Research Article **IJFNR (2019) 3:23**

International Journal of Food and Nutrition Research
(ISSN:2572-8784)

EFFECT OF HIGH POLYPHENOLS BEVERAGES ON mRNA LEVELS FROM TP53 AND ATM GENES

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ABSTRACT

Introduction: In addition to its anti-cancer action, p53 and ATM play an important role in oxidative balance control, promoting cell repair and survival. High fat diets can lead to increased production of reactive oxygen species (EROS). Grape polyphenols seem to reduce EROS and restore oxidative balance, favoring the performance of p53 and ATM. **Objetive**: The aim of this study was to investigate the antioxidant properties of high polyphenols beverages associated with a high fat diet in mRNA levels of p53 and ATM. **Methods**: Fifty female rats were divided into five groups: Control Group (CG) - control diet (4% fat); High fat diet group (HFD) - high fat diet (20% fat); Grape Juice Group (GJ) – grape juice (15 ml/day) + high fat diet; Red Wine Group (RW) – red wine (10 ml/day) + high fat diet; Resveratrol Solution Group (RS) – resveratrol solution (15 ml/day) + high fat diet. Eight weeks later, muscular and adipose tissue were collected and subjected to PCR analysis. **Results**: In muscular tissue, the highest p53 mRNA expression was found in the GJ and VT group, and not in the SR as expected. In adipose tissue, GJ presented the highest expression among all groups. TpATM expression was higher in the HFD, both in adipose and muscle tissue. Treatment with high polyphenols beverages normalized TpATM expression, especially in adipose tissue. **Conclusion**: In this experimental model, high fat diet alters ATM mRNA levels, but does not change p53 mRNA levels. Grape juice and red wine showed to be the most effective to increase TP53 mRNA levels, possibly due to a set of bioactive compounds that acts synergistically. Additionally, rich polyphenols beverages normalizes ATM mRNA levels, mainly in adipose tissue.

Keywords: p53, ATM, high fat diet, grape polyphenols

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How to cite this article:
Vânia Mattoso; Gabrielle Rocha, Sérgio Barroso, Vilma Blondet de Azeredoa Adenilson de Souza da Fonseca. EFFECT OF HIGH POLYPHENOLS BEVERAGES ON mRNA LEVELS FROM TP53 AND ATM GENES.International Journal of Food and Nutrition Research, 2019; 3:23.

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Website: http://escipub.com/

Introduction

High fat diets have been implicated in the generation of reactive oxygen species (ROS), leading to oxidative stress (OZAKI; NAKAGAWARA, 2011). Constant cell exposure to oxidative stress can cause mutations, translocations and deletions in DNA bases, leading to pre-mutagenic states (OZAKI; NAKAGAWARA, 2011).

High levels of oxidative stress and/or DNA damage alters the expression of ataxia-telangiectasia mutated (ATM) and p53 transcription factor, which play a crucial role in genome integrity (OZAKI; NAKAGAWARA, 2011). ATM and p53 act synergistically, inducing cells to senescence or apoptotic events (LEE et al., 2014; ZHAN et al, 2010; LIU; XU, 2011; DITCH; PAUL, 2012).

ATM phosphorylates p53 transcription factor, which, in turn, activates the transcription of several genes involved in redox modulation, such as glutathione peroxidase and members of Sestrin family 1 and 2, protecting the cell from damage induced by hydrogen peroxide (PAN et al, 2012; RODRIGUEZ et al, 2012; KANG, 2013). Several studies suggest that not only p53 but also ATM controls ROS levels and they can be activated even without DNA damage, participating in a set of signaling pathways involved in antioxidant defense and metabolic regulation (DITCH; PAUL, 2012; KANG, 2013; GUO et al., 2010).

The effect of polyphenols on oxidative stress management is well documented in the literature, since they can prevent lipid peroxidation, oxidative damage and apoptosis through free radicals scavenger action. Epidemiological studies have demonstrated that polyphenol consumption is a protective factor in the development of chronic diseases (RAHAL et al., 2014). They are the largest and the most widespread phytochemical found in planted-derived products, such as fruits, grains and vegetables (XIA et al, 2010). In grapes, these compounds are mainly located in the bark and seeds and are the main contributor of biological activities found in grapes and their products (FLAMINI et al., 2013). The main polyphenols families found in this fruit are flavonoids (anthocyanidins, proanthocyanidins, flavonols and quercetins) and stilbenes, such as resveratrol (FLAMINI et al., 2013).

Previous studies showed that polyphenols influence expression and activity of both p53 and ATM. Thus, the consumption of high polyphenols foods and beverages may have a synergistic effect on p53 and ATM, acting in the regulation of intracellular redox homeostasis, upregulating antioxidant genes, preventing oxidative stress-induced DNA damage and tumor development under low-stress conditions (KANG, 2013; GUPTA, 2012).

The purpose of this study was to evaluate the effects of a high fat diet associated to high polyphenols beverages consumption on TP53 and ATM mRNA levels in muscular and adipose tissues of *Wistar* rats.

Materials and Methods

Animals and diet

Animals

The study was conducted in the Experimental Nutrition Laboratory of the Department of Nutrition and Dietetics, School of Nutrition Emilia Jesus Ferreiro at the Federal Fluminense University (LabNE-UFF). The experimental protocol was approved by the Brazilian Society of Science in Laboratory Animals (SBCAL) of the Federal Fluminense University, according to the guidelines of the Brazilian College on Animal Experimentation (protocol number 473).

Fifty female *Rattus novergicus Wistar albino*, 90 days, 200±20g, from LabNE-UFF were housed in plastic cages in a controlled environment (24°±2°C, with a 12 h daylight cycle), with free access to food and water. The experiment lasted for 8 weeks.

The animals were randomly divided into five groups (n=10/group):

1) control group (CG): fed a control diet (4%

of total calorie intake from fat) based on the American Institute of Nutrition Recommendations for adult-rodents (AIN 93M).

2) high fat diet group (HFD): fed high fat diet (20% of total calorie intake from fat)

3) grape juice group (GJ): fed a high fat diet (20% of total calorie intake from fat) and received red grape juice (15 mL/day)

4) red wine group (RW): fed a high fat diet (20% of total calorie intake from fat) and received red wine (10 mL/day)

5) resveratrol solution group (RS): fed a high fat diet (20% of total calorie intake from fat) and resveratrol solution (15 mL/day).

Table I show the ingredients used for formulation of high fat and control diet.

Table I: Ingredients used for formulation of control and high fat diets (g/100g chow)

Ingredients	Control	High fat
Casein*	14.0	14.0
Starch	62	46.07
Soybean oil	4.0	-
Lard	-	20
Celulose	5.0	5.0
[1]Vitamin mix	1.0	1.0
[2]Minerals mix	3.5	3.5
B-colin	0.25	0.25
L-cystine	0.18	0.18
Sugar	10.0	10.0
Total	100	100

Subtitle: (*) % protein in casein = 92.5% protein/100g casein; (1) Vitamin mix (mg/Kg dieta): retynil palmitate 2.4, cholecalciferol 0.025, benadiona sodium bisulfite 0.8, biotin 0.22, cyanocobalamin 0.01, riboflavin 6.6, thiamine hydrochloride 6.6 and tocopherol acetate 100; (2) Minerals mix (g/Kg dieta): copper sulphsate 0.1, ammonium molybdate 0.026, sodium iodate 0.0003, potassium chromate 0.028, zinco sulfate 0.091, calcium hydrogen phosphate 0.145, iron sulfate 2.338, magesium sulfate 3.37, manganese sulfate 1.125, sodium chloride 4.0, calcium carbonate 9.89 and potassium diidrogenophosphate 14.75.

Samples collection and preparation

At the end of the experiment, all animals were submitted to vaginal smear procedure to identify the phase of the estrous cycle.

The rats in the estrous' phase were fasted for 6 hours prior to sacrifice and anesthetized with ketamine chloride (90 mg/kg) and xylazine hydrochloride (10mg/kg). Blood samples were collected by cardiac puncture into tubes with EDTA. Adipose and muscular tissue samples were collected, homogenized in TRIzol® and frozen at -80^{0}C.

The choice of tissues to be used was due to their different physiological characteristics. Muscle tissue expresses more Tp53 and ATM genes and presents several ways of managing oxidative stress. On the other hand, adipose tissue presents less expression of the afore mentioned factors (CRUZAT *et al*., 2007).

Total RNA extraction

Adipose and muscular tissue samples were macerated into microcentrifuge flex tubes with TRIzol® reagent and centrifuged (12,000×g, 4 °C, 10 minutes). Supernatants were transferred to other tubes, chloroform was added, mixtures were centrifuged (12,000×g, 4 °C, 15 minutes), aqueous phases were transferred to other tubes, and isopropanol was added. After incubation (room temperature, 15 minutes), mixtures were centrifuged (12,000×g, 4 °C, 10 minutes), supernatants were discarded, and precipitate was washed with ethanol-DEPC (80% ethanol, DEPC 0.1 %) solution added and centrifuged. Supernatants were withdrawn and total RNA was reconstituted in water-DEPC (0.1 %) solution and stored (−80°C).

Complementary DNA synthesis

RNA concentration and purity were determined on a spectrophotometer by calculating the optical density ratio at a 260 nm/280 nm wavelength ratio. Complementary DNA (cDNA) synthesis was carried out using a two-step cDNA synthesis kit (Promega, USA). Four micrograms of RNA were reverse transcribed into cDNA using GoScript™ reverse transcriptase (Promega, USA), according to the manufacturer's protocol, using a total 20 µl reaction. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using 5 µl of GoTaq qPCR Master Mix (Promega) for a final volume of 10 µl volume containing 50 ng of cDNA. To determine the initial relative of cDNA quantity, samples were amplified with TP53, ATM and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. Reactions, in duplicate for each sample, were run on an Applied Biosystems 7500 RT-qPCR machine (Applied Biosystems, USA). The mixtures were initially denatured at 94 °C for 10 minutes. The PCR consisted of 40 cycles at the following conditions: denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and an extension period at 72 °C for 30 s. Melt curve analyses were performed for all genes and PCR product specificity, as well as integrity, were confirmed by the presence of a single peak. Relative expression was normalized by reference gene levels (GAPDH), using non-exposed rats as control group. Duplicate CT values were analyzed in Microsoft Excel (Microsoft) using the comparative CT (2−ΔΔCT) method.

PCR analysis

cDNA synthesis was carried out using a two-step cDNA synthesis kit (Promega®), using TP53 and ATM primers. One microgram of RNA was reverse transcribed into cDNA using GoScript™reverse transcriptase (Promega®) according to the manufacturer's protocol using a total reaction of 20 µl. Real time quantitative PCR (RT-qPCR) was performed using 5 µL of Gotaq qPCR Master Mix (Promega). For determination of the initial relative quantity of cDNA, samples were amplified with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (reference gene) (LIVAK, 2001)

Statistical analysis

Data were expressed as mean ± standard deviation. For comparison between the means, one-way analysis of variance and Duncan post-test were used. For data, correlations between p53 and ATM were analyzed with Pearson’s correlation coefficient. The assumption of normality (Gaussian distribution) was verified by Kolmogorov-Smirnov test to support the use of the statistical methods described above. The analyses were performed using Graphpad Prism software for Windows. Values were considered statistically significant if $p<0.05$.

Results

TP53 mRNA levels in muscle tissue of GJ group were higher ($p<0.05$) when compared to CG (Figure 1). Between the groups that received rich polyphenols beverages, the highest TP53 mRNA levels were found in GJ ($p<0.001$) and RW ($p<0.05$) groups, and not in the RS, as expected, when compared to HFD. In adipose tissue, GJ presented the greatest expression of TP53 mRNA when compared to CG, RS and HFD groups ($p<0.001$). However, RW showed higher expression in relation to RS

and CG (p<0.05) but lower than to GJ (p<0.01) (Figure 2).

In muscle tissue, HFD group showed ATM mRNA levels significantly higher than CG (p<0.001). Between the groups treated with rich polyphenols beverages, the RW group presented lower ATM mRNA levels in relation to the HFD (p<0.01) (Figure 3). Similarly, in adipose tissue, HFD also presented greater (p<0.001) ATM mRNA levels when compared to CG (Figure 4). Additionally, treatement with rich polyphenols beverages, normalized ATM mRNA levels (p<0.001).

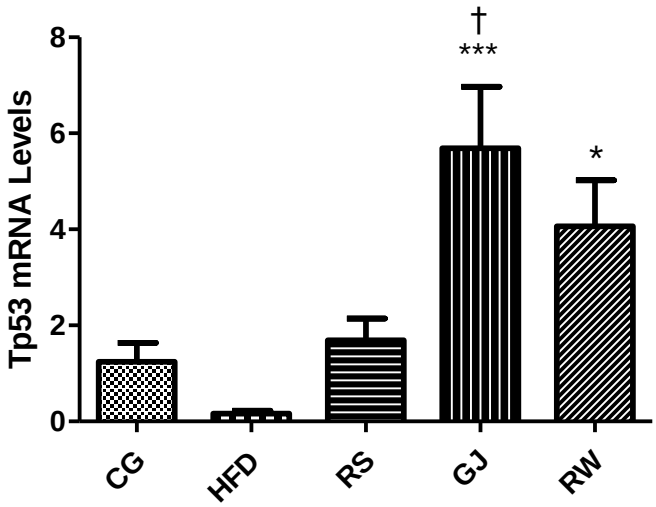

Figure 1. Relative TP53 mRNA levels in muscle tissue. Control group (CG) - control diet (4% fat); high fat diet group (HFD) - high fat diet (20% fat); grape juice group (GJ) - received 15 mL/day grape juice + high fat diet; red wine group (RW) - received 10 mL/day red wine + high fat diet; resveratrol solution group (RS) - received 15 mL/day resveratrol solution + high fat diet. (†) p<0.05 when compared with control group (CG). (***) p<0.001 when compared with high fat diet group (HFD). (*) p<0.05 when compared with high fat diet group (HFD).

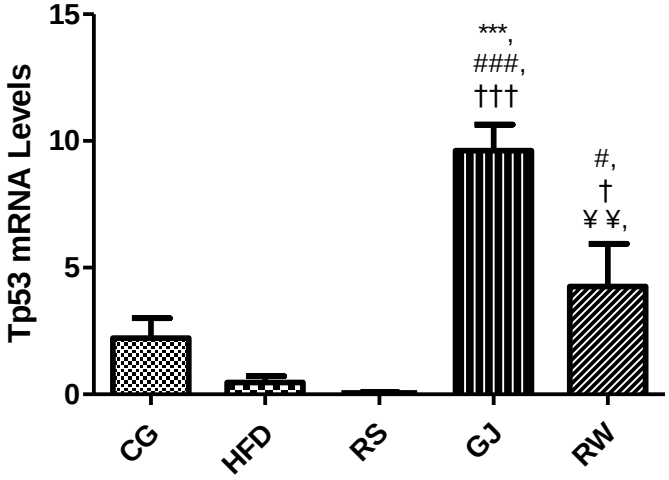

Figure 2. Relative TP53 mRNA levels in adipose tissue. Control group (CG) - control diet (4% fat); high fat diet group (HFD) - high fat diet (20% fat); grape juice group (GJ) - received 15 mL/day grape juice + high fat diet; red wine group (RW) - received 10 mL/day red wine + high fat diet; resveratrol solution group (RS) - received 15 mL/day resveratrol solution + high fat diet. (#) p<0.05 when compared to high fat diet group (HFD). (###) p<0.001 when compared to high fat diet group (HFD). (***) p<0.001 when compared to control group (CG). (†††) p<0.001 when compared to resveratrol solution group (RS). (†) p<0.05 when compared to resveratrol solution group (RS), (¥¥) p<0.01 when compared to grape juice group (GJ).

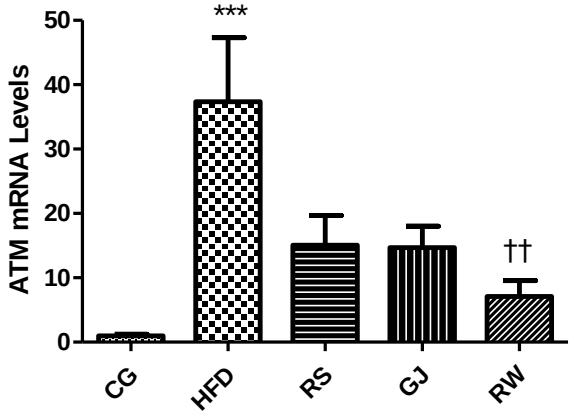

Figure 3. Relative ATM mRNA levels in muscle tissue. Control group (CG) - control diet (4% fat); high fat diet group (HFD) - high fat diet (20% fat); grape juice group (GJ) - received 15 mL/day grape juice + high fat diet; red wine group (RW) - received 10 mL/day red wine + high fat diet; resveratrol solution group (RS) - received 15 mL/day resveratrol solution + high fat diet. (***) $p<0.001$ when compared to control group (CG). (††) $p<0.01$ when compared to high fat diet group (HFD).

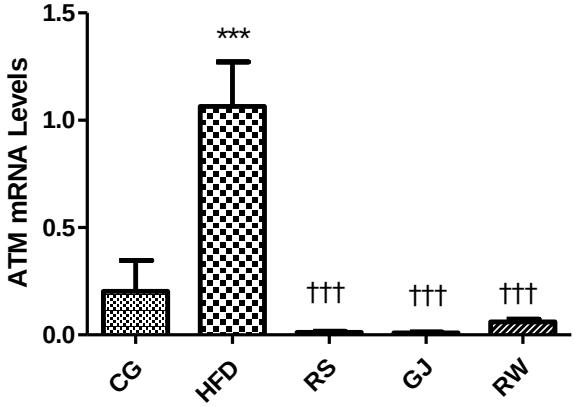

Figure 4. Relative ATM mRNA levels in adipose tissue. Control group (CG) - control diet (4% fat); high fat diet group (HFD) - high fat diet (20% fat); grape juice group (GJ) - received 15 mL/day grape juice + high fat diet; red wine group (RW) - received 10 mL/day red wine + high fat diet; resveratrol solution group (RS) - received 15 mL/day resveratrol solution + high fat diet. (***) $p<0.001$ when compared to control group (CG).(†††) $p<0.001$ when compared to high fat diet group (HFD).

Correlations were observed between p53 and ATM mRNA levels in control group ($r = 0,493$, $p = 0,020$). No other associations were observed.

Discussion

Oxidative stress is considered a potent inducer of p53 and ataxia telangiectasia mutated (ATM) expression. p53 is a transcription factor, also known as the "guardian of the genome" and coordinates cell-cycle arrest, DNA repair, apoptosis, and senescence, maintaining genome stability, integrity and regulating longevity and aging process (SHIMIZU, 2012; VOUSDEN & LANE, 2007). ATM also acts maintaining genomic stability by activating a cell-cycle checkpoint in response to DNA damage and/or telomeric instability (ITO et al., 2004). Maintenance of genomic stability is crucial for prevention of cell death and/or neoplasic process (SHILOH & ZIV, 2012).

Recent findings suggest that both p53 and ATM acts as a redox sensor in human cells,

controlling ROS levels (DITCH; PAULL, 2012; VOUSDEN & LANE, 2007; HOMAYOUNFAR et al., 2015). In accordance with the literature, GUO *et al.,* 2014, showed in their study, that untransformed human fibroblasts treated with hydrogen peroxide present higher levels of ATM and p53. In the same way, high fat diets are known to induce ROS production due to increased oxidative stress (DITCH; PAULL, 2012). Thereby, the present study proposes to verify if a high fat diet would be able to change p53 and/or ATM expression.

Considering the recommendations of 4% of the total lipids by the AIN-93M, for adults rats, and that high fat diets would be those that use fat above this value, the dietary model with 20% of fat used at the present study can be classified as high fat diet, and thus, could induces the production of ROS.

In this experimental model (high fat diet – 20%), no change in p53 mRNA expression was observed in adipose or muscle tissue due to a high fat diet (Figure 1 and 2). Otherwise, other studies using an obesogenic model (40-70% of total lipid) during 8 to 16 weeks, has found a positive correlation between high fat diets and p53 expression. Possibly this can be explained by the higher lipid content of the diet used, which provide a more prooxidative and EROS-rich environment, altering the transcription factor expression (SHIMIZU et al., 2012; FORD et al., 2013; HOMAYOUNFAR et al., 2015; HATANAKA et al., 2017).

On the other hand, in the present study, animals fed with 20% of fat, presented higher expression of Tp ATM genes (Figure 3 and 4). Corroborating with this study, Daugherity *et al.* (2012) showed, in a similar experimental model using 18% of total fat during 8 weeks, an elevation of ATM mRNA levels in hepatic tissue and commented that ATM expression and activation play an important role in cellular response due to ROS levels (Daugherity et al., 2012). Thus, it is evident that high fat diets, such as those commonly consumed in developed and developing countries, provide a pro-oxidative environment in the body. Possibly, in a long term, adopting diets with this profile can increase the risk of developing chronic diseases.

One of the most widespread classes of constituents present in plant kingdom is polyphenols. Previous *in vitro* studies using cancer cells from breast, colon, prostate and other tissues pointed the importance of polyphenols in upregulating p53 protein levels (ETIENNE-SELLOUM et al., 2013). Pre-clinical studies present similar results to the *in vitro* studies: animals that received polyphenols from teas, juices or extracts presented highest expression of p53 mRNA, also in a dose and time-dependent manner. These compounds have been widely used in studies as potent antioxidants and cellular protectors (GU, 2013).

In the present study, treatment with grape juice led to higher TP53 mRNA expression, followed by red wine, in both tissues (Figure 1 and 2). Corroborating with this study, Dolara *et al*., 2005, using high fat diet (50% total lipid) and red wine and Roy *et al*., 2007, using grape seed extract (GSE) in cell cultures, found that animals treated with wine and GSE upregulates p53 expression with a concomitant increase in p53 phosphorylation. Same results in p53 was observed by Chang *et al*., 2015, using mulberry extract in muscle cells and by Gu et al., 2013, using green tea polyphenols in lung cancer model.

However, resveratrol supplementation alone does not caused any effect on TP53 expression, in this experimental model (Figure 1 and 2). Possibly, the positive results found in GJ and RW groups, and not in RS group, could be explained by a complex matrix of polyphenols present in grape juice and red wine, which includes proanthocyanidins, ellagic acid, kampferol, myricetin, quercetin, malvidin, peonidine, cyanidin and catechin and resveratrol (LIANG, 2014). It is expected that greater expression of p53 in groups treated with grape juice and red wine, bring benefits on controlling oxidative stress and, possibly, at a

long term, promoting genomic stability. It is worth to note that the use of natural matrices or food products, instead of supplements, may represent a more reliable and effective alternative in nutritional and therapeutic approaches.

It is important to note that p53 protein has two important and opposing effects: in low levels of stress and DNA damage, such as those naturally in the day-by-day life-span, p53 protein acts lowering ROS levels and promoting cell repair and survival. With high, severe and sustained stressful conditions, such as oncogene activation, p53 protein acts inducting apoptosis. Thus, the classification of "good" or "bad", and the dichotomy between promoting cell survival or apoptosis, depends on environment, cell type and other contributing factors (VOUSDEN & LANE, 2007).

On the other hand, ATM expression showed opposite results when compared to p53 expression. Treatment with rich polyphenols beverages normalized ATM expression in adipose tissue, leading to ATM mRNA levels similar to CG. Although, in muscle tissues, only red wine normalized levels of ATM when compared to HFD group (Figure 3 and 4). Different results was observed by Lee *et al.,* 2014, that found no change in the expression and activation of ATM in normal human fibroblasts treated with resveratrol under oxidizing conditions.

The correlation between p53 and ATM expression is crucial to maintaining cell cycle and genomic stability. As demonstrated in previous studies, ATM and p53 proteins work together (ETIENNE-SELLOUM et al., 2013; Kubota et al., 2014) and alterations between the correlation of p53 and ATM expression/activation seem to be related to a greater probability of developing cancer (Kubota *et al*., 2014). In this study, a positive correlation was found between p53 and ATM expression in CG, but no correlation was found in the other groups. Lavin *et al*. (2015), described that ATM protein phosphorylates numerous downstream target proteins involved in genome stability. Possibly, a diet with 20% of fat may have altered some point of the signaling cascade and, other pathways, rather than p53 pathway, could have been phosphorylated, justifying the fact that, altough there was an increase of ATM mRNA levels, there was not a consequent increase of TP53 mRNA levels in the other groups.

Since higher concentrations of ATM are reported to increase oxidative stress (GUO *et al.,* 2014), the normalization in ATM expression modulated by polyphenols consumption may have a positive effect in cell metabolism, protecting against the deleterious effects of consumption high amounts of fat. Thus, the exchange between a diet rich in fats for a diet rich in vegetables and fruits and, consequently, higher content of polyphenols could present an important modulating factor of oxidative stress, preventing the development of diseases and increasing longevity in a long term. As mentioned above, polyphenols presents greater antioxidant properties, modulating ROS production, controlling oxidative stress and acting as a key modulator in cellular signaling pathways, in a variety of cell culture and *in vivo* systems (Ito *et al*., 2004; Reliene; Schiestl., 2007; Lee et al., 2014)

However, it is important to note that was not possible to find references using all the variables that was used in this study (high fat diet and high polyphenols beverages in healthy animals), which hindered the discussion of the results.

CONCLUSIONS

In this experimental model, high fat diet did not altered Tp53 mRNA levels. However, treatment with grape juice and red wine showed to be effective to increase TP53 mRNA levels in both tissues, possibly due to a set of bioactive compounds that acts synergistically.

In ATM mRNA levels, high fat diet is able to increase it expression, which are reverted by

high polyphenols beverages, mainly in adipose tissue.

Conflict of interest

The authors declare that they have no conflict of interests.

Acknowledgments

We would like to thank FAPERJ and FOPESQ for research founding and the PPG-CAPS of the Federal Fluminense University.

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