Effect of Maca (Lepidium Meyeni W.) on Levels of Induced Hsp72 Deregulation

Medina Pérez J.[1,2], Villanueva Salas J.[1,2], Carpio Carpio J. [1,2], Villanueva Sardón L.[1], Julitza Paredes-Fuentes [1,2], y Bernabé-Ortiz Julio C.[2]

[1] Laboratorio de Investigación en Biología molecular y farmacología experimental, Universidad Católica de Santa María, Arequipa, Perú.

[2] Escuela de posgrado de Universidad Católica de Santa María, Arequipa, Perú.

Correspondencia: E-mail: jbernabe@ucsm.edu.pe

Resumen

Heat shock protein (Hsp) 72 is a molecular chaperone that is overregulated under conditions of stress and proteotoxic damage, associated with cellular protection. The Peruvian tuber maca Lepidium Meyeni W. has pharmacological properties associated with neuroprotection. To determine their relationship, this study examines the effect of H2O2 and β-amyloid as toxic agents on the differentiated SH-SY5Y cell line causing deregulation of Hsp72 and the effect presented by pentanic extracts of red, yellow and black maca hypocotyls at concentrations of 0.5 and 1 ppm by RT-PCR and immunofluorescence. The expression of Hsp72 caused by toxicants was high, accompanied by a translocation process, evidencing the protective effect on the nucleus of neuroblastoma cells by Hsp72 under stress conditions, the increase of Hsp72 expression has been regulated with the previous application of maca extracts, evidencing the permanence in the presence and concentration of Hsp72 in cytoplasm, thus showing the protective effect of maca by inhibiting the need for the action of Hsp72 as neuroprotective of the nucleus.

Keywords: Hsp72, maca, neurotoxicants, neuroprotection

1. Introduction

Heat Shock Proteins (HSPs) are involved in the repair and degradation of damaged proteins following a stress process, they also act in the stabilization and folding of nascent proteins [1,2], have similar biologically important roles in many organisms [3], maintain cellular homeostasis and cellular stress response, including molecular chaperones and high temperature induced proteins as well as proteases, ubiquitin and dehydrins [4]. HSPs follow a classification according to their molecular weight, such as HSP90 (85-90 kDa), HSP70 (68-73 kDa), HSP60, HSP47 and small HSP (12-43 kDa) [5] and are located in various cellular compartments, HSP10, HSP60 are located in mitochondria while there are others in the cytoplasm, reticulum, cytosol, endoplasm and nucleus [6].

HSPs shows a structure consisting of 4 regions: an N-terminal domain site of regulation by ATP activity, a glycine- and the phenylalanine-rich region adjacent to the N-terminal domain, a cysteine-rich region, and finally, the C-terminal region, their chaperone activity is achieved by binding the hydrophobic segments of the peptides to the proteins [7].

HSP70 represents a large subfamily, encoded by 13 genes in humans, located on chromosomes 1, 5, 6, 9, 14 and 21, abundant in eukaryotic cells, where they act as chaperones together with others such as Hsp40, Hsp90 and Hsp110. They participate in the elimination of damaged or defective proteins by interacting with the C-terminal end of Hsp70 (CHIP protein), which is an E3 ubiquitin ligase [8,9].

Among them, Hsp72 constitutes the classical stress-inducible cytoplasmic Hsp, which serves as a helper during stressful situations, with cytoprotective properties; its overexpression has been proven to protect against the formation of protein aggregates in neurodegenerative disorders or obesity-induced insulin resistance [10].

Maca (Lepidium meyenii Walpers) is an Andean brassica grown between 3,950- and 4,500 meters altitude, considered a nutritional, functional, nutraceutical food and even a candidate adaptogen. The hypocotyl, which represents the edible part of maca is distinguished according to different colors, but in general, it contains: proteins, lipids, hydrolyzable carbohydrates, the good profile of essential amino acids, saturated and unsaturated fatty acids, contains secondary metabolites such as glucosinolates, imidazole alkaloids, macaenes, macamides, macahidanto-inas and meyeniinas [11,12,13].

Macamides have been linked to the pharmacological activity of maca [14], as well as its fatty acid amide hydrolase (FAAH) inhibitory action [15].

The study evaluated the effect of extracts of 3 phenotypes of maca on the downregulation of HSP72 induced by hydrogen peroxide or β-amyloid application on retinoic acid-differentiated SH-SY5Y human neuroblastoma cells. These results show that maca extracts can protect from the effect of both neurotoxicants in neuroblastoma cells.

2. Materials and Methods

2.1 Materials

yellow (MA) maca hypocotyls were obtained from the Laboratory of Research in Molecular Biology and Experimental Pharmacology (Catholic University of Santa Maria, Arequipa, Peru), which were dissolved

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in DMSO 0.5% (dimethyl sulfoxide) provided by Sigma Aldrich as well as hydrogen peroxide used as a toxic stressor. The retinoic acid (ATRA all trans retinoic acid) used for cell differentiation to neuronal phenotype was obtained from Merck KGaA, and the amyloid peptide of 25-35 amino acid fraction requested from AnaSpec was dissolved in PBS 1x (Merck KGaA phosphate buffered saline).

2.2 Cell culture and treatments

The cell line used was human neuroblastoma SH-SY5Y (ATCC 94030304), the DMEM medium (Dubelco's modified Eagle's medium), trypsin-EDTA, fetal bovine serum (FBS) and penicillin-streptomycin and neomycin solution were obtained from Sigma Aldrich. Cells were cultured in DMEM medium supplemented with 10 % SBF and 1 % antibiotic, maintained in an environment with 5 % CO2 and 37 oC until reaching 80 % confluence, then cultured with DMEM medium with 2 % SBF and 10 µM retinoic acid for 7 days to allow differentiation [16].

On a cell density of 5x105 cells/mL, maca extracts dissolved in DMSO 0.5 % were applied and divided into groups as follows: Red Maca 0.5 ppm (Group I), Red Maca 1 ppm (Group II), Yellow Maca 0.5 ppm (Group III), Yellow Maca 1 ppm (Group IV), Black Maca 0.5 ppm (Group V), Black Maca 1 ppm (Group VI) and DMSO 0.5 % (Control) for 18 hrs.

On the other hand, in the same groups described above, 2 treatment schemes were applied with 2 neurotoxic agents after 24 hours of contact with the macro extracts: hydrogen peroxide 25 µM-18 hr and β-amyloid peptide 10 µM-18 hr and control groups, after the treatment time, the total RNA was extracted from the cells for the evaluation of Hsp72 expression.

2.3 Hsp72 expression by RT-PCR

Total RNA was isolated from the cell homogenate by the Trizol method (TRI Reagent Sigma Aldrich) using the Cold Spring Harbor Laboratory Press protocol [17], the concentration obtained was determined on a ®NP80 nanophotometer (All Work ©IMPLEN), and a volume corresponding to a final concentration of 25 ng/µL in the PCR mix was transcribed for cDNA synthesis following the protocol of the OneScript cDNA synthesis kit (Applied Biological Materials Inc. abm). The cDNA was then tested by conventional and real-time PCR for expression of the HSPA2 gene (HspA2-F 5'TTGTTGGAAGTCCTTGGTATA3' and HspA2-R 5'CATTTGCATTTATGCATGCATTTGTGT3') concerning GAPDH (GAPDH-F 5'GAAGGTGAAGGTCGGAGTC3' and GAPDH-R 5'GAAGATGGTGATGGATGGGATTGATTTC3').

Amplification of both genes was carried out using GoTaq® qPCR Master mix (©Promega Corporation) and 2X PCR Taq Plus Master mix (Applied Biological Materials Inc. abm).

2.4 Immunofluorescence

To evaluate the localization of Hsp72, SH-SY5Y cells were cultured on slides in wells of plates at a concentration of 1x105 cells/mL, which were fixed with 4% paraformaldehyde for 30 min at room temperature, then neutralized with 50 mM ammonium chloride in PBS for 20 min, washed 3 times with 1X PBS, and incubated for 2.5h, were washed 3 times with 1X PBS, permeabilized with a mixture of 0.05 % Triton 20 % and 3 % SBF for 50 min, incubated for 2.5 h with 1:500 primary anti-Hsp72 (HPA000798 Sigma-Aldrich®). The anti-Hsp72 was detected using alexa fluor® 568 labeled goat anti-mouse secondary antibody 1:1000 (© Abcam plc.) incubated for 1.5 h in the dark, together with hoechst 33342 (© Abcam plc.) to observe the cellular genetic material, the slides were washed 3 times by PBS and placed in the fluorescent mounting medium. Cells were observed on an inverted microscope with an integrated camera ZOE Fluorescent cell image (© Bio-Rad Laboratories, Inc.).

2.5 Statistical analysis

Data were expressed as mean ± standard deviation of at least 3 experiments for each experiment. Data were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's test using Graph Pahd Prism 9.4.0 for Windows, differences were considered with a significance value of $p<0.05$.

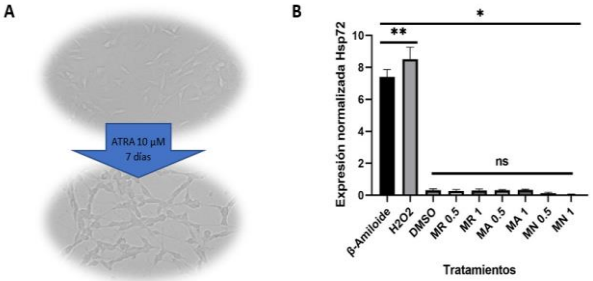

*Figure 1. Morphology of undifferentiated and differentiated SH-SY5Y cells (A), images obtained at 20x magnification showing non-differentiated cells (top) lacking neuritic projections as opposed to the long projections observed from day 3 of differentiation (bottom) with 10 µM ATRA in 2% SBF culture medium. Quantification of normalized expression of Hsp72 (B) in SH-SY5Y cells treated with 10 µM β-amyloid, 25 µM H2O2 and 0.5 or 1 ppm of MR, MA and MN in DMSO 0. 5 % for 18 hrs, data are shown as mean ± ds after normalization with GAPDH, where there is increased expression of Hsp72 versus neurotoxicants, there being significant evidence among all groups *<0.0001 and among neurotoxicants **0.0064, but between DMSO and macaques there is no difference ns p>0.9660.*

3 Results and Discussions

3.1 Hsp72 upregulation altered by hydrogen peroxide and β-amyloid and not by maca extracts.

The evaluation of Hsp72 expression was performed on cells differentiated with retinoic acid in 7 days since a neuronal phenotype model capable of having morphological and biochemical properties of a neuron is required to evaluate neurotoxicity and neuroprotection, which is the subject of this study.

In **Figure 1 (A),** the morphological characteristics of differentiated cells versus non-differentiated cells were observed, clearly noting the extension of neurites evidenced from the 3rd day of contact with ATRA, in addition, a decrease in the speed of proliferation was noted, remaining in contact with ATRA for at least 7 days

to be used in the experiments. This is because it is known that during this differentiation process, especially from the 4th day, there is an increase in neuromarkers such as thyroxine hydroxylase TH, neuronal nucleic protein (NeuN) and neuron-specific enolase (NSE) and only on the 7th day the levels of Nestin typical of a non-differentiated cell begin to decrease considerably [18].

In the RT-PCR analysis of Hsp72 for the first treatments Figure 1 (B), it is revealed that in comparison of all groups there is a significant difference *<0.0001, even between both neurotoxicants **0.0064, however, the marked increase in upregulation caused by β-amyloid, and H2O2 is noticeable. One of the primary intracellular responses as a defense mechanism is the response to heat shock caused by stressors such as oxidative stress or ROS, this response happens mainly through HSF (Heat shock transcription factor), which links environmental stress and cellular response [19]. The β-amyloid peptide is a neurotoxicant associated with neurodegenerative diseases due to the activation of pathogenic mediators such as oxidative stress; recognized for having favorable effects as an antioxidant at very low concentrations (<1 μM) [20]; but reported as an agent that promotes inflammatory response at higher concentrations, activating nuclear factor κB/p65 (NF-κB/p65), increasing the expression of cytokines and mediators such as protein kinase (ERK)-regulated MAPKs, Jun N-terminal kinase (JNK) and p38 [21].

Hsp72 belongs to a highly conserved family of Hsp, it is highly inducible in response to various stressors [22], it was demonstrated that the application of β-amyloid 10 μM and H2O2 25 μM on human neuroblastoma cells differentiated to neuronal phenotype, induced the upregulation of Hsp72 in 18 hr.

Maca (Lepidium meyenii Walpers), considered a nutritional, functional and nutraceutical food, has been studied for its antioxidant action for a long time and proposed as a neuroprotective agent; however, it is important to highlight the type of extraction performed on this plant material, given that the choice of the extraction method as well as its solvent directly affects the isolated metabolome [23,24] and may cause important actions to be omitted by using, for example, a hydrophilic solvent when the metabolites of interest have a lipophilic character [25]. Since the aim of the study was to evaluate the neuroprotective activity of maca extracts, pentanic extracts of red, yellow and black maca hypocotyls were used, and it was observed that the addition of these extracts at low concentrations, as shown in **Figure 1 (B),** do not induce the overregulation of Hsp72, which would indicate that they do not represent a stressor that produces the need to activate cellular defense, with no difference *** p>0.9660 between the different extracts and the control (DMSO).

3.2 Protective effect of maca on Hsp72 regulation

A marked difference was found in **Figura 2**, between Hsp72 expression shown by SH-SY5Y cells with exposure to maca extracts before contact with stressors versus cells that received only β-amyloid or H2O2 application *p<0.0001, demonstrating that maca extracts regulate the toxicity caused by stressors.

Hsp72 possesses a housekeeping function and is associated with a variety of cellular processes, under stress conditions its activation reduces cellular stress, oxidative stress, promotes cell survival and refolding of misfolded proteins and degradation of misfolded protein aggregates by ubiquitin proteasome system (UPS) and autophagy-lysosomal pathways [26]. This cellular protective function of Hsp72 has been revealed by the application of β-amyloid in cells, since amyloid peptides can accumulate as oligomers and aggregate as fibrils [27], Hsp72 could recognize them as toxic forms and trigger their overexpression inhibiting amyloid aggregation, also, Hsp72 has been linked as a regulator of tau homeostasis, preventing abnormal tau aggregation directly [28] protecting neural pathways by inhibition of apoptotic activity via SAPK(JNK) [29].

*Figure 2. Regulation of Hsp72 in SH-SY5Y cells; Quantification of normalized Hsp72 expression in SH-SY5Y cells pretreated with 0.5 or 1 ppm MR, MA and MN for 18 hr and subsequently with 25 μM H2O2 for 18 hr. (A) the data show a difference between cells treated only with H2O2 and those that received pretreatment with maca extracts *p<0.0001, evidencing a lower expression of Hsp72 in all cells that received a pretreatment, noting the same trend for each type of maca as there was no difference between them (ns, p>0. 6224), Quantification of normalized Hsp72 expression in SH-SY5Y cells pretreated with 0.5 or 1 ppm of MR, MA and MN. for 18 hr and subsequently with 10 μM of β-amyloid for 18 hr. (B), difference is demonstrated between neurotoxicant application alone and with pretreatment *<0.0001, all data are shown as mean ± ds after normalization with GAPDH However, the initial exposure with maca extracts before contact with H202 25 μM Figure 2 (A) significantly decreased (*p<0.0001) the expression of Hsp72 reaching levels close to the control, indicating the protective effect exerted by the maca extracts, which between ecotypes showed a difference, but not between concentrations for each ecotype p<0.14, noting a protective effect up to a concentration of 1 ppm against the toxic action of H202.*

A similar effect was shown by exposure with β-amyloid 10 μM after the application of maca extracts at the same concentrations, shown in Figure 2 (B), again showing a difference between the expression caused by the neurotoxicant and the probable protectors *p<0.0001, with higher expression values in the case of the groups with red maca and black maca and lower in both concentrations of yellow maca, it can be concluded that yellow maca has a neuroprotective effect at concentrations of up to 1 ppm, while in the case of the other ecotypes it would be necessary to evaluate whether they have a protective effect at higher concentrations.

The effect provided by the pentatonic extracts of maca would be exerting a protective role against neurotoxicants such as β-amyloid and H202, it could be presumed that having in its contents secondary metabolites such as macamides which have structural similarity with the endocannabinoid agonist anandamide and being considered as inhibitors of fatty acid amide hydrolase (FAAH), could be promoting its protective action by blocking the hydrolysis of anandamide to increase its endogenous levels and maintain its action [15], in

addition to the characteristics of some electron-donating macamides that could neutralize the action of pro-oxidant agents.

Figure 3. Indirect immunofluorescence for Hsp72 in SH-SY5Y cells. The A, B, C control cells were compared with treatments performed with exposure to neurotoxicants H2O2 25 µM D, E, F and β-amyloid 10 µM G, H, I, as well as pre-treatment with MN 0.5 ppm for 24 hours and subsequent contact with H2O2 25 µM J, K, L and H2O2 25 µM M, N, O, and then treated with specific anti-Hsp72 antibody.

3.3 Localization of Hsp72 in SH-SY5Y neuroblastoma cells

Immunofluorescence images at 40x magnification show a translocation effect produced by the contact of neuroblastoma cells with the neurotoxicants H2O2 25 µM and β-amyloid 10 µM, where the initial cytoplasmic position of Hsp72 (B) is observed in unstressed cells and, after exposure to the neurotoxicants the distribution is directed towards the nucleus evidencing the protective effect on the nucleus (E, H); however, the localization of Hsp72 remains intact in cytoplasm when cells are exposed to previous treatment with maca for both toxicants (K, N), which could indicate that the translocation has been unnecessary as Hsp72 levels have been regulated to control values.

4 Conclusions

Maca extracts have a protective role against the toxic action of H2O2 25 µM and β-amyloid 10 µM at concentrations of 0.5 and 1 ppm, being greater its protective effect against H2O2, this could be due to the presence of electron donor groups present in the structures of some secondary metabolites of maca. The neurotoxic effect of H2O2 and β-amyloid in SH-SY5Y neuroblastoma cells was confirmed by the up-regulation of Hsp72, which by indirect immunofluorescence indicates a translocation process as a mediator of nuclear protection.

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