

Mitochondrial Energy Metabolism in Baby Hamster Kidney (BHK-21/C13) Cells Treated with Karnozin EXTRA®

Metabolismo Energético Mitocondrial en Células de Riñón de Hámster Bebé (BHK-21 / C13) Tratadas con Karnozin EXTRA®

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SUMMARY: Carnosine is known as a natural dipeptide, which inhibits the proliferation of tumor cells throughout its action on mitochondrial respiration and cell glycolysis. However, not much is known about its effects on the metabolism of healthy cells. We explored the effects of Karnozin EXTRA® capsule with different concentrations of L-carnosine, on the cell viability and the expressions of intermediate filament vimentin (VIM) and superoxide dismutase (SOD2) in normal fibroblasts BHK-21/C13. Furthermore, we investigated its action on the energy production of these cells. Cell viability was quantified by the MTT assay. The Clark oxygen electrode (Oxygraph, Hansatech Instruments, England) was used to measure the “intact cell respiration rate”, state 3 of ADP-stimulated oxidation, maximum oxidation capacity and the activities of complexes I, II and IV. Results showed that Karnozin EXTRA® capsule in concentrations of 2 and 5 mM of L-carnosine did not induce toxic effects and morphological changes in treated cells. Our data revealed a dose-dependent immunofluorescent signal amplification of VIM and SOD2 in the BHK-21/C13 cell line. This supplement substantially increased the recorded mitochondrial respiration rates in the examined cell line. Due to the stimulation of mitochondrial energy production in normal fibroblasts, our results suggested that Karnozin EXTRA® is a potentially protective dietary supplement in the prevention of diseases with altered mitochondrial function.

KEY WORDS: Carnosine; Fibroblasts; Energy metabolism; Vimentin; Superoxide dismutase 2.

INTRODUCTION

Carnosine is a natural dipeptide, widely used as a dietary supplement (Caruso *et al.*, 2019). Numerous physiological and pharmacological effects of carnosine have been investigated in vitro and in vivo (Renner *et al.*, 2010; Ding *et al.*, 2018). There are several important carnosine roles, such as a neurotransmitter, free radical scavenger, mobile organic pH buffer and metal chelator (Sarrami *et al.*, 2017; Miceli *et al.*, 2018). Thus, its potential effects on mitochondria are mentioned in several researches (Bao *et al.*, 2018; Cheng *et al.*, 2019).

Mitochondria are highly dynamic cellular organelles that participate in cellular homeostasis and are considered the “powerhouse” of the cell's energy production (Spinelli and Haigis, 2018). However, it was observed that energy production differs depending on the cell type. For their metabolism, healthy cells use mitochondrial ATP generation coupled to oxygen consumption known as oxidative phosphorylation (OXPHOS) (Zick *et al.*, 2009). The baby hamster kidney fibroblasts (BHK-21/C13) are an adherent cell line often used in drug research (Elmosallamy *et al.*,

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2021; Shen *et al.*, 2021). Noteworthy, it is recognized that carnosine acts both on cell metabolism through oxidative phosphorylation and on the process of glycolysis. A number of studies have suggested that carnosine is involved in the glycolysis metabolism of tumor cells (Shen *et al.*, 2014; Bao *et al.*). On the other hand, there is no data on the effects of carnosine on the mitochondria of normal fibroblasts.

Therefore, in the current study, we explored the effects of L-carnosine obtained from the capsule of Karnozin EXTRA® on cell proliferation, morphological characteristics and energy production in normal fibroblasts. We also investigated the immunofluorescence positivity of structural (vimentin-VIM) and oxidative (superoxide dismutase 2 - SOD2) markers of cells treated with this supplement.

MATERIAL AND METHOD

Cell culture. BHK-21/C13 cells were supplied from the European collection of authenticated cell cultures (Salisbury, UK). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 2 mM glutamine and 1 % penicillin-streptomycin (all from Capricorn Scientific, GmbH; Germany). Cells were kept at 37 °C in an atmosphere containing 5 % CO₂, subcultured twice a week. After reaching 70-80 % of confluency, cells were harvested using 0.05 % trypsin/EDTA to obtain single-cell suspension and counted using the trypan blue dye exclusion test. After seeding, cells were allowed to stabilize for 24 h before the treatment.

Experimental design. Karnozin EXTRA® capsule is a commercially available, L-carnosine-based formulation, enriched with vitamin E, coenzyme Q10, L-carnitine, northern blueberries and grape seed extracts (CarnoMed, LLC, Novi Sad, Serbia). The capsule was dissolved in phosphate buffer saline (PBS) immediately before use, vortexed and centrifuged. The supernatant was filtered through a bacteriological filter. The cells were treated for 24 h with a supernatant to reach final concentrations of 2, 5 and 10 mM of L-carnosine.

Morphological characterization of cells. To examine morphological features, cells were harvested and plated in a 24-well plate (5x10⁴ cells/well) containing glass coverslips. After the treatment, BHK-21/C13 cells were imaged by the Leica MC 190 camera on an inverted microscope (Leica DMIL) with phase contrast at 200x magnification. Afterward, the cells were stained with classical hematoxylin-eosin staining (H&E). These sections were analyzed using Leica

DMLB 100T microscope and photographed on Leica MC 190 camera.

The MTT assay. Cell proliferation assay was performed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method, according to Mosmann (1983), with modifications. Exponentially growing cells were plated into a 96-well plate (10 x10³ cells/well) and preincubated for 24 h at 37 °C. The cells were treated for an additional 24 h with the capsule supernatant, to reach final concentrations of 2, 5 and 10 mM of L-carnosine. The wells containing cells without the treatment were used as control. Afterward, MTT solution (0.5 mg/ml) was added to each well and the plates were incubated for 3 h. Acid-isopropanol was added to all the wells and mixed thoroughly to dissolve formazan crystals. The absorbances were measured using a spectrophotometer plate reader (Multiscan MCC340, Labsystems) at 540/690 nm. Cell viability was calculated as the percentage of viable treated cells against the control group treated with culture medium only. Two independent experiments were set out with quadruplicate wells for each concentration.

Immunofluorescence staining. In order to examine the expressions of VIM and SOD2 the cells were seeded onto glass coverslips in a 24-well plate (5x10⁴ cells/well). After the treatment period, the cells were fixed with 4 % paraformaldehyde for 10 min at room temperature and permeabilized with 0.3 % Triton X-100 for 5 min. Next, the cells were incubated in 10 % normal goat serum for 30 minutes and then primary antibodies were added: anti-VIM (rabbit polyclonal, 1:200 dilution), anti-SOD2 (rabbit polyclonal, 1:50 dilution). Thereafter, the slides with secondary anti-rabbit antibody linked to Alexa Fluor® 555 (goat, polyclonal) were incubated for 30 min in the dark and the nuclei were stained with DAPI. Images were analyzed using Leica DMLB 100T fluorescence microscope and photographed on Leica MC 190 camera. Immunofluorescence evaluations were performed in ten random fields at 400x magnification using ImageJ 1.45 software (USA). An outline was drawn around all cells visualized on the stained slides. The total area of selected cells and the mean fluorescence (integrated density) were measured, along with several adjacent background readings. The total corrected cellular fluorescence (TCCF) was calculated as integrated density – (area of selected cell × mean fluorescence of background readings).

Measurement of mitochondrial respiration. For the evaluation of mitochondrial respiration, BHK-21/C13 cells were cultured in a T75 flask (SARSTEDT, Numbrecht, Germany) at a density of 6x10⁵ cells. After 24 h of treatment with different concentrations of L-carnosine, cells were

trypsinized and counted by trypan blue dye exclusion test. A cell suspension (2×10^6 viable cells) was rinsed twice in PBS and resuspended with prewarmed (37°C) MiR05 medium (mitochondria respiration medium: 110 mM sucrose, 60 mM potassium lactobionate, 0.5 mM ethyleneglycoltetraacetic acid [EGTA], 3 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 20 mM taurine, 10 mM KH_2PO_4 , 20 mM Hepes and 2 mg/ml bovine serum albumin, pH 7.1) (Gnaiger, 2020). The Clark oxygen electrode (Oxygraph, Hansatech Instruments, England) was used to measure the “intact cell respiration rate”, state 3 of ADP-stimulated oxidation, maximum oxidation capacity and activities of complexes I, II and IV. All these parameters were measured by substrate-inhibitor titration as described earlier (Kuznetsov *et al.*, 2008). We used $5\ \mu\text{g/ml}$ as the effective digitonin concentration for the plasma membrane permeabilization. All respiration rates were expressed in nM O_2/min per million cells. All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Statistical analysis. All analyzed data were expressed as mean \pm standard deviation (SD). Significance analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test, while significant difference was set at $P < 0.05$ and $P < 0.001$. All statistical analyses were performed using a prism software program (GraphPad Software 8).

RESULTS

Changes in proliferation and morphology of Karnozin EXTRA® treated cells. To determine the effects of L-carnosine treatment on cell morphology, we used H&E staining and phase-contrast microscope. As shown in Figure 1A, control cells and cells treated with 2 and 5 mM L-carnosine were adherent and normal in shape and size. On the other hand, BHK-21/C13 cells treated with 10 mM L-carnosine showed morphological alterations. Cells treated with 10 mM L-carnosine became spherical, shrunken and loss of cell volume was also observed. An increase in the number of floating cells in the culture medium was also observed in phase contrast microphotographs (data not shown).

We used the colorimetric MTT assay for measuring cell proliferation. The viability of BHK-21/C13 cells after 24 h treatment is shown in Figure 1B. It was apparent that no toxic effect of 2 and 5 mM L-carnosine was present in normal fibroblasts. On the contrary, administration of L-carnosine in the concentration of 10 mM led to a significant reduction in cell number compared to control ($p < 0.001$).

Effect of Karnozin EXTRA® on vimentin expression in BHK-21/C13 cells. We investigated the effect of L-carnosine on vimentin expression in normal fibroblasts, a predominant intermediate filament in most cell types and tissues. The data showed that treatment with L-carnosine induced a dose-dependent expression of vimentin (Fig. 1C). The highest TCCF was observed in cells treated with 10 mM carnosine, compared to the control and other experimental groups ($p < 0.001$). As shown in Figure 1D, Karnozin EXTRA® treated cells displayed changes in the arrangement and appearance of intermediate filaments compared to control.

Effect of Karnozin EXTRA® on SOD2 expressions in BHK-21/C13 cells. In the present study, we analyzed SOD2 expression in BHK-21/C13 cells upon the treatment with Karnozin EXTRA® (Fig. 1E). Cells treated with 5 and 10 mM L-carnosine obtained from the capsule showed a statistically significant difference compared to the untreated control group ($p < 0.001$). The highest TCCF was observed in the cells treated with 5 mM L-carnosine (Fig. 1F).

Changes in mitochondrial oxidative phosphorylation under the action of Karnozin EXTRA®. We investigated the effects of Karnozin EXTRA® on oxidative metabolism in cultured BHK-21/C13 cells. The “intact cell respiration rate” was recorded in the respiration medium without additional substrates or inhibitors. As shown in Figure 1G, after treatment with 2 mM and 5 mM L-carnosine, the intact cell respiration rate was significantly higher compared to control ($p < 0.001$).

Next, we used digitonin, which disrupted the cell membrane. After the addition of exogenous substrates fuelling mitochondrial complex I respiration (pyruvate and malate), a significant effect of 2 and 5 mM carnosine on complex I activity was observed (Fig. 1H, $p < 0.001$ vs. control).

The rate of ADP - stimulated oxidation (state 3) is presented in Figure 1I. Treatment with L-carnosine in 2 and 5 mM concentrations led to a significant increase in oxygen consumption rate ($p < 0.001$ vs. control).

To determine the effect of different L-carnosine concentrations on the maximum mitochondrial oxidative capacity, we used the uncoupler carbonylcyanide-*p*-trifluoromethoxy-phenylhydrazone (FCCP) (Fig. 1J). The results showed that cells treated with 2 and 5 mM L-carnosine increased mitochondrial respiration rates compared to control ($p < 0.001$).

After adding rotenone, which inhibited the activity of complex I, succinate was used as a substrate for the mitochondrial complex II (succinate dehydrogenase) (Fig.

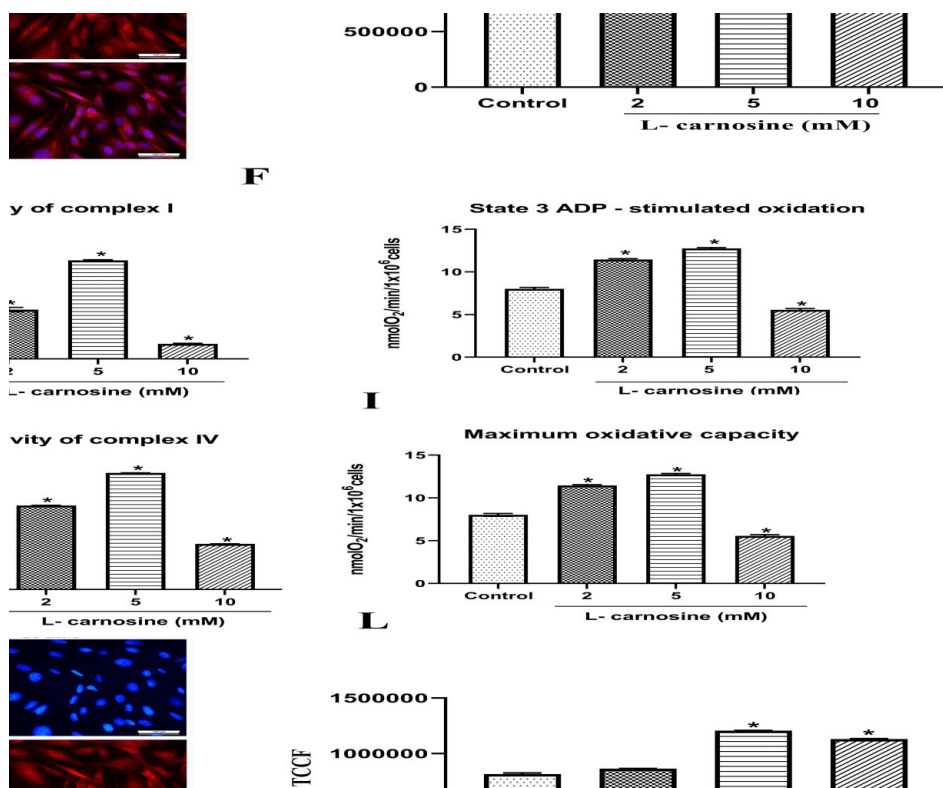


Fig. 1. Effects of Karnozin EXTRA® on BHK-21/C13 cells. A) Morphological features of cells exposed to different concentrations of L-carnosine for 24 h, H&E staining (400x magnification, scale bars represent 30 μ m) and phase contrast (200x magnification, scale bars represent 100 μ m); B) cell viability following the treatment with L-carnosine assessed by MTT assay; C) total corrected cellular fluorescence (TCCF) of vimentin (Vim) in BHK-21/C13 cells following the treatment; D) arrangement of intermediate filament Vim (green) in BHK-21/C13 cells following the treatment, nuclear DAPI staining is shown in blue (400x magnification, scale bars represent 100 μ m); E) immunofluorescence staining of SOD2 (red) in BHK-21/C13 cells following the treatment, nuclear DAPI staining is shown in blue (400x magnification, scale bars represent 100 μ m); F) total corrected cellular fluorescence (TCCF) of SOD2 in BHK-21/C13 cells following the treatment; G) bioenergetic characterization of BHK-21/C13 cell line following the treatment in terms of the «intact cell respiration rate»; H) the activity of complex I; I) state 3 after administration of the ADP substrate; J) maximum oxidative capacity of the electron transport system (ETS) after application of FCCP; K) the activity of complex II; L) the activity of complex IV.

1K). Under these conditions, in BHK-21/C13 cells treated with 2 and 5 mM L-carnosine, a significant increase in mitochondrial respiration rates was detected compared to the control ($p < 0.001$).

Cytochrome c oxidase (Complex IV) activity was also determined in examined cells using tetramethyl-p-phenylenediamine (TMPD) and ascorbate as energy substrates (Fig. 1L). This resulted in a substantial increase in the rate of O_2 consumption, especially in the group treated with concentrations of 5 mM L-carnosine compared to other groups ($p < 0.001$).

However, in cells treated with 10 mM L-carnosine, a significant decrease in the recorded mitochondrial respiration rates was observed ($p < 0.001$ vs. control).

DISCUSSION

To our knowledge, this is the first report to evaluate the effects of Karnozin EXTRA® supplement and L-carnosine in general on a healthy fibroblast cell line. Our principal findings are that Karnozin EXTRA® supplement in 2 mM and 5 mM concentrations has no toxic effect on viability and does not cause morphological changes in the examined cell line. Furthermore, VIM and SOD2 expressions in examined cells are associated with an increase in the tested supplement concentration in the growth medium. Finally, this supplement significantly increases mitochondrial respiration rates of BHK-21/C13 cells.

It is well known that L-carnosine has growth inhibitory effects on some cancer cells. Shen *et al.* (2014) studied the effect of carnosine on human gastric cancer (SGC-7901) cell viability. They tested carnosine concentrations of 5 and 20 mM, applied during 24 and 48 h treatment. The authors showed that carnosine markedly reduced cell viability to 84.0 % and 57.9 % of control at 24 h, and to 73.5 % and 45.9 % of control at 48 h, respectively. Moreover, carnosine showed an inhibitory effect on the growth of human cervical gland carcinoma cells (HeLa) (Bao *et al.*). After 48 h of treatment with carnosine concentrations of 5, 20, and 50 mM, this cell line exhibited reduced cell viability to 88.09 %, 67.82 %, and 21.89 % of control, respectively. It is believed that this effect of carnosine on cancer cells is not accompanied by apoptosis or necrosis, but may be caused by suppression of cell metabolism (Gaunitz & Hipkiss, 2012). In this study, we found that treatment with L-carnosine in 2 and 5 mM concentration had no negative effect on BHK-21/C13 cells viability during the treatment period for 24 h (96.07 % and 94.03 % of control, respectively). These results

are also in accordance with the treated cells' morphological features that retained normal shape and size after treatment with 2 and 5 mM L-carnosine.

Early studies described the role of vimentin filaments as structural proteins that are the major players contributing to cytoarchitecture and tissue integrity. Vimentin, as a multifunctional protein, also has the ability to interact with a large number of other proteins, which makes it a potential regulator of several physiological functions (Tang *et al.*, 2008). Studies have also shown that vimentin has a functional role in cell migration (Eckes *et al.*, 1998; Gilles *et al.*, 1999). We found that treatment with Karnozin EXTRA® supplement resulted in inducing dose-dependent expression of this intermediate filament in healthy cells. The effect of carnosine on vimentin expression was also observed in a study by Ikeda *et al.* (1999). The cultured rat fibroblasts (3Y1) were grown in the presence of 30 mM L-carnosine for 7 days. It was found that carnosine strongly stimulates the synthesis of vimentin filaments, which is in accordance with our data. Furthermore, there is a strong relationship between vimentin-based intermediate filaments and mitochondrial morphology and function (Tang *et al.*). Thus, it can be assumed that stimulation of vimentin synthesis by carnosine affects the processes within the mitochondria (Zakharchenko *et al.*, 2003).

It is well known that superoxide dismutase is one of the most important enzymes responsible for scavaging free radicals. The administration of L-carnosine significantly increased SOD2 expressions in a dose-dependent manner. Our findings are in agreement with Rezzani *et al.* (2019), where the role of carnosine on SOD2 expression was evaluated in L6 rat skeletal myoblasts treated with 10, 20 and 30 mM L-carnosine for 24 h. L-carnosine in all of the used concentrations exerted a moderate/strong expression of SOD2 in the mentioned cell line. This association between carnosine administration and increased SOD2 expression could be explained by the antioxidant role of carnosine in free radical scavenging but also due to its effects on the regulation of mitochondrial functions (Xie *et al.*, 2017).

It has long been considered that carnosine exerts its effects only through glycolysis, especially in cancer cells (Iovine *et al.*, 2012; Shen *et al.*, 2014; Bao *et al.*). However, in recent years, carnosine was also linked to mitochondrial metabolism to inhibit the proliferation of these cells (Shen *et al.*, 2014; Bao *et al.*; Cheng *et al.*). Several investigations revealed that different concentrations of L-carnosine inhibit the activity of mitochondrial respiratory chain complexes in human gastric cancer cells and cervical gland carcinoma cells (Shen *et al.*, 2014; Bao *et al.*; Cheng *et al.*). However, most studies were conducted on cancer cell lines, so little is

known about L-carnosine effects on the mitochondrial respiratory chain of normal cells. In the current study, we measured mitochondrial respiration rate and the respiratory chain complex activity in normal fibroblasts. We found that the administration of 2 and 5 mM L-carnosine stimulates mitochondrial respiration rate.

In addition, Karnozin EXTRA® supplementation stimulates the “intact cell respiration rate” by increasing the respiration rates of complexes I, II, IV. Changes in respiration rates were also observed after the addition of ADP and FCCP. Some researchers believe that carnosine effectively preserves mitochondrial membrane potential (Hansen *et al.*, 2010; Shen *et al.*, 2010). It is known that the pH gradient and the membrane potential are important for mitochondrial function (Hansen *et al.*). Namely, some drugs cross the mitochondrial membrane and act in the mitochondrial matrix, potentially changing the pH gradient and mitochondrial membrane potential. It is known that carnosine has an important role in mitochondrial matrix pH regulation (Hansen *et al.*; Bao *et al.*). At this point, it should be noted that carnosine could affect the function of respiratory chain complexes through changes in the pH gradient of the mitochondrial matrix. Another possibility that could explain the effect of carnosine on mitochondria metabolism is that carnosine stimulates the activity of complex I, II and IV in normal fibroblasts by direct interaction with respiratory chain complexes.

Interestingly, administration of L-carnosine in 10 mM concentration led to a significant reduction in cell proliferation and recorded mitochondrial respiration rates. Having in mind the complexity of the capsule composition, we could not determine whether these results are due to the high concentration of L-carnosine, the activity of other ingredients of the capsule, or both.

Our data provide evidence about the effects of L-carnosine supplement on the healthy fibroblast cell line. Due to the stimulation of mitochondrial energy production in normal fibroblasts, our results suggested that Karnozin EXTRA® is a potentially protective dietary supplement in the prevention of diseases with altered mitochondrial function. However, further research is needed for evaluating the effects of each ingredient of the capsule.

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RESUMEN: La carnosina se conoce como dipéptido natural, que inhibe la proliferación de células tumorales a través de su acción sobre la respiración mitocondrial y la glucólisis celular. Sin embargo, no se sabe mucho de sus efectos sobre el metabolismo de las células sanas. Exploramos los efectos de la cápsula Karnozin EXTRA® con diferentes concentraciones de L-carnosina, sobre la viabilidad celular y las expresiones de vimentina de filamento intermedio (VIM) y superóxido dismutasa (SOD2) en fibroblastos normales BHK-21 / C13. Además, estudiamos su acción sobre la producción de energía de estas células. La viabilidad celular se cuantificó mediante el ensayo MTT. Se utilizó el electrodo de oxígeno Clark (Oxygraph, Hansatech Instruments, Inglaterra) para medir la “tasa de respiración de células intactas”, el estado 3 de oxidación estimulada por ADP, la capacidad máxima de oxidación y las actividades de los complejos I, II y IV. Los resultados mostraron que la cápsula de Karnozin EXTRA® en concentraciones de 2 y 5 mM de L-carnosina no indujo efectos tóxicos ni cambios morfológicos en las células tratadas. Nuestros datos revelaron una amplificación de señal inmunofluorescente dependiente de la dosis de VIM y SOD2 en la línea celular BHK-21 / C13. Este suplemento aumentó sustancialmente las tasas de respiración mitocondrial registradas en la línea celular examinada. Debido a la estimulación de la producción de energía mitocondrial en fibroblastos normales, nuestros resultados sugirieron que Karnozin EXTRA® es un suplemento dietético potencialmente protector en la prevención de enfermedades con función mitocondrial alterada.

PALABRAS CLAVE: Carnosina; Fibroblastos; Metabolismo energético; Vimentina; Superóxido dismutasa 2.

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