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Original article

Physalin F, a seco-steroid from *Physalis angulata* L., has immunosuppressive activity in peripheral blood mononuclear cells from patients with HTLV1-associated myelopathy

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ABSTRACT

Human T-lymphotropic virus type 1 (HTLV-1) induces a strong activation of the immune system, especially in individuals with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Physalin F is a secosteroid with potent anti-inflammatory and immunomodulatory activities. The present study aimed to investigate the effects of physalin F on peripheral blood mononuclear cells (PBMC) of HAM/TSP subjects. A concentration-dependent inhibition of spontaneous proliferation of PBMC from HAM/TSP subjects was observed in the presence of physalin F, as evaluated by ^{3}H-thymidine uptake. The IC_{50} for physalin F was 0.97 ± 0.11 μM. Flow cytometry analysis using Cytometric Bead Array (CBA) showed that physalin F (10 μM) significantly reduced the levels of IL-2, IL-6, IL-10, TNF-α and IFN-γ, but not IL-17A, in supernatants of PBMC cultures. Next, apoptosis induction was addressed by using flow cytometry to evaluate annexin V expression. Treatment with physalin F (10 μM) increased the apoptotic population of PBMC in HAM/TSP subjects. Transmission electron microscopy analysis of PBMC showed that physalin F induced ultrastructural changes, such as pyknotic nuclei, damaged mitochondria, enhanced autophagic vacuole formation, and the presence of myelin-like figures. In conclusion, physalin F induces apoptosis of PBMC, decreasing the spontaneous proliferation and cytokine production caused by HTLV-1 infection.

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1. Introduction

Human T-lymphotropic virus type 1 (HTLV-1) infects 5–10 million people, mainly in Latin America, the Caribbean, South and Central Africa, and Japan [1]. The virus is the etiological agent of two major diseases, adult T-cell leukemia and lymphoma (ATL) and a progressive neurologic disease, known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), which occur in less than 5% of infected individuals[2,3]. Less frequently, the virus causes uveitis and infective dermatitis [4,5]. In addition, HTLV-1-infected individuals are more prone to other infectious diseases such as disseminated strongyloidiasis, severe scabies, and tuberculosis, suggesting an impairment in the immune response [6–10].

HTLV-1 preferentially infects memory CD45RO$^+$ CD4$^+$ and CD8$^+$ T-lymphocytes, monocytes, and dendritic cells, leading to persistent infection and strong immune activation [11–13]. Spontaneous proliferation of CD4$^+$ and CD8$^+$ T-cell subsets, as well as NK cells, are found in HTLV-1-infected individuals in both in vitro and in vivo [14–17]. T-lymphocyte activation, reduced lymphoproliferative response to recall antigens in vitro and high production of proinflammatory cytokines such as IFN-γ, TNF-α, IL-2, IL-6 and IL-10 are mainly reported in patients with HAM/TSP [15,18,19]. It has been proposed that immune activation and increased levels of cytokines play a role in both pathology and progression towards HAM/TSP [18–20].

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Since HAM/TSP was first described, little progress has been made in the development of treatment options. Due to their anti-inflammatory properties, steroids are widely used for the treatment of HAM/TSP, although few benefits have been observed [21]. *Physalis angulata* L. (Solanaceae) is a broadly distributed indigenous herb found in areas of Africa, Asia, and the Americas. It is widely used in popular medicine because of its analgesic, anti-inflammatory, and antirheumatic properties [22]. Physalins are steroid derivatives isolated from *Physalis* spp. with potent anti-inflammatory and immunomodulatory activities [22–26]. Physalin F prevents mortality induced by lethal injection of lipopolysaccharide (LPS), and inhibits rejection of allogeneic transplants in mice [22–24]. The anti-inflammatory activity of physalin F was also demonstrated in intestinal ischemia, reperfusion injury, and arthritis models [27,28]. In addition, physalin F inhibits the production of TNF-α, IL-6, IL-12, and of NF-kB, a key inflammatory transcription factor [23,26]. The purpose of this study was to investigate the immunomodulatory effects of physalin F on peripheral blood mononuclear cells (PBMC) obtained from subjects with HAM/TSP. Given the immunopathological mechanisms of HTLV-1 infection, the effects of physalin F were evaluated on spontaneous cell proliferation, cytokine profile, apoptosis, and ultrastructural changes of PBMC.

2. Material and methods

2.1. Subjects

Twenty one HTLV-1-infected subjects with HAM/TSP diagnosis defined according to World Health Organization criteria followed at Bahiana School of Medicine and Public Health reference center for HTLV in Salvador, Northeast Brazil were included in the study [29]. Samples were screened for HTLV-1/2 antibodies, using enzyme-linked immunosorbent assay (Ab-Capture ELISA test system; Ortho-Clinical Diagnostics, Inc., Raritan, NJ) and confirmed by using western blotting (HTLV Blot 2.4; Genelabs Technologies, Singapore). The group had a mean age of 61 years and consisted of 14 women (67%) and 7 men (33%). Informed consent was obtained from all enrolled subjects, and the Institutional research boarding of the Oswaldo Cruz Foundation (FIOCRUZ) approved this study (Protocol 1.011.669.)

2.2. Culture conditions and PBMC isolation

Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood samples by Ficoll-Hypaque density gradient centrifugation (Pharmacia Biotech; Uppsala, Sweden). Cells were cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MD) supplemented with 2 mM L-glutamine (Sigma–Aldrich), 1% nonessential amino acids (Gibco Laboratories, Gaithersburg, MD), 1 mM sodium pyruvate (Sigma–Aldrich), 100 U/mL penicillin (Sigma–Aldrich), 100 μg/mL streptomycin (Sigma–Aldrich), 100 μg/mL HEPES (Invitrogen, Eugene, OR), and 10% fetal bovine serum (FBS; Gibco Laboratories).

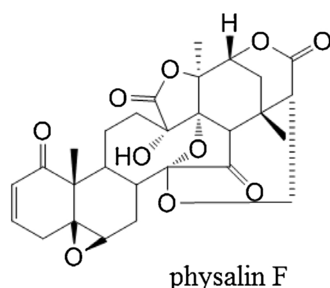

Fig. 1. Chemical structure of physalin F isolated from *P. angulata*.

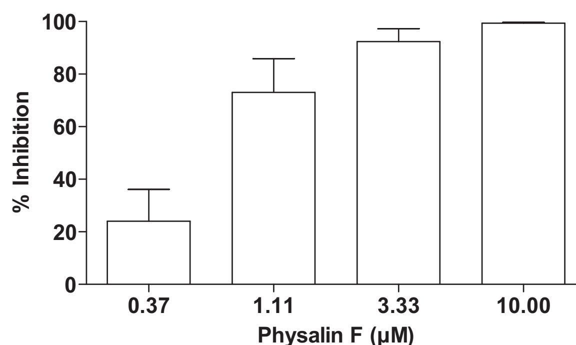

Fig. 2. In vitro inhibition of spontaneous proliferation of PBMC from subjects with HAM/TSP by physalin F. PBMC isolated from three patients with HAM/TSP diagnosis were cultured in the absence or presence of different concentrations of **1** for three days. Cell proliferation was measured by ^{3}H-thymidine incorporation using a β-radiation counter. Values represent the means ± SEM of three patients.

2.3. Test substance

Physalin F was isolated from *Physalis angulata* L. collected in Bele'm do Para', Brazil, as described previously [22]. Preparation of **1** (97.8% purity by HPLC) was dissolved in DMSO (Sigma–Aldrich) and then diluted in cell culture medium. The final concentration of DMSO was below 1% in all experiments.

2.4. In vitro cellular toxicity assay

PBMC (10^5 cells/well) from HTLV-infected patients and uninfected controls were cultured in 96-well plates in the absence or presence of serial dilutions of **1** (ranging from 0.62 to 20 μM) at 37 °C in a 5% CO_2 humidified atmosphere. After 24 h of culture, cells were pulsed for three h with 20 μL of 5 mg/mL MTT (3-[4,5-Dimethylthiazol 2yl]-2,5 diphenyltetrazolium bromide; Thiazolyl blue; Sigma–Aldrich). The optical density (OD) was determined by Versamax photometer (Molecular Devices Inc., Menlo Park, CA) at 570 nm. The toxicity was evaluated by the ratio of OD of a well in the presence of physalin F with the OD of control wells in the presence of medium. Concentrations associated to cellular viabilities equal or greater than 80% were considered non-toxic.

2.5. Lymphoproliferation assay

PBMC (10^5 cells/well) from three subjects with HAM/TSP diagnosis were cultured in the absence or presence of different concentrations of physalin F on RPMI 1640 medium supplemented with 10% FBS. Cells were seeded in triplicate on 96-well plates and cultured at 37 °C in a 5% CO_2 humidified atmosphere for 72 h. A 1 μCi/well amount of [methyl-^{3}H]thymidine (PerkinElmer, Waltham, MA) was added to the cultures, which were then incubated for 18 h at 37 °C and 5% CO_2. After this period, the content of the plate was harvested to determine the ^{3}H-thymidine incorporation using a β-radiation counter (Chameleon, Hydex; Turku, Finland). Results of cell proliferation were expressed as mean counts per minute.

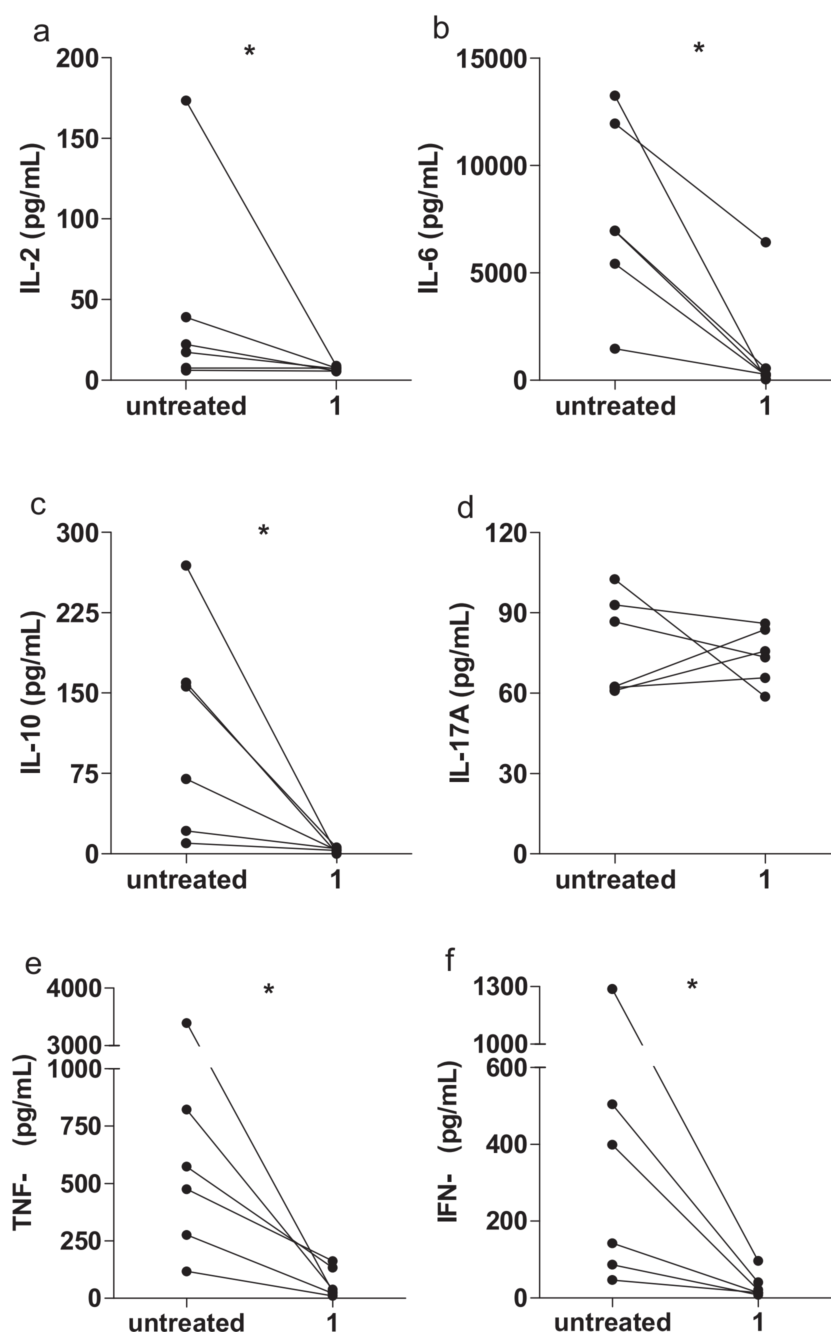

Fig. 3. Assessment of cytokines in culture supernatants of HTLV-1-infected cells. PBMC cultures were treated with 10 μM physalin F or left untreated for 18 h. Cytokine levels were measured in supernatants by Cytometric Bead Array flow cytometry. (a) IL-2; (b) IL-6; (c) IL-10; (d), IL-17α, (e), TNF, (f), IFN-γ. $^{*}P = 0.03$ (Wilcoxon and Mann–Whitney tests).

2.6. Detection of phosphatidylserine translocation by annexin V

PBMC from six HTLV-1-infected subjects were seeded on 96-well plates at a cell number of 2×10^5 and cultured in the absence or presence of 10 μM of physalin F for 18 h at 37 °C and 5% CO_2. Cells were then stained with Alexa Fluor® 488 annexin V and propidium iodide (PI), using the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen, Eugene, OR) according to the manufacturer's instructions. The cells were acquired by flow cytometry (FACSAria, Becton Dickinson, Mountain View, CA) and the analysis was performed using the FlowJo software (Ashland, OR). A total of 50,000 events were acquired in the region previously established as that corresponding to PBMC.

2.7. CBA assay

The PBMC from six HTLV-1-infected patients were plated in 96-well plates at 2×10^5 cells/well in the presence of 10 μM of physalin F at 37 °C for 24 h. Supernatants were then harvested and cytokines were measured using BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit, according to the manufacturer 's instructions (BD Biosciences, San Jose, CA). The concentrations of cytokines in the samples were calculated by extrapolating the mean fluorescence intensity (MFI) on the respective standard curves. The cells were acquired using flow cytometry (FACSAria) and analysis was performed using FlowJo and GraphPad Prism 5.02 software (Graph Pad Software, San Diego, CA).

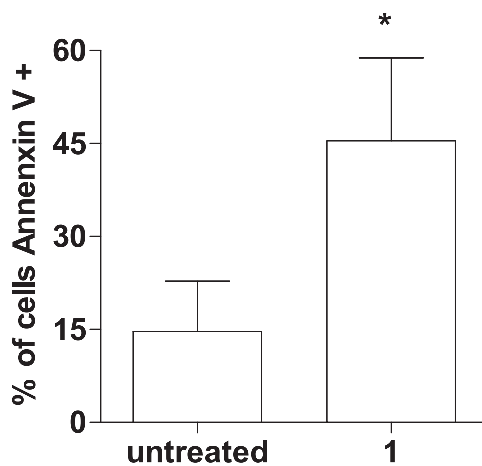

Fig. 4. Analysis of phosphatidylserine surface exposure in cells from HTLV-infected subjects. PBMC cultures were either treated with 10 μM physalin F or left untreated for 18 h. Cells were incubated with propidium iodide and annexin V and analyzed using flow cytometry. Values represent the mean ± SEM of six determinations. *$P = 0.01$ (Wilcoxon and Mann–Whitney tests).

2.8. Transmission electron microscopy analysis

For transmission electron microscopy (TEM) analysis, PBMC (2×10^6) were treated with physalin F (10 μM) and incubated for 18 h at 37 °C. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, washed in the same buffer and then treated with 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM calcium chloride, for 60 min in the dark. Cells were dehydrated in acetone series and infiltrated in polybed epoxy resin (Polysciences, Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate. The ultrastructure analysis was performed in a transmission electron microscope (Jeol JEM 1230 or Zeiss EM109).

2.9. Statistical analyses

Data are expressed as percentages, means and standard errors. Groups were compared using the Mann–Whitney test and Wilcoxon signed-rank test. A P value of less than 0.05 denoted a statistically significant difference. GraphPad Prism 5.02 software was used for all statistical analyses.

3. Results and discussion

Initially, the effects of physalin F (Fig. 1) were evaluated in cultures of PBMC obtained from HTLV-1$^+$ subjects with HAM/TSP diagnosis. The *ex vivo* system using PBMC from HTLV-1$^+$ subjects was chosen instead a cell line, such as MT2—which is a transformed HTLV-1 T-cell line, because it better represents the clinical model of HTLV-1-infected individuals. Indeed, T-cell lines are represented by a monoclonal population, while PBMC from patients have several clones of infected cells [15]. The addition of physalin F caused a concentration-dependent reduction of spontaneous proliferation characteristic of HTLV-1-infected cells (Fig. 2). The estimated IC_{50} for physalin F was 0.97 ± 0.11 μM, and this compound was not toxic to PBMC cultures when tested in concentrations below 20 μM (data not shown). Then, the effects of physalin F on cytokine production was evaluated. Cultures of PBMC obtained from HTLV-1$^+$ subjects spontaneously produced several cytokines, including IL-2, IL-6, IL-10, IL-17A, TNF-α and IFN-γ (Fig. 3). Addition of physalin F at 10 μM significantly reduced IL-2, IL-6, IL-10, TNF-α and IFN-γ production. No differences were observed in the levels of IL-17A in cultures treated with physalin F (Fig. 3). IL-4 levels were below the detection limit of the assay (data not shown).

The immunomodulatory potential of physalin F has been extensively investigated in multiple studies [22,24]. This compound has a potent inhibitory activity on mouse lymphocytes, reducing proliferation and cytokine production in cultures stimulated with mitogen or in mixed lymphocyte reaction [24]. Additionally, the effects of physalin F were investigated in vivo, in collagen-induced arthritis, allergic airway inflammation, and allogeneic transplant murine models [24–28]. Moreover, physalin F also showed antiproliferative effects on cancer cells [30]. Thus, the results obtained herein reinforce the antiproliferative potential of physalin F on human cells infected with HTLV-1.

The proviral load of HTLV-1 (amount of provirus incorporated into the genome) is mainly maintained by mitotic division of infected cells, which is in part regulated by the activation and secretion of cytokines such as IL-2 and IL-15 [17]. Herein, it was also found that physalin F strongly inhibited IL-2 production, which suggests that the reduction of PBMC proliferation may be due in part to diminished IL-2 production. HTLV-1 proviral load could be considered a marker of HAM/TSP development [31,32]. It is possible that physalin F also has an impact on HTLV-1 proviral load by reducing cell proliferation and cytokine production, however one limitation of the present study was that HTLV-1 proviral load was not measured.

Next, the effects of physalin F on apoptosis in PBMC cultures was investigated. Addition of physalin F to PBMC cultures from HTLV-1-infected subjects enhanced the percentage of apoptotic cells in a concentration-dependent way, as shown by annexin V staining (Fig. 4). Ultrastructural analysis by transmission electron microscopy revealed that, in the absence of physalin F, PBMC from HTLV-1-infected subjects presented subcellular alterations, including extensive mitochondrial damage and pyknotic nuclei (Fig. 5a–c). Cells incubated with 10 μM of physalin F showed a higher frequency of the changes described above. In addition, a remarkable swelling of the endoplasmic reticulum cisternae was observed, culminating in long ridges of cytoplasm, and autophagic vacuole-like compartments (Fig. 5d–g). Nuclei of physalin F −treated cells also presented myelin-like membranes and intranuclear membranes, presumably formed by juxtaposed autophagic vacuole or hypertrophied nucleoplasmic reticulum cysternae.

Increased percentages of apoptotic cells after treatment with physalin F may also be a factor in the reduction of PBMC proliferation as observed by both flow cytometry (increased annexin V staining) and ultrastructural alterations indicative of apoptosis and autophagy (mitochondrial damage, presence of pyknotic nuclei and myelin figures). Previous reports have shown that physalin F triggers apoptosis of cancer cells by activating caspase 3 and c-myc pathways as well as inhibiting NF-kB activation and accumulating reactive oxygen species [30,33]. Further studies are required to demonstrate the mechanisms by which physalin F induces apoptosis of HTLV-1-infected cells. The reported increased resistance of HTLV-1-infected cells to apoptosis suggests that treatment with apoptosis-inducing agents, such as observed herein with physalin F, is a relevant therapeutic approach [34].

HTLV-1 induces cell activation and intense proliferation of both subpopulations of CD4$^+$ and CD8$^+$ T-lymphocytes [14,15]. It has been reported that cells from subjects with HAM/TSP show higher levels of proliferation and pro-inflammatory cytokines production than asymptomatic carriers [18,19]. Thus, immunomodulatory strategies to reduce immunological alterations promoted by the virus may result in a useful therapy for HTLV-1-infected individuals.

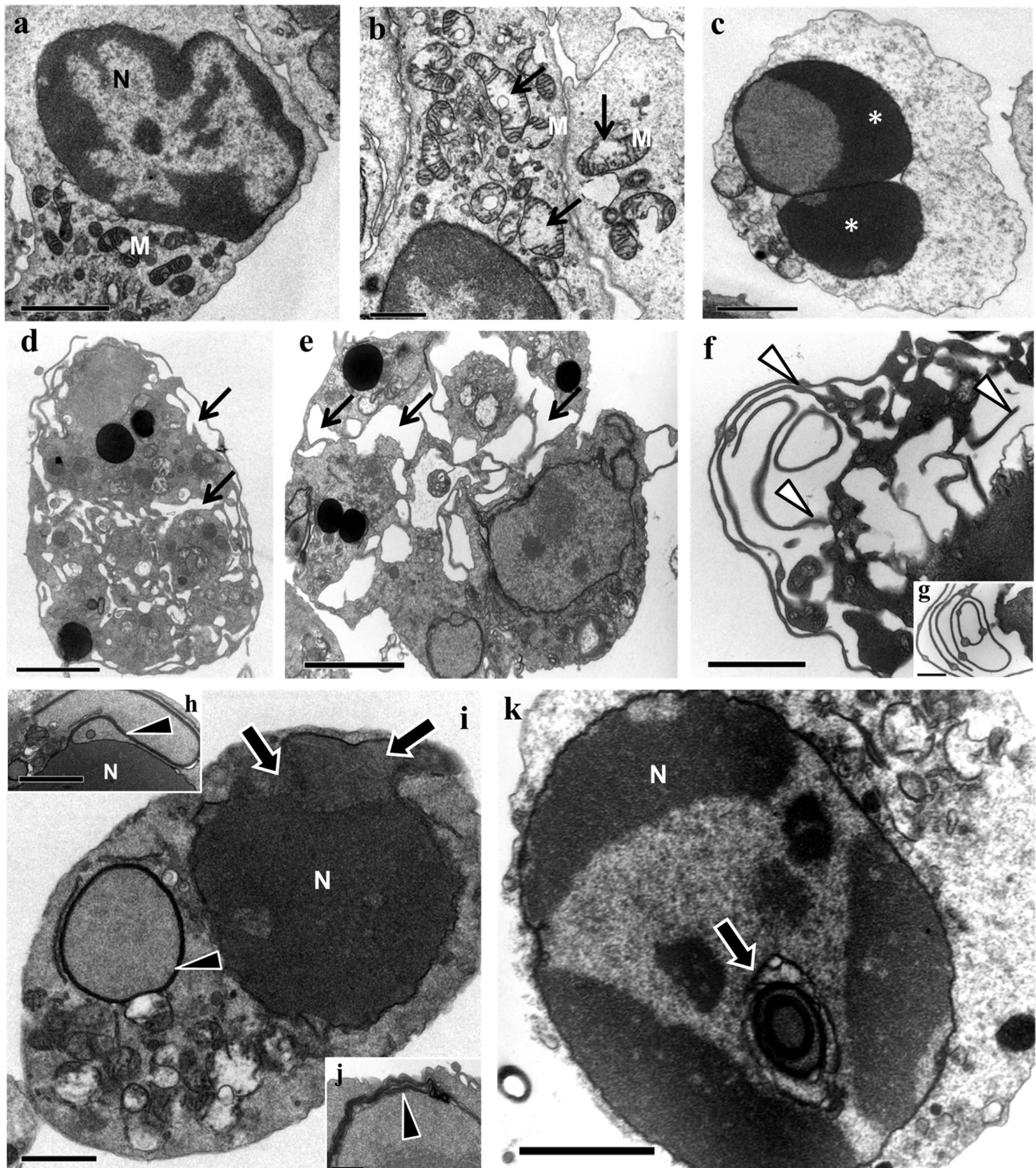

Fig. 5. Transmission electron microscopy of PBMC obtained from HTLV-infected subjects before (a–c) and after (d–j) physalin F treatment. The majority of the cells (a) displayed intact mitochondria (M) and normal nuclei (N), whereas some cells showed disrupted mitochondria (b arrows) and pyknotic nuclei (c, *). Pyknosis was eventually associated with the loss of cytoplasmic contents, indicating concomitant necrotic cell death mechanism. **1** triggered remarkable swelling of cisternae presumably of the endoplasmic reticulum (d, e arrows), culminating in the formation of long cytoplasmic ridges (f arrowheads, g). The nuclear envelope formed thin (h, arrowhead) or broad (i arrows) reticulum cisternae, presumably sectioned transversally and tangentially, respectively as well as autophagic vacuole-like compartments (i arrowhead). Nuclei of physalin F—treated cells also presented myelin-like membranes (j arrowhead) and intranuclear membranes, presumably nuclear juxtaposed autophagic vacuoles or nucleoplasmic reticulum cisternae (j arrow). Scale bars: a, c, d and $e = 2\ \mu m$; b, f, h, i and $k = 1\ \mu m$; g and $j = 0.5\ \mu m$.

4. Conclusion

In conclusion, the present work showed that the natural compound physalin F has a potent immunosuppressive effect on HTLV-1-infected cells, which reinforces this class of compounds as antiproliferative agents. Due to the lack of therapeutic approaches for HTLV-1-infected patients, these results also support the search for complementary therapies, in addition to the development of antiretroviral drugs.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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