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Preclinical safety assessment of β-hydroxyphospho-carnitine

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Abstract

β-hydroxyphospho-carnitine(β-HPC) has been shown therapeutic potential. However, safety information is lacking. Acute toxicity, chronic toxicity, embryotoxicity, teratogenicity and genotoxicity of β-HPC were evaluated. For the acute toxicity, β-HPC was orally administered to mice at a dose range of 5-9000 mg/kg. For the repeated dose toxicity study, male rats were orally administered with β-HPC at the doses of 100 mg/kg for a period of 24 weeks. The effects on body weight, food and water consumption, organ weight, hematology, clinical chemistry as well as histology were studied. For embryiotoxicity and teratogenicity, β-HPC was administered to fertile chicken eggs (range 60-480 µg/mL). The genotoxic potential was tested using a comet assay. Data of each parameter were analyzed by oneway ANOVA followed by Tukey-Kramer test to compare the difference between treated groups. In general toxicity studies, orally administered β-HPC showed no sign of toxicity in any of the analyzed parameters involved in rodent single- and repeated-dose studies. Chicken embryos exposed to β-HPC *in ovo* showed alterations in growth and development during the early stage and at the end of their development in a dose dependent manner. In some cases, embryonic development terminated at stages 40/45 on the Hamburger-Hamilton scale and there were no associated malformations. β-HPC was not genotoxic to THP-1 cells. The results of the toxicological research confirmed that the safety profile of β-HPC. This safety evaluation results support the implementation of an exploratory Phase I clinical trial and underscore the potential of β-HPC as a new drug.

Keywords: Acute oral toxicity, chronic toxicity, β-hydroxyphospho-carnitine, L-carnitine, preclinical, safety

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1. Introduction

Metabolic syndrome is characterized by obesity, insulin resistance, atherogenicdyslipidemia, and hypertension.[1] Pharmacological therapy becomes a critical step in the management of patients with metabolic syndrome when lifestyle modifications fail to achieve the therapeutic goals.[2,3] Currently, there is no single therapeutic approach and treatment should target individual components of metabolic syndrome.For this reason, considerable effort has been placed into achieving new treatments.

L-Carnitine is well known for its role in the transportation of activated long-chain fatty acyl groups across the inner mitochondrial membrane and into the mitochondrial matrix for subsequent ß-oxidation.[4] In humans, L-carnitine supplementation improves glucose tolerance and partially facilitates body weight loss, in particular during insulin-resistant states.[5] Hence the scientific community's interest in finding new derivatives with improved pharmacological properties over L-carnitine. Some carnitine derivatives have remarkably improved metabolic function under pathological conditions.[6]

It has been shown that β-hydroxyphospho-carnitine (β-HPC), an analogue of L-carnitine, can be potentially used in the treatment of metabolic syndrome.[7] According to previous results, the pharmacological profile of β-HPC is more potent and efficient than that of L-carnitine to improve both carbohydrate and lipid metabolism in experimental models. At a dose of 100 mg/kg during 14 weeks, orally administered β-HPC

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reduced cholesterol and triglyceride levels in liver and serum; reduced hepatic steatosis, and improved liver architecture in obese Zucker$^{fa/fa}$ rats.[8] Thus, β-HPCis a promising candidate in the discovery and development of novel therapeutic agents against metabolic syndrome. Preliminary results in animals have shown a favorable pharmacokinetic profile and nosafety issues were identified after treatment with any β-HPC dosage.[9]

Although safety evaluation is a prerequisite for the drug development process, few studies on the toxicity and pharmacological safety of L-carnitine derivatives have been performed. Therefore, the objective of the present study was to investigate acute oral toxicity, 24-weeks repeated dose (Chronic) toxicity, embryotoxicity, teratogenicity and genotoxicity of β-HPC.

2. Material and Methods

2.1 Test material and animals.

β-HPC (β-hydroxy-γ-aminophosphonate; CAS number 1220955-20-3) was obtained from NUCITEC S.A. de C.V. (Qro., Mexico) and dissolved in sterile deionized water prior to experimental use. Male adult Wistar ratsweighing 100g, and female and male adult Balb-c mice (Harlan Laboratories Inc. Mexico) were used. The animals were housed in a temperature and humidity controlled environment and were allowed food (Standard Purina Chow Diet, Mexico) and water *ad libitum*.The experiments were conducted in accordance with the Guide for the Care and Use for Laboratory Animals.[10] All procedures were approved by the Institutional Animal Care and Use Committee of the Veterinary Medical School at the National Autonomous University of Mexico.

2.2 Acute oral toxicity study.

This study employed 140 Bal-c mice, 10-12 weeks old. Animals were obtained from random breeding in a closed colony. At least ten mice were used at each dose level and they were all of the same sex. After completingstudies on one sex we started with the other. Females were nulliparous and non-pregnant. Groups of animals of a single sex were dosed in a stepwise procedure using the fixed doses of 5, 50 300, 5000 and 9000 mg/kg. β-HPC was orally administered once to mice that had fasted for 18 h. Animals were observed for signs of toxicity during the first 0.5, 1, 2, 4, 8, 12 h and 24 h. Signs recorded during acute toxicity included: motor activity, anaesthesia, tremor, arching, and rolling, clonicseizures, ptosis, tonic extension, lacrimation, exophthalmos, pilo-erection, salivation, depression, ataxia, sedation, hypnosis, cyanosis and analgesia. Behaviour parameters, death, weight, and the amount of water and feed were analysed. All animals that survived after 24 h were killed for humane reasons and 200 mg/mL sodium pentobarbitone was used.

2.3 Chronic (24-weeks repeated dose) toxicity study.

For the toxicological evaluation, the animals were divided in two groups, each one consisting of 10 rats,after acclimatization. The control group was under a controlled diet and had water *ad libitum*. The treated group received β-HPCat 100 mg/kg body weight/day p.o. in 500 µL of water. Dosage was chosen based on previous pharmacological studies.[8] All treatments were administrated 5 days a week for 24 weeks. After time, all animals were killed for humane reasons and 200 mg/mL sodium pentobarbitone was used.

Toxicity indices consisted of daily clinical observations, body weight, food consumption, clinical pathology, organ weight, and histopathology. Necropsies included examination of external body orifices; abdominal, thoracic, and cranial cavities, and organs. Tissue fragments from liver, intestine, lung, kidney, heart, thymus, spleen and brain were fixed in 10% formaldehyde solution, dissolved in phosphate-saline buffer (pH 7.4), dehydrated in alcohol and embedded in paraffin. Four-micrometer paraffin sections were stained with Hematoxylin& Eosin (H&E) and subjected to histopathological examination.

Serum was separated by centrifugation at 3000 rpm for 15 min. Biochemical parameters of serum enzyme activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glucose, cholesterol and triglycerides (TG) were measured using a commercial reagent kit (ELITech, Mexico); urea, creatinine and uric acid were measured using a commercial reagent kit (ELITech, Mexico).Insulin was quantified using ELISA kits (Rat/ Insulin Kits, Millipore, Missouri, U.S.A). Hematological parameters (total and differential leukocyte count, hematocrit, hemoglobin, platelets and erythrocyte count)were measured using a Clinical Chemistry Analyzer (Becton, Dickinson, USA).

2.4 Embryotoxicity and teratogenicity studies.

Fertile White Leghorn chicken eggs were obtained from A.L.P.E. S.A. (Puebla, Mexico) and stored at 6 °C. Thirty-six fertilized eggs were weighed, sterilized, and divided into six groups. The first group served as a non-treated control and was considered the negative control. The other groups received β-HPC in different concentrations (60 μg/mL, 120 μg/mL, 240 μg/mL and 480 μg/mL). The doses were chosen based on the plasma concentrations found in previous pharmacokinetic studies.[9]The last group received caffeine (10 mg/mL) and was considered the positive control. Given that L-carnitine has been used previously in pregnant animals as supplement, authors did not consider necessary included as an additional group. A embryotoxicity assay was carried out as described previously.[11] Test solutions (1 mL) were added to the air sac under sterile conditions. Each solution was injected after drilling into the shell at the blunt end of the egg; after injection, the holes were immediately sealed with melted paraffin wax. The eggs were then transferred to and maintained in a forced draft incubator at 37.5 °C with a relative humidity of 55% until the desired stage of development was reached.

To determine the concentration dependency of analogembryotoxicity, a histological analysis was carried out. Embryos in each group were fixed in 4% formaldehyde, stained with acetocarmineand dehydrated with alcohols. After time embryos were included in eugenolfor routine histological examination. The embryo was examined and staged according to the morphological criteria previously outlined.[12] Embryonic stages at the time of the analog application varied from 14 to16, which corresponded approximately to developed somites numbered 22-28.

To determine the teratogeniceffect of β-HPC, we treated thirty-six fertilized eggs, as described above, were and let the chick fully develop and hatch. The chickens were examined and we determined viability and hatchability. Development stages and the presence of malformations were measured according to the Hamburger-Hamilton scale.

2.5 Genotoxicity study

We used the comet assay to evaluate whether β-HCP induces DNA damage.[13] THP-1 cells (TIB-202 ATCC). Cells were cultured in RPMI-1640, supplemented with nonessential amino acids, 10% FCS, L-glutamine (2 mol/L), and antibiotics (100 U/mL penicillin and 100 mg/ml streptomycin). 5 X 10^5 viable cells were plated in 25 cm^2 culture bottles and maintained at 37°C under an atmosphere of 5% CO_2 and 95% air. Fresh medium was added every 2 d and the cells were harvested and diluted 5-fold every 7 d. Cells were treated with β-HPC at 10, 100 and 1000 μM during 24 h. We used H_2O_2 300 μM as positive control. After some time, the cells were harvested using 5 min centrifugation at 1000 rpm, washed with phosphate-saline buffer (PBS) for 20 min on ice, and then washed twice with PBS. Slides were incubated overnight in lysis solution (30 mMNaOH, 1,2 M NaCl, 1% (w/v) laurylsarcosine, 0.05% triton X 100, 1% DMSO, pH 12.4). The slides were rinsed three times for 20 min each in electrophoresis buffer (30 mMNaOH, 10 mM EDTA, pH 12.4) to remove lysis solution and unwind the nuclear DNA. The samples were subject toagarose gel electrophoresis for 20 min at 25 V and 300 mA in the same buffer. Following electrophoresis, the gels were neutralized in 400 mMTris-HCl, pH 7.5 for 15 min. For visualization in a fluorescence microscope, the slides were stained with ethidium bromide (10 μg/mL) and 60 randomly selected nuclear images of each slide were taken with an epifluorescent microscope (Olympus IX81) using a BP 515-560 nm excitation filter at 100X magnification. Microscopic images of comets were captured by a digital camera(Olympus Camedia C-5060, USA). The results were used to analyze the nuclear DNA damage.For DNA damage analysis, 100 cells were scored per slide.[19] Cells were assigned various damage degrees, visually, based on their tail intensities (Class 0 = no damage; Class 1 = mild damage; Class 2 = moderate damage; Class 3 = high damage; and Class 4 = severe damage). The percentage of damaged cells was calculated manually. An arbitrary unit (AU) was used to express the extent of DNA damage.

3. Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). Significant differences were detected by one-way analysis of variance using the GraphPadInstatprogramme (GraphPad Software V2.03) (GraphPadSoftwware Inc., San Diego, CA). The Tukey-Kramer multiple comparisons test was applied when significant variations were found. Differences were considered significant at $P < 0.05$.

4. Results

4.1 Acute oral toxicity study

The single-dose toxicity test was performed in Balb-c mice. No animals died during the test. Drug administration was not associated with abnormal clinical signs or abnormal body weight changes. In addition, there were no abnormal necropsy findings. All dose levels (5, 50 300, 5000 and 9000 mg/kg) of β-HPC were well tolerated, with no signs of toxicity (data not shown).

4.2 Chronic (24-weeks repeated dose) toxicity study

No animal deaths, no clinical signs, and no abnormal changes were noted in the 24-week repeated-dose toxicity study. All dose levels were well tolerated. Figure 1 shows the growth curve of animals treated with β-HPC after 24 weeks. Animals treated with β-HPC showed a growth reduction, but this was not statistically significant. Organ weight was similar to that of control animals (Figure 2). Histopathological analysis of organs did not show morphological alterations in intestine, liver, lung, kidney, heart, and brain. A larger number of germ nodules of lymphocytes were observed in thymus and spleen tissue (Figure 3). The analyzed clinical chemistry parameters did not show changes in glucose, cholesterol and triglyceride serum levels. Hepatic and renal functions were not altered (Table 1). The hematological parameters analyzed did not show any changes, and all animals showed values similar to those of the control group (Table 2). During the time of study, the animals did not exhibit signs of motor activity, anaesthesia, tremor, arching, and rolling, clonic seizures, ptosis, tonic extension, lacrimation, exophthalmos, pilo-erection, salivation, depression, ataxia, sedation, hypnosis, cyanosis, or analgesia.

Table 1. Analyzed clinical chemistry parameters

Parameters	Control	β-HPC (100 mg/Kg)
Glucose (mg/dL)	109.07 ± 23.87	123.32 ± 18.66
Cholesterol (mg/dL)	61.24 ± 12.25	61.12 ± 12.83
Triglycerides (mg/dL)	49.36 ± 16.96	57.14 ± 8.78
Uric acid (mg/dL)	1.81 ± 0.16	1.75 ± 0.37
Creatinine (mg/dL)	0.74 ± 0.06	0.70 ± 0.07
Urea (mg/dL)	49.63 ± 6.78	47.33 ± 5.47
AST (U/L)	187.32 ± 21.36	188.12 ± 18.90
ALT (U/L)	65.91 ± 8.85	49.41 ± 10.38
ALP (U/L)	102.32 ± 9.43	117.38 ± 29.45

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase. Data are means ± S.D., n = 10

* p< 0.05 as compared with control group

Table 2. Analyzed hematological parameters

Parameter	Control	β-HPC (100 mg/Kg)
Hemoglobin (g/dL)	15.88 ± 0.59	15.80 ± 0.41
Hematocrit (%)	48.90 ± 2.87	49.74 ± 1.58
Erythrocytes (10^6 /μL)	8.66 ± 0.19	9.05 ± 0.47
MCV (fl)	56.45 ± 2.28	55.01 ± 1.61
MCH (pg)	18.35 ± 0.48	17.50 ± 0.86
CMHG (g/dL)	32.50 ± 0.93	31.78 ± 0.80
Leucocytes (10^3 /μL)	7.20 ± 0.87	7.94 ± 0.91
Platelets (μL)	720250 ± 81957	876166.67 ± 63565
Lymphocytes (%)	78.50 ± 2.52	83 ± 4.31
Monocytes (%)	2 ± 0.58	1.85 ± 0.85
Eosinophils (%)	0 ± 0	0 ± 0
Neutrophyls (%)	19.50 ± 3.11	15.75 ± 4.65

MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular haemoglobin concentration. Data are means ± S.D., n = 10

* p< 0.05 as compared with control group

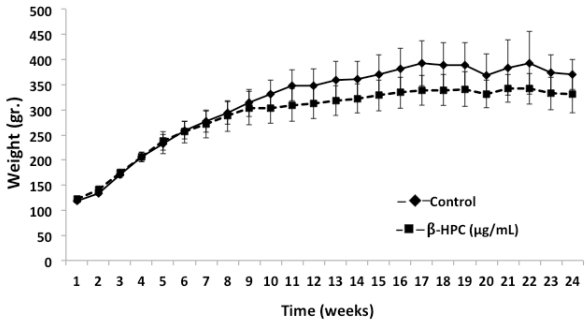


Figure 1. Growth curve of animals treated with β-HPC. Animals were treated with 100mg/Kg β-HPC p.o. for 24 weeks. Results are represented as mean ± d.s. n=10 * p<0.05 as compared with control group.

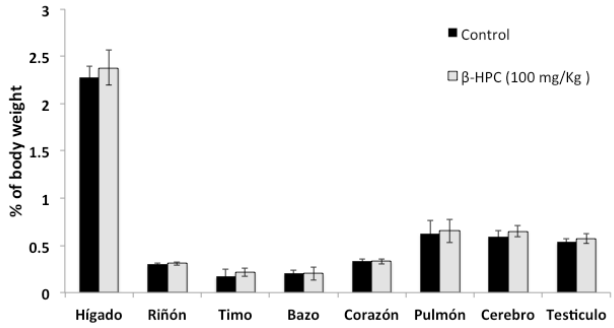


Figure 2. Organ weight in proportion to body weight. Animals were treated with 100mg/Kg β-HPC p.o. for 24 weeks. The values are the mean for each group ± d.s. n=10 * p<0.05 as compared with control group.

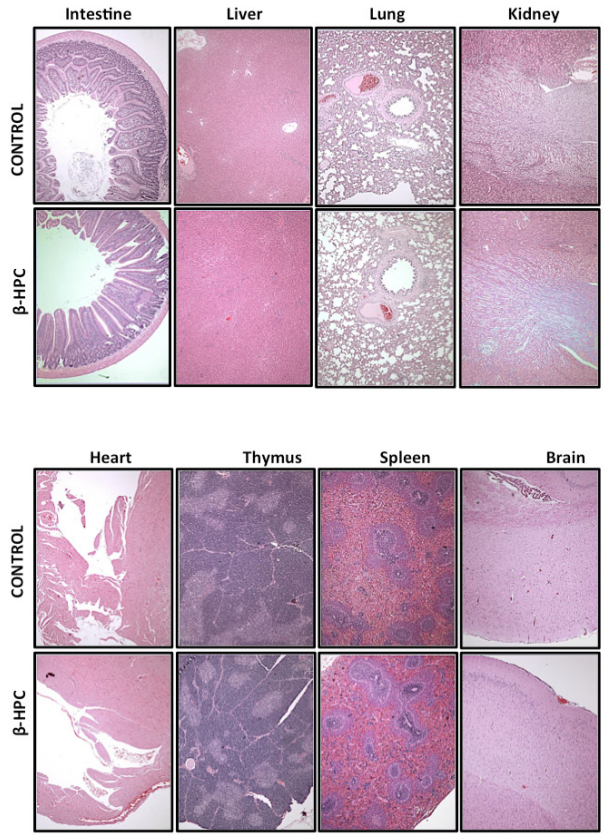


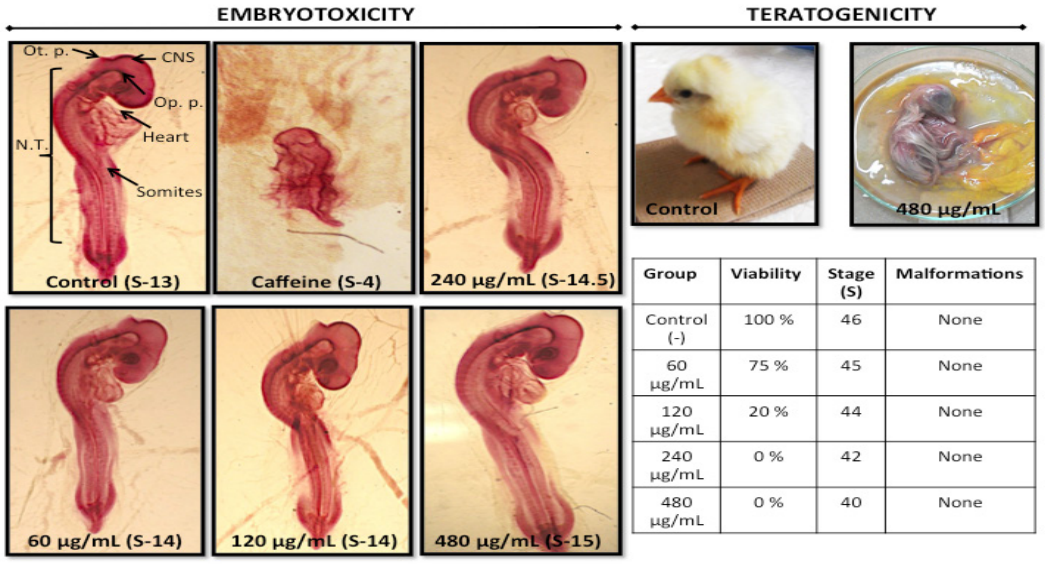
Figure 3. Morphological architecture of intestine, liver, lung, kidney, heart, thymus, spleen and brain after treatment with 100 mg/Kg of β-HCP p.o. for 24 weeks. Hematoxiline & eosinestain, n= 6 animals. Magnification 10X.

4.3 Embryotoxicity and teratogenicity studies

We used a chicken embryo model to assess the embryotoxicity of β-HCP. Figure 4 shows a representative chicken embryo treated with β-HCP. β-HCP-treated chicken embryos did not show any morphological change when compared with the control group. However, although there were no malformations, we observed faster development in embryos that were in Stages 14-15 when compared with untreated eggs on Stage 13 of the Hamburger-Hamilton scale. The effect was dose-dependent. The teratogenic effect of β-HCP was also evaluated using a chicken embryo model. The results showed that the treatment of chicken embryos with β-HCP for 21 days produced a reduced chicken viability (Figure 4). Only 75% of the chickens were alive and able to hatch when they were treated with 60 µg/mL ofβ-HCP. Chickens treated with 120, 240 and 480 µg/mL β-HCP were not viable and did not get to hatch. Although the hatched chicken did not show apparent malformations, they did not attain full development (32-36 on the Hamburger-Hamilton scale). The delay in chicken development was dose dependent.

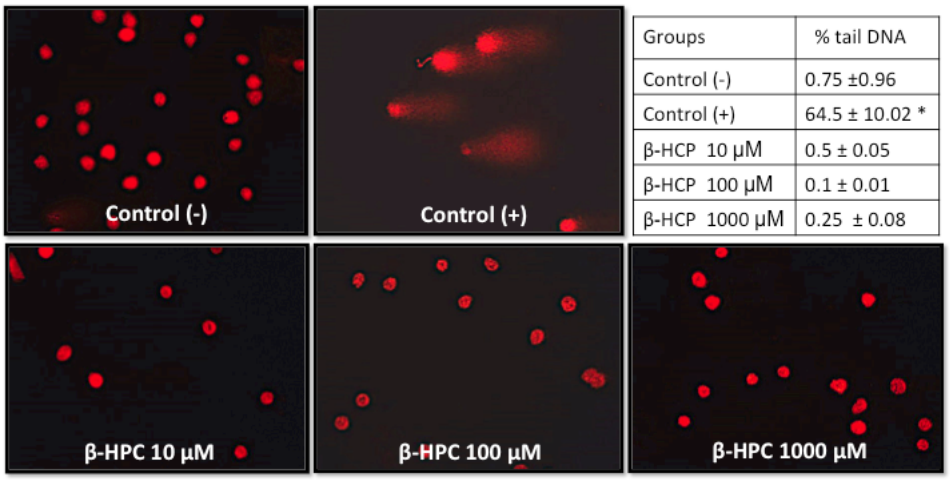
4.4 Genotoxicity studies

To evaluate whether β-HCP can induce DNA damage we employed the comet assay. In the control group (Control (-), the recorded damage was in the 0 and 1 category,



Group	Viability	Stage (S)	Malformations
Control (-)	100 %	46	None
60 µg/mL	75 %	45	None
120 µg/mL	20 %	44	None
240 µg/mL	0 %	42	None
480 µg/mL	0 %	40	None

Figure 4. Embryotoxicity and teratogenicity analysis for β-HCP in chicken embryo. Embryos were stagedin accordance with the Hamburger-Hamilton scale.Ot. p.=oticplacode; Op. p.=optic placode; CNS = Central nervous system; NT= neural tube (Magnification 4X).



Groups	% tail DNA
Control (-)	0.75 ±0.96
Control (+)	64.5 ± 10.02 *
β-HCP 10 μM	0.5 ± 0.05
β-HCP 100 μM	0.1 ± 0.01
β-HCP 1000 μM	0.25 ± 0.08

Figure 5. Analysis of β-HCPgenotoxicity.THP-1 cells were treated with β-HCP (10, 100 and 1000 μM). H2O2 300 μM was used as positive control (control +). Results are expressed as average percentage of tail DNA ± d.s. n=10 * $p<0.05$ as compared with control group.

meaning no damage or mild damage. The positive control group (Control +) has a remarkable DNA damage of 64.5% and included all the categories of DNA damage: this confirms the sensitivity of the experimental conditions employed. When THP-1 cells were treated with β-HCP at 10, 100 1000 μM, no DNA-damage showed in THP-1 cells (Figure 6). Recorded DNA damage was in the 0 and 1 category, which were classified as no damage or mild damage.

5. Discussion

β-HCP is currently being considered as a promising agent to treat metabolic syndrome. However, no safety evaluation, including *in vitro*, *in ovo*, and *in vivo* toxicological tests, has been reported. Our study is the first one to describe the general toxicology of β-HCP.

L-Carnitine is well tolerated by humans and all animal species, possibly because of its decreasing bioavailability with increasing dietary L-carnitine concentrations. However, there are currently no reports on their general toxicology.

Previous reports by this group showed that the absolute bioavailability of orally administered β-HCP was 60%, and although this bioavailability is higher than the bioavailability of L-carnitine, β-HCP was well tolerated without adverse events. This study shows that, when β-HCP was orally administered to rodents, no signs of toxicity were observed after single dose and after repeated-dose administration during a time-frame of 24 weeks. Multiple dose toxicity studies did not show alterations in any of the parameters analyzed. This suggests this drug might be safely used in long-term treatment, such as those involving metabolic syndrome.

Exposure to chemicals during different stages of development like the pre- and peri-conception, fetal, perinatal, peripubertal and adult stages has varying impact on health. This study evaluated the embryotoxic and teratogenic effect of β-HCP in a chicken embryo model. Chickenshave been extensively used to assess chemical embryotoxicity. [14] Although opinion is divided on the usefulness of the chicken embryo model in predicting teratogenicity, this system has gained popularity for reasons such as inexpensiveness, sensitivity, and parallelism to mammalian morphological development. In the chicken embryo test the chemicals affect the embryo directly, so that both deaths and malformations may be monitored.[12] The occurrence of embryonic effects after β-HCP treatment shows an inverse relationship between concentration and the embryonic stage. During the early stage (48 h incubation), β-HCP induced accelerated embryo development; during the last stage (21 days incubation), it delayed embryonic development. However, β-HCP did not cause malformations in embryos.

The chicken embryo may have a limited capability to synthesize L-carnitine during incubation and the growing embryo derives most of its energy from the β-oxidation of fatty acid reserves within the egg.[15] It has been reported that the presence of L-carnitine in the fertile egg may decrease embryonic mortality by

reducing oxidative stress during the hatching process, thereby increasing hatching rate,and that the effects may be most evident in various muscle groups. [16,17] appear to be that carnitine has an active participation in the intermediary metabolism both in the pregnant woman and in the neonate.[18] However, it has also been shown that L-carnitine supplementation via *in ovo* injection resulted in growth, food intake, and muscle development impairments.[19] This study demonstrated that β-HCP was no teratogenic, reduced chickdevelopment, and that there was a high incidence of dead embryos in a dose dependent manner. Our hypothesis is that the administration of β-HCP accelerated the β-oxidation of fatty acids in the mitochondria in order to generate ATP. Because ATP reserves were larger, the embryo used them and accelerated its growth and development, which was reflected in a more advanced stage according to the Hamburger and Hamilton scale. As the development of the embryo progressed, the quick growth depleted the food and energy reserves contained in the egg and these were not enough to complete development (Stage 46). Therefore, according to this study, there is positive evidence of human foetal risk. However, embryotoxicity could be explained by the fact that we employed a chicken embryo model and that β-HCP was added directly into the egg. Further studies using mammalian species are required in order to determine its safety during pregnancy.

The DNA of THP-1 cells in the presence of different β-HCP concentrations was analysed usinga comet assay. The assay has manifold applications in fundamental research for DNA damage and repair, genotoxicity testing for novel chemicals and pharmaceuticals, environmental biomonitoring, and human population monitoring. It has been reported that L-carnitine did not induce genotoxic effects but, on the contrary,exhibited pronounced antigenotoxic effects, protecting DNA against oxidative damages induced by genotoxic agents. [20, 21]According to the present results, β-HCP did not show genotoxic effects on THP-1 cells at any of the employed concentrations, which indicates that β-HCP is not a genotoxic agent.

6. Conclusions

The results of present study showed that single and 24-weeks repeated dose oral administration ofβ-HCP produced no significant toxic effects with the median LD50 > 9000 mg/Kg. β-HCP did not produce embryotoxicnorteratogenic effects, however, produces development delayed in chicken embryos; as well as, has not genotoxic potential. The present study demonstrated excellent safety margins of β-HCP and suggested potential for further development after appropriate clinical studies.

6.1 Finnancial support and sponsorship

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7. Conflict of interests

The authors declare that there are no conflicts of interest.

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