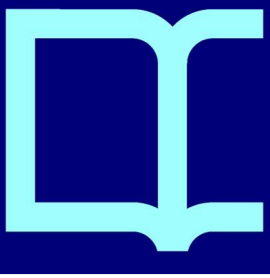

Effects of red wine polyphenols and alcohol on glucose metabolism and the lipid profile: a randomized clinical trial

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Short title: Red wine, polyphenols and alcohol and HOMA Index.

Non-standard abbreviations: Dealcoholized Red Wine: DRW; Red Wine: RW.

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Abstract

Background & Aims: Epidemiological data suggest that moderate red wine consumption reduces cardiovascular mortality and the incidence of diabetes. However, whether these effects are due to ethanol or to non-alcoholic components of red wine still remains unknown. The aim of the present study was to compare the effects of moderate consumption of red wine, dealcoholized red wine, and gin on glucose metabolism and the lipid profile.

Methods: Sixty-seven men at high cardiovascular risk were randomized in a crossover trial. After a run-in period, all received each of red wine (30g alcohol/d), the equivalent amount of dealcoholized red wine, and gin (30g alcohol/d) for 4 week periods, in a randomized order. Fasting plasma glucose and insulin, homeostasis model assessment of insulin resistance (HOMA-IR), plasma lipoproteins, apolipoproteins and adipokines were determined at baseline and after each intervention.

Results: Fasting glucose remained constant throughout the study, while mean adjusted plasma insulin and HOMA-IR decreased after red wine and dealcoholized red wine. HDL cholesterol, Apolipoprotein A-I and A-II increased after red wine and gin. Lipoprotein(a) decreased after the red wine intervention.

Conclusions: These results support a beneficial effect of the non-alcoholic fraction of red wine (mainly polyphenols) on insulin resistance, conferring greater protective effects on cardiovascular disease to red wine than other alcoholic beverages.

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Key words: Red wine, polyphenols, alcohol, insulin resistance, HOMA Index, lipoprotein(a).

1. Introduction

Consistent epidemiological data suggest that moderate alcohol consumption is associated with a reduced risk for fatal and nonfatal coronary heart disease and for cardiovascular disease (CVD) mortality, independently of the type of alcoholic beverage consumed[1]. The effect of alcohol in moderation on reducing the incidence of diabetes[2-4], a strong risk factor for CVD, may be a mediating mechanism. A meta-analysis of 20 cohort studies comprising 477,200 subjects indicated that moderate alcohol consumption (<60 g/d in men and <50 g/d in women) was inversely associated with diabetes risk. The dose-response trend showed that the strongest inverse association was observed for 22-24 g/d[2]. Furthermore, in a meta-analysis of 15 prospective studies, the relative risk of developing type-2 diabetes was lower in moderate alcohol drinkers than in abstainers or heavy drinkers, independently of the type of alcoholic beverage consumed[3]. Nevertheless, in a prospective study in healthy women, an inverse association between moderate alcohol intake and lower diabetes risk was most apparent in those who reported wine or beer drinking compared to women who reported liquor intake[4].

A salient feature of alcohol consumption is the increase in HDL-cholesterol (HDL-C) and apolipoprotein (Apo) A-I concentrations[5]. HDL-C and ApoA-I positively affect insulin secretion and pancreatic β-cell survival, thereby enhancing insulin sensitivity (IS)[6]. Since insulin resistance increases the risk of both CVD and diabetes[7], moderate alcohol consumption could possibly decrease these risks by improving IS. However, clinical trials assessing the short-term effects of moderate consumption of different alcoholic beverages on IS are few and the results are contradictory, as some studies have shown a positive effect[8,9] while most have reported no benefit[10-14].

Among alcoholic beverages, red wine (RW) is of note because it provides both alcohol and abundant polyphenolic compounds, which are thought to provide additional benefits on lowering CVD risk[15]. To determine the possible differential effects on risk markers of alcohol and polyphenols in RW, dealcoholized red wine (DRW) or grape extracts, which are rich in grape polyphenols but devoid of ethanol, may be used. Consumption of DRW in two studies had no effect on fasting concentrations of lipids and lipoproteins or IS[12,13]. In another study lyophilized grape powder (an analog of a polyphenolic extract of wine) decreased LDL-cholesterol (LDL-C) and ApoB concentrations in women[16]. Furthermore, concentrated red grape juice decreased LDL-C and ApoB and increased HDL-C and ApoA-I concentrations in healthy volunteers as well as in hemodialysis patients[17], but IS was not assessed in these studies using grape products[16,17]. Thus, it remains unclear whether the protective effects of alcoholic beverages on the risk of CVD and diabetes are due to ethanol or to their non-alcoholic components (mainly polyphenols). Therefore, we designed a randomized clinical trial to compare the effects of moderate alcohol consumption (30 g alcohol/d) through the ingestion of gin, a non-polyphenolic alcoholic beverage, RW, a high polyphenolic alcoholic beverage, and the equivalent amount of DRW, a high-polyphenol non-alcoholic beverage, on IS, serum lipids, and other cardiometabolic markers in subjects at high risk of CVD.

2. Subjects and Methods

2.1. Subjects

A total of 73 male moderate alcohol consumers aged between 55 and 75 years were recruited for the study in the outpatient clinic of the Internal Medicine Department of our institution from January 2008 to December 2010. The subjects included were at high risk for CVD because of family history of premature CVD and/or the presence of diabetes, hypertension, dyslipidemia, and overweight/obesity. Exclusion criteria included documented CVD, human immunodeficiency virus infection, chronic liver disease, malnutrition, neoplastic or acute infectious diseases, and customary use of vitamin supplements. Participants were offered free beverages but no monetary compensation. The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent.

2.2. Study design and diet monitoring

The study was an open, randomized, controlled, crossover trial with three intervention periods (**Figure 1**). Two weeks prior to the study the subjects were asked to maintain their usual diet and to refrain from consuming any alcoholic beverage. Baseline data were collected after this run-in period. Following this, participants were individually randomized by the dietitian in a crossover design among three treatment sequences lasting 4 weeks each, in which the test beverages were provided. Randomization was based on a computer-generated random number table, resulting in six possible diet sequences. A dietitian assigned participants to interventions and

instructed them to consume gin (100 mL/day, containing 30 g of ethanol), RW (272 mL/day, containing 30 g of ethanol and 798 mg of total polyphenols), or DRW (272 mL/day, containing 1.14 g of ethanol and 733 mg of total polyphenols). No washout periods were included between the interventions. The phenolic composition of the RW and DRW used in the study is detailed in **Table 1**. The total phenolic content of the three beverages was determined with the Folin-Ciocalteu method, and the phenolic profile was determined by HPLC-DAD, as described previously[18]. No significant differences were observed in the phenolic content of RW and DRW, while gin contained no detectable phenolic compounds.

Throughout the study the participants were asked to maintain their usual dietary habits, physical activity level and medications and to abstain from alcohol-free beer or alcoholic beverages except for those provided by the investigators. Natural foods rich in antioxidants, especially fruit and vegetables, were carefully monitored in order to achieve a similar dietary antioxidant content during the interventions. Participants were not blinded to the type of drink they ingested. After the run-in period and the day after each intervention period, a medical record and the Minnesota Leisure Time Physical Activity Questionnaire, which has been validated in Spain, were administered. In addition, the last week of the run-in period and the last week of each intervention period, subjects were asked to fill in a validated 7-d food record questionnaire of 5 week-days and 2 week-end days (**Figure 1**). The food records were used to assess nutrient intake and to monitor adherence to the study protocol. Compliance with the test drinks was also assessed by measures of urinary biomarkers of both alcohol and polyphenol intake. Foods were converted into nutrients by using the Food Processor Nutrition and Fitness Software (*esha* Research, Salem, OR), adapted to local foods. At

the end of the study, a clinician assessed any possible adverse effects from the interventions by administering a checklist of symptoms.

2.3. Clinical and laboratory measurements

Fasting blood and 24-h urine samples were collected at baseline and after each intervention. Serum, EDTA-plasma, and urine samples were stored with a blinded code at –80°C until assayed. The clinical investigators and laboratory technicians were blinded to the interventions. For each subject, the parameters determined in thawed samples of whole serum or plasma, as appropriate, were as follows: blood glucose with the glucose oxidase method; cholesterol and triglycerides with enzymatic procedures; HDL cholesterol after precipitation with phosphotungstic acid and magnesium chloride; and homocysteine and vitamin B_{12} by an automated electrochemiluminiscence immunoassay system (Advia-Centaur, Siemens, Barcelona, Spain). Plasma ApoA-I, ApoA-II, ApoB, ApoC-I, ApoC-III, lipoprotein(a), growth hormone (GH), insulin, adiponectin and leptin concentrations were quantified in whole serum samples by a customized Human Multi Analyte Profiling assay (Human MAP, Rules Based Medicine Inc., Austin, TX). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by multiplying the fasting insulin concentration (mIU/L) by the fasting glucose concentration (mM) and dividing by 22.5[19]. The effects of the interventions on inflammatory biomarkers related to atherosclerosis have been published elsewhere[18].

Ethylglucuronide, a biomarker of alcohol intake, was measured in 24-h urine samples by liquid chromatography, performed on an Agilent 1200 apparatus coupled with a hybrid quadrupole time-of-flight QSTAR Elite (Applied Biosystems/MDS Sciex). Resveratrol metabolites from phase II metabolism, as a biomarker of RW and

DRW intake[20], were also measured in 24-h urine samples using the validated methodology of Urpi-Sarda et al.[21] with slight modifications[18].

2.4. Statistical analyses

Sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline, Brentford, United Kingdom) assuming a maximum loss of 10% participants. To detect mean differences for the HOMA Index of 0.1 with a conservative SD of 0.15, 26 subjects would be needed to complete the study (α risk = 0.05, power = 0.9). However, to obtain greater differences, the sample size was more than doubled. The HOMA Index was used to determine the sample size, but changes in all endpoints were of equal interest.

Statistical analyses were performed using SAS Statistical Analysis Systems (version 9.2, SAS Institute Inc, Cary, North Carolina). Descriptive statistics [mean ± SD or n (%)] were used to describe the baseline characteristics of the participants and the outcome variables. Variables with a skewed distribution [GH, Insulin and lipoprotein(a)] were transformed to their natural logarithms for analyses and are shown as antilogarithmic values to facilitate the interpretation of the results. To compare changes in outcome variables from baseline in response to the interventions, one-factor analysis of variance (ANOVA) for repeated measures was used. To compare among-treatment changes in outcome variables, analysis of covariance (ANCOVA) for repeated measures with the previous intervention value (or the baseline value in the first intervention) as the covariate was used. To exclude the presence of a carryover effect for the three periods, the interaction between the type of treatment (RW, DRW and G) and the period sequence (1^{st}, 2^{nd} or 3^{rd}) was analyzed in the repeated measures ANCOVA analyses. The

Bonferroni post hoc test for multiple comparisons was used in both the ANOVA and ANCOVA analyses. For urinary biomarkers, results are expressed as means and 95% CIs for the among-intervention differences in the ANOVA analyses. *P* was considered significant when <0.05.

3. Results

3.1. Characteristics of study subjects

Of the 73 subjects included, six withdrew before completing the study. The reasons for withdrawal were intercurrent illness (n = 2), need to travel (n = 2), and refusal to drink DRW after tasting it (n = 2). Therefore, 67 subjects completed the study. The baseline characteristics of the completers (**Table 2**) shows that they had a sizeable burden of cardiovascular risk factors, with a high prevalence of family history of early-onset CVD, hypertension, overweight or obesity (with a maximum BMI of 40.5 Kg/m^2) and ≈25% prevalence of smoking, dyslipidemia, and diabetes. None of the diabetics was treated with insulin. The baseline characteristics of non-completers were similar to those of completers (independent samples Mann-Whitney U test; $P > 0.170$, all).

3.2. Measures of compliance and dietary control

There were no individual deviations from the interventions according to the participants' dietary reports. Urinary ethylglucuronide concentrations increased significantly after the RW and gin periods compared to baseline and DRW (**Table 3**). In addition, after consumption of RW and DRW, the 24-h urinary excretion of total resveratrol metabolites was higher than after the gin intervention and the baseline values (**Table 3**). According to these findings, compliance with the three interventions was excellent.

No significant differences in energy and nutrient intake (**Table 4**) or energy expenditure in physical activity were observed before and after each intervention according to food records and physical activity questionnaires. No individual changes in drug intake were reported and no adverse effects were observed. No carryover effect was observed.

3.3. Effects on glucose metabolism and adipokines

Changes in glucose metabolism are shown in **Table 5**. Fasting glucose concentrations did not change in any intervention, while the mean adjusted insulin values decreased significantly after the RW and DRW interventions compared to both baseline (21% and 20%, respectively) and the gin period (15% and 13%, respectively). Therefore, HOMA-IR decreased 30% and 22% from baseline after the RW and DRW interventions, respectively, as well as in comparison to gin (22% and 14%, respectively). On exclusion of the 15 participants with diabetes the results remained practically unchanged (adjusted HOMA-IR 1.47 ± 0.07, 1.08 ± 0.06, 1.13 ± 0.06, and 1.32 ± 0.07 at baseline and after RW, DRW, and gin, respectively). No significant changes were observed in GH, leptin and adiponectin from baseline or among the three interventions.

3.4. Effects on lipoproteins and apolipoproteins

Changes in the lipid profile are also detailed in **Table 5**. The mean adjusted LDL-C concentrations decreased 4.5% from baseline after the RW intervention. HDL-C concentrations increased from baseline after the RW and gin periods and in comparison

to the DRW intervention (7% and 5%, respectively, for both). Therefore, the LDL/HDL ratio decreased by 8% and 5% after the RW and gin interventions compared to the DRW period and after the RW intervention compared to baseline (7%). The mean adjusted lipoprotein(a) concentration decreased by 12% after RW compared to DRW and gin. ApoA-I and ApoA-II concentrations increased in parallel after RW and gin compared to DRW (12% for ApoA-I both, and 12% and 8% for ApoA-II), but only ApoA-II increased from baseline after RW and gin (9% and 5%, respectively). The mean adjusted ApoB concentration decreased by 5% after DRW compared to baseline. No significant changes were observed in total cholesterol, triglycerides, ApoC-I, and ApoC-III from baseline and among the three interventions.

3.5. Changes in other cardiovascular risk factors and B_{12} vitamin

As shown in **Table 5**, no significant changes were observed for homocysteine, and vitamin B_{12}. The mean adjusted serum folic acid concentrations were lower compared to baseline after RW (-12%) and DRW (-11%) and versus the gin period (-18% and -17%, respectively).

4. Discussion

The main findings of our study are that RW rich in polyphenols with or without alcohol (RW and DRW interventions) but not gin, an alcoholic beverage devoid of polyphenols, improved glucose metabolism, as measured by HOMA-IR, and that RW but not DRW or gin decreased lipoprotein(a) in men at high cardiovascular risk.

The results of the few prior clinical studies examining the effects of moderate alcohol consumption on IS have been inconsistent[8-14]. Two studies reported no significant improvement in IS after 17 days of whisky in 23 healthy men[11] or 4 weeks of RW or DRW in 17 healthy men[12]. A third study compared RW and vodka for 8 weeks in 20 insulin-resistant individuals and found little improvement of IS with either beverage[14]. In contrast, a fourth study[9] reported a 43% improvement in IS after 2 weeks of RW in 9 diabetic men. In the female population, short-term consumption of RW in middle-aged overweight women[10] or of RW and DRW in postmenopausal women[13] had no benefit on IS. In another trial in 51 post-menopausal women[8], consumption of 30 g/d alcohol (ethanol in orange juice) was associated with a 7.2% improvement in IS compared with 0 g/d, while 15 g/d had no effect. In our study both RW and DRW improved IS which, together with prior findings, suggests that both ethanol and polyphenols are responsible for this beneficial effect. Nonetheless, further well-controlled clinical trials are required to prove this contention.

The effects of moderate alcohol consumption on plasma lipoprotein(a), an independent risk factor for CVD and atherosclerosis, with a strong genetic basis that is relatively refractory to both lifestyle and drug intervention[22], are controversial. A recent meta-analysis concluded that moderate alcohol consumption had no effect on lipoprotein(a)[5]. A similar conclusion was reached in another study using RW for 4

weeks in healthy subjects[23]. However, in a clinical trial performed in healthy men, RW but not gin was associated with reduced lipoprotein(a)[24] and in another study lipoprotein(a) decreased after 10 days of RW but not white wine[25]. Furthermore, social alcohol consumption was associated with lower plasma lipoprotein(a) in a cross-sectional study of middle aged Finnish men[26]. In our study mean adjusted lipoprotein(a) was reduced by 12% after RW (ethanol plus polyphenols) but not after the DRW or gin interventions. This reduction led to a lipoprotein(a) concentration that coincides with the margin between very high and high risk of cardiovascular disease (1.785µmol/L; 50mg/L), limiting the clinical relevance of the observed results. Nonetheless, given the paucity of effective therapy for elevated lipoprotein(a), the potential lowering efficacy of ethanol and/or polyphenols deserves further research.

In the present study, LDL-C and ApoB were slightly, albeit significantly, reduced from baseline after RW and DRW, respectively. However, post-treatment LDL-C and ApoB values were similar across the three interventions, although the oxidized LDL fraction may differ between the interventions, as red wine polyphenols decrease postpandrial lipid oxidation[27]. As expected[5], moderate consumption of RW and G, but not DRW, increased plasma HDL-C and ApoA-I and ApoA-II concentrations and decreased the LDL/HDL ratio. This increase in Apo–II concurs with data from a prior study of RW in healthy men and women[23]. Therefore, the beneficial effects of moderate RW intake on lipid and lipoprotein metabolism are dependent on its alcohol component. Nevertheless, regarding glucose metabolism, we observed that the increased IS (in the interventions with polyphenols) does not correlate with the increase in HDL-C and ApoA-I concentrations (in the interventions with alcohol), as proposed by von Eckardstein A *et al*[6].

Regarding the effects of alcohol on plasma homocysteine, Gibson et al.[28] and Marcucci *et al.*[29] found that RW increased its plasma concentrations. No differences in homocysteine were observed after the three interventions in our study. Nevertheless, after RW and DRW consumption, we observed a significant decrease in serum folic acid, albeit within the physiological range. This can be explained by the fact that some RW polyphenols inhibit intestinal folate uptake[30].

This study has some limitations. First, consumption of alcohol was not blinded, which is difficult to achieve given the known physiologic effects and distinct taste of alcoholic beverages. Second, our study sample was made up of older men at high cardiovascular risk, thus the results may not be extrapolated to other populations. Third, gin may contain some bioactive aromatic and other substances derived from the aging process. We measured the total polyphenols in gin and these were under the lower limit of detection (**Table 1**). Given the low bioavailability of these compounds it seems reasonable to assume that gin contains only ethanol and no other interfering substances. In addition, DRW contains 0.42% of alcohol (**Table 1**). This means that individuals ingested approximately 1g ethanol/day during that intervention. This limitation could be arranged by administering grape or red fruit juice but another limitation would be added. DRW has the same phenolic composition as red wine, whereas the juices do not have exactly the same composition and phenolic profile as red wine, adding more confounding variables to the analyses and impeding comparison of the effects of red wine polyphenols in an alcoholic and a non-alcoholic matrix. Lastly, our study duration of 4 weeks may not represent the potential beneficial effects of long-term moderate alcohol consumption.

In conclusion, while ethanol itself exerts a protective effect on the lipid profile, the non-alcoholic fraction of RW (mainly polyphenols) has a beneficial effect on insulin

resistance and RW appears to decrease lipoprotein(a) plasma concentrations. These findings suggest that RW has greater protective effects than other alcoholic beverages on cardiovascular risk.

Statement of Authorship

RE, CA-L, RML-R and MG were involved in the conception and design of the research; GC-B, MU-S and RL in the conduction of the research; GC-B, ER, SA, PV-M and RE in the statistical analysis and interpretation of data; GC-B, MU-S, ER and RE wrote the paper and RC, MG, RML-R and CA-L, critically revised and finally approved the manuscript.

Conflict of interests

Dr. Ramon Estruch and Rosa M Lamuela-Raventos are members of the FIVIN (Foundation for the study of wine and nutrition), and in the past they received grants from this foundation and the Spanish Foundation of Beer and Health. Nevertheless, these foundations had no involvement in the study design, the collection, analysis and interpretation of data, the writing of the manuscript or the decision to submit the manuscript for publication. The other authors declare no conflict of interests.

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FIGURE LEGENDS

Figure 1. Flow chart of the subjects included in the study. RW: red wine; DRW: dealcoholized red wine.