



Tau Protein and Its Phosphorylation in Alzheimer's Disease When Lepidium Meyenii (Maca) Acts as A Neuroprotective Agent

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Abstract

The early stages of Alzheimer's disease (AD) are characterized by the accumulation of β -protein and high phosphorylation of the microtubule-associated protein tau. There is still no cure for this neurodegenerative disease. So, medicinal plants of the territory are used, as well as secondary metabolites that can help to decrease or probably stop this process. *Lepidium meyenii* (maca) can have a great influence on this problem. The authors worked with the human neuroblastoma cell line SH-SY5Y, which after being conditioned and differentiated with 10 μ M Retinoic Acid (RA) for 5 days, the following working groups were formed: Group 1 (cells + β -A 10 μ M); Group 2 (cells + MA 5ppm/24h, cells + MR 5ppm/24h, cells + MN 5ppm/24h); Group 3 (cells + MA 5ppm/24h + β -A 10 μ M/24h, cells + MR 5ppm/24h + β -A 10 μ M/24h, cells + MN 5ppm/24h + β -A 10 μ M/24h). Three ecotypes of *Lepidium meyenii* (macaamarilla, red and black) 5pp, were plated on differentiated cells for 24 hours, then peptide β -Amyloid (42) was added at a concentration of 10 μ M for 24 hours. Tau protein expression was evaluated by immunoblotting, using antibodies that recognized endogenous tau (Tau 46) and phosphorylated tau (Phospho-Tau Ser396). It was observed that exposure of SH-SY5Y cells to β -A 10 μ M, increased Tau phosphorylation. The three ecotypes of maca failed to exert a neuroprotective effect by inhibiting Tau hyperphosphorylation, however, there was evidence of increased Tau protein expression when exposed to the three different types of maca. 866

Key Words: Tau protein, Phosphorylation, *Lepidium meyenii*, Ecotypes, β -Amyloid

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Introduction

Alzheimer's disease (AD) is a neurodegenerative process marked by two neuropathological features: the formation of neurofibrillary tangles (NFT) and senile plaques (SP) (1). The formation of the aforementioned NFTs results in AD due to the accumulation of tau proteins in their phosphorylated form called NFTs or paired helical filaments (PHFs) (1). Tau is a highly soluble protein, not normally found in misfolded aggregates. It is expressed mainly, but not exclusively, in neurons (2). Tau binds to microtubules and stabilizes them. It appears to regulate microtubule assembly and function. The interaction between microtubules and tau is

regulated by the degree of tau phosphorylation. Phosphorylated tau dissociates from microtubules and can aggregate and form paired helical filaments (PHFs) and eventually form NFTs (3). The dissociation of tau from microtubules in a soluble cellular pool destabilizes microtubules (4). Under normal physiological conditions, tau can be phosphorylated at various epitopes that probably serve to regulate its interaction with microtubules (5). Now under pathological conditions, tau becomes hyperphosphorylated and becomes more prone to dissociate from microtubules and self-aggregate. NFTs contain large amounts of hyperphosphorylated tau, although it is unclear

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whether phosphorylation is incidental to aggregation (6).

Phosphorylation is extensively studied and is quite common in the tau protein. It has been observed that dozens of amino acids are phosphorylated, including serines, threonines and tyrosines in all areas of the protein (2).

In Peru as worldwide, there is no successful cure for this neurodegenerative disease, according to the World Health Organization (WHO) estimates that by the year 2030 the number of people who will develop Alzheimer's will be approximately 78 million; also, the increase in the cost of care worldwide has increased from 1.3 to 2.8 billion dollars worldwide. That is why it is important to resort to the Peruvian flora and focus on the *Lepidium meyenii* (maca) for its properties already known at the neurological level, it is known that there are 3 ecotypes or varieties of maca, Yellow, Red and Black, and that some of its active principles such as Macamides (7) could act by inhibiting the abnormal phosphorylation of the tau protein, thus allowing this protein not to destabilize, preventing it from forming neurofibrillary tangles and senile plaques, resulting from the activation of AD.

This review focuses on the tau protein associated with the microtubules of the cytoskeleton, the main element involved in the formation of PHF and the trigger of AD. We tested the activity of macamides extracted from 3 ecotypes of maca (yellow, red and black), which could exert a neuroprotective effect on the human neuroblastoma cell line SH-SY5Y, preventing the abnormal phosphorylation of Tau when these cultures are exposed to the β -A amyloid-peptide (β -A).

Methodology

Cell Culture and Treatments

In the first stage, human neuroblastoma SH-SY5Y cells (ATCC 94030304) were used, which were cultured in flasks at a concentration of 1.5×10^5 cells/mL in Dulbecco's modified Eagle medium, 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, 1% essential amino acids, were obtained from Sigma Aldrich; they were incubated at 37°C in a humid atmosphere with 95% aeration and 5% humidity and incubated at 37°C in a humidified atmosphere with 95% aeration and 5% humidity CO₂ for 24 hours. For cell differentiation, Retinoic Acid (RA) was added at a final concentration of 10 μ M(8), the day after seeding in the flask, with SFB also at 2% and exposed for 5

days to RA, then the cells were washed (DMEM) and the culture medium was changed by removing the SFB, 24 hours before starting the treatments and assays.

Subsequently in SH-SY5Y cells (3×10^5 cells/mL) treatments based on 3 ecotypes of *Lepidium meyenii* (yellow (MA), red (MR) and black (MN) maca) were added at a concentration of 5ppm in Dimethyl Sulfoxide (DMSO, Sigma Aldrich) and β -Amyloid (β -A). Group 1 (cells + β -A 10 μ M); Group 2 (cells + MA 5ppm/24h, cells + MR 5ppm/24h, cells + MN 5ppm/24h); Grupo 3 (cells + MA 5ppm/24h + β -A 10 μ M/24h, cells + MR 5ppm/24h + β -A 10 μ M/24h, cells + MN 5ppm/24h + β -A 10 μ M/24h).

Lysate preparation and protein extraction

After preparation of the study groups, the samples were collected, the cells were washed in cold (4°C) phosphate buffered saline (PBS) 3 times and NP-40 Lysis Buffer (containing phosphatase and protease inhibitors) with 2% Triton was added, then incubated for 2 minutes at 4°C. Cells were removed and collected by centrifugation at 4°C at 16000 rfk for 30 minutes. The supernatants were collected and the protein concentration was measured. The supernatants of the upper phase were resuspended in Laemmli buffer (50 mM Tris-HCl, pH 6.8, 2% sodium dodecylsulfate) (SDS), 2 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1 combined protease inhibitor tablet "Protease solution" per 55 ml (BIOSEARCH TECHNOLOGY), 1 mM phenylmethylsulfonyl fluoride (added from a 100x concentrated solution in DMSO), 10 mM sodium fluoride, β -glycerophosphate 20 mM and 2 mM sodium orthovanadate). in 2x Laemmli buffer without a pre-wash step. The β - mercaptoethanol (2-5 %) was present in the lysis buffer and samples were denatured at 90 °C or 95 °C for 5-15 min. Samples were centrifuged at 20,000 g for 10 min at 4 °C, the supernatant was collected, mixed with Laemmli buffer containing 5 % β -mercaptoethanol and incubated at 95 °C for 5 min.

Immunotransfer

Proteins from cell extracts extracted from each study group were separated on 10% SDS-PAGE gels (Merck) and transferred to a PVDF membrane in transfer buffer (25 mM Tris, 192 mM glycine) using a voltage of 120 V for 150 min. The membrane was blocked in 1% milk for non-phosphorylated



proteins and 4% BSA for phosphorylated proteins, both diluted in TBS-T. Subsequently, groups G1, G2 and G3 were incubated with primary antibody diluted in TBS with Tween 20 (TBS-T) and 0.1% milk, and groups G1, G2 and G3 were also prepared with a primary antibody with TBS-T and 0.1% fetal bovine serum. Both were incubated under constant agitation for 3 hours (4°C) and then overnight refrigerated and stored at 4°C. The primary antibodies and their working dilutions used were: Tau46 (ThermoFisher SCIENTIFIC, Cat. 13-6400, 0.75:1000 dilution) and Phospho-Tau Ser396 (ThermoFisher SCIENTIFIC, Cat. 44-752G, 1:1000 dilution). The membranes were washed 3 times with TBS-T for 10 minutes, then the membranes were exposed to the secondary antibody, and the preparation was performed according to the manufacturer's recommendations.

The secondary antibody was horseradish peroxidase (HRP)-bound IgG (Cell Signaling, cat. no. 7074 and 7076), used at a dilution of 1:80000, mixed with TBST 0.1% milk (for Tau) and 0.1% BSA (for p-Tau) both in TBS-T. Proteins were visualized using 3,3 Diaminobenzidine at a concentration of 0.60 mg/mL (Sigma Aldrich, D8001) and the

GelDoc Go Imaging System Features imager with Image Lab Touch software (BioRad Laboratories, Hercules, CA, USA).

Results

Retinoic Acid Cell Differentiation (AR)

It should be recalled that the human neuroblastoma cell line SH-SY5Y is a third successive subclone of the SK-N-SH line, originally established from a bone marrow biopsy of a neuroblastoma patient (9). SK-N-SH comprises at least two biochemically and cytologically distinct phenotypes: one neuroblastic (N-type) and one substrate-adherent (S-type), which can undergo transdifferentiation (10). Although derived from a neuroblastic subclone, the SH-SY5Y line retains a low proportion of S-type cells. Human neuroblastoma SH-SY5Y cells have been exposed to Retinoic Acid (10uM) in a 2% DMEM medium for 5 days and fully differentiated cells were obtained. Short treatments with RA inhibit proliferation and allow cell differentiation; however, cells exposed to more than 8 to 10 days of RA progressively increase cell growth (Figure 1).

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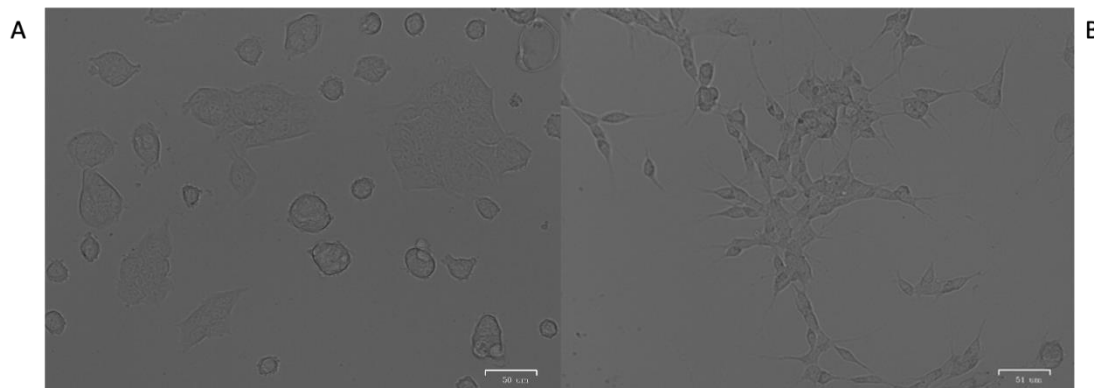


Figure 1. Human Neuroblastoma Cell Line SH-SY5Y, A. Undifferentiated cells 24 hours after culture. B. Differentiated cells with Retinoic Acid (10uM) after 5 days of treatment.

SH-SY5Y cells treated with 3 ecotypes of maca (MA, MR and MN 5ppm)

Phosphorylation was evaluated in cells treated with three different types of maca (MA, MR and MN), all at a concentration of 5ppm. Maca ecotypes were added to SH-SY5Y human neuroblastoma cells for 24 hours, proteins from total protein lysates were separated using denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and T46 antibodies were used that recognize the endogenous tau state in SH-SY5Y. It was observed

that cells translated with (MA, MR and MN 5ppm), slightly increased Tau expression, evidenced by the formation of protein bands between 50-65kDa, for the control (cells+DMSO) (Figure 2-A), when phosphorylation was detected using primary antibodies that recognize specific tau sites (S396), the formation of 2 protein bands between 50-65kDa was observed, showing higher expression in the control (cells+DMSO) and lower expression in the cells exposed to the three ecotypes of Maca (Figure 2-C).

The effect of the three ecotypes of maca (MA, MR and MN 5ppm) on SH-SY5Y cells, exposed to β -Amyloid

Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the components of total cellular protein lysates. Phosphorylation of tau at specific epitopes was assessed by immunoblotting using phosphospecific tau antibodies. Using the antibody that recognizes phosphorylated tau (S396), protein bands with an

apparent molecular weight of 50-65kDa were observed in samples from treated (with MA, MR and MN + β -A 10uM +) and untreated (β -A 10uM -) cells (Figure 2-D). An increase in Tau protein expression was observed in the different ecotypes of maca. It can be seen that the black maca ecotype presents a lower signal, compared with the yellow and red ecotypes, with the control (cells+ β -A 10uM) having a lower signal than the other three samples already mentioned.

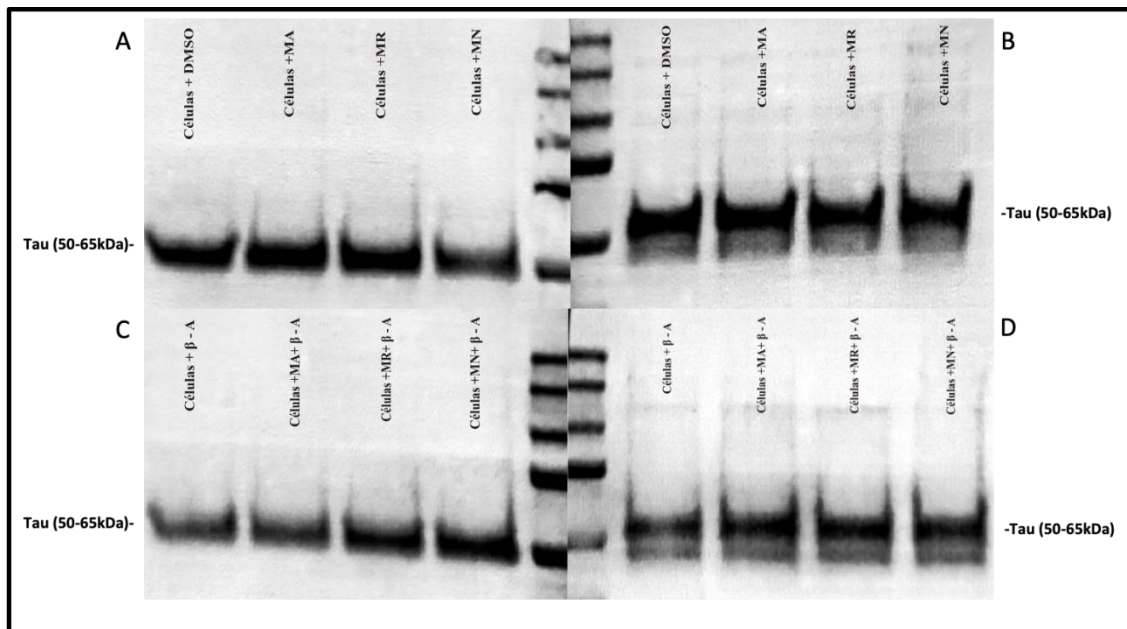


Figure 2. Immunoblotting of Tau (46) and Phospho-Tau (Ser396). A. SH-SY5Y cells exposed to three ecotypes of maca (MA, MR and MN) at 5ppm, as a result, endogenous Tau (T46) is expressed. B. Represents cells exposed to three ecotypes of maca, where the expression of phospho-Tau (Ser396) is evidenced, phosphorylated Tau protein is found with higher expression. C. SH-SY5Y cells with 5ppm (MA, MR and MN) for 24h and β -A 10uM for 24h later, higher expression of endogenous Tau is observed. D. Yellow, Red and Black Maca at 5ppm were added to cells for 24h with β -A 10uM for 24h also, no inhibition of phosphorylation but increased Tau expression was observed.

The same samples were analyzed with T46 tau antibody that recognizes endogenous tau (Figure 2-B). In cells treated with β -A (10uM) a less intense signal is visualized at ~ 50-65 kDa, than those cells treated with MA, MR and MN (5ppm/24h) + β -A (10uM/24h), the intensity levels in the bands corresponding to MA, MR and MN have increased for control.

Conclusions

The cell culture model SH-SY5Y is related to Tau protein phosphorylation when this cell line is exposed to a stressor agent such as β -Amyloid. This leads to the appearance of 50 to 65 kDa protein

species immunoreactive to phospho-tau S396 and Tau 46 specific antibodies. We have been able to determine that the concentration of cells used for immunoblotting assays is 3×10^6 cells/flask.

Regarding the cell differentiation process, the use of Retinoic Acid (10uM) is already known and the effects of RA on SH-SY5Y are well documented. These include attenuation of the proliferation rate, the extension of neuritic processes and the development of a slightly increased choline acetyltransferase activity (11); this occurs when SH-SY5Y cells are incubated with RA (10uM) for 5 days in 2% SFB DMEM culture medium, this information is based on existing studies indicating incubation with RA for 5 to 7 days.

Subsequent studies mention that SH-SY5Y cells are susceptible to developing cellular stress (14), one of the physiological conditions of cellular stress is the overproduction and accumulation of β -A (15), forming neurofibrillary plaques. In vitro studies have stressed neuronal cells using H_2O_2 (14), β -A has also been frequently used in other investigations (13) The β -A synthetic peptide at a concentration of 10uM, has been used as a stressor agent in SH-SY5Y cells, showing a cellular response at the protein level, i.e., this stressor agent would be increasing the expression of the Tau protein.

Lepidium meyenii (Maca) has been par excellence one of the most studied Peruvian tubers in matters related to neurodegenerative diseases, extracts of Maca have been analyzed which produce different pharmacological effects in animals and humans (12). The hypocotyls of *Lepidium meyenii* have proven to be the most interesting at the pharmacological level; lipophilic extracts of maca concentrate most of the active components of this Andean tuber. Our study has been developed to demonstrate the possible neuroprotective properties of the pentanic extracts of the three ecotypes of Maca in vitro models trying to demonstrate a protective effect at the cellular level. The ecotype Maca Negra has been one of the most studied types of maca, demonstrating in some works the reduction of stress induced by H_2O_2 (12). The present study used a 5ppm concentration of three pentanic extracts of *Lepidium meyenii*, trying to find the inhibition of tau hyperphosphorylation upon stress with β -A(10uM). However, the neuroprotective effect of the three ecotypes of maca (MA, MR and MN) has not been demonstrated, but it has been verified that cells treated with *Lepidium meyenii* (5ppm) for 24 h, and then exposed to the β -A(10uM) stressor agent, have a neuroprotective effect, increase the expression of Tau protein in the Western Blot analysis for the control, which would demonstrate that *Lepidium meyenii* would favor other functions of the Tau protein such as the regulation of axonal transport of nutrients, neurogenesis, regulation of signaling pathways into the neuronal interior and neuronal polarity.

Finally, the neuroprotective effect of pentanic extracts of maca inhibiting Tau hyperphosphorylation is not ruled out, which may occur using other concentrations of *Lepidium meyenii*, but further studies with different concentrations of maca are recommended. The neuroprotective effect may be found upstream or

downstream of tau hyperphosphorylation events, suggesting a broader plane of investigation. In addition to phosphorylation, other posttranslational modifications in the tau protein are known. It is clear that these mechanisms that cause tau aggregation are not yet elucidated, but it is possible that posttranslational modifications other than phosphorylation also regulate this process. These modifications with acetylation, nitration, and glycosylation (19, 20), could interact with phosphorylation events or may affect tau function independently of phosphorylation.

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