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Aminochrome induces microglia and astrocyte activation

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ABSTRACT

Aminochrome has been suggested as a more physiological preclinical model capable of inducing five of the six mechanisms of Parkinson's Disease (PD). Until now, there is no evidence that aminochrome induces glial activation related to neuroinflammation, an important mechanism involved in the loss of dopaminergic neurons. In this study, the potential role of aminochrome on glial activation was studied in primary mesencephalic neuron-glia cultures and microglial primary culture from Wistar rats. We demonstrated that aminochrome induced a reduction in the number of viable cells on cultures exposed to concentration between 10 and 100 μM. Moreover, aminochrome induces neuronal death determined by Fluoro-jade B. Furthermore, we demonstrated that aminochrome induced reduction in the number of TH-immunoreactive neurons and reactive gliosis, featured by morphological changes in $GFAP^+$ and $Iba1^+$ cells, increase in the number of $OX\text{-}42^+$ cells and increase in the number of NF-κB p50 immunoreactive cells. These results demonstrate aminochrome neuroinflammatory ability and support the hypothesis that it may be a better PD preclinical model to find new pharmacological treatment that stop the development of this disease.

1. Introduction

Neuroinflammation is one of the mechanisms involved in the loss of dopaminergic neurons in PD (Herrero et al., 2015). In substantia nigra of PD patients have been revealed reactive microglia expressing complement receptor 3 (CD11b/OX-42) (McGeer et al., 1988) and increase in the number of amoeboid immunoreactive cells for ionized calcium-binding adaptor molecule 1 (Iba1) (Doorn et al., 2014). A lot of evidences of neuroinflammation have been obtained with exogenous neurotoxins. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine has been shown to activate microglia and astrocytes (Tonouchi et al., 2016). 6-Hydroxydopamine stimulates mRNA and protein levels of TNF-α, interleukin-6 and -1β (Yan et al., 2015). Rotenone increases TNF-α, interleukin-1β and -6 in an animal model (Sharma et al., 2016) and another study with microglia cells showed that rotenone stimulated secretion of interleukin 1β -6, 12 and TNF-α (Ye et al., 2016).

It was demonstrated that microglial activation by neuromelanin is NF-κB dependent and involves p38 mitogen-activated protein kinase (Wilms et al., 2003). However, there is no information about neuroinflammation by using the endogenous neurotoxin aminochrome. The dopamine oxidation to neuromelanin is a pathway that involves the formation of several *o*-quinones. The most stable *o*-quinone formed during dopamine oxidation is aminochrome that can be neurotoxic by forming adducts with proteins or when it is reduced by one-electron to leukoaminochrome *o*-semiquinone radical (Segura-Aguilar et al., 2016). Therefore the question being addressed here is whether aminochrome induce microglia and astrocytes activation.

2. Materials and methods

2.1. Synthesis and purification of aminochrome

Aminochrome was produced by incubation of Dopamine (7.5 mmol) with 10 ng of tyrosinase in potassium phosphate buffer (25 mM, pH 6) for 15–20 min at room temperature. To purify, the incubation solution was loaded on a CM-Sephadex C50-1000 (18 × 0.7 cm) column (Sigma-Aldrich, C25120). The red-orange solution corresponding to aminochrome was collected and detected by spectrophotometry method measuring the absorbance at 480 nm. Aminochrome concentration was determined by the molar extinction coefficient of 3058 M^{-1} cm^{-1} (Segura-Aguilar and Lind, 1989).

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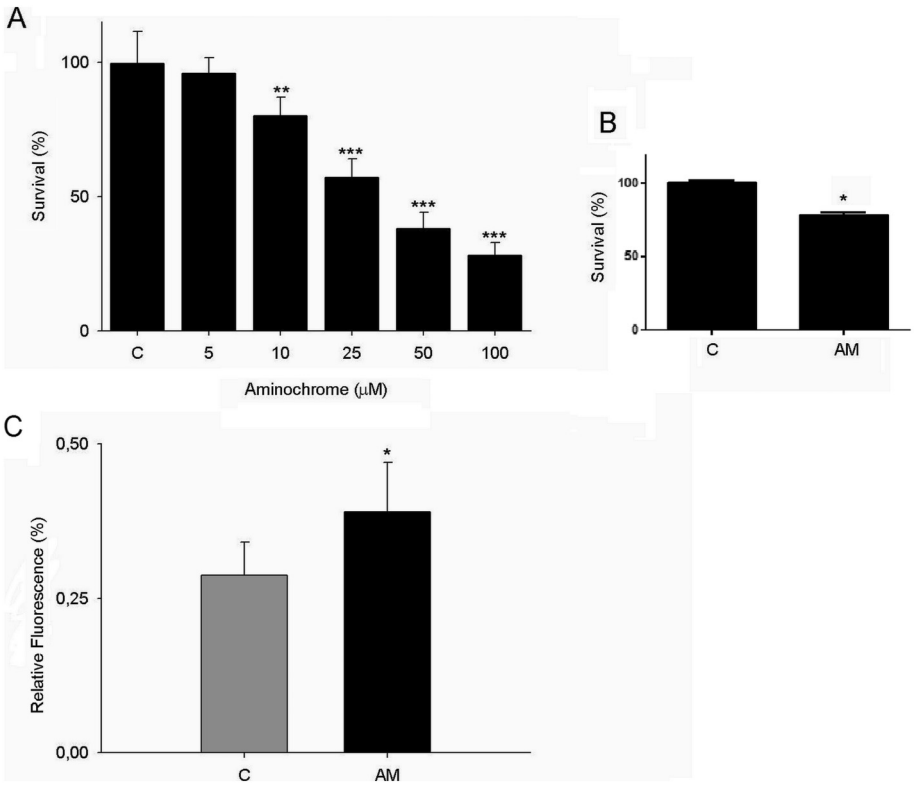

Fig. 1. The effect of aminochrome on cell viability in PMNG cultures. (A) Aminochrome induces decrease in cell survival in a concentration-dependent manner (10 to 100 μM, for 48 h) determined by Trypan blue staining. (B) Aminochrome induces decrease in cell survival since 24 h (10 μM) determined by Trypan blue staining. (C) Aminochrome induces neuronal cell death determined by Fluoro-jade B staining. The values are the mean ± SD (n = 8). In A, the statistical significance was standardized with one-way analysis of variance and postdate by post hoc Student-Newman-Keuls test, in B and C the statistical significance was assessed by using Student's *t*-test of variance between control and one sample (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Primary cultures

Primary cultures from Wistar rats were performed according to Brazilian guidelines for production, maintenance and use of animals for teaching activities and scientific research and the local Ethical Committee for Animal Experimentation, protocol number (0272012, ICS - UFBA).

2.3. Mesencephalic neuron-glia (PMNG)

Primary mesencephalic neuron-glia cultures were prepared according to Zhang et al. (2007). Ventral mesencephalic tissues from E14 Wistar rats were dissected out and forced through a sterile 75 μm Nitex mesh. Cells were suspended in DMEM/HAM-F12 medium (Cultilab, Brazil) supplemented with glucose 1 g/L, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin, 100 μg/mL streptomycin, 10% SFB (Cultilab) and 10% horse serum (HS) (Cultilab). After that, they were seeded at 5×10^5/well in 24-well culture plate precoated with poly-D-lysine (20 μg/mL) and laminin (2.5 μg/mL) and incubated in a humidified atmosphere with 5% CO_2 at 37 °C. Seven-day cultures were used for treatment with the composition about 55% astroglia, 7% microglia, and 37% neurons, of which about 3% was TH-immunoreactive neurons.

2.4. Microglial cultures

Microglial cells were obtained from cortex of Wistar newborn rats (0–2 days old) Microglial isolation was performed according to Mecha et al. (2011). In brief, after decapitation, the forebrains of newborn Wistar rats were dissociated mechanically and resuspended in DMEM supplemented with 10% FBS, 10% HS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured on poly-D-lysine (25 μg/mL)-coated flasks. Upon reaching confluence (7–10 days), adherent microglial cells were harvested by shaking at 165 rpm at 37 °C for 3 h. Isolated microglia were seeded into 24-well plates at a density of $3 \times 10^4/cm^2$, and experiments performed after 24 h. Cells were cultured at 37 °C in 5% CO_2.

2.5. Cell viability

Cell viability was accessed by Trypan blue exclusion test in cultures exposed to aminochrome at different concentrations (5–100 μM) or control conditions (without aminochrome), for 48 h. Floating and adherent cells were harvested after trypsinization (trypsin 0.05%, EDTA 0.02%) and centrifuged at 1300 × *g* for 5 min. The cells were suspended in 200 μL PBS and stained with Trypan blue (0.1%). The proportion of viable cells was determined.

Moreover, floating and adherent cells, cultured in 35-mm Ø plates (TPP Switzerland), were harvested after trypsinization (trypsin 0.025%, EDTA 0.50%) and centrifuged for 5 min at 1300 × *g*.

2.6. Neurodegeneration

Neurodegeneration was assessed using FJ-staining performed ac-

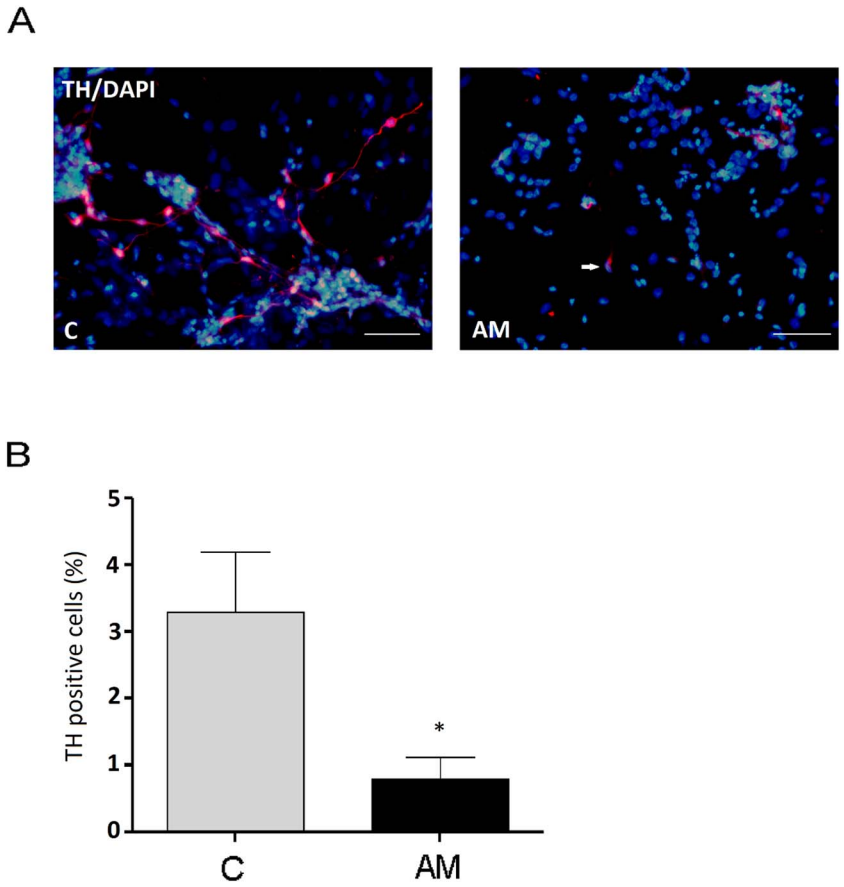

Fig. 2. Aminochrome reduces the number of TH-immunoreactive neurons in PMNG cultures. (A) Aminochrome reduces the number of TH-immunoreactive neurons determined by ratio of TH-immunoreactive cells (red) to chromatin stained with DAPI (blue). The arrow indicates neuron presents low TH expression. Obj. 20 × 0.70, scale bars = 50 μm. (B) Proportion of TH-immunoreactive neurons quantified and plotted. The values are the mean ± SD (n = 8) and the statistical significance was assessed by using Student's *t*-test of variance between control and one sample (*p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cording to Schmuck and Kahl (2009) in PMNG cultures exposed to aminochrome at 10 μM or control conditions, for 48 h. Cultures were fixed with cold methanol for 10 min. After, cells were rehydrated with PBS for 30 min, and permeabilized with Triton X-100 (0.3%) for 10 min. Then, cells were incubated for 30 min at room temperature with 0.001% FJ (Histo-Chem Inc., Jefferson AR) followed by rinse with PBS and chromatin was staining with DAPI (Sigma, St. Louis, MO) at a final concentration of 5 μg/mL in PBS. Thereafter, cells were analyzed by quantitative fluorometry at Varioskan™ Flash Multimode Reader (Thermo Plate) measured at 480 nm (excitation) and 530 nm (emission) to green (FJ) and 370 nm (excitation) and 470 nm (emission) to blue (DAPI).

2.7. Immunocytochemistry

After 24 h exposure, control and treated with 10 μM aminochrome PMNG cultures were fixed with cold methanol for 10 min. Following, cells were incubated with primary antibody (polyclonal rabbit anti-TH antibody (1:1000; ABCAM); polyclonal rabbit anti-GFAP antibody (1:300; DAKO); polyclonal rabbit anti-Iba1 antibody (1:300; WAKO); mouse monoclonal anti-OX 42 antibody (1:200, ABCAM)) for overnight at 4 °C following wash with PBS. After 12 h exposure, control and treated with 10 μM aminochrome microglial cell cultures were fixed with cold methanol for 10 min. After, these cells were incubated with primary antibody polyclonal rabbit anti- Iba1 antibody (1:300; WAKO) and monoclonal mouse anti-NFκB p50 antibody (1:200; Santa Cruz). Following they were incubated with secondary antibody (ALEXA FLUOR 594 goat anti-rabbit; ALEXA FLUOR 488 goat anti-mouse or ALEXA FLUOR 594 goat anti-mouse; ALEXA FLUOR 488 goat anti-rabbit) for 2 h. Chromatin was staining with DAPI. Thereafter, cells were analyzed by fluorescent microscopy and photographed (Olympus AX70).

2.8. Statistical analysis

Data were presented as mean ± SEM. Statistical significance was standardized with one–way analysis of variance and postdate by post hoc Student-Newman-Keuls test between control and multiple samples or standardized with Student's *t*-test of variance between control and one sample. p-Values < 0.05 were considered statistically significant.

3. Results

On PMNG cultures Trypan blue exclusion test demonstrated that 48 h exposure aminochrome induces decrease in cell survival in a concentration-dependent manner (10 to 100 μM) ($p < 0.01$; $p < 0.001$ Fig. 1A). The decrease in cell survival induced by 10 μM aminochrome was also visualized at 24 h exposure ($p < 0.001$ Fig. 1B). Moreover, 10 μM aminochrome for 48 h exposure induces neuronal death since FJ fluorescence intensity increases of 1.4 times ($p < 0.05$; Fig. 1 C). This

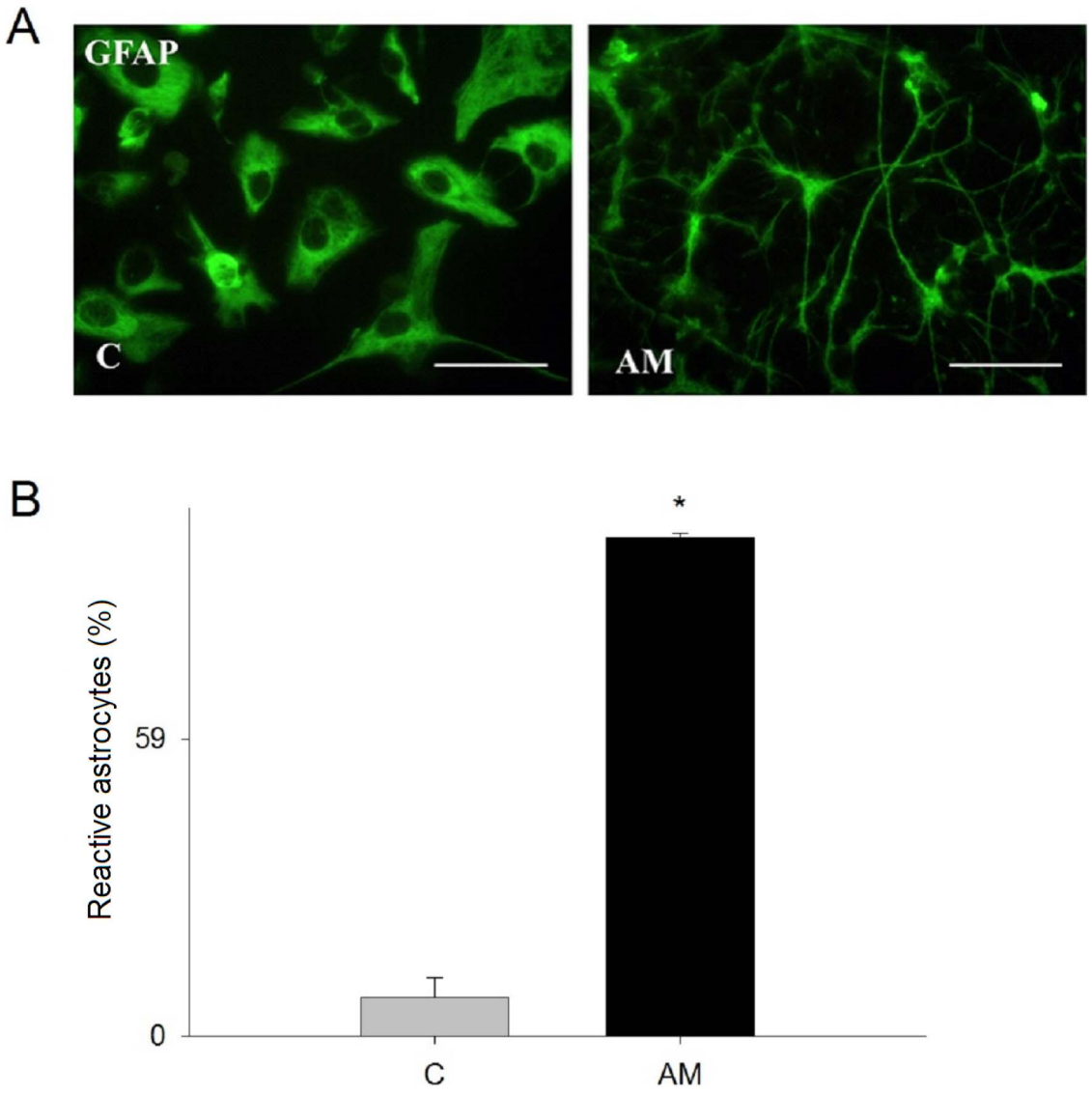

Fig. 3. Aminochrome induces astrogliosis in PMNG cultures. (A) Aminochrome induces astrogliosis determined by morphological changes in GFAP$^+$ cells. Obj. 20 × 0.70, scale bars = 50 μm. (B) Proportion of reactive astrocytes quantified and plotted. The values are the mean ± SD (n = 8) and the statistical significance was assessed by using Student's *t*-test of variance between control and one sample (*p < 0.05).

aminochrome concentration (10 μM) was considered for all experiments.

Immunocytochemistry assay in PMNG cultures revealed in control cultures 3.3 ± 0.9% TH-immunoreactive neurons that were reduced to 0.78 ± 0.3% ($p < 0.05$) after 24 h exposure to 10 μM aminochrome (Fig. 2A and B). Moreover, aminochrome induces morphological changes in GFAP$^+$ astrocytes, presenting cell bodies retraction and long and thin cytoplasmic processes in proportion of 99.3 ± 0.97% ($p < 0.001$) compared to control cells (7.9 ± 3.9%; Fig. 3A and B), and morphological changes in Iba1$^+$ microglia presenting amoeboid morphology in proportion of 100% ($p < 0.001$) compared to control cultures (7.9 ± 3.9%; Fig. 4A and B). Furthermore, increase in the proportion of OX-42$^+$ cells to 13.5 ± 1.6% ($p < 0.05$) compared to control cultures (2.5 ± 0.9%; Fig. 4C).

Immunocytochemistry assay in microglia cultures also revealed morphological changes in Iba1$^+$ cells presenting amoeboid morphology in proportion of 90.1 ± 1.7% ($p < 0.001$) after 24 h exposure to 10 μM aminochrome, compared to control cultures (15.7 ± 6.7%; Fig. 5A and B). Moreover, it was visualized increase in proportion of NFκB p50 positive microglia induced by aminochrome ($p < 0.05$; Fig. 5A and C).

4. Discussion

There is a general agreement in the scientific community that the degeneration of nigrostriatal neurons involves mitochondrial dysfunction, aggregation of alpha-synuclein, oxidative and endoplasmic reticulum stress, dysfunction of protein degradation systems and neuroinflammation (Ebrahimi-Fakhari et al., 2012; Mercado et al., 2013; Mullin and Schapira, 2013; Taylor et al., 2013). However, it is unknown the identity of the neurotoxin that trigger these mechanisms in PD. It has been proposed that aminochrome is the neurotoxin that triggers the degeneration of nigrostriatal neurons (Segura-Aguilar et al., 2016; Segura-Aguilar et al., 2014) since aminochrome has been reported to induce mitochondrial dysfunction, oxidative stress (Arriagada et al., 2004); dysfunction of both proteasomal and lysosomal systems (Huenchuguala et al., 2014; Zafar et al., 2006); formation of neurotoxic

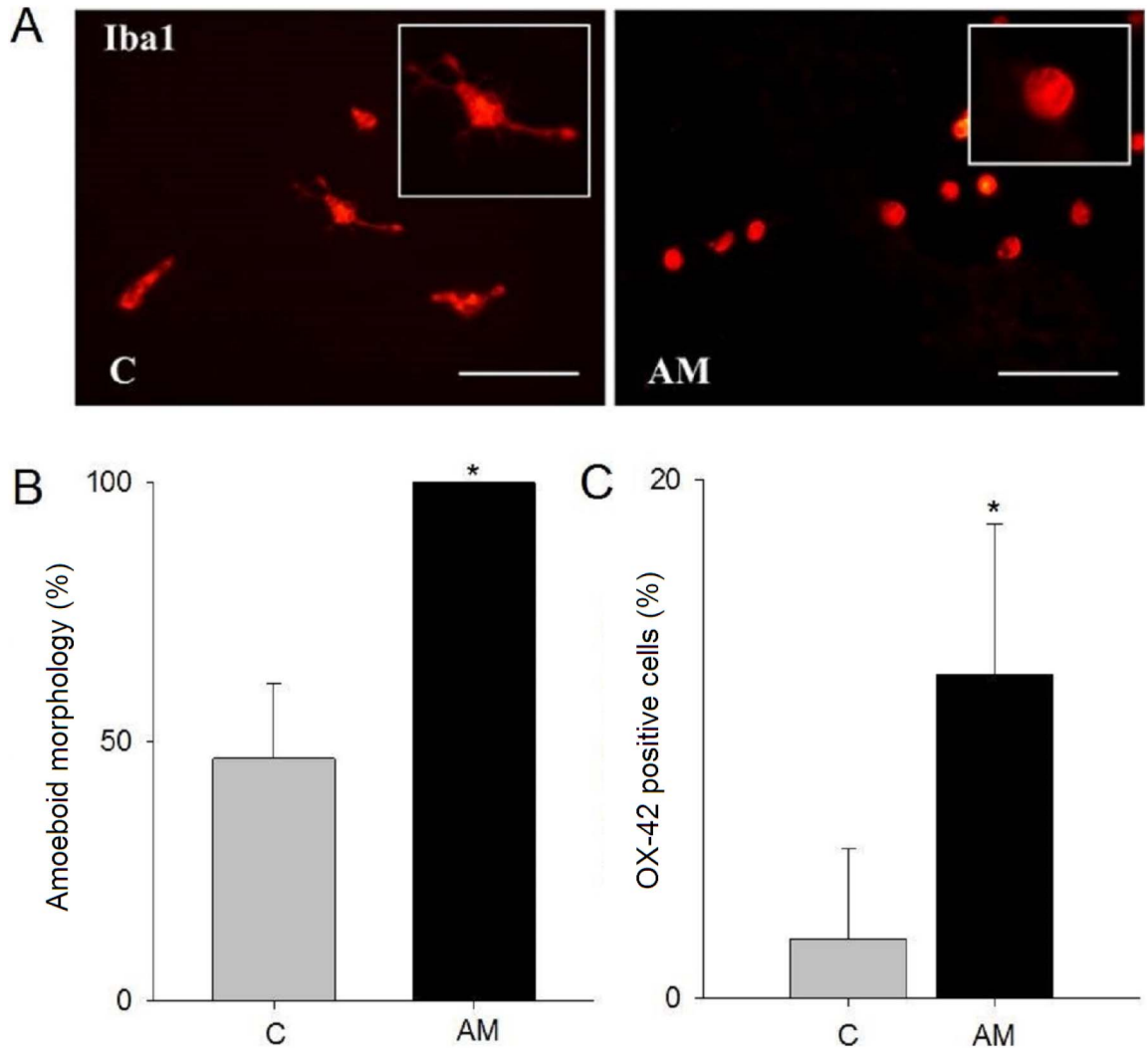

Fig. 4. Aminochrome induces microgliosis in PMNG cultures. (A) Aminochrome induces microgliosis determined by morphological changes in Iba1$^+$ cells presenting amoeboid morphology. Obj. 20 × 0.70, scale bars = 50 μm. (B) Proportion of amoeboid Iba1$^+$ cells quantified and plotted. (C) The proportion of OX-42$^+$ cells quantified and plotted. The values are the mean ± SD (n = 8) and the statistical significance was assessed by using Student's *t*-test of variance between control and one sample (in B, *p < 0.05; in C, *p < 0.001).

alpha-synuclein oligomers (Munoz et al., 2015); endoplasmic reticulum stress (Xiong et al., 2014). In this study we have evidences that aminochrome induce activation of both microglia and astrocytes supporting the role of aminochrome in the neurodegenerative process of the nigrostriatal neurons in PD.

Astrogliosis is associated with changes in the expression of many genes and characteristic morphological hallmarks, and has beneficial or harmful influence in CNS disorders (Pekny et al., 2014). Indeed, it is well characterized that astrocytes exert an important microglia cross-talk associated to neuroinflammation that implies in progression of neurotoxins-induced PD-like degeneration (Hirsch and Hunot, 2009).

There are many different kinds of evidence that support the hypothesis that microgliosis play by production of ROS and NO (Peterson and Flood, 2012) or via secretion of pro-inflammatory cytokines (Hirsch and Hunot, 2009) an important function in the progression of dopaminergic neurons degeneration. Furthermore, NF-κB seems to be crucially involved in regulating inflammatory responses in microglial cells (Rosenstiel et al., 2001). Both, astrogliosis and microgliosis induced by aminochrome support the importance of non-neuronal cells on pathological mechanisms in PD and demonstrated that aminochrome is directly involved in all mechanisms of the disease. Furthermore, it lay emphasis on hypothesis that aminochrome may be a better PD preclinical model to find new pharmacological treatment that stop the development of this disease.

5. Conclusion

We concluded that besides neuronal death, aminochrome induces astrogliosis and microgliosis, which is a first data related to aminochrome-induced neuroinflammation.

Transparency document

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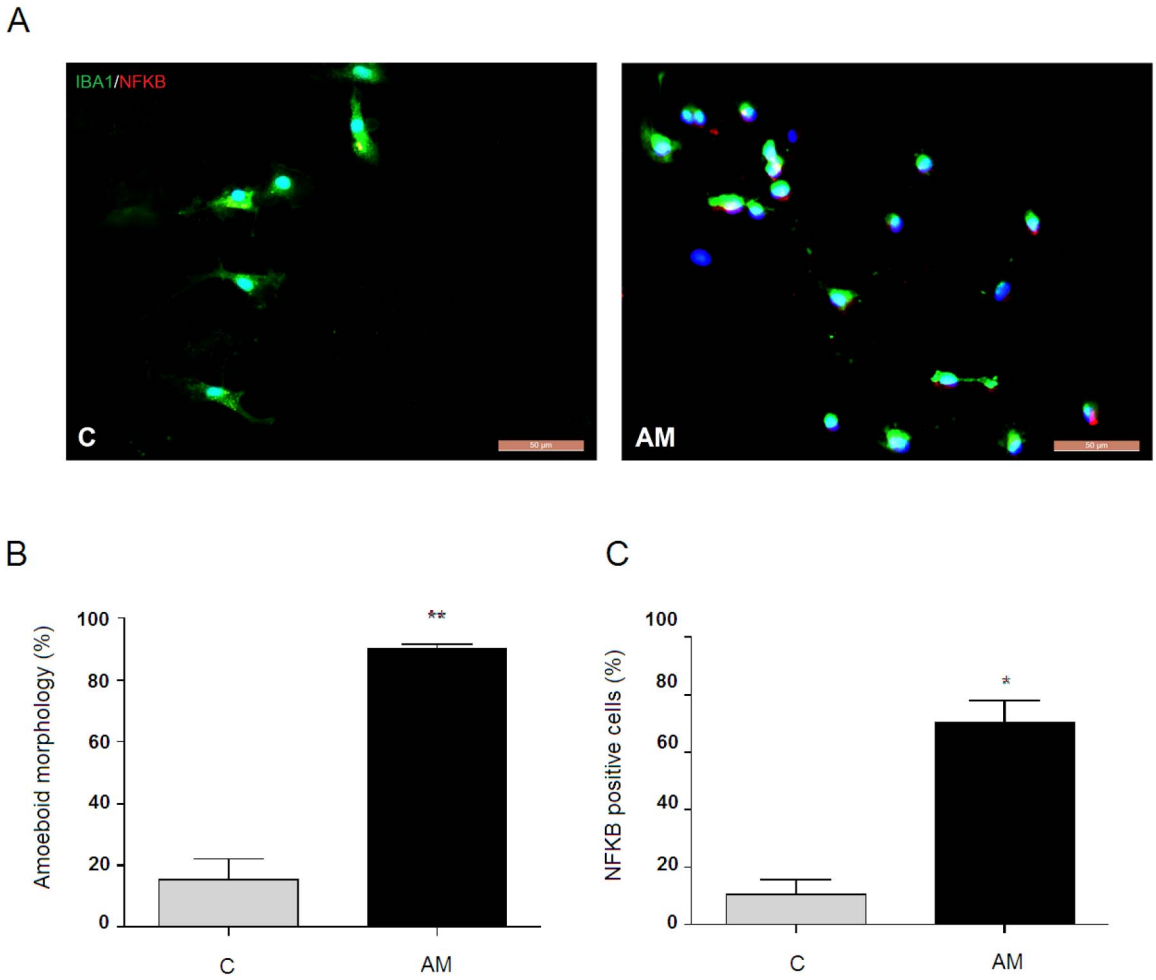

Fig. 5. Aminochrome induces microgliosis in isolated primary cultures. (A) Aminochrome induces microgliosis determined by morphological changes in Iba1$^+$ cells (green) presenting amoeboid morphology and increase of NF-κB p50 expression (red). Obj. 20 × 0.70, scale bars = 50 μm. (B) Proportion of amoeboid Iba1$^+$ cells quantified and plotted. (C) The proportion of NF-κB p50$^+$ cells quantified and plotted. The values are the mean ± SD (n = 8) and the statistical significance was assessed by using Student's *t*-test of variance between control and one sample (in B, *p < 0.001; in C, *p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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