Nanocapsules With Naringin And Naringenin Affect Hepatic and Renal Energy Metabolism Without Altering Serum Markers of Toxicity in Rats

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ABSTRACT

Naringin and naringenin are flavonoids found in citrus fruits and have several health benefits, however these compounds are susceptible to degradation, limiting their therapeutic application. To solve this problem, an alternative is to incorporate them into nanocapsules. The aim of this work was to evaluate the toxicity of these nanocapsules against renal and hepatic serum markers and also on the activities of pyruvate kinase, Mg^{2+}-ATPase, and creatine kinase. Nanocapsules containing naringin and naringenin, nanocapsules without the active compounds and the compounds in their free form were administered orally, once a day, for 28 days. After treatment, the serum levels of hepatic and renal markers were not altered, nor the activities of pyruvate kinase tissue, however, the treatment of nanocapsules with flavonoids increased the activities of mitochondrial creatine kinase in the kidney and hepatic Mg^{2+}-ATPase. Thus, renal and hepatic serum markers, which are normally used as indicators of toxicity, did not change after the period of administration of the nanoparticles. However, the activities of important enzymes of the energy metabolism in these organs were affected. Our findings reinforce that nanomaterial testing for toxicity needs to go beyond traditional methods to ensure the safe use of nanoparticles for therapeutic purposes.

Keywords: nanoparticles, flavonoids, nanotoxicity, creatine kinase, Mg^{2+}-ATPase.

1. Introduction

Naringin (NA) and naringenin (NG) are flavonoids found in various citrus fruits, especially grapefruit, and are used by the population because they have antioxidant, anti-inflammatory, anticancer activities and several other effects that bring medical interest in their use (Alam et al. 2014). Despite the health benefits of NA and NG, they are moderately soluble in water, unstable and have extensive first-pass metabolism, resulting in low bioavailability when administered orally. To overcome these problems, it is necessary to improve the preparation and administration of these compounds, an alternative is the use of nanocarrier systems, such as nanocapsules, which improve the pharmacological stability of substances and increase their bioavailability and efficacy (Ferreira et al. 2015).

For these nanocapsules to be used in medicine for therapeutic purposes, previous tests are necessary, and toxicity is one of the most important factors to be considered (Novo et al. 2013). A study by Pohl et al. (2017) demonstrated that oral administration of nanocapsules containing NA and NG, for 28 days, did not cause oxidative stress in stomachs of Wistar rats, indicating that these nanoparticles may not be toxic, but

other analyzes need to be performed to corroborate the safe use of these nanoparticles. Therefore, to verify the safety of these nanocapsules we analyzed the parameters of renal and hepatic toxicity.

The kidneys are excellent markers of toxicity because they act as a filter, eliminating metabolites and toxins from the blood through the urine (Nelson and Cox 2014). The liver has the ability to detoxify, that is, remove many toxic compounds such as alcohol and drugs that are ingested or produced by the body (Motta 2009). One of the most affected organs and one of the main targets of most chemicals that cause toxic effects after environmental exposure is the liver, but new studies have shown that it is also possible to find lesions after this chemical exposure in the kidneys, but there are still doubts regarding specific aspects of renal function (Kataria et al. 2015).

In order to verify the effect of the nanocapsules on renal and hepatic function we decided to carry out a more in-depth investigation in addition to the first triage clinical analyzes, where serum markers of toxicity are usually measured, investigating key enzymes in energy metabolism so that the results are more accurate, thus guaranteeing the safety of the use of these nanoparticles. We evaluated the cellular energy metabolism, which is responsible for the energy supply to cells through the transformation of molecules and nutrients into adenosine triphosphate (ATP) and other molecules capable of providing energy to the cellular biological work. These processes of energy generation are performed by several enzymes that are fundamental in cellular energy metabolism (Nelson and Cox 2014; Mussoi and Rech 2019). Among these enzymes are pyruvate kinase (PK) and magnesium ATPase (Mg^{2+} -ATPase) and creatine kinase (CK) (Shuch et al. 2013), however, CK is not found in hepatocytes (Wallimann and Hemmer 1994) and therefore we did not evaluate it in this study.

PK is an important enzyme of the metabolic pathway of glucose, one of the main ways of obtaining energy from the renal and hepatic cells. This enzyme catalyzes the dephosphorylation of phosphoenolpyruvate (PEP) in pyruvate, producing two molecules of ATP/glucose (Shuch et al. 2013; Israelsen and Vander Heiden 2015). CK is another enzyme extremely crucial for the maintenance of cellular energy homeostasis. It catalyzes the reversible transfer of the phosphoryl group from phosphocreatine to ADP, regenerating ATP. For this enzyme there is the mitochondrial isoform (mitCK), which uses ATP to form phosphocreatine and ADP, and the cytosolic isoform (cytCK) that forms creatine through phosphocreatine, generating ATP (Ferreira, 2014; Rech et al. 2006, 2018). Mg^{2+}-ATPase is localized in the plasma membrane of renal and hepatic cells and is essential because it carries the Mg^{2+} that is the cofactor of several enzymes involved in energy metabolism (Nozadze et al. 2015). PK, CK and Mg^{2+}-ATPase are important enzymes for the formation of energy. We also evaluated the hepatic markers AST and ALT and the renal markers Cr and U which, when at high levels, indicate tissue damage (Baaij et al. 2015).

Therefore, the objective of this study was to evaluate renal and hepatic toxicity through detection of serum urea (U), creatinine (Cr), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) serum levels, as well as parameters of energy metabolism in liver and kidney.

2. Methodology

2.1 Animals

Twenty-eight male Wistar rats (300-400 g) were used in accordance with ethical standards approved by the Ethics Committee on Animal Use of the Franciscan University (CEUA/UFN) under protocol number 05/2016. The animals had free access to water and standard commercial chow.

The animals were randomly separated into four groups and received by intragastric gavage 2,5 µL per g of body weight of water to control group (C), 5 µg of naringin + 5 µg of naringenin in free form per g of body weight to F group, 5 µg of blank nanocapsules without active compounds (BN) and nanocapsules with 5 µg of naringin + 5 µg naringenin per g of body weight to (NN) group. On the 28th day, the rats were euthanized by decapitation, the kidneys and liver were removed and stored in freezer -80 ºC. Blood from the animals was collected in tubes with separator gel, centrifuged at 800 x *g* for 15 minutes at room temperature. The serum was removed and stored in the freezer at -80 ºC for further analysis of AST, ALT, creatinine and urea.

2.2 Nanoparticles

NA + NG nanocapsules were prepared by Pohl et al. (2017) containing 0.05 g naringin/mL + 0.05 g naringenin/mL, according to the methodology described by Cordenonsi et al. (2016). The aqueous phase consisted of water and polysorbate 80, the organic phase composed of naringin and naringenin, Eudragit L100, sorbitan monostearate, diisopropyl adipate, and ethanol was added in an aqueous phase composed of water and polysorbate 80. The suspensions of blank nanocapsules (NB) were prepared in the same manner without the presence of the drugs. For free form (F), there was no presence of Eudragit L100 in the composition.

The physical-chemical characterization of the nanocapsules showed that the particles containing NA and NG have 102.40 ± 6.6 nm in diameter, polydisperation index of 0.182 ± 0.01, Zeta potential of -13.03 ± 2.5 mV and pH of 3.81 ± 0.01 (Pohl et al. 2017) being in agreement with Cordenonsi et. al (2016).

2.3 Tissue Preparation

The kidneys and liver were weighed and homogenized in a buffer with KCl 50 mM. They were then centrifuged at 800 x *g* for 15 min and after a part of the supernatant was removed for the Mg^{2+}-ATPase assay and another part was centrifuged at 12.000 x *g* for 10 min at 4 ºC, a fraction of the supernatant was removed for the PK and cytCK assay, and the pellet corresponding to the mitochondrial fraction of CK was washed with Tris-sucrose isotonic buffer and resuspended in 100 mM Tris-HCl buffer, pH 7.5, containing 15 mM $MgSO_4$.

2.4 Serum preparation

After decapitation, whole blood was collected in tubes containing separator gel. The tubes were centrifuged at 800 x g for 15 min and the supernatants were used to evaluate serum levels of urea, creatinine, AST and ALT which were determined on a CELM SBA 200 automated analyzer using Labtest kits.

2.5 Magnesium-atpase assay

The activity of Mg^{2+}-ATPase was determined according to a method adapted from Fiske and Subbarow (1925). The reaction contains 5,0 mM $MgCl_2$, 80 mM NaCl, 20 mM KCl e 40 mM of Tris-HCl

buffer, pH 7,4 in a final volume of 200 µL. The inorganic phosphate (Pi) released was measured by the method of Chan et al. (1986). The activity was expressed as nmol Pi/min/mg protein.

2.6 Pyruvate kinase assay

PK activity was measured by the method of Leong et al. (1981). The incubation medium contains 0.1 M Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.16 mM NADH, 75 mM KCl, 5.0 mM ADP, 7 units of lactate dehydrogenase (LDH), 0.1% (v/v) Triton X-100, and 10 µL of the mitochondria-free supernatant in a final volume of 0.5 mL. The reaction was started after 30 min of preincubation at 37 ^{o}C by addition of 1.0 mmol/L phosphoenolpyruvate (PEP). All assays were performed in duplicate. Results were expressed as nmol of pyruvate formed per min per mg of protein.

2.7 Creatine kinase assay

CK was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 2% α-naphtol and 0.1 mL 0.05% diacetyl in a final volume of 1 mL and read after 20 minutes at 540 nm. All assays were performed in duplicate. Results were expressed as nmol of creatine formed per min per mg protein.

2.8 Determination of proteins

The protein content was determined by the method of Lowry et al. (1951). The tests were performed in triplicate. Bovine serum albumin was used as a standard. All determinations were expressed in mg protein/mL.

2.9 Statistical analyses

Data were analyzed using one-way ANOVA. Post hoc analysis of a significant interaction, when required, was performed by the Dunnett test. All data were analyzed by the Statistical Package for Social Sciences software (SPSS 12.0 for Windows). Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1 Renal energy metabolism

The analysis of the results of the renal energy metabolism (Figure 1) shows that the treatments (F, NB and NN) did not change the PK activity (Figure 1a) when compared to that of control animals ($p > 0.05$). However, NB administration increases Mg^{2+}-ATPase activity ($p < 0.001$) when compared to control group ($p < 0.05$) (Figure 1b).

Figure 1c shows that NB ($p < 0.05$) and NN ($p < 0.01$) administrations increased the renal activity of mitCK when compared to that of control animals. The F ($p < 0.001$) and NB ($p < 0.05$) administrations reduced significantly the activity of cytCK, when compared to the control group (Figura 1d).

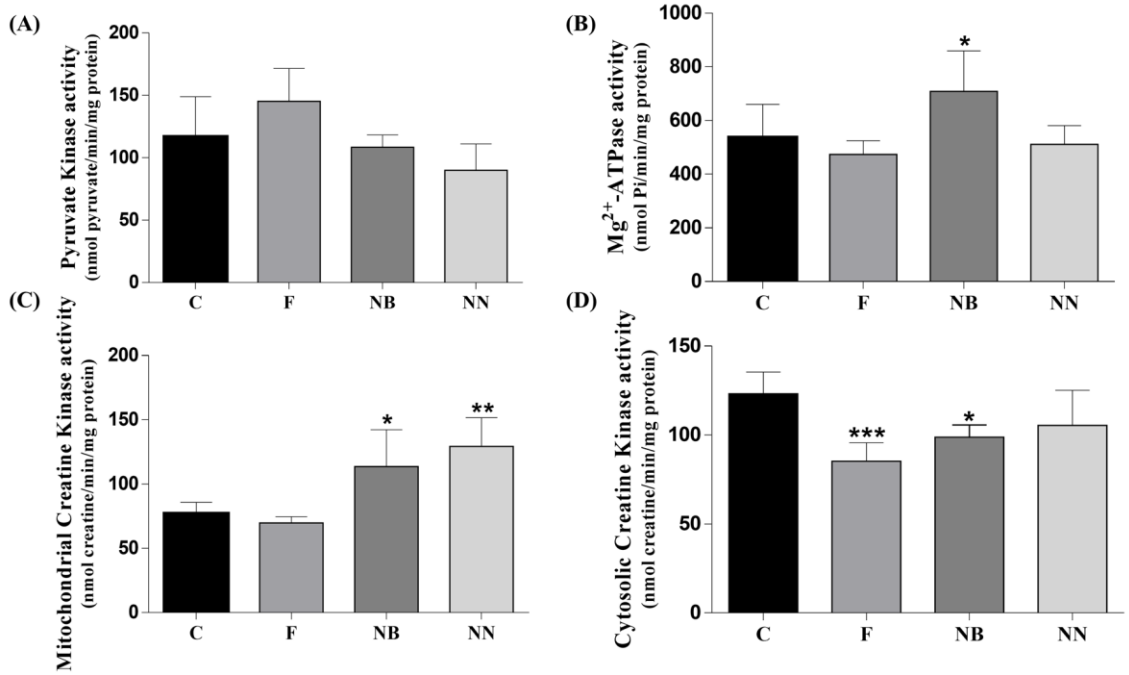

Figure 1. Effect of nanocapsules containing naringenin and naringin on **(a)** PK activity of kidney. **(b)** Mg^{2+}-ATPase activity of kidney. **(c)** mitCK activity of kidney. **(d)** cytCK activity of kidney. Data are mean ± SD (n = 5 to 7 animals per group). *p < 0.05; **p < 0.01; ***p < 0.001 compared to the control group (Dunnett test).

3.2 Hepatic energy metabolism

Regarding to the hepatic energy metabolism enzymes measures (Figure 2), the administrations (F, NB and NN) also did not affect pyruvate kinase activity (Figure 2a) when compared to that of control animals ($p > 0.05$).

On the other hand, F ($p < 0.01$), NB ($p < 0.001$) and NN ($p < 0.001$) treatments did not change the activity of Mg^{2+}-ATPase, in comparison with that of animals from control (Figure 2b).

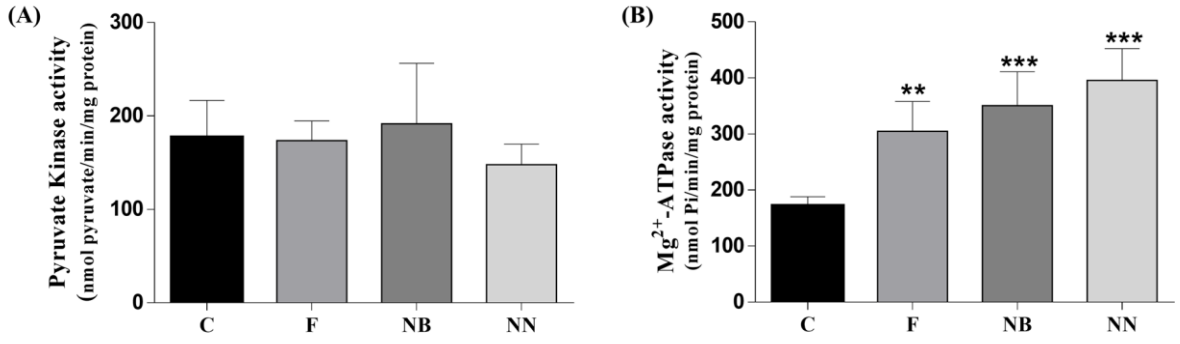

Figure 2. Effect of nanocapsules containing naringenin and naringin on (a) PK activity of liver. (b) Mg^{2+}-ATPase activity of liver. Data are mean ± SD (n = 5 to 7 animals per group). *p < 0.05; **p < 0.01; ***p < 0.001 compared to the control group (Dunnett test).

3.3 Serum markers of renal and hepatic function

Table 1 shows that animals submitted to all treatments did not change the creatinine levels when compared to control animals, however, showed an increase in serum urea levels to F group ($p < 0.05$)

compared to control group (Table 1). Whiles serum AST and ALT activities were not altered by treatments (F, NB and NN) when compared to control animals.

Table 1: Results of renal and hepatic serum markers.

	Urea (mg/dL)	*Creatinine (mg/dL)*	*AST (U/L)*	*ALT (U/L)*
C	35.57 ± 2.7	1.128 ± 0.58	192.85 ± 34.6	59.86 ± 18.1
F	38.86 ± 1.7*	1.31 ± 0.39	187.66 ± 23.6	72.17 ± 7.7
NB	36.43 ± 2.4	1.747 ± 0.90	173.29 ± 51	59.71 ± 16.5
NN	36.29 ± 2.2	0.848 ± 0.57	171.7 ± 33.6	64.86 ± 14.6

The groups are represented by control (C), free (F), nanocapsules without active compounds (NB) and nanocapsules with naringin and naringenin (NN). Data were expressed as mean ± SD. * $p < 0.05$ compared to the control group (Dunnett test).

4. Discussion

Production of the nanoparticles with therapeutic application search the refinement of the pharmacological effects, increasing bioavailability and targeting the active ingredient to the specific location, decreasing toxicity (Nishihira et al. 2019). *In vivo* tests are necessary to evaluate the effect of nanocapsules in a living organism that can simulate the human organism, for that aim is chosen the animal model that, although not completely similar to the human organism, helps us to understand the action mechanisms of the particles and their toxicity thus, it can be applied safely in humans (De Franceschi et al. 2013; Pohl et al. 2017). Therefore, in this study, we investigated whether oral administration of nanocapsules containing NA and NG could affect the activities of energy metabolism enzymes and renal and hepatic markers suggesting toxicity, since the kidneys are essential in drug elimination and the liver the main organ responsible by the detoxification of the organism, thus being important markers of toxicity (Kriz and Kaissling 2008; Nelson and Cox 2014).

A very important enzyme for maintaining cell viability is PK, this enzyme plays a very important role in glycolysis, the main way of obtaining energy from renal and hepatic cells, where it catalyzes the dephosphorylation of phosphoenolpyruvate (PEP) in pyruvate, generating two molecules of ATP by glucose (Shuch et al. 2013). So that the inhibition of the PK is related with decrease intracellular levels of ATP so result damage in the cell viability, in addition the oxidative stress can cause decrease PK due to pyruvate is an antioxidant endogenous molecule, this a very important for the cell homeostasis (Baldissera et al. 2015; Rech et al. 2017). Adenylate kinase (AK), CK and PK are key enzymes for balance the homeostasis energy. These systems operate together, when there a decrease in one enzyme the increase activity of the other can be compensated, for this reason is necessary evaluation for the system complete (Kolling et al. 2019). The results of our study demonstrated that no changes were found in the PK activity on the groups compared to the control in both organs. Thus, none of the treatments altered the mechanism of action of this enzyme.

The main enzyme responsible for the transport of Mg^{2+}, that is a cofactor in the synthesis of ATP, is the Mg^{2+}-ATPase and therefore it was investigated in this study (Silva et al. 2013; Pilchova et al. 2017).

The results showed a significant increase in Mg^{2+}-ATPase activity in the NB group compared to the C groups in the kidney. In the liver, a significant increase was observed in the F, NB and NN groups compared to the control. Studies performed by Ferreira et al. (2015) demonstrate that, at the concentration of 500 μg/mL, the nanocapsules without the compounds and the nanocapsules containing NA and NG reduced the cell viability, therefore showing some toxicity, this toxicity was not found in this parameter in our study because the activity of this enzyme was increased and not decreased. Mg^{2+} -ATPase is a marker of the cell impairment when exposed the toxic compounds, hence when there a reduction in the activity this enzyme is possible show a toxic action these compounds (Sun et al. 2018). Therefore, is possible observe in the ours results what NN and NB don't caused toxicity and kept the effect of the group F. A study by Amudha et al. (2015) demonstrated that water-diluted Naringin flavonoid was able to prevent the reduction of Mg^{2+}-ATPase activity in rats brains under oxidative stress induced by exposure to nickel, thereby helping to preserve cell membrane integrity by inhibiting lipid peroxidation.

Another enzyme that plays an important role in energy metabolism is a CK that catalyzes the reversible transfer of a phosphoryl group from ATP to creatine producing PCr and ADP and, conversely, generates ATP. This enzyme is divided into two isoforms: mitCK and cytCK (Rech et al. 2008; Rowe et al. 2013). The results showed that there was an increase in mitochondrial CK activity in the NB and NN groups compared to the C group, which can also be explained by the results found by Ferreira et al. (2015) that were previously mentioned, once the two nanoparticles demonstrated significant increases in mitCK activity, thus increasing phosphocreatine production, using more ATP and compromising energy metabolism (Ferreira 2014). This detection, as well as the result found in the enzyme Mg^{2+}-ATPase, may be related to the Eudragit polymer that was used in the composition of the nanocapsules but was not used in the F group. However, studies conducted showed that Eudragit obtained results that show safety in its use and do not demonstrate toxicity (Yen et al. 2009; Mustafin 2011).

The results of cytosolic CK demonstrate significantly decrease in the activity in the F and NB groups when compared to the control group. Studies performed in rats with induced cardiomyopathy demonstrated that administration of NA at doses of 100 and 200 mg/kg significantly decreased the activity of CK (Papasani and Annapurna 2014). In another study performed in rats with diabetes, a decrease in CK activity was also found in rats treated with NA, thus demonstrating that this compound and its product NG may decrease the activity of this enzyme, corroborating the discovery of this study (Ahmed et al. 2012). The decrease of the cytosolic CK can cause a reduction of the phosphocreatine transformation in creatine and ATP, therefore occurring a decrease of the ATP production by this enzyme (Ferreira 2014). The results of the cytCK activity demonstrate that the administration of nanocapsules containing NA and NG does not compromise the correct functioning of this enzyme, in addition the nanocapsules prevent the effect of decrease of the CK found in the compounds NA and NG, demonstrating that the nanoencapsulation of these substances decreases the toxicity of the active compounds in free form (Ahmed et al. 2012; Ferreira 2014).

The serum levels of AST and ALT did not show significant differences in either group, this shows that the treatments do not cause hepatic toxicity since the AST and ALT markers are considered sensitive indicators of hepatocellular damage once the damaged liver cells release the enzyme aminotransferase into the circulation, resulting in liver damage (Motta 2009; Ahmed et al. 2019). According to Pari and Amudha (2011), NA supplementation significantly reduced cadmium-induced hepatic toxicity in Wistar rats,

dramatically decreasing lipid peroxidation and restoring antioxidant defense levels. Thus, a possible hepatic protection of the nanoparticles containing NA and NG should be further investigated. Ahmed and collaborators (2019) demonstrated then NA and NG, containg in the navel orange peel hydroethanolic, induced potential hepatopreventive effects, may be mediated via enhancement of the antioxidant defense system, suppression of inflammation and apoptosis.

Here, we demonstrate that serum creatinine and urea levels did not show significant differences, except in the F group compared to the control group showed a significant increase of urea. The increase in urea levels by group F shows that the compounds in the free form may be nephrotoxic, because urea as well as creatinine is a marker of renal damage, since both molecules are filtered by the kidneys and excreted in the urine, thus high serum levels of these markers demonstrate a deficiency in renal function. (Diaz Gonzalez and Scheffer 2003, Guyton and Hall 2006, YU et al. 2007, Deignan et al. 2008). This increase was not found in the NN group, demonstrating that nanoencapsulation of such compounds can prevent such toxicity. A study by Li et al. (2013) showed that the administration of NA for 13 weeks did not alter serum creatinine and urea levels but a study by Arumugam et al. (2016) show that the administration of NA increased urea levels in hyperammonemic mice induced by ammonium chloride.

5. Conclusion

In summary, it can be concluded that the nanocapsules containing naringin and naringenin did not produce renal and hepatic toxicity when investigating the serum markers of toxicity. In addition, the activities of important enzymes of the energy metabolism in these organs were affected by the administration of these nanocapsules demonstrating that nanomaterial toxicity testing needs to go beyond traditional methods. Because of these findings, more studies need to be performed to be sure about the safety of their use.

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