

## Albumin attenuates DNA damage in primary-cultured neurons

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### ABSTRACT

Human serum albumin (HSA) is an effective therapeutic agent that protects neurons after cerebral ischemia or related injuries by means of its antioxidant capacity. Our aim was to test whether bovine serum albumin (BSA) might also provide protection, especially against DNA damage. Rat cortical neurons were cultured in both the presence and absence of BSA. To test the neuroprotective role of BSA against DNA damage and neuronal death, primary cultures were investigated using both  $\gamma$ -H2AX and pATM immunocytochemistry, and the TUNEL assay, respectively. Quantitative analyses revealed that the cultures in the absence of BSA had a higher number of apoptotic neurons. Additionally, neurons showing DNA strand breaks were fewer when BSA was added to the medium. BSA acts as a neuroprotective molecule, reducing both the DNA damage and apoptosis rates. This effect is similar to that described for HSA, probably due to its antioxidant activity. Hence, we have demonstrated that BSA provides a neuroprotective role when DNA damage occurs. Additionally, we suggest that BSA probably shares similarities with HSA in its antioxidant activity, opening new ways in the study of stroke and related brain diseases.

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It is well documented that human serum albumin (HSA) confers neuroprotection in animal models of ischemic stroke [2,9,11]. Stroke treatment with high doses of HSA results in diminished brain injury, such as decreased edema and infarct size [11]. Neuroprotective properties, such as increased local cerebral perfusion or the maintenance of microvascular integrity after stroke, have also been described for HSA [9]. Moreover, it has also been attributed an antioxidant activity, which seems to be partly essential for its neuroprotective effects [9].

Previous studies have reported that bovine serum albumin (BSA) promotes neuronal survival [3,7,15]. However, the involvement of BSA in decreased neuronal death remains unclear. The aim of this work was to analyze, in an *in vitro* model of neuronal death, the neuroprotective role of BSA, in particular against DNA damage. Our results provide a basis for successive studies in the treatment for brain injuries and diseases in which DNA damage is probably produced when oxidant species are released.

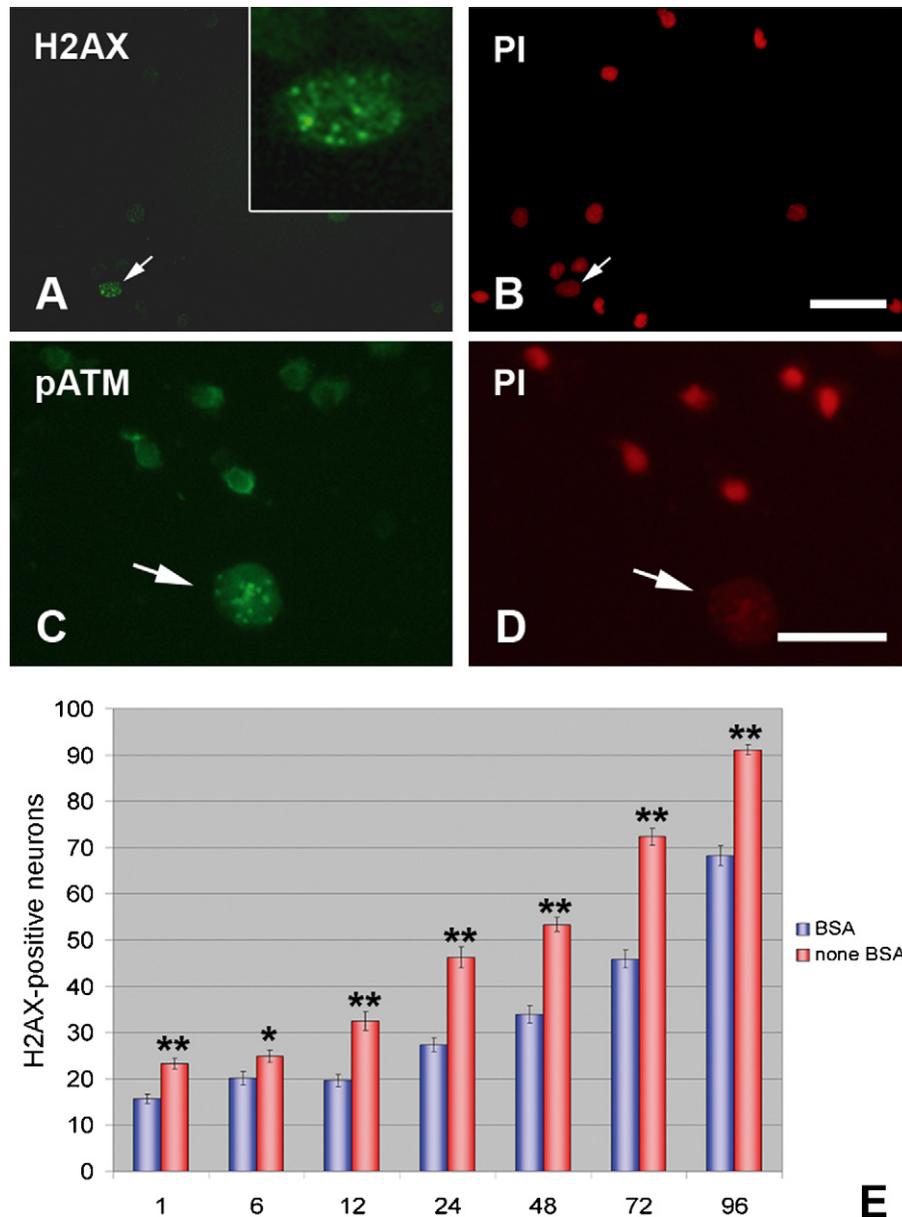
Four albino pregnant Wistar rats were used. In order to obtain neurons in primary culture, fetuses of 17.5 days of gestation were extracted by sacrifice of the mother, following the animal care rules of the Council of the European Communities (86/609/EEC) and current Spanish legislation (RD 1201/2005).

Cells were mechanically dissociated from the forebrains (cerebral neocortex) and plated at a density of  $1.25 \times 10^5$  cells per 3.5 cm diameter Petri dish, previously treated with  $10 \mu\text{g}/\text{mL}$  of poly-L-lysine. For survival experiments, they were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12, both in the absence and presence of 2% (w/v) fatty acid-free BSA (A7030). This neuronal death model is based on trophic/survival factor deprivation. BSA concentration was chosen based on previous report [15]. BSA was dialyzed in Elliot buffer containing 122 mM NaCl, 4.8 mM KCl, 0.4 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 1.3 mM  $\text{CaCl}_2$ , pH 7.4, for 24 h and filtered through a  $0.2\text{-}\mu\text{m}$  filter (Pall Gelman Laboratory, Ann Harbor, USA) before use as previously described [15]. Then, cultures were maintained for 1 h, 6 h, 12 h, 24 h, 48 h, 72 h, and 96 h at  $37^\circ\text{C}$ . Reagents were purchased from Sigma–Aldrich Chemical (Madrid, Spain).

We analyzed the expression pattern of the phosphorylated forms of the histone variant H2AX ( $\gamma$ -H2AX), and the Ataxia Telangiectasia Mutated gene (pATM), both sensors of DNA strand breaks (DSBs). Neurons were fixed in 4% paraformaldehyde for 20 min and rinsed in phosphate buffered saline (PBS). Then, they were treated with ethanol at  $-20^\circ\text{C}$  for 5 min and washed in PBS, prior to an incubation in blocking serum containing 5% normal goat serum and 0.2% Triton X-100 in PBS for 1 h. Mouse anti- $\gamma$ -H2AX antibody (1:300, Millipore) or mouse anti-pATM (1:400, Rockland) were incubated in the same solution overnight at  $4^\circ\text{C}$ . Fluorescent secondary antibody was then applied for 2 h. Finally, neurons were counterstained with 1:2000 propidium iodide (PI) for 15 min and coverslipped with antifading solution.

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**Fig. 1.** Staining for  $\gamma$ -H2AX (A) and pATM (C), both counterstained with PI (B and D). The arrow shows a cultured neuron exhibiting DNA damage (A) and pATM-reactive nuclear foci (C). In (A), the insert shows a higher magnification of a neuron with many  $\gamma$ -H2AX-positive foci (B). Bar chart (E) shows the percentage of neurons exhibiting DNA damage from BSA- and non-BSA-added cultures. Scale bar = 20  $\mu$ m.

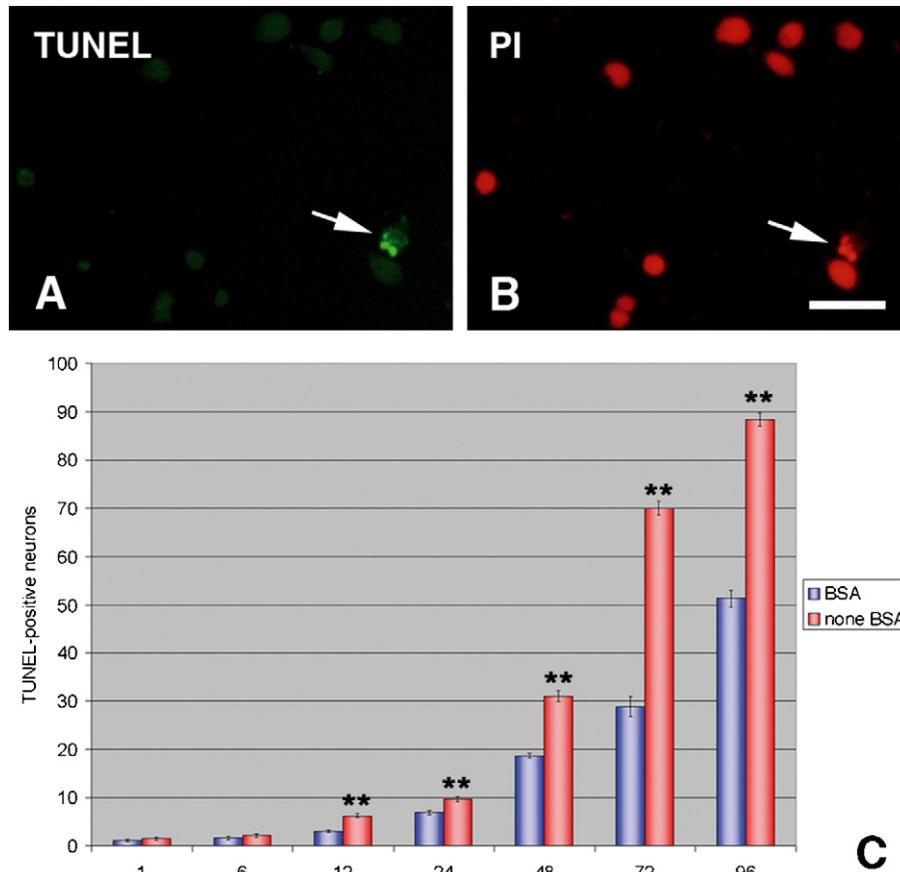
The TUNEL assay was used as indicative of apoptotic cell death. Neurons were fixed as described above, and pre-incubated in TUNEL buffer containing 1 mmol/L  $\text{CoCl}_2$ , 140 mmol/L  $\text{C}_2\text{H}_6\text{AsO}_2\text{Na}$  and 0.3% Triton X-100 in 30 mmol/L Tris buffer pH 7.2, for 30 min. After incubation at 37 °C with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (800 U/mL) and nucleotide mixture (1  $\mu$ mol/L) for 90 min, neurons were rinsed with saline sodium citrate (3  $\times$  10 min), PBS (3  $\times$  10 min) and counterstained as described above.

Four primary cultures were analyzed using ImageJ (v.1.38x, Java-based image processing program developed at the National Institutes of Health, USA). The percentage of neurons exhibiting DNA damage and apoptotic fate was estimated by counting 500 neurons of multiples areas of each culture. Values are means  $\pm$  S.E.M. Statistical analyses were carried out using the Student's *t*-test to compare both experimental groups. Significant differences were considered at \* $P$  < 0.05 and \*\* $P$  < 0.01.

Immunohistochemical assays for  $\gamma$ -H2AX and pATM revealed the formation of foci of nuclear DNA damage (Fig. 1A and C). In both cases, cultured neurons were counterstained with propidium iodide (Fig. 1B and D). BSA deprivation afforded a significant increase in the percentage of neurons containing DSBs as compared to those in the presence of BSA from 1 h to 96 h, as seen in the chart (Fig. 1C).

In order to analyze the apoptotic ratio of cultured neurons, we performed the TUNEL assay (Fig. 2A and B). Our results clearly revealed the neuroprotective effect exerted by BSA under the culture conditions employed. Quantitative analysis demonstrated a decrease in the percentage of apoptotic neurons from 12 h onwards under BSA treatment (Fig. 2C).

The goal of this study was to determine the neuroprotective efficacy of BSA in a neuronal model of apoptosis. Under the culture conditions used, BSA decreased both the percentage of neurons containing DSBs and the apoptotic cell death ratio.



**Fig. 2.** (A) An apoptotic neuron (TUNEL stained; arrow) surrounded by non-apoptotic neurons, both counterstained with PI (B). (C) The apoptotic cell ratio expressed as a percentage, elicited by the presence or absence of BSA. Scale bar = 20  $\mu$ m.

These beneficial properties have been previously reported for HSA [1,8]. It has been proposed that the copper-chelating tetrapeptide aspartate-alanine-histidine-lysine (DAHK), located on the HSA N-terminal, attenuates both DSBs and telomere shortening, conferring neuroprotective effects to HSA after stroke. Moreover, analogues of this peptide exhibit superoxide dismutase-like activity by inhibiting superoxide formation and reducing lipid peroxidation [1]. Therefore, HSA may block oxidant-induced neuronal death by means of its DAHK sequence [8].

Interestingly, BSA has a similar N-terminal aspartate-threonine-histidine-lysine (DTHK) tetrapeptide to that described for HSA [4], where alanine has been replaced by threonine. It has been reported that both the affinity and the specificity of DTHK to chelate copper is very similar to that described for DAHK [12,14]. Nevertheless, BSA should exert its neuroprotective role not only through the copper-chelation property of DTHK, but also through other mechanisms as well.

It should be taken into account that the BSA used in the present work, which has been also handled in previous studies [7,8,15,16], could exhibit post-translational modifications. However, whether its neuroprotective properties are also maintained by the native form of BSA, or whether it has other physiological roles remains to be elucidated.

Previous reports have described that BSA is involved in glucose metabolism [15] and that it promotes neuronal survival by increasing the synthesis and release of glutamate [16]. It is well known that glutamate both has trophic effects at low concentrations and decreases neuronal death through the activity of the NGF receptor TrkA [10,15,16]. In addition, it has been described that BSA prevents

both mitochondrial depolarization and apoptosis by a reduction of excessive cytosolic  $Ca^{2+}$  concentrations [7], thus diminishing the activation of caspase pathways. Moreover, BSA acts as a cytoprotective antioxidant through the activation of catalase – a hydrogen peroxide inactivating enzyme [13] – decreasing oxidative stress in the cell. Both the activation of caspase pathways and oxidative stress are crucial pathological signals that precede both the DNA fragmentation and apoptosis processes. Additionally, BSA exhibits antimutagenic effects against certain genotoxic compounds [3].

In the present report, by means of the nuclear expression of proteins involved in the signalling of DNA damage (pATM and  $\gamma$ -H2AX) we have demonstrated that BSA provides a reduction in the activation of those sensors, and that therefore appears to be a decrease in DNA damage. Considering that DNA damage is likely involved in promoting several neurodegenerative processes related to oxidative stress processes [5], including experimental stroke [6], a reduction in oxidative stress should result in a decrease in the ratio of both DSBs and neuronal death.

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