INTRODUCTION

Alcohol is a damaging agent for all cells, from the liver to nervous system (1-3); however, epidemiological evidences suggest that moderate alcohol consumption, particularly with red wine, reduces the mortality due to coronary heart disease and may have a protective effect also on the liver (4-7). The possible protective effect of red wine has been attributed to its content in polyphenols such as flavonoids and resveratrol, dietary antioxidants able to counteract, both in vitro and in vivo, oxidative-mediated cell damage (8-10).

Oxidative stress plays a major pathogenic role in a number of gastrointestinal tract diseases (11). In particular, generation of radical oxygen species (ROS) is involved in the pathogenesis of gastric epithelial cell damage. Therefore, this study was designed to evaluate whether different alcoholic beverages, at a similar ethanol concentration, exerted different damaging effect in gastric epithelial cells in vitro. Moreover, we evaluated whether pre-treatment of gastric epithelial cells with alcoholic beverages prevented oxidative stress-induced damage to gastric cells. Cell damage was assessed, in MKN-28 gastric epithelial cells, by MTT assay. Oxidative stress was induced by incubating cells with xanthine and xanthine oxidase. Gastric cell viability was assessed following 30, 60, and 120 minutes incubation with ethanol 17.5-125 mg/ml or different alcoholic beverages (i.e., beer, white wine, red wine, spirits) at comparable ethanol concentration. Finally, we assessed whether pre-incubation with red wine (with or without ethanol) prevented oxidative stress-induced cell damage. Red wine caused less damage to gastric epithelial cells in vitro compared with other alcoholic beverages at comparable ethanol concentration. Pre-treatment with red wine, but not with dealcoholate red wine, significantly and time-dependently prevented oxidative stress-induced cell damage. Conclusions: 1) red wine is less harmful to gastric epithelial cells than other alcoholic beverages; 2) this seems related to the non-alcoholic components of red wine, because other alcoholic beverages with comparable ethanol concentration exerted more damage than red wine; 3) red wine prevents oxidative-stress-induced cell damage and this seems to be related to its ethanol content.

Key words: alcohol, gastric epithelial cell viability, oxidative stress, red wine, xanthine oxidase, flavonoids

MATERIAL AND METHODS

Chemicals

Absolute ethanol was provided by Sigma Aldrich. Beer, spirits and wine used were selected from different commercial types. Beers: Tuborg 5.0% ethanol and Ec28 11.0% ethanol; Spirits: whisky 40% ethanol; White wine: Falanghina 11.0%; Red wines: Toscano 11.5% ethanol; Aglianico 12.0% ethanol; Solopaca 12.5% ethanol.

DMEM F:12 medium (Dulbecco's modified Eagle's medium), penicillin, streptomycin, fetal bovine serum (FBS), L-glutamine and trypsin/EDTA were obtained from Life Technologies Inc., (Gaithersburg, Maryland, USA). MTT [3-
(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and xanthine and xanthine oxidase were purchased by Sigma Aldrich. All solvents were for HPLC grade.

**Design of the study**

1. **Preparation of alcoholic samples and dealcoholate red wine for cell assays**

All samples were appropriately diluted with DMEM F:12 medium serum-free before addition to harvested cells to reach the final concentration (17.5 to 125 mg mL<sup>-1</sup>) in each well. Red wines were dealcoholated by room temperature vacuum evaporation using a Rotavapor (Heidolph mod. WB 2000).

2. **Cell culture and experiments of cytotoxicity**

MKN-28 cells were derived from a human well differentiated gastric tubular adenocarcinoma and showed gastric-type differentiation (16). Cells were grown as monolayers in DMEM F:12 medium supplemented with 10% fetal calf serum, 1% antibiotic solution and 1% L-glutamine 200 mM at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Harvested cells were plated in 6-well microplates (1 x 10<sup>4</sup> cells/well) and allowed to grow to confluence over 24 hours before use. Cells were incubated with or without alcoholic beverages (17.5 to 125 mg mL<sup>-1</sup> of ethanol) for 30, 60 and 120 minutes.

3. **Induction of oxidative stress**

Oxidative stress was induced by incubating MKN-28 cells with xanthine oxidase (XO) (50 mU mL<sup>-1</sup>) in the presence of its substrate xanthine (X) (1 mM) for 120 minutes. Exposure of gastric cells in culture to X-XO causes significant cell injury and this has been demonstrated to be due to generation of ROS and in particular of OH produced from H<sub>2</sub>O<sub>2</sub> by the iron catalysed Fenton reaction (17).

4. **Evaluation of cell viability**

Cell viability was determined by the MTT assay. Briefly, 200 µL of MTT (5 mg mL<sup>-1</sup>) were added to each well, and samples were incubated for 180 minutes at 37°C and centrifuged. After aspiration of supernatant, in each well was added 1 mL of 1 M HCl containing isopropanol to solubilized formazan produced. Absorbance of each sample was analyzed at 540 nm using Microplate Reader. Cell viability (%) was calculated by dividing the absorbance of samples obtained from cells incubated with ethanol, red wine, white wine or beer by the absorbance of samples obtained from cells incubated with culture medium only (control) and multiplying this ratio by 100. Data are presented as the mean of three experiments run in duplicate.

5. **Identification of red wine phenolic compounds**

Two classes of polyphenols, the flavan-3-ols (catechins and proanthocyanidins) and the anthocyanins, are the natural antioxidants present at the highest concentration in wine. For the separation and structural analysis of individual phenolic compounds, a liquid chromatography-mass spectrometry (LC-MS) instrument (Perkin Elmer Sciex API 3000), which combines the separation of LC with the selectivity and sensitivity of the MS detector, was used.

For the anthocyanin moiety analytical separation was performed on a Supelcosil LC-18 (5 µ) column (250x4.6 mm). Column temperature was 40°C. The mobile phase (flow rate: 0.800 mL/minutes) consisted of acidified water (0.5% of trifluoroacetic acid, solvent A) and methanol (solvent B). Separation was obtained by the following gradient: at 0 min 20% B; at 12 min 60% B; at 15 min 70% B; at 25 min 70% B; at 30 min 100% B; at 35 min 100% B; at 38 min 20% B. The compounds were detected at 520 nm. The samples of red wine and dealcoholated red wine were diluted 1:5 with acidified water (0.1% trifluoroacetic acid) prior injection.

To quantify the other polyphenols (phenolic acids and catechin) Prodigy ODS 3 100A C18 (5 µ) column (250x4.6 mm) and gradient elution with A (water/0.1% formic acid) and B (methanol/water 0.1% formic acid 80/20) solutions, were used. Column temperature was 40°C. Separation was obtained by the following gradient: at 0 min 10% B; at 3 min 30% B; at 6 min 35% B; at 20 min 45% B; at 22 min 50% B; at 24 min 55% B; at 25 min 60% B; at 27 min 80% B; at 30 min 90% B; at 33 min 10% B at a flow rate of 1.0 mL/minutes. The compounds were detected at 280 nm.

**Statistical analysis**

Data are expressed as mean±SD. Significance of differences was assessed by one way analysis of variance (ANOVA) and (when the F value was significant) by the Tukey-Krammer test for multiple comparisons or by the Student’s t-test for comparison between two means. Differences were considered to be significantly different if p<0.05.

**RESULTS**

**Dose- and time-related effects of ethanol on cell viability**

Pure ethanol decreased MKN-28 cell viability in a dose and time dependent manner, as reported in Fig. 1. Pretreatment with ethanol at a concentration lower than 35 mg mL<sup>-1</sup> did not affect cell viability, whereas increasing doses of ethanol caused a progressive reduction of cell viability. At 60 minutes, 85 mg mL<sup>-1</sup> of ethanol induced a cell injury close to 90%. Fig. 2 compares data obtained by incubating cells with pure ethanol, red wine, white wine, spirits and beer, at the same ethanol concentration (i.e. 125 mg mL<sup>-1</sup> of ethanol) for up to 120 min. Cell viability was differently affected by ethanol and other alcoholic beverages in a time and dose-depending manner. Pure alcohol, beer, spirits and, to a lesser extent, white wine reduced cell viability in a dose and time dependent manner, whereas other alcoholic beverages including spirits, white wine and beer reduced cell viability in a time but not dose dependent manner.
viability already at 30 minutes and the damaging effect increased overtime (Fig. 2; p<0.05 vs. controls). On the other hand, red wine only slightly reduced cell viability at 30 minutes incubation whereas no significant damage was observed at longer time period (Fig. 2).

On the basis of these results, we compared the effects on cell viability of three different red wines: Toscano, Aglianico and Solopaca at the same ethanol concentration. As shown in Fig. 3 the effect of the three wines was similar. All of them had a significantly lower damaging effect on gastric cells as compared to equal concentration of ethanol (Fig. 3). This result was more evident at relatively high concentration of ethanol. Also, Toscano red wine, especially at lower ethanol concentrations, seemed to be less damaging to cultured cells (p<0.05 vs. ethanol) compared with Aglianico and Solopaca.

Because the results of these experiments suggested that red wines were somewhat less damaging than solutions containing equal concentration of ethanol, we evaluated whether dealcoholated red wine caused even less damage to cultured cells. Both red wines (i.e. with and without alcohol) were less damaging to cells than ethanol (Fig. 4). No significant differences as to this damaging effects were observed between alcoholated and dealcoholated red wine.

**Effect of oxidative stress on MKN-28 cell viability after exposition to ethanol and red wine**

We then examined whether pre-treatment with alcoholic beverages (pure ethanol, red wine, white wine, spirits and beer) exerted any effect on damage induced by oxidative stress in MKN-28 gastric cells. We have previously reported (16, 18) that 2 hour incubation with X (1 mM) and XO (50 mU mL⁻¹) caused a significant dose-dependent (p<0.05) reduction in cell viability.

---

**Fig. 2.** MKN-28 cell viability was differently affected by ethanol and other alcoholic beverages in a time and dose-dependent manner. Cells were incubated at the same doses (125 mg mL⁻¹ of ethanol) and times of observation. Means±SD from three separate experiments run in duplicate. * p<0.05 vs. control 30 min; # p<0.05 vs. control 60 min; † p<0.05 vs. control 120 min. Black bars: 30 min; grey bars: 60 min; white bars: 120 min.

**Fig. 3.** Effects of three red wine samples on MKN-28 cell viability (120 min). Means±SD from three separate experiments run in duplicate. (* p<0.05 vs. ethanol treated cells) Black bars: Ethanol; grey bars: Toscano red wine; white bars: Aglianico red wine; diagonal bars: Solopaca red wine.

**Fig. 4.** Effects on MKN-28 cells viability of red wine (whole and dealcoholated) after incubation of 120 min. Means±SD from three separate experiments run in duplicate. (* p<0.05 vs. ethanol treated cells) Black bars: Ethanol; grey bars: red wine; white bars: dealcoholated red wine.
In order to study the ability of selected alcoholic beverages to prevent X-XO oxidative stress, MKN-28 cells were pre-incubated with alcoholic solutions containing different amounts of ethanol (17.5 to 125 mg mL\(^{-1}\)) and then incubated with X-XO for 2 hours. Sixty and 120 minutes pre-incubation with red wine significantly prevented X-XO-induced cell injury increasing cell viability from 45% (without pre-treatment) to 65% and 70% (after 60 and 120 minutes pre-incubation, respectively) (Fig. 5). On the other hand, white wine, spirits and beer did not prevent X-XO induced oxidative stress (data not shown).

We then sought to evaluate whether dealcoholated red wine also exerted any protective effect against oxidative stress-induced cell injury. Pre-treatment with dealcoholated red wine did not prevent oxidative stress-induced damage to gastric cells.

In order to study the ability of selected alcoholic beverages to prevent X-XO oxidative stress, MKN-28 cells were pre-incubated with alcoholic solutions containing different amounts of ethanol (17.5 to 125 mg mL\(^{-1}\)) and then incubated with X-XO for 2 hours. Sixty and 120 minutes pre-incubation with red wine significantly prevented X-XO-induced cell injury increasing cell viability from 45% (without pre-treatment) to 65% and 70% (after 60 and 120 minutes pre-incubation, respectively) (Fig. 5). On the other hand, white wine, spirits and beer did not prevent X-XO induced oxidative stress (data not shown).

We then sought to evaluate whether dealcoholated red wine also exerted any protective effect against oxidative stress-induced cell injury. Pre-treatment with dealcoholated red wine did not prevent oxidative stress-induced damage to gastric cells.
Anthocyanins and flavonoids wine composition

Because Toscano red wine was somewhat less damaging to cells compared with Aglianico and Sopoloaca, especially at lower ethanol concentration (Fig. 3), we assessed whether this was partially due to a different bioactive compound profile.

Anthocyanins profiles of three red wine are shown in Fig. 6. It should be noted that the anthocyanins composition of Aglianico clearly differs from that observed in Toscano and Sopoloaca wines. Although malvidin-3-O-glucoside and two acylated derivatives of malvidin (malvidin-3-O-acetylglucoside and malvidin-3-O-coumarylglucoside) are the main components present in all wines; in Aglianico, delphinidin-3-O-glucoside, delphinidin-3-O-malonylglucoside and cyanidin-3-O-glucoside, not detected in other types of wine, were present. On the other hand, Toscano and Sopoloaca contain petunidin-3-O-glucoside, not detected in Aglianico.

Gallic acid is the main flavonoid present in red wine. The presence of high concentration of gallic acid in red wines would be expected since this phenolic acid is principally formed by hydrolysis of flavonoid gallate esters, which are largely absent in white wines, due to the lack of skin extraction. Other polyphenols identified are catechins and caffeic and chlorogenic acids as shown in Fig. 7. No significant differences were observed for these compounds among the different red wines used.

DISCUSSION

Several in vitro studies indicate that ethanol can exert a differential regulation on cell viability in a dose-dependent manner. High concentrations of ethanol lead to necrotic cell death, mediated by a high production of reactive oxygen species (ROS) (19-21). Interestingly, ethanol at low concentration preferentially causes apoptosis and reduces cell necrosis, probably through a reduction of intracellular oxidative stress (22-24).

Our data show that the cytotoxic effect of ethanol on MKN-28 gastric cells is directly correlated with the time of exposure and the concentration, but cell viability is differently affected by ethanol and other alcoholic solutions. This study shows that red wine causes less injury to gastric epithelial cells in tissue culture compared to white wine, spirits and beer at comparable ethanol concentration, thus suggesting that components of red wine other than ethanol may contribute to its lower damaging effect. Also, red wine, but not other alcoholic beverages, significantly counteracted damaging effect of X-OH to gastric epithelial cells, and this was strictly dependent on its ethanol content. In fact, this protective effect was not observed with dealcoholated red wine.

A great attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, but the mechanism whereby dietary antioxidants protect the gastric mucosa against injury is not completely elucidated. Phenolic compounds have been shown to exert direct antioxidant effects acting as ROS scavenger, hydrogen donating compounds, singlet oxygen quenchers and metal ion chelators (25, 26). It is generally assumed that the higher is the total polyphenols content of the beverage or food, the greater is its antioxidant activity (27).

Red wine can be an important dietary source of polyphenols because contains over 200 different polyphenolic compounds and a close relationship between total phenolic content and total antioxidant potential for red wines is documented (28). In this study, only red wine counteracted the damaging effect of xanthine-xanthine oxidase generated ROS in gastric epithelial cells. Interestingly, this protective effect was not observed with dealcoholated red wine. This indicates that the presence of ethanol is necessary for this protection against oxidative stress to occur. We may speculate that this protection exerted by red wine might be part of the concept of "adaptive cytoprotection", that is the protective effect exerted by a mild irritant given before a damaging agent to the gastric mucosa (29). The amounts of polyphenolic composition is different considerably in different types of wine, depending on the grape variety, environmental factor and the wine processing techniques (30, 31). Generally, the phyto phenolic compounds and polyphenols exhibit strong antioxidizing activity and the inhibitory effect on breast cancer (32).

We have selected three types of red wine with dissimilar antioxidant profiles to evaluate their effect on cell viability both in basal condition and after an oxidative stress. Our results confirm a variation in polyphenolic content among wine samples tested; however, the wine composition does not explain the different effects on cell viability obtained with different wine samples. Moreover, all types of red wine significantly reduced the damage brought about by oxidative stress in a similar way. We focused our attention on anthocyanin moiety, because this class of compounds differentiates red wine from white wines. Ghiselli et al., measured the antioxidant activity of whole and dealcoholated red wine and relative polyphenolic fractions extracted from an Italian wine and observed that in spite of the strong loss in antioxidant capacity after dealcoholization, wine still maintained an appreciable radical scavenging activity (33). In our study, pre-treatment with dealcoholated red wine did not prevent oxidative stress-induced damage to gastric cells.

In conclusions, red wine is less harmful to gastric epithelial cells than other alcoholic beverages. This seems related to the non-alcoholic components of red wine, because other alcoholic beverages with comparable ethanol concentration exerted more damage. Red wine prevents oxidative stress-induced cell damage and this seems to be related to its ethanol content.

Acknowledgement: this work was supported by a grant of Assessorato all’Agricoltura della Regione Campania.

REFERENCES

4. Lieber CS. Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. Alcohol Alcohol 2004; 34: 9-19.

Received: October 15, 2009
Accepted: December 20, 2009

Author’s address: Prof. Carmela Loguercio, Via Foria 58, 80131 Naples, Italy; Phone: +39 (0) 81 5666718; Fax: +39 (0) 81 5666718; E-mail: carmelina.loguercio@unina2.it