS formation and 8.0 μg/ml for the lipid peroxides formation.

Acute inflammation – paw edema

Seven days pretreatment with RGC at a dose 400 mg/kg or with indomethacin 2 mg/kg significantly inhibited the carrageenan induced paw edema during 4 h after carrageenan administration (Figure 2). Paw volume in the vehicle-treated rats showed a 2-fold increase at both 2 and 4 h post-treatment. At the same time point, the increase in paw volumes in both indometachin- and RGC-treated rats was significantly lower ($p < 0.001$) – 1.5- and 1.4-fold, respectively.

Table 1. Composition of RGC powder in comparison with agricultural grapes.

	RGC (100gr)	Agricultural Grapes (100gr)
Energy (kcal)	17	108
Protein (g)	1.3	1.3
Carbohydrate (g)	3.7	26.1
Fat (g)	0.10	0.09
Ash (g)	0.86	0.84
Sodium (mg)	3.0	2.0
Potassium (mg)	320	190
Moisture (g)	94.1	71.7
Total proximate composition	100.00	100.00

Values are normalized to 100 g fresh weight of edible food.

Table 2. Polyphenols composition of RGC in comparison with agricultural red grape.

	RGC (mg/kg fresh weight) Range	Agricultural Red Grapes (mg/kg fresh weight) Range
Total Polyphenols	2333–2600	727–4204
Anthocyanins	266–667	614–3103
Tannins	1840–3133	2389–5088
Resveratrol	726–916	0–42.5

Inflammatory hyperalgesia

As illustrated in Figure 3, the injection of carrageenan into the right hind paw of rats caused a reduction in the latency of their withdrawal response to heat stimulation as compared with baseline, referred to as hyperalgesic response. In this model, RGC significantly increased the latency induced by carrageenan 4 h after carrageenan administration, compared with the vehicle-treated control ($p < 0.01$). Indomethacin also significantly increased the latency induced by carrageenan 2 ($p < 0.01$) and 4 h ($p < 0.05$) after carrageenan administration, compared with vehicle-treated rats. The latency after 4 h was higher than after 2 h in the RGC group but this difference did not reach statistical significant. In contrast, a strong statistical trend ($p = 0.06$) was revealed in the latency decrease observed after 4 h in comparison with the 2 h time point in the indomethacin group.

LC/MS

LC-MS analysis detected four derivatives of RES (m/z −227.0701 to −227.0737) in RGC, all of which show the UV absorbance at 306 nm. The most prominent derivative (detected at retention time of 5.3 min) and two additional derivatives (detected at 4.6 and 6.1 min) were hexose glycosides of trans-RES isomers. The fourth derivative was of *trans*-RES, detected at a retention time of 6.9 min (Table 3 and Figure 4).

Solubility

The solubility of RGC-RES was compared with that of two RES products, S-RES and plant-RES. All three products that were tested completely dissolved in 80% methanol. However, when the three RES sources were dissolved in water, either acidified (pH = 2), mimicking the stomach pH conditions or at pH 7.0, dissolution of RGC-RES was more than 6-fold higher, 44% for

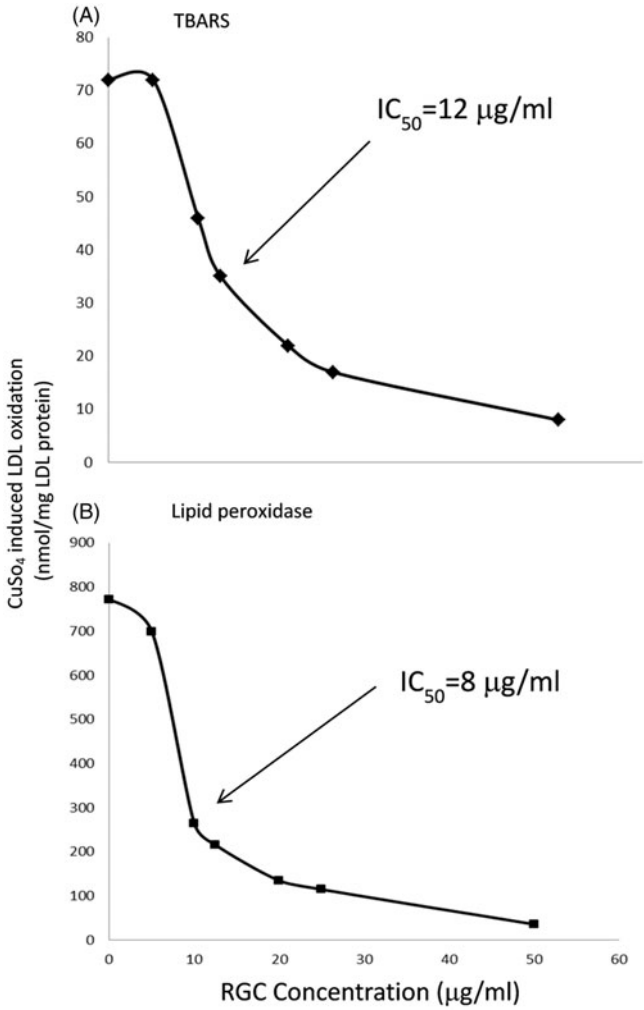

Figure 1. The effect of RGC powder on LDL oxidation induced by copper ion. LDL oxidation was measured as TBARS (A) or as lipid peroxidase (B) formation. Values represent mean of three samples.

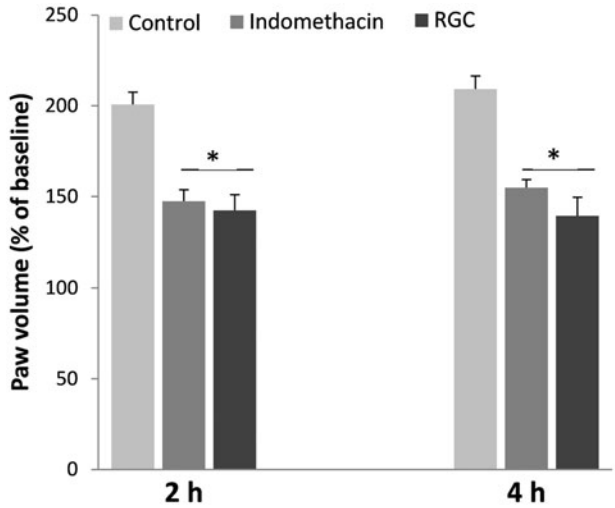

Figure 2. RGC (400 mg) and indomethacin (2 mg/kg) effect on carrageenan-induced edema. Values represent means ± SEM ($n = 8$ per group). *$p < 0.05$.

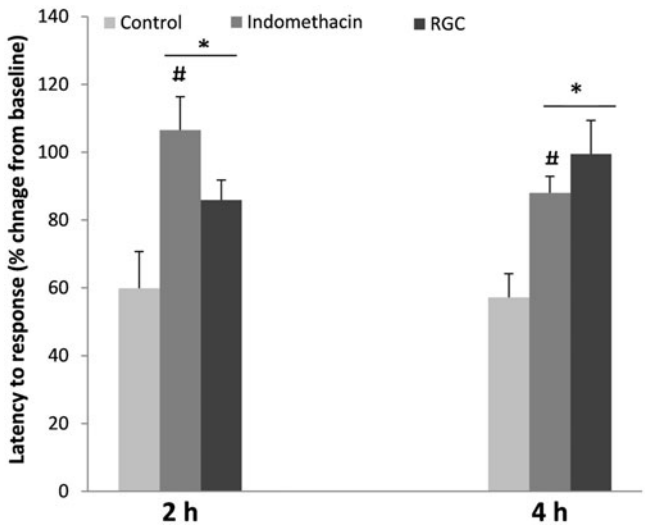

Figure 3. RGC (400 mg) and indomethacin (2 mg/kg) effect on latency to hot stimulus response. Values represent means ± SEM ($n = 8$ per group). *$p < 0.05$; # $p = 0.06$.

RGC-RES versus 7% for the two other RES sources ($p < 0.0001$; Figure 5).

Bioavailability

Demographics and safety

Fifteen healthy male volunteers participated in the study. Subjects age range was 28–55 years (mean 42.1 years) and BMI range was 21.4–30 kg/m^2 (mean 25.8 kg/m^2). All subjects were tested negative to drugs and alcohol with no clinically significant abnormalities in laboratory parameters and vital sign measurements at screening and admission. No adverse events were observed or reported throughout the study for both RGC and plant-RES.

Plasma pharmacokinetics of free and total RES

Mean trans-RES plasma concentration versus time curves for total RES and free RES are displayed in Figure 6. Free RES concentrations after ingestion of the 50 mg dose were low, in many cases below the LLOQ (0.5 ng/ml) and, therefore, are not displayed. RGC profile at both concentrations demonstrates two clear concentration peaks, the first at 1 h and a second higher peak at 5 h. Mean pharmacokinetic parameters of *trans*-RES are summarized in Table 4. Importantly, measurable quantities of RES were detected as early as 20 min post-administration in all subjects in the RGC groups (except 1 in the RGC 150 mg group).

Discussion

Red grapes are an important source of polyphenols, especially of RES, one of the most investigated plant secondary metabolites with myriad of beneficial health effects. RGC is an innovative, patent protected, RES-rich nutraceutical. While RGC-RES concentration is high and stable, RES content of agricultural grapes was found to be scarce due to factors such as different climates, geographical growing regions, type of soils, and exposure to diseases (Roldán et al., 2003). Moreover, during harvesting, preservation and storage processed RES might be decomposed as a result of enzyme systems found in red grape cell (Regev-Shoshani et al., 2003; Versari et al., 2001). Furthermore, RGC production is a controlled process favoring the production of RES while preventing its destruction in the cells.

A growing body of evidence has accumulated on the biological activities of RES including inhibition of lipid peroxidation, free-radical scavenging, inhibition of platelet aggregation,

Table 3. Identification of resveratrol derivatives in RGC.

Molecular weight	Calculated atomic composition	Retention time (min)	Comment
389.1250	$C_{20}H_{21}O_8$	4.6	Glycoside of *trans*-resveratrol
389.1250	$C_{20}H_{21}O_8$	5.3	Glycoside of *trans*-resveratrol
389.1250	$C_{20}H_{21}O_8$	6.1	Glycoside of resveratrol. Chromatographic separation was not complete but it also can be a derivative of *cis*-resveratrol (according to UV)
227.0719	$C_{14}H_{11}O_3$	6.9	*trans*-Resveratrol

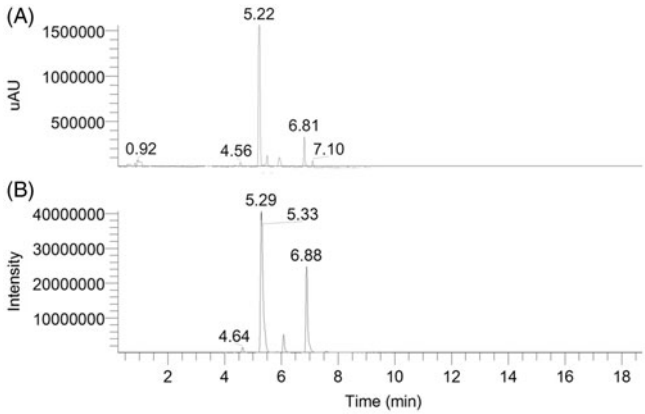

Figure 4. LC-MS analysis of RES in RGC: UV chromatogram ($\lambda = 306$ nm) (A) and extracted ion chromatogram (m/z −227.0701 to −227.0737) (B).

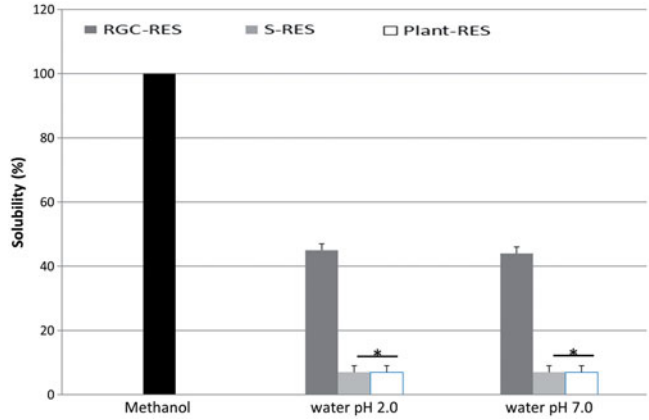

Figure 5. Solubility in water expressed as percentage of solubility in methanol of RES derived from RGC (RGC-RES), synthetic RES (s-RES), and RES extracted from plants (plant-RES). Values represent means ± SEM ($n = 3$ per group). *$p < 0.001$.

anti-inflammatory activity, and blood pressure lowering effect (Cottart et al., 2014).

It is widely accepted that lipid peroxidation in general and that of LDL in particular plays a central role in the development of cardiovascular diseases. Therefore, dietary intervention in an effort to inhibit LDL oxidation is agreed to be beneficial (Aviram & Vaya, 2001). RGC has shown a strong inhibitory effect on LDL oxidation *in vitro*. The novel RGC-RES may have contributed to the observed effect. However, as the origin of RGC-RES is whole cells containing a matrix of polyphenols, the strong inhibition of LDL oxidation seems closely related with RGC polyphenolic constituents and their synergistic effect (Aftab et al., 2010; Rayalam et al., 2011). Nevertheless, a freeze-dried powder prepared from varieties of white, red, and purple grapes in some of which RES is either lacking or not the predominant polyphenol, tested for inhibition of LDL oxidation under similar conditions of the current study, demonstrated a smaller inhibitory effect, as represented by higher IC_{50} compared with RGC (Fuhrman et al., 2005). The next logical step would be to test the inhibition of LDL oxidation following RGC consumption.

Anti-inflammatory and analgesic effects have been shown to be exerted by different kinds of plant polyphenolic matrices in various animal models (Deng et al., 2011; Liao et al., 2012; Sheu et al., 2009). Specifically, RES analgesic properties have been documented both centrally and peripherally with possible underlying mechanisms that include AMPK-pathways activation and inhibition of cyclooxygenase-2 (COX2), an enzyme involved in inflammation and pain, mRNA expression (Bazzo et al., 2013; Falchi et al., 2010; Tillu et al., 2012). In addition, RES has been shown to possess anti-inflammatory activity, probably via suppression of pro-inflammatory agents (Manna et al., 2000). This model of carrageenan-induced inflammation was originally described by Winter (Winter et al., 1962) and was extensively studied and found to be highly reproducible. Similar to our results, *trans*-RES decreased hyperalgesia induced by carrageenan injected into the rat hind paw; however, in contrast to our results, no anti-inflammatory effect as expressed by reduction in inflamed paw volume has been observed (Gentilli et al., 2001). Such differences could be explained by different modes of administration. While in our study RGC was administered for 8 consecutive days via oral gavage, *trans*-RES was given as a single dose injected i.p. Also, the glycoside form of RES found in RGC with other types of polyphenols that could act synergistically may have contributed to the anti-inflammatory effect. Interestingly, while indomethacin analgesic effect decreased after 4 h, the effect of RGC increased or at least has not been changed throughout the same period of time. While possible synergism between RGC-RES and other molecules in RGC powder may contribute to the observed antioxidant and anti-inflammatory effects, we cannot rule out that it may also influence otherwise the tested properties.

RES demonstrated relatively high oral absorption but rapid and extensive metabolism, as determined by LC/MS, resulting in overall poor bioavailability (Walle et al., 2004). As reported by Boocock et al. (2007a), even doses as high as 5 g resulted in plasma free RES peak concentrations of only 538 ng/ml. A possible reason for this poor bioavailability is the low solubility of RES attributed to its tendency (like other polyphenol aglycones) to form aggregates or hydrogen bonds via hydrophobic interaction of aromatic groups or hydroxyl groups, respectively (Biasutto et al., 2009). One of the factors that may improve RES solubility and hence its bioavailability is glycosylation of the RES parent compound (Biasutto et al., 2009). Indeed, here we showed that in RGC, the glycoside form of *trans*-RES, with one hexose moiety is the predominant form of RES. This finding coincides with previous analysis showing up to seven different glycosylated stilbenoids (monomeric and dimeric) detected in a *vitis vinifera* cell culture (Mulinacci et al., 2010).

Table 4. Plasma pharmacokinetic values following supplementation with RGC ($n = 15$ in each group).

	Mean AUC_t (ng h/ml)	%CV	Mean C_{max} (ng/ml)	%CV	Median T_{max} (h)	Range (h)
Total RES						
RGC 150 mg	10 404	29.9	1684	33.1	4.00	0.67–6.00
RGC 50 mg	2694	52	458.4	52.4	1.00	0.33–8.00
Free RES						
RGC 150 mg	9.85	74.3	6.89	56.9	1.00	0.33–4.00

This glycosylated structure may render RGC-RES more stable to enzymatic oxidation (Regev-Shoshani et al., 2003), but more importantly, it may enable RGC-RES to be more soluble in aqueous media such as body fluids. Accordingly, in line with its RES-glycoside chemical nature, RGC-RES presented higher water solubility compared with other sources of RES. The glycosyl groups in RGC-RES with its higher water solubility may also explain its significantly high gastrointestinal absorption rate as demonstrated in RES found in the plasma samples of most subjects that received RGC already after 20 min.

Several studies have reported peripheral blood RES levels after oral ingestion of different quantities of RES. Consistent with the reported bioavailability of *t*-RES (Amri et al., 2012; Brown et al., 2010; Cottart et al., 2014; Walle, 2011) RGC-RES also had low bioavailability. RGC-RES pharmacokinetic profile revealed AUC and C_{max} in the low range of what is known for free RES (Almeida et al., 2009; Boocock et al., 2007b; Brown et al., 2010), while well within the range reported for total RES (Goldberg et al., 2003; Walle et al., 2004). Although we cannot rule out some methodological differences, the total RES C_{max} reported herein exceeded equivalent published results of others with other sources of RES (Amiot et al., 2013; Poulsen et al., 2013; Wong et al., 2011).

Administration of RGC-RES resulted in a T_{max} value of 4 h, which is substantially longer than the T_{max} of 0.5–1 h reported following the administration of pure t-RES (Brown et al., 2010; Cottart et al., 2010). Longer T_{max} can be found following the administration of whole grape products such as grape juice or grape extract (Ortuno et al., 2010; Rotches-Ribalta et al., 2012). Nevertheless, due to limit of detection concerns, RGC in our experiment was administered in an amount aimed to achieve high level of RES comparable with experiments done with pure *t*-RES. Despite the closer similarities of RGC to grape products, it is reasonable to assume that RGC in doses that resemble normal consumption of grape product would have resulted with slightly different pharmacokinetic profile. However, consistent with the phramacokinteic behavior of RES in natural grape sources, RGC-RES yielded two very distinct concentration peaks appearing at 1 and 5 h (Figure 6). This phenomenon is most likely attributed to entero-hepatic recirculation as has been shown using a rat model (Marier et al., 2002). The first peak may represent extensive glucoronidation and sulfation of RES already in the enterocytes of the small intestines (Teng et al., 2012). The second peak occurs as a result of bile containing metabolites flowing from the liver to the intestines normally after food and may serve to prolong the pharmacological effect of certain substances and their metabolites (Roberts et al., 2002). Plausibly, longer presence of RGC-RES in the digestive tract and circulation contributed to the observed higher second concentration peak. Importantly, concentration time curves of synthetic or yeast fermentation source of RES (Poulsen et al., 2013) as well as of plant derived RES (Amiot et al., 2013) show a distinct single concentration peak.

Interestingly, a pharmacokinetic analysis of one form of glycosylated RES (*t*-RES-3-*O*-β-D-glycoside; piceid) ingested by healthy volunteers revealed a similar profile of double peaks presenting by two of the sulfate derivatives of RES: 3,4-disulfate and 3,5-disulfate (Burkon & Somoza, 2008). It is possible, therefore, that the metabolite profile demonstrated for RGC-RES results from the existence of sulfate derivatives as major metabolites. In this regard, the question of low bioavailability should be considered in light of the possible role played by RES metabolites. One hypothesis is that RES metabolites serve as an inactive pool of RES in the blood. Despite the lack of *in vitro* activity of glucurinated and sulfated RES, they can be converted to active *trans*-RES by β-glucuronidase existing in humans either locally or systematically (Henry-Vitrac et al., 2006), or by hydrolysis of the sulfate groups at target tissues (Walle et al., 2004; Wang et al., 2004). Alternatively, a growing body of evidence suggests that RES metabolites themselves may be active, as has been shown for RES-glucuronides (Lu et al., 2013; Wang et al., 2004), RES-sulfates (Calamini et al., 2010; Hoshino et al., 2012) and speculated for RES-glycosides (Soleas et al., 2001). The latter are uniquely important when glycoside–RES found in RGC is considered. It has been shown recently that forms of glycoside–RES escaped the enzymatic hydrolysis and metabolism occurring in the gut and liver and detected in human plasma more than 5 h after administration (Rotches-Ribalta et al., 2012). It is possible that in our study these forms of RES have escaped not

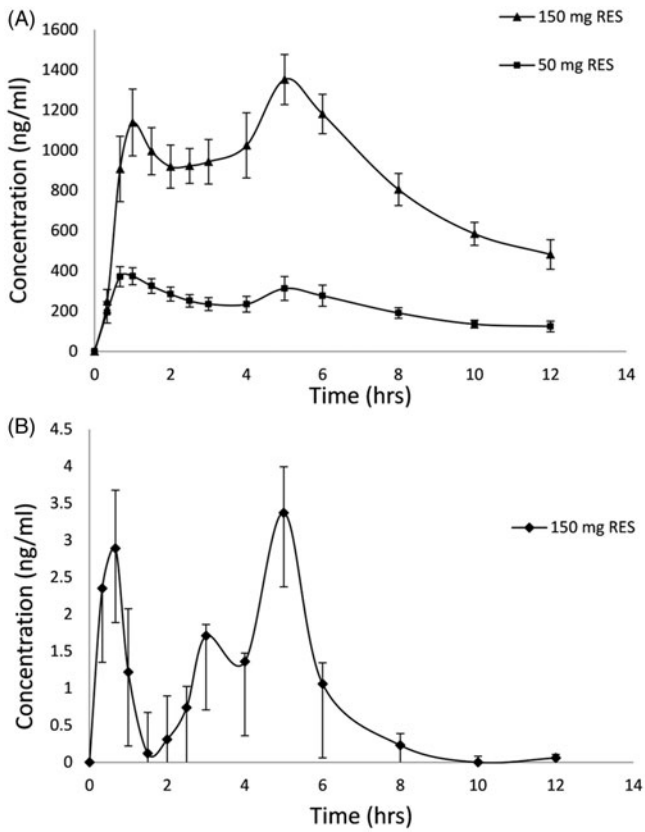

Figure 6. Plasma concentrations of *trans*-RES after the administration of a single dose of RGC-RES (equivalent to 50 and 150 mg RES). Total (A) and free (B) *trans*-RES ($n = 15$).

only the *in vivo* hydrolysis, but also the *in vitro* treatment of plasma samples. Therefore, the concentrations of both free and total RES could be biased and may not represent the full spectrum of RES in plasma after RGC concentration.

Conclusions

We have carried out a study demonstrating the anti-oxidative and anti-inflammatory properties of RGC as well as the characteristics and pharmacokinetic profile of RES-rich red grape cell product. RGC-RES was absorbed relatively quickly and had unique concentration/time curves that have not been reported previously for RES of different sources. Further investigation is needed to elucidate the exact type of the metabolites formed in human plasma and the clinical significance of RGC beneficial effects.

Declaration of interest

Fruitura Bioscience Ltd. supplied the study material and financial support. M. A., R. Y., A. K., and Y. H. are employees of Fruitura Bioscience Ltd., Rehovot, Israel. A. D. declares that he has no conflict of interest in relation to this work.

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