



Genotoxic risk in humans and acute toxicity in rats of a novel oral high-dose coenzyme Q10 oleogel

Natalia Ehrenhaus Masotta^{a,e,1}, Fabian Martinez-Perafan^{b,1}, Marta Ana Carballo^b, Susana Beatriz Gorzalczany^c, Ana M. Rojas^{a,e}, Valeria P. Tripodi^{d,e,*}

^a Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Industrias, ITAPROQ (CONICET-UBA), Int. Güiraldes 2620, Ciudad Universitaria, C1428BGA, Buenos Aires, Argentina

^b Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Instituto de Fisiopatología y Bioquímica Clínica (INFIBIOC), Departamento de Bioquímica Clínica, CIGETOX (Citogenética Humana y Genética Toxicológica), C1113AAD, Buenos Aires, Argentina

^c Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Farmacología, C1113AAD, Buenos Aires, Argentina

^d Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Tecnología Farmacéutica, Junín 954, C1113AAD, Buenos Aires, Argentina

^e CONICET, Argentina

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ABSTRACT

Coenzyme Q₁₀ (CoQ₁₀) supplementation has demonstrated to be safe and effective in primary and secondary CoQ₁₀ deficiencies. Previously, we have designed a high-dose CoQ₁₀ oleogel (1 g/disk) with excipients used in quantities that do not represent any toxic risk. However, it was necessary to demonstrate their safety in the final formulation. Following this purpose, an acute toxicity study of the oleogel in rats was performed. Furthermore, the genotoxic risk was evaluated in human volunteers after CoQ₁₀ supplementation with oleogel and compared to the solid form (1 g/three 00-size-capsules). In addition, the general health status and possible biochemical changes of the participants were determined using serum parameters. Results suggested the absence of adverse effects caused by the interaction of the components in the oleogel formulation. Therefore, we conclude that the designed novel high-dose CoQ₁₀ oleogel was safe for oral consumption.

1. Introduction

Mitochondrial respiratory chain disarrays are a diverse group of multisystemic illnesses which arise from either nuclear or mitochondrial DNA mutations. Formerly thought to be rare, these hereditary disorders can be described as one of the most common groups of innate errors of metabolism with a birth predominance of 1 in 5000 [1]. At the moment, no clear evidence to establish any pharmacological interventions for the majority of mitochondrial pathologies exists, except for coenzyme Q₁₀ (CoQ₁₀) insufficiencies [2]. CoQ₁₀ is a lipid-soluble cofactor, which acts as an electron-transfer carrier and it is naturally synthesized by mammals and plants. In most mammals, including humans, CoQ₁₀ is the predominant form with a ten isoprenyl unit side chain in *trans*

configuration at C6 of the 2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone (ubiquinone) or -1,4-benzoquinol (ubiquinol, the reduced form). It is mostly found in heart, kidney and liver [3]. CoQ₁₀ deficiencies can be caused by a primary disorder in its biosynthetic pathway. This primary form is a very infrequent autosomal recessive alteration whereby ataxia, myopathy or multisystem disease are the main features. It was especially associated with the infantile multi-systemic and cerebellar ataxic phenotypes [2]. Patients who develop renal dysfunction due to a CoQ₁₀ insufficiency respond successfully to high-dose CoQ₁₀ supplementation when the therapy is started rapidly in the illness development with gradual restoration of renal function and declined proteinuria [1].

Nevertheless, most of the CoQ₁₀ deficiency published cases are

Abbreviations: ALT, alanine aminotransferase; ALKP, alkaline phosphatase; AST, aspartate aminotransferase; CoQ₁₀, coenzyme Q₁₀; CBMNcyt, cytokinesis-block micronucleus cytome; EC, ethylcellulose; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; MCT, Medium-chain Triglycerides; MNi, micronuclei; NBUDs, nuclear buds; NPBs, nucleoplasmic bridges.

* Corresponding author at: Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Tecnología Farmacéutica, Junín 954, C1113AAD, Buenos Aires, Argentina.

E-mail address: vtripodi@ffy.uba.ar (V.P. Tripodi).

¹ These authors contributed equally to this work.

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originated by secondary disorders, where some genetic defects not related to the biosynthesis of the CoQ₁₀ are manifested in the patients, moreover an underlying condition of them can generate a wide range of dysfunctions of unknown etiology [1]. The secondary CoQ₁₀ deficiency is a common attribute among different mitochondrial diseases, including oxidative phosphorylation dysfunctions, and CoQ₁₀ has been broadly used in their therapies [4]. Additionally, it is associated to other kind of diseases such as oculomotor apraxia type I, multiple acyl-CoA dehydrogenase deficiency, cardiolipinosis, methylmalonic aciduria, or mucopolysaccharidosis type III [5]. Altered mevalonate route in familial hypercholesterolemia, associated with raised expression of cholesterologenic enzymes and declined expression of CoQ₁₀ biosynthetic enzymes, was remedied by CoQ₁₀ supplementation as well [6].

Even though supplementation with CoQ₁₀ is beneficial in both primary and secondary deficiencies, the design of oral formulations containing CoQ₁₀ has become a challenge. CoQ₁₀ shows very low solubility in water because of their high lipophilicity and molecular weight [7,8]. The low CoQ₁₀ apparent density is another major disadvantage for the development of oral forms, especially to satisfy daily high-dose (50 mg/kg/day) regimens in the treatment of adult patients with the deficiency, who also suffer from dysphagia as another secondary neurological consequence. A high-dose CoQ₁₀ oleogel (1 g/disk) was successfully developed by Ehrenhaus Masotta et al. [9] as an alternative formulation for day-to-day intake of high doses of CoQ₁₀ (1 g/5 g oleogel-disk), which could be used for oral therapy of adolescent and adult patients. This form permits to reduce the dosing frequency in these high therapeutic requirements, while alleviates the discomfort felt by patients due to the dysphagia associated to the CoQ₁₀ deficiency, maintaining then the adherence of patients to the oral therapy. The formulation was demonstrated to be stable for at least 1 year [9]. Recently, we have also demonstrated that CoQ₁₀ oleogel bioavailability in healthy volunteers was similar in comparison to those who took the solid form (1 g/three 00-sized capsules) [10].

The CoQ₁₀ oleogel excipients were used in quantities that do not represent any toxic risk. Similar oleogels, but without CoQ₁₀ were also reported and proposed as fat replacers in food processing [11]. However, since there is no previous evidence about the design of this kind of oleogels containing CoQ₁₀, the toxicity of this novel oleogel has to be determined, in which the CoQ₁₀ is dissolved in a medium-chain triglyceride (MCT) oil containing sorbitan monostearate (SMS) as surfactant and an aromatizing compound as flavoring agent, all entrapped by the ethylcellulose gel network.

Animal models such as rats are widely used in toxicology in order to determine the relative risks to humans associated with exposure to new drugs or xenobiotics [12]. After analysis in rodents, the safety of a drug is evaluated in humans through different biomarkers of biofluid samples such as blood and urine. In genotoxic risk assessment, for example, the use of peripheral blood lymphocytes to evaluate markers of DNA damage is common. In addition, clinical chemistry parameters are considered as indicators of an individual's general health status and some of them show possible adverse effects associated with a drug, for example, the activity of the enzymes alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and gamma-glutamyl transferase (GGT) is used as a biomarker of hepatic damage, whereas irregular concentrations of serum lipids would indicate a risk of heart disease [13].

Accordingly, the aim of this research was to assess the safety of the consumption of the novel oral high-dose coenzyme Q10 oleogel previously described [9] by carrying out an acute toxicity test in rats and a genotoxicity assay in lymphocytes from healthy volunteers in addition to monitoring the general health status of the participants and the determination of possible biochemical changes using serum biochemical parameters.

2. Materials and methods

2.1. Acute toxicity in rats

2.1.1. Animals

Healthy female and male Sprague-Dawley rats (aged 6–8 weeks) were purchased from the Laboratory Animal Facility of the Facultad de Farmacia y Bioquímica (Universidad de Buenos Aires) and used following international guidelines and local regulations concerning the care and use of laboratory animals for biomedical research (NIH Publication N° 85–23, Revised 1985). The Institutional Animal Care and Use Committee of Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (Ethics approval: Exp-FFyB 39414/19) authorized the trial. The animals were acclimatized to laboratory conditions for 5 days and housed in groups of five in standard conditions (22 ± 1 °C; 12 h light/dark cycle). The animals were fed with a standard diet and tap water *ad libitum*.

2.1.2. Study design

An acute toxicity study was conducted with the novel oleogel formulation which was administered as a single oral dose of 12000 mg/kg body weight (BW) containing 2400 mg of CoQ₁₀ and 9600 mg/kg BW of excipients. The period of observation was 14 days.

Forty rats, 20 males and 20 females, were randomly distributed into 4 groups of 10 animals each (5 females, 5 males) using computer generated randomization. All females were nulliparous and nonpregnant. The experimental animal groups were designed as followed:

- i **Vehicle control group (n = 10):** 10 mL/kg BW of MCT oil
- ii **CoQ₁₀ control group (n = 10):** 2400 mg/kg BW of CoQ₁₀ (powder) solubilized in 10 mL/kg BW of MCT oil.
- iii **Excipients control group (n = 10):** 9600 mg/kg BW of placebo oleogel (included all excipients, except for the CoQ₁₀)
- iv **Oleogel with CoQ₁₀ group (n = 10):** 2400 mg/kg BW of CoQ₁₀ provided in 9600 mg/kg BW of placebo oleogel.

A single dose of each solution was administered to the animals. Mortality and clinical signs were monitored twice a day (in particular, immediately and up to 4 h after administration) for 14 days. During this period, the body weight was recorded.

2.1.3. Clinical signs

Animals' clinical signs and mortality were daily observed during the 14-day study. These observations comprised modifications in sensory organs (such as eyes, nose, skin), body secretions, autonomic activities (respiratory rate, piloerections, among others), hunched posture, decreased motor activity, ataxia, muscle tremor and general behavior [14]. Any abnormality was recorded.

2.1.4. Body weight

The body weight was recorded using a calibrated balance on day 0, 3, 7, 10 and 14.

2.1.5. Gross necropsy and organ weights

On the 14th day, the rats fasted overnight and were euthanized in a CO₂ chamber. A gross necropsy observation was carried out to find the presence of lesions on the external surface of the body, skeletal systems, body cavities (such as cranial, thoracic, abdominal and pelvic), as well as all orifices [15]. Pathological observation was done systematically by external reflection and internal examination of organs and tissues. Liver, kidney, spleen, heart, brain and ovary/testicles were excised, free of fat, washed in cold saline solution, dried with clean tissue paper and observed for gross pathological changes. The organ weights were recorded using a calibrated balance.

2.1.6. Histological evaluation

Histological studies of liver and kidneys taken from all animals of each group were performed. They were fixed in 10 % formalin in phosphate buffer, dehydrated in ethanol and finally embedded in paraffin wax. Histological sections of 5 μm thickness were obtained and subsequently stained with hematoxylin and eosin (HE) and were viewed under light microscope at 4X, 10X and 40X (Carl Zeiss Axioskop 2 Plus). In order to unbiased analysis, pathologist examined histopathological material blind to treatment.

2.2. Genotoxicity in healthy volunteers

2.2.1. Pharmaceutical forms

Two different oral forms containing high doses of CoQ₁₀ were considered for this study. Oleogels with a dose of 1 g of CoQ₁₀ (ubiquinone 98 % w/w, Zhejiang Medicine Co. Ltd., Xinchang Pharmaceutical Factory, China) per disk were developed as previously described [9]. Every oleogel disks had a final individual mass of 5 g and consisted of an edible gel in which the CoQ₁₀ was solubilized in hot oil. Briefly, ethylcellulose (EC) was mixed with the MCT oil while stirring at a constant speed of 9500 rpm with an Ultraturrax T25 (IKA, Germany) on a hot plate (Velp, Italy). Then, when 130 °C was reached, the SMS surfactant was added and heating was continued until 155 °C for complete dissolution of EC. Afterwards, when the temperature decreased to 140 °C, 1 g of CoQ₁₀ per 5 g of total system was added. Once the solution reached 25 \pm 2 °C (mean \pm SD), the action of EC caused the gel to be properly formed. The final system had the following composition: 19.9 % w/w CoQ₁₀, 66.0 % w/w of MCT oil, 12.7 % w/w of EC, 0.7 % w/w SMS and 0.7 % w/w aromatizing.

Three hard-shell gelatin capsules of 00 size (Magel S.A., Argentina) were needed to load 1 g of CoQ₁₀ powder in order to obtain the same CoQ₁₀ dose of one oleogel disk. Thermal properties of the CoQ₁₀ powder were determined in Ehrenhaus Masotta et al. [9].

2.2.2. Subjects

The study was carried out in 10 healthy volunteers of both sexes: 6 women and 4 men range: [mean (\pm SD) age: 35.50 (\pm 11.36) y; range: 26–57 y]. It was carried out a purposive sampling including human volunteers from the school environment to decrease the bias due to possible school and social factors. All subjects replied to a food frequency questionnaire and an anamnesis for screening their health status, lifestyle and diet. Individuals with any infection or chronic disease were excluded from the investigation, as well as those who were recently exposed (up to 3 months) to ionizing or non-ionizing radiation for therapeutic or diagnostic purposes, and who consumed dietary supplements or medications.

Prior to the study, the approval of the ethics committee of the Facultad de Farmacia y Bioquímica of the Universidad de Buenos Aires was obtained (N° 90132/2017 and 48891/2016), and all the participants signed an informed consent form.

2.2.3. Study design

This study was a repeated-dose, comparative, unblinded, cross-over design consisting of two phases. Each phase involved two successive periods: treatment and non-treatment. As an even number of individuals of each sex was recruited, the participants were distributed between A and B groups (2 men and 3 women in each group) in a manner that each participant in group A had their counterpart in group B with the same sex and approximately the same age (\pm 2 years). In the first phase, each participant of group A was orally supplemented with a dose of 1 g CoQ₁₀/5 g oleogel-disk per day during 14 days. Meanwhile, each participant of group B received the same dose supplementation but as the capsules (1 g CoQ₁₀/three 00-size-capsules) under the same conditions. Following a washout period of two weeks, the second formulation was then taken per each group.

According to the weight of the participant, the equivalent of the

treatment dose was 10–14 mg/kg/day.

2.2.4. Sample collection

Peripheral blood samples were taken from each participant at three different periods for each trial phase: before treatment (basal period), the last day of treatment (treatment period) and 14 days after treatment (recovery period). Blood samples (~10 mL) were drawn by venipuncture using two vacutainer tubes: one heparin tube (~6 mL) for the cytochrome assay in peripheral blood lymphocytes (PBL), and one serum separator tube (~3 mL) to evaluate biochemical parameters. All samples were processed immediately after their collection.

2.2.5. Genotoxicity biomarkers in peripheral blood lymphocytes

Biomarkers of alterations in the DNA of lymphocytes were evaluated using the cytokinesis-block micronucleus cytochrome (CBMNcyt) trial according to the protocol [16] with some modifications as described by Martínez-Perafán et al. [17]. Briefly, isolated lymphocytes were incubated at 37 °C under 5% CO₂ in a multiwell plate (two wells per individual). Cytochalasin B (4.5 $\mu\text{g}/\text{mL}$; Sigma-Aldrich, Steinheim, Germany) was added after 44 h as a cytokinesis blocker. Then, the lymphocytes were harvested at 72 h of incubation, fixed and dropped onto slides. Cells were then stained with a 10 % Giemsa (Merck, Darmstadt, Germany) solution. A number of 2000 binucleated cells were scored per subject (1000 cells for each of two duplicate wells) using a transmission light microscope (Olympus CX31, Japan). The biomarkers reported were frequency of micronuclei (MNI), nuclear buds (NBUDs) and nucleoplasmic bridges (NPBs).

2.3. Serum biochemical parameters

The Cobas® 6000 analyzer (Roche, Switzerland) was employed to assess the concentration of glucose, creatinine, urea, total proteins, albumin, total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, phosphate, calcium and magnesium in serum. Also, to determine the activity of the following enzymes: ALKP, ALT, AST, LDH, and GGT.

2.4. Statistical analysis

The results were evaluated by the statistical functions of the SPSS software (Chicago, IL, U.S.) whereas the graphs were created with the GraphPad Software (San Diego, CA, U.S.). Normality and variance homogeneity of data were performed by Shapiro Wilks and Levene's tests, respectively. Chi-square and Student's *t*-test were used to compare participants' demographic characteristics and habits. Regarding the results of genotoxicity biomarkers and biochemical parameters, the two-way ANOVA test with repeated measures was used to evaluate the main effects of the "pharmaceutical form" and "sampling period" variables. In addition, the Bonferroni correction was applied for multiple comparisons. In relation to the acute toxicity, one-way ANOVA was used for the measured parameters, whereas the post-hoc Bonferroni test was carried out for the comparisons between the treatment groups and the vehicle control group. Significance was established at $p < 0.05$.

3. Results

3.1. Oleogel acute toxicity

3.1.1. Clinical signs

The single oral administration of the oleogel formulation containing 2400 mg/kg BW of CoQ₁₀ did not produce clinical signs, adverse effects, abnormal behavior or mortality in any of the animals assayed. Normal morphological characteristics (fur, skin, eyes and nose) were observed. No prominent clinical signs such as vocalization, lacrimation, salivation, irregular respiratory pattern, convulsion, tremors, diarrhea, lethargy and no unusual behavior were observed in any of the animals during the

observation period. As no mortality was recorded after CoQ₁₀ supplementation through the novel formulation, the LD₅₀ value was considered to be higher than the upper limit test dose of 12000 mg oleogel/kg BW.

Moreover, no signs of toxicity were observed in the animals that belonged to other three groups (vehicle, excipients, and CoQ₁₀). In order to perceive any mortality and adverse toxic effects, special emphasis was set on the first hours after administration.

3.1.2. Body weight

The BW of experimental groups was similar, considering the same sex at the start of the experiment. If we analyze the progression of BW over time in each group, differences are seen due to the natural growth of the animals increasing significantly since day 5 for male rats and day 7 for female rats, compared with their respective basal level. However, according to the data summarized in Table 1, the BW of rats of the same sex belonging to each group showed non-significant differences at the time studied ($p < 0.05$). Therefore, non-significant changes in the BW were produced by the treatments during the observation period ($p < 0.05$).

3.1.3. Gross necropsy and organ weight

Macroscopic observations showed no irregularities in gross anatomical features of the studied organs for all animals. The organ weights are presented in Table 2. No significant differences were observed between the oleogel formulation with CoQ₁₀ and each of the other three groups (vehicle, excipients, and CoQ₁₀) assayed for males or females in a given organ.

3.1.4. Histological evaluation

Microscopical images obtained from the histology of the liver and kidney of male and female rats are shown in **Supplementary material (S1)**. Histological examination of the kidney of male and female rats in all treatments showed a normal histostructure. Signs of nephritis, glomerulitis or tubular changes were not observed in any of the studied groups. No signs of hepatitis or hepatocyte changes were observed in any group.

3.2. Oleogel toxicity evaluation in healthy volunteers

As a high value of LD₅₀ was determined in animals (12000 mg oleogel/kg BW), the toxicity study in human volunteers was then performed.

3.2.1. Volunteer characteristics

Based on anamnesis data, 30 % of participants were smokers and 70 % were moderate alcohol drinkers (<20 g ethanol/day) according to the definition of U.S. Department of Health and Human Services and U.S. Department of Agriculture [18]. Non statistically significant differences

were found between men and women respecting the smoking habit or alcohol consumption ($p > 0.05$; data not shown). On the other hand, none of the participants was obese and they had a balanced diet, which means that none of them manifested high, low or no consumption of a particular food group. The body mass index of the volunteers showed no significant differences ($p = 0.76$) when considered both groups of men and women ($24.4 \pm 0.8 \text{ kg/m}^2$ and $23.4 \pm 1.5 \text{ kg/m}^2$ and, respectively). Both mean values are in the optimum range ($18.5\text{--}24.9 \text{ kg/m}^2$) conforming to the World Health Organization [19].

3.2.2. Genotoxicity biomarkers

Regarding genotoxicity, Fig. 1 shows the line charts related to the frequency of biomarkers evaluated through the cytome test in peripheral blood lymphocytes during the treatment with both pharmaceutical forms of CoQ₁₀ (capsules and oleogel). According to the data, no significant differences ($p > 0.05$) existed between the pharmaceutical forms, sampling period (basal, treatment, recovery) or the interaction of these factors for each biomarker (MNi, NBUDs, and NPBs). In particular, the mean baseline frequency of each biomarker was not altered after oleogel supplementation, in other words, no significant differences were found between these sampling periods for the mean frequencies of MNi ($p = 0.233$), NBUDs ($p = 1.000$) and NPBs ($p = 1.000$). A similar trend in the capsule supplementation scheme was observed. These results suggested that there was no genotoxic risk associated with the use of CoQ₁₀ in either of the two pharmaceutical forms, since the frequency of genotoxicity markers did not change after treatment.

3.3. Biochemical parameters

According to the reference ranges, the analysis of the blood biochemical parameters revealed that the mean concentrations found for all the analytes corresponded to values of healthy adults (Fig. 2–4). Significant differences were not observed between pharmaceutical forms, sampling period or the interaction of these factors for the blood levels of glucose, urea, creatinine, total proteins, albumin (Fig. 2), total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides (Fig. 3). The same result was found for the enzymatic activity of ALKP, AST, ALT, GGT, and LDH (Fig. 4).

Regarding the levels of phosphate, calcium and magnesium ions (Fig. 5), the only statistical significance was observed for the interaction of the factors “pharmaceutical form” and “sampling period” for calcium: in the treatment sampling period the concentration was significantly higher with the capsules ($p = 0.032$), whereas after the recovery period it was with the oleogel supplementation ($p = 0.027$) (Fig. 5B). Otherwise, a significant difference ($p = 0.033$) between treatment period and recovery period was observed only with the oleogel (Fig. 5B).

Table 1

Acute toxicity study of CoQ₁₀ oleogel in rats: body weights (BW)¹ recorded.

	Vehicle control group ²		CoQ ₁₀ control group ³		Excipients control group ⁴		Oleogel formulation with CoQ ₁₀ ⁵	
	Females	Males	Females	Males	Females	Males	Females	Males
Initial weight (g)	204.0 ± 3.0	284.4 ± 7.6	203.2 ± 2.5	272.4 ± 8.3	207.2 ± 2.6	277.0 ± 7.6	208.4 ± 2.8	278.0 ± 7.6
Weight at day 3 (g)	208.0 ± 3.0	295.2 ± 8.9	207.6 ± 2.5	286.0 ± 7.6	212.0 ± 3.0	292.0 ± 7.0	212.2 ± 3.0	289.4 ± 4.6
Weight at day 5 (g)	210.8 ± 2.9	308.0 ± 9.3*	211.2 ± 2.4	306.2 ± 5.5*	215.2 ± 2.9	307.2 ± 5.0*	215.2 ± 3.1	303.2 ± 3.6*
Weight at day 7 (g)	213.6 ± 2.8*	323.6 ± 8.4*	214.2 ± 2.8*	319.4 ± 6.0*	218.4 ± 3.3*	326.8 ± 4.3*	218.2 ± 3.5*	317.8 ± 3.3*
Weight at day 10 (g)	218.0 ± 2.9*	348.4 ± 9.5*	222.2 ± 3.4*	346.6 ± 9.1*	223.4 ± 2.9*	344.8 ± 5.6*	222.2 ± 3.4*	340.8 ± 8.0*
Weight at day 14 (g)	225.6 ± 5.2*	385.0 ± 12.1*	224.2 ± 5.4*	382.2 ± 10.6**	229.2 ± 4.3*	371.2 ± 10.0*	233.0 ± 5.5*	374.0 ± 15.1*

¹ Values are means ± SEM, (n = 40, 10 per group, 5 per sex in each group).

* Significantly different when compared to initial weight from each group, $p < 0.05$.

² 10 mL of medium-chain triglyceride (MCT) oil (vehicle) per kg BW.

³ 2400 mg of CoQ₁₀ (powder), solubilized in 10 mL/kg BW of MCT oil, per kg BW.

⁴ 9600 mg of excipients per kg BW (placebo).

⁵ 2400 mg of CoQ₁₀ (provided in 9600 mg/kg BW of placebo oleogel) per kg BW.

Table 2
Acute toxicity study of CoQ₁₀ oleogel in rats: organ weights¹ recorded.

	Organs											
	Liver		Kidney		Spleen		Heart		Brain		Ovary or testicle	
	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males	Females	Males
Vehicle control group (g) ²	6.39 ± 0.31	12.83 ± 0.37	1.79 ± 0.07	3.03 ± 0.15	0.48 ± 0.03	0.73 ± 0.04	1.01 ± 0.04	1.44 ± 0.07	1.54 ± 0.09	1.61 ± 0.10	0.11 ± 0.01	3.21 ± 0.07
CoQ ₁₀ control group (g) ³	6.61 ± 0.17	11.45 ± 0.66	1.78 ± 0.06	2.92 ± 0.10	0.51 ± 0.02	0.70 ± 0.04	0.94 ± 0.03	1.44 ± 0.08	1.63 ± 0.06	1.71 ± 0.09	0.13 ± 0.01	3.13 ± 0.21
Excipients control group (g) ⁴	6.65 ± 0.22	11.80 ± 0.37	1.81 ± 0.04	2.97 ± 0.25	0.51 ± 0.02	0.67 ± 0.06	0.98 ± 0.02	1.34 ± 0.09	1.57 ± 0.07	1.64 ± 0.09	0.12 ± 0.01	3.07 ± 0.13
Oleogel formulation with CoQ ₁₀ (g) ⁵	6.88 ± 0.29	12.19 ± 0.62	1.89 ± 0.06	2.72 ± 0.10	0.52 ± 0.03	0.61 ± 0.04	0.95 ± 0.04	1.34 ± 0.07	1.51 ± 0.09	1.60 ± 0.10	0.12 ± 0.01	2.96 ± 0.06

¹ Values are means ± SEM (n = 40, 10 per group, in each group 5 per sex).

² 10 mL of medium-chain triglyceride (MCT) oil (vehicle) per kg BW.

³ 2400 mg of CoQ₁₀ (powder), solubilized in 10 mL/kg BW of MCT oil, per kg BW.

⁴ 9600 mg of excipients per kg BW (placebo).

⁵ 2400 mg of CoQ₁₀ (provided in 9600 mg/kg BW of placebo oleogel) per kg BW.

4. Discussion

The pharmaceutical regulatory entities usually require the acute toxicity test report for labeling and classification of substances for human use [20]. Acute systemic toxicity evaluates adverse effects that could be induced by a tested formulation or substance when it is administered in a single dose to estimate the potential hazard on humans.

As many authors who evaluated CoQ₁₀ acute toxicity of different pharmaceutical forms in animals [21–23], in our study the body weight, gross necropsy and organ weight, as well as the liver and kidney histopathology and other evaluated clinical signs were not affected by the administration of the CoQ₁₀ oleogel formulation when compared with a control group of animals that received only excipients (excipients control group), suggesting that the novel combination does not induce deleterious effects on growth or health in the experimental conditions used.

The liver and kidney are highly susceptible organs, and although macroscopic evidence of toxicity was not observed in any of the organs studied, histopathology was nevertheless performed in these two crucial organs, in control and treated animals, confirming no organ damage.

Besides, although acute exposure is more related to functional imbalance rather than changes in gross architecture of an organ system, none of them was observed in our study, indicating that the developed oleogel could be considered as an acceptable and harmless strategy to treat CoQ₁₀ deficient conditions.

On the other hand, the Food and Drug Administration agency [24] mentions in its “Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use”, that the assessment of genotoxic risk is an essential aspect in order to characterize the potential hazard for carcinogenic effects associated to pharmaceuticals. In addition, they highlight the micronucleus test as one of the most reliable and robust assays in the standard genetic toxicology battery. It is worth mentioning that the increased frequency of micronuclei in peripheral blood lymphocytes is considered a predictive marker of cancer risk [25]. There is also evidence of this parameter as a prognostic/predictive biomarker for monitoring the treatment response in some types of cancer [26]. For these reasons, in this work the latest version of the MN test known as cytome assay (CBMNcyt) was used, which includes NBUDs and NPBs as additional genotoxicity biomarkers [27].

CoQ₁₀ is an endogenously produced compound so it is presumed not to be genotoxic [28,29]. It is important to emphasize that not only CoQ₁₀ toxicity aspects should be evaluated when new and promising formulations are developed, but it is also fundamental to assess the possible interactions between the CoQ₁₀ and the other excipients contained in the formulae which could represent a toxic risk. It is currently known that some chemical compounds that are presumed safe for human health are genotoxic when they are mixed even using supposedly safe low concentrations. This is the case of the majority of active components in agrochemicals, in which an additive or synergistic effect is observed [30–32]. It should be noted that in the literature there is no evidence of previous studies relating CoQ₁₀ oral formulations and genotoxicity effects in humans. Therefore, the present work is the first one in this field since it evaluates in healthy volunteers the possible genotoxicity of a novel high-dose CoQ₁₀ oleogel previously developed in Ehrenhaus Masotta et al. [9]. Regarding the validity of the results presented in this study, we had to assure that a 14-days-supplementation with the high-dose oleogel allowed CoQ₁₀ to reach the systemic circulation.

In the present study the results of a multiple dose CoQ₁₀ supplementation (1 g/day, 14 days) via oleogel (1 g CoQ₁₀/disk) or the solid form (1 g CoQ₁₀/three 00-size-capsules) are compared. The CBMNcyt assay in peripheral blood lymphocytes showed that there are no genotoxic effects in the human volunteers during the treatment with both pharmaceutical forms. These findings are in accordance with those obtained by Kitano et al. [33] who demonstrated no chromosome damage, no mortality or atypical clinical signs in an *in vivo* MNi test when

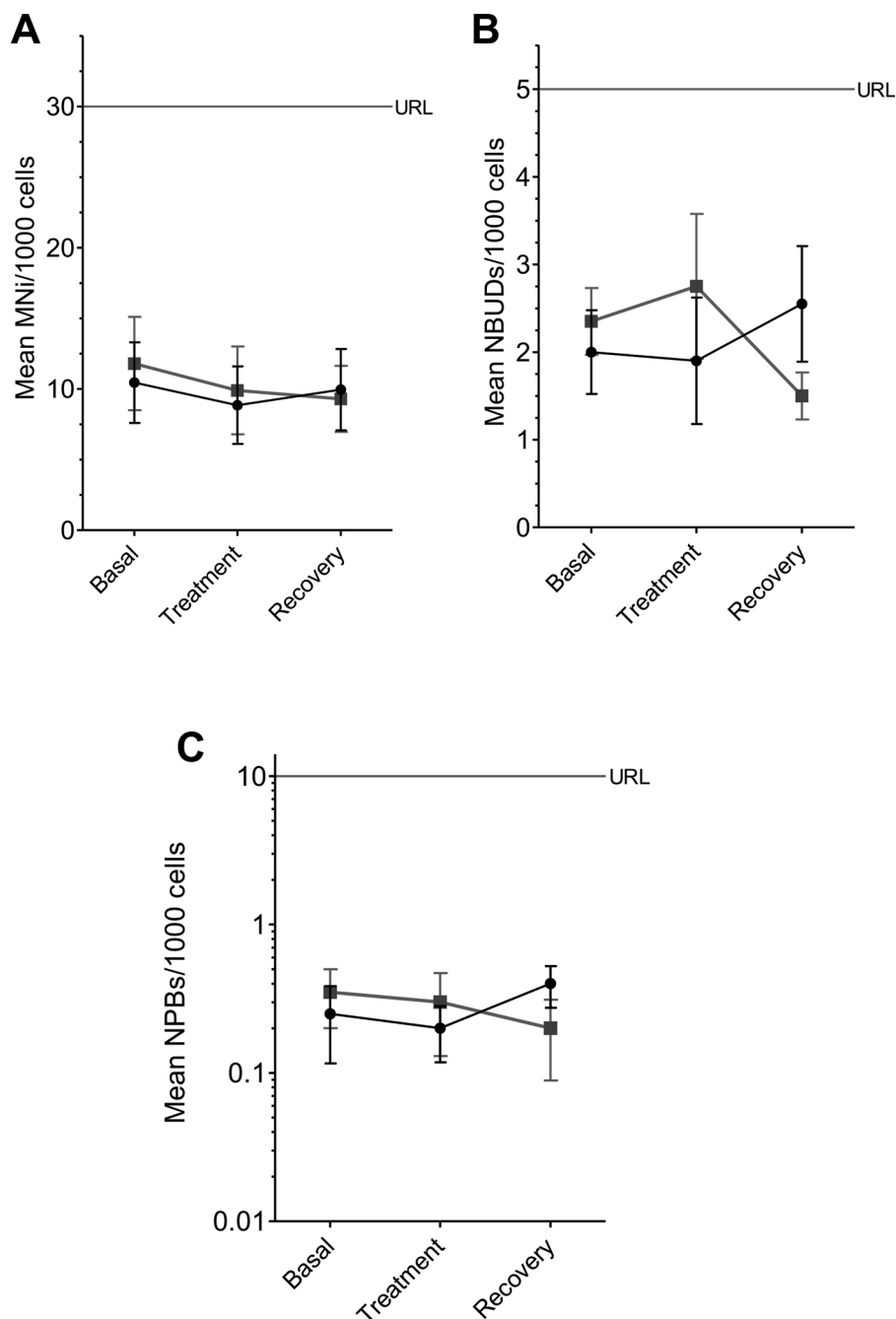


Fig. 1. Genotoxicity biomarkers evaluated by the cytome assay in peripheral blood lymphocytes obtained from participants at the basal, treatment and recovery periods of sampling after oleogel (■) or capsules' (●) consumption: micronuclei (A); nuclear buds (B); nucleoplasmic bridges (C). Bars correspond to SEM; URL: upper reference limit.

ubiquinol was administered orally to rats at daily doses up to 2000 mg/kg.

In addition, the general health status of the participants is simultaneously evaluated in the present work by clinical chemistry tests which are complementary to the genotoxicity assay because they provide a broader analysis of the possible effects associated with the administration of new drugs. It is worth noting that the metabolic products of pharmaceutical compounds or supplements could not only interact with genetic material, but also with other molecules and metabolic pathways. Therefore, a comprehensive evaluation of the response to a new drug is especially necessary if the ideal of personalized and translational medicine is to be achieved. Although in our study the biochemical parameters are maintained within reference ranges, it is relevant to mention

the calcium changes produced during CoQ₁₀ treatment with the oleogel and the capsules. We cannot explain the differential response between formulations; however, calcium elevation due to CoQ₁₀ supplementation was not surprising. A significant raise in serum calcium was also determined in a recent investigation carried out on a rat model of osteoporosis, in which the animals were treated with 20 mg CoQ₁₀/kg every 5 days for 3 months until reaching calcium levels of the control animals [34]. The increased serum calcium levels following CoQ₁₀ supplementation have been also previously observed in humans, being this enhancement attributed to the role of the coenzyme in increasing the production of vitamin D in the mitochondria of renal proximal tubule [35]. This information should be borne in mind for future investigations to achieve a better comprehension of the relationship

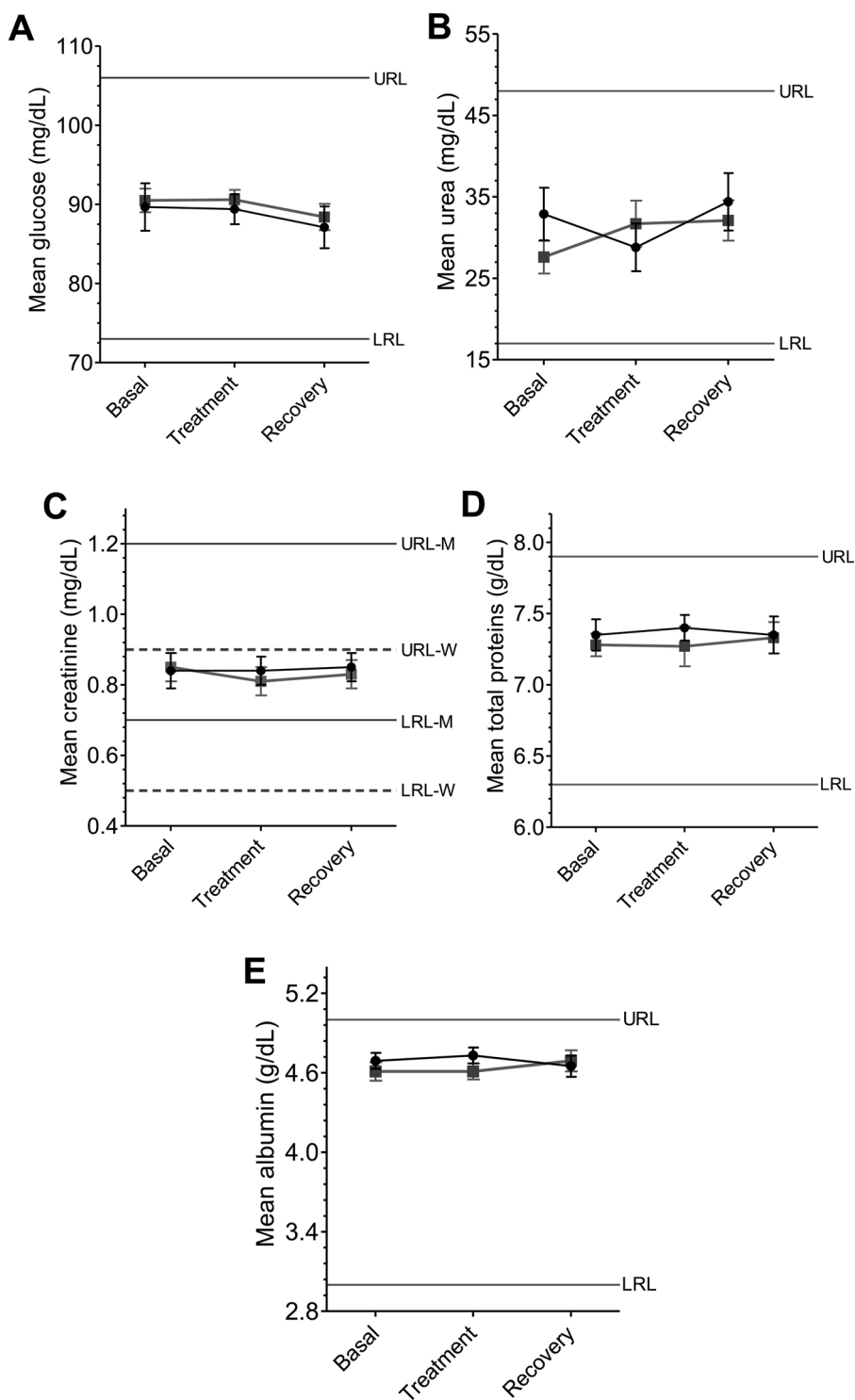


Fig. 2. Glucose (A), urea (B), creatinine (C), total proteins (D), and albumin (E) biochemical parameters in serum obtained from participants at the basal, treatment and recovery periods of sampling after oleogel (■) or capsules' (●) consumption. Bars correspond to SEM. URL: upper reference limit; URL-W: upper reference limit for women; URL-M: upper reference limit for men; LRL: low reference limit. LRL-W: low reference limit for women; LRL-M: low reference limit for men.

between ubiquinone and calcium metabolism.

Regarding studies on the CoQ₁₀ effect in healthy people, only one work was found wherein several blood biochemical parameters of healthy volunteers were evaluated after supplementation with ubiquinol doses of 90, 150 and 300 mg/day for 28 days [36]. The results of this trial were consistent with ours, since no clinically relevant differences were found in biochemical parameters tested after ubiquinol treatment.

Moreover, we have previously evaluated vitamins with antioxidant

capacity (vitamin A, C and E) together with oxidized and reduced glutathione, one of the most representative redox state markers, following oleogel supplementation [10]. None of the above mentioned parameters were found to be diminished, hence it can be interpreted that the oleogel matrix did not interfere in the normal redox-state of the volunteers.

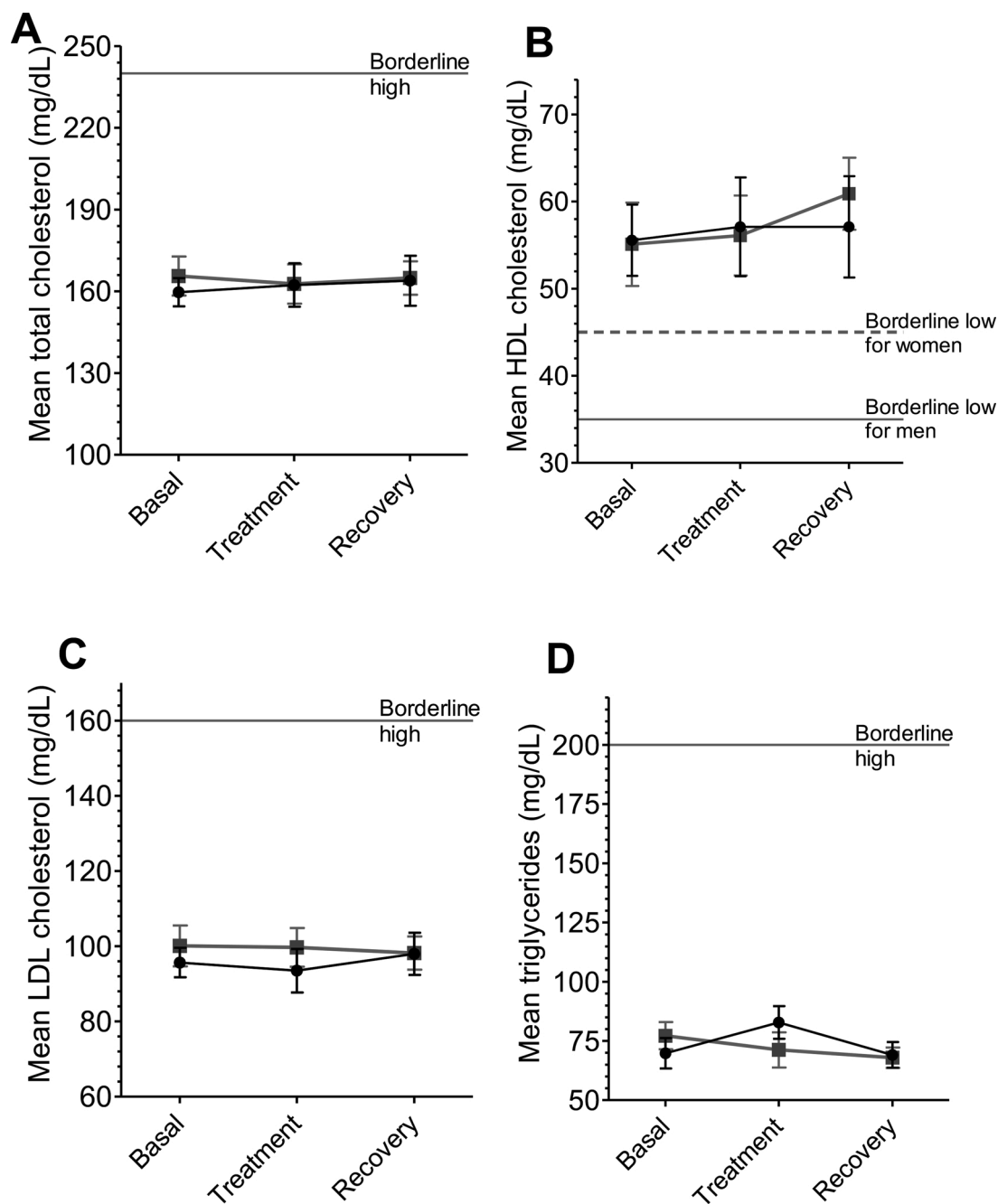


Fig. 3. Total (A), HDL (B) and LDL (C) cholesterol as well as triglycerides' (D) levels in serum of participants at the basal, treatment and recovery periods of sampling after oleogel (■) or capsules' (●) consumption. Bars correspond to SEM.

5. Conclusions

In summary, the results describe the absence of acute toxicity and genotoxic risk of the novel high-dose CoQ₁₀ oleogel in rats and healthy human volunteers, respectively. No effect was observed in any of the life clinical signs, body weight changes, macroscopical observations and weight of organs, and liver and kidney histopathology in rats, neither in any of the measured biochemical parameters evaluated in the serum of human volunteers. These results suggested the absence of detrimental effects generated by the interaction of the excipients used in the CoQ₁₀ oleogel formulation. In the light of the presented results, it can be concluded that the novel high-dose CoQ₁₀ oleogel (1 g/disk) formulation designed, which is easily swallowed by CoQ₁₀-deficient patients who suffer from secondary dysphagia, is safe for oral therapy.

CRediT authorship contribution statement

Natalia Ehrenhaus Masotta: Development of pharmaceutical formulation. Investigation. Formal analysis, Data acquisition. Optimized analytical methods. **Fabian Martinez-Perafan:** Genotoxic analysis. Investigation. Formal analysis and Data acquisition. **Marta Ana Carballo:** Genotoxic study design. Interpretation of analytical data. **Susana Beatriz Gorzalczany:** Toxicity study design. Animal care and management. Data acquisition and interpretation. **Ana M. Rojas:** Development of pharmaceutical formulation. Study concept and design, interpretation of data. Drafting of the manuscript and Critical revision of the manuscript for important intellectual content. Writing - Review & Editing. Visualization. Funding acquisition. **Valeria P. Tripodi:** Development of analytical methods. Study concept and design, interpretation

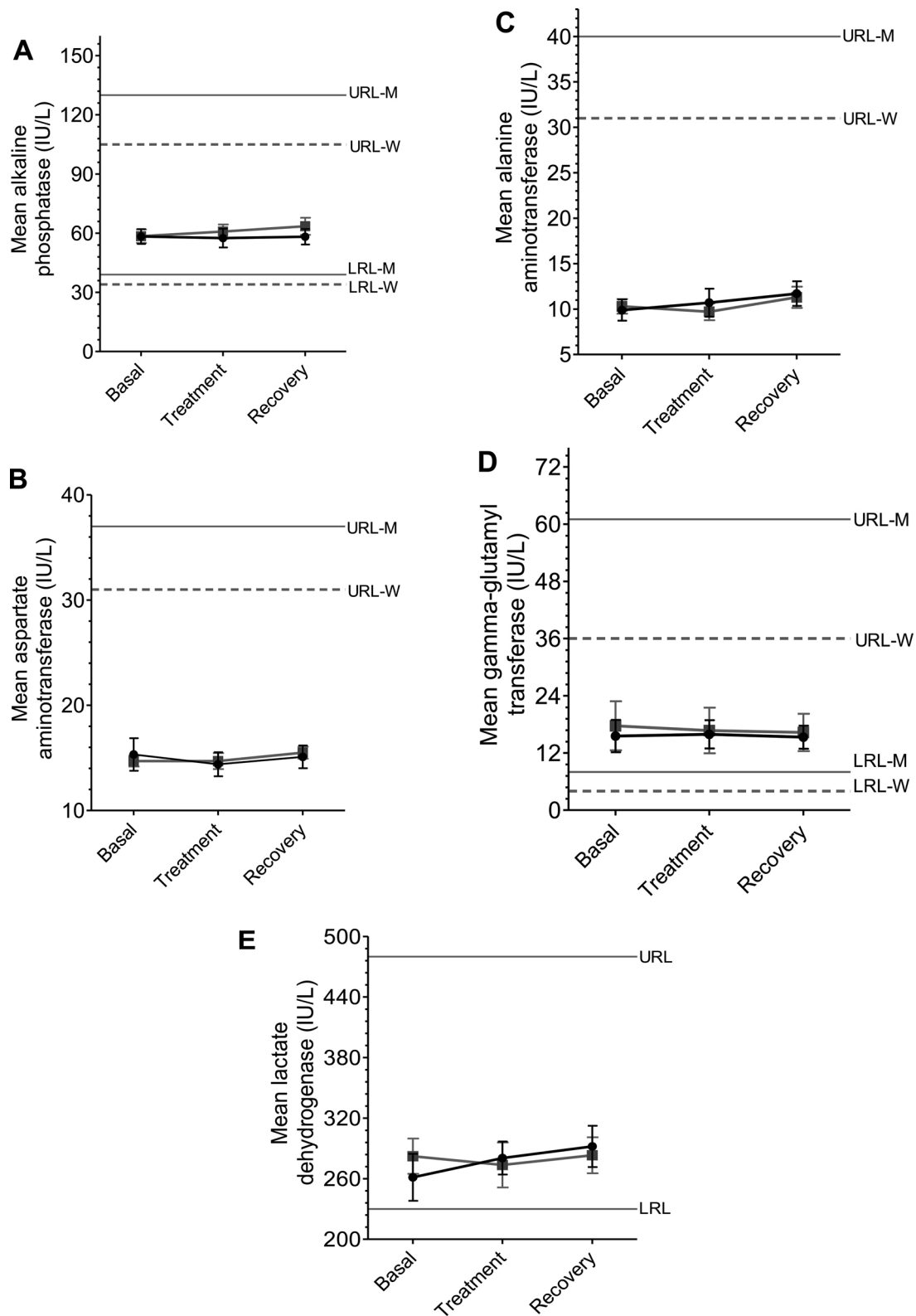


Fig. 4. Alkaline phosphatase (A), aspartate (B) and alanine (C) aminotransferases, gamma-glutamyl transferase (D), and lactate dehydrogenase (E) enzymatic parameters in serum from participants at the basal, treatment and recovery periods of sampling after oleogel (■) or capsules' (●) consumption. Bars correspond to SEM. URL: upper reference limit; URL-W: upper reference limit for women; URL-M: upper reference limit for men; LRL: low reference limit. LRL-W: low reference limit for women; LRL-M: low reference limit for men.

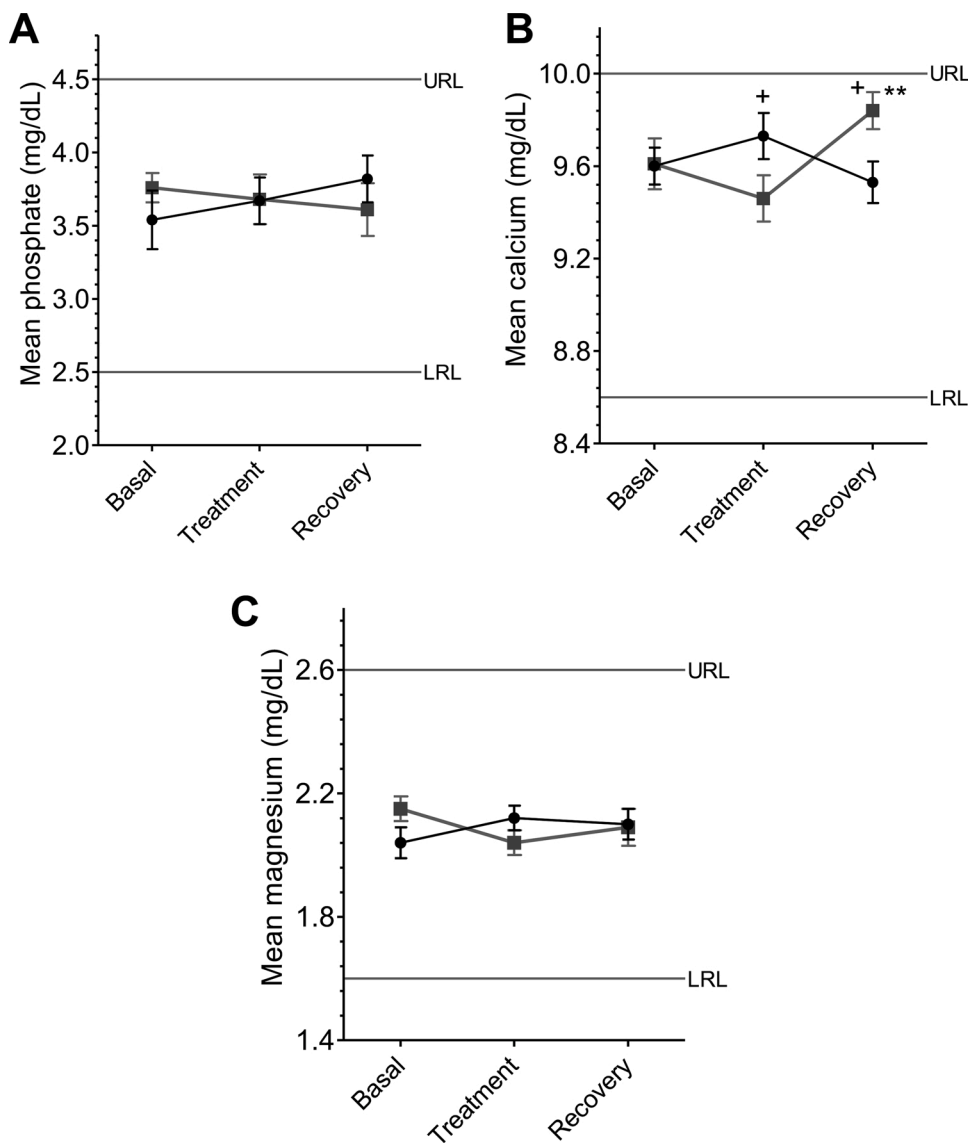


Fig. 5. Phosphate (A), calcium (B) and magnesium (C) concentrations in serum from participants at the basal, treatment and recovery periods of sampling after oleogel (■) or capsules' (●) consumption. Bars correspond to SEM, (+) indicates a significant difference between both pharmaceutical forms at the treatment and recovery periods, whereas (**) denotes a significant difference between treatment and recovery periods only for the oleogel scheme. URL: upper reference limit; LRL: low reference limit.

of data. Drafting of the manuscript and Critical revision of the manuscript for important intellectual content. Writing - Review & Editing. Visualization. Funding acquisition. Supervision.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

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