Disruptive Effect of Epigallocatechin-3-Gallate on the Proliferation/Apoptosis Balance in the Olfactory Epithelium

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Abstract: The polyphenol epigallocatechin-3-gallate (EGCG), is the main active catechin of the green tea. It exerts a wide range of biochemical and pharmacological effects on different animal cells. EGCG acts as a scavenger of growth factors and it was demonstrated its antiproliferative and proapoptotic role in different cancer cells. Nevertheless, little is known about the effect of EGCG on normal cells. In the present work, we have studied the EGCG effect on the proliferation/apoptosis balance in the olfactory epithelium of amphibian tadpoles. Animals treated with EGCG showed an increase in the number of apoptotic cells and a decrease in the basal proliferation. In addition, EGCG showed differential effect on the two classical growth factors signalling pathways, MAPK and PI3K route. EGCG treatment triggered a drop in phospho-ERK1/2 level without affecting the phospho-AKT level. These findings demonstrate the antiproliferative and proapoptotic effect of EGCG on normal olfactory cells and postulate the olfactory epithelium as a good experimental model for the study of the factors involved in neurogenesis process.

Keywords: Olfactory neurons, tadpoles, growth factors.

Epigallocatechin-3-gallate (EGCG), the main active component of the green tea, is a polyphenol that exerts a wide range of biochemical and pharmacological effects and it has been shown to possess antiproliferative and proapoptotic properties in different tumoral cells [1-3]. It was demonstrated that EGCG exhibits a potent antioxidant effects, an antitumourgenic function and a suppressive effects on angiogenesis [2, 4, 5]. Also, EGCG acts as a scavenger of the growth factors that promote carcinogenesis and other proliferative disorders [6]. This catechin irreversibly binds with growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF), turn them not available for binding with their respective cognate tyrosine kinases receptors. In other cell systems, these receptors have at least two major signaling pathways: the mitogen-activated protein kinase (MAPK) [7] and phosphatidylinositol-3-kinase-AKT pathways [8].

Among the MAPKs, the extracellular signal-regulated kinases (ERK1/2) are mainly activated by mitogen and growth factors [9]. The involvement of ERK1/2 signalling in neuronal survival has been demonstrated by the observed decrease in its activity in primary cortical neurons intoxicated with glutamate [10]. Moreover, it has been confirmed that EGCG inhibits the ERK and PI3-Kinase/AKT pathways activated by different growth factors [11, 12].

EGCG has been shown to inhibit growth and to induce apoptosis in different human cancer cells, both *in vivo* and *in vitro*, perhaps related to the inhibition of VEGF [13, 14]. Although there is a lot of evidence about the effects of EGCG on pathological cells, little is known about their effects on normal cells. Some investigators demonstrated the effects of EGCG on neuronal activity [15, 16]. Jeong *et al.* [17] reported the inhibitory action of EGCG on neuronal activity in rat medial vestibular nuclear neurons using patch-clamp recordings. Recently, it has been confirmed that treatment of hippocampal neurons with EGCG resulted in an elevation of caspase activities providing further evidence that apoptosis was the dominant mode of EGCG-induced cell death in cultures of hippocampal neurons [18].

Our attention was focused over the effect of EGCG on neurons, in particular over olfactory neurons. The olfactory epithelium (OE) is an excellent model to investigate regulation of neuronal stem cell expansion and differentiation, as it undergoes continuous neuronal replacement. This cellular replacement involves stages of proliferation and maturation governed by the sequential and combinatorial exposure of cells to extrinsic factors and regulated by autocrine and paracrine signals such as peptide growth factors. *In vitro*, it was demonstrated that three growth factors exert well-defined effects in the OE: FGF-2; transforming growth factor-p2; and PDGF; these growth factors and their receptors play a significant role in the dynamic state of proliferation, differentiation and cell death [19, 20]. As tadpoles have a completely differentiated and functional olfactory system and, considering that their neurons are in contact with the environment facilitating the drugs exposition, they may be postulated as useful model for the study of the factors which regulate neurogenic process. Recently, Hassenklöver *et al.* [21] demonstrated the effect of purinergic receptors on the proliferation rate in the olfactory epithelium of *Xenopus laevis* tadpoles. This work certainly strengthen the fact that the tadpole olfactory epithelium is an excellent model for studying neuronal proliferation and differentiation processes. In the present work, we studied the effect of EGCG on the

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proliferation/apoptosis balance in amphibian tadpoles OE exhibiting a decrease in the proliferation of neuronal precursor cells and an increase in the apoptosis of mature olfactory receptor neurons.

ANIMALS TREATMENT

Rhinella (Bufo) arenarum tadpoles were obtained by *in vitro* fertilization according to Paz *et al.* [22]. Larvae were maintained in dechlorinated tap water, with constant photoperiod and temperature (12:12 h, 22°C). Tadpoles ranging from stages 35 to 37 according to Gosner [23] were used. All experiments were performed in accordance with the principles of laboratory animal care of the Institutional Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA Res CD: 140/00, and the principles of the NIH (publication 8523, revised 1985). Tadpoles (N=40, stages 35-37) were immersed in an EGCG solution containing 0, 12.5, 25, 50, 100 µg/ml of dechlorinated water and maintained 24 h. After EGCG treatment, tadpoles (N=8, for each treatment) were treated by immersion in phosphate buffered saline with 20 mM 5-Bromo-2´-deoxyuridine (BrdU, Sigma B5002, St. Louis, MO), for thirty minutes at room temperature (22-24°C) as described by Quick and Serrano [24]. Tadpoles were maintained for three hours after BrdU exposure prior to euthanasia; and processing the tissue for BrdU incorporation using immunohistochemical methods. After fixation in Bouin's solution (24 hr, 4°C), animals were dehydrated in graded concentrations of ethanol, cleared in xylene and embedded in Histoplast (Biopack, Argentina). Serial horizontal sections were cut at 7 µm and mounted onto *Superfrost* Plus (Erie Scientific Co, New Hampshire, USA) slides.

IMMUNOHISTOCHEMISTRY

Sections were deparaffined in xylene, rehydrated through a series of graded alcohols and washed in Phosphate Buffer Saline (PBS). For Caspase 3-activated immunodetection, sections were incubated 10 min. in 10 mM sodium citrate buffer, pH 6.0 at sub-boiling temperature. Tissue sections were treated with 3% hydrogen peroxide (H_2O_2) solution to quench endogenous peroxidase activity. Nonspecific antigen binding sites were blocked by preincubation with TNB blocking solution (Cat. FP1020, NEN Life Science Products, Boston, MA) and subsequently incubated overnight at 4°C with the primary antibody rabbit anti-Caspase 3-activated, 1/50 (Cell Signalling, Boston, MA). Then, sections were treated with the appropriate biotinylated antibody (Jackson Immunoresearch Laboratories, Baltimore, MD) followed by avidin–horseradish peroxidase–biotin complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). The reaction was visualized by exposure to 3,3´-diaminobenzidine tetrahydrochloride (DAB) staining kit (Dako, Carpinteria, CA). The slides were mounted in Permount (Fisher Scientific, Pittsburgh, PA).

For BrdU-labeled nuclei detection, the DNA was denatured with 2 N HCl for 20 min at 37° C, and subsequently incubated overnight with mouse anti-BrdU, 1/200 (Novocastra, New Castle, Upon Tyde, UK). Then sections were treated with anti-mouse biotinylated antibody (Jackson Immunoresearch Laboratories, Baltimore) followed by streptavidin-Alexa 488 (Invitrogen, Carlsba, CA). Tissue was counterstained with propidium iodide and fluorescence image was analyzed by confocal laser microscopy (Olympus FV-30 attached to a microscope Olympus Bx-61).

Omission of the primary antibody eliminated all staining and served as a negative control. Control slides from animals that did not receive a BrdU injection revealed no staining (data not shown).

PROLIFERATION ANALYSIS

Slides containing comparable sections of OE from each animal were selected, coded so that cell counts results were obtained in blind condition. It was defined a region of interest (250 µm of length) in the medial region of the OE. The number of BrdU-positive (BrdU+) nuclei in each selected area was observed and counted. Because the diameter of anuran olfactory cells are approximately of 10 µm and serial sections were cut at 7 µm, we counted BrdU+ cells leaving one section between each, so there is low probability that labeled nuclei would be counted twice, causing an overestimation of proliferating cells. We counted the number of BrdU+ cells in the basal cells zone (BC-Z) where olfactory progenitor cells are located; olfactory receptor neuron zone (ORN-Z) and total BrdU+ cells (BC-Z plus ORN-Z) (see Fig. **1A**).

APOPTOSIS ANALYSIS

For Caspase 3-activated quantification, slides containing comparable sections of OE from each animal were selected and it was counted the total of DAB- positive cell along the OE section. The TUNEL technique was used to confirm the results obtained with Caspase 3-activated. Apoptotic cells were detected using a commercial kit (ApogTag, Chemicon, Millipore, Billerica, MA). Briefly, tissue sections were deparaffined, rehydrated and treated with Proteinase K (Dako) 20 µg/ml, 15 min at room temperature, washed in PBS and then incubated with Formaldehyde 4%, washed again in balance buffer. Immediately, they incubated in the reaction mixture containing the TdT enzyme for 60 min at 37º C. Finally, they were incubated with anti-digoxigenin-fluorescein antibody. Images of the sections were captured by a confocal laser microscope.

IMMUNOBLOTTING ANALYSIS

Twenty olfactory systems (olfactory epithelia, nerves and bulbs), resulting from 0 and 25 µg/ml EGCG treatments, were homogenized in 5% (w/v) of ice-cold lysis buffer (100 mM NaCl, 10 mM Tris–HCl, pH 7.4, 1mM EDTA, 0.5% NP40, 1% Triton, 1mM sodium orthovanadate, 1 mM PMSF) with 1X protease inhibitor cocktail (Sigma, St. Louis, MO). After homogenization, total protein extracts were maintained 1 hr at 4° C in constant agitation. Then, it was centrifuged at 8000xg for 10 min at 4°C and the supernatant was frozen at 80 °C. Protein extracts were separated on 15% SDS-polyacrylamide gels electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Arlington Heights, IL) and then blocked overnight with a 5% solution of non-fat dry milk and incubated with the primary antibody. The antibodies used were: rabbit anti-phospho-ERK 1/2 1/1000 (Cell Signaling), mouse anti-phospho-AKT 1/1000 (Cell Signaling) and mouse anti-β-tubulin, 1/1000 (E-7, Developmental Studies Hybridoma Bank, University of Iowa). Membranes were incubated overnight at 4°C and then they were washed with

Tris Buffer Saline–Tween and then further incubated with an ABC kit (Vectastain ABC kit, Vector Labs) following the instructions of the manufacturer. The membranes were developed using an enhanced chemiluminescence detection system to visualize peroxidase activity (Chemicon) and the image was captured with a Luminescent Image Analyzer LAS-1000 plus (Fuji Photo Film Co. Ltd). Controls for non-specific binding included incubation in the absence of the primary antibody.

STATISTICAL ANALYSIS

The results were exhibited as media ± standard error (SEM). For the quantification of apoptotic or proliferating cells, analysis was carried out using a *nested analysis of variance (ANOVA)*. The homogeneity of variances within groups was verified with Bartlett´s test. When ANOVA demonstrated significant differences, a multiple comparison using the Tukey test was made. For the analysis of Western blot a t-test for unmatched samples was used. Results were considered significantly different when $p< 0.05$.

First, it was examined the effect of different doses of EGCG on the tadpoles survival. For this, animals were immersed in different concentrations of EGCG (0, 12.5, 25, 50 and 100 µg/ml), during 24 hr. EGCG up to 50 µg/ml did not affect the survival but the highest concentration of EGCG was toxic to tadpoles (data not shown). Based on these results, we used EGCG up to 50 µg/ml for all the experiments.

The exposition of tadpoles to 25 and 50 µg/ml EGCG significantly reduced the number of proliferating cells in the OE compared to the control (mean±SEM: 14.95±0.71; 13.25±1.53 ; 20.37±2.82, respectively) (Fig. **1A-D**). These differences in the number of proliferating cells was not observed in the low concentration of EGCG (12.5 µg/ml; 19.94±2.74) compared to the control (Fig. **1B**). On one hand, the decrease in the number of total cells in proliferation for the two higher concentrations of EGCG could be fundamentally related to a decrease in the number of BrdU+ cells in the BC-Z, since differences in the number of BrdU+ cells were not observed in the ORN-Z between treatments (Fig. **1E**).

We analysed the effect of EGCG on the apoptosis by two apoptotic markers: The activation of Caspase-3 and TUNEL. The identification of apoptotic cells was made by the Caspase 3-activated enzyme immunodetection. This enzyme is responsible for nucleus and cytoplasm proteolytic activity during apoptosis. The activation of this enzyme has shown to be an effective approach to identify apoptosis in olfactory receptor neurons [25]. EGCG treatment provoked an increase of apoptotic cells in the OE (Control: 5.25±0.46; EGCG 12.5: 6.08±0.57; EGCG25: 18±1.8; EGCG 50: 14.25±1.05) (Fig. **2A-D**). Significant differences in the number of apoptotic cells were observed in animals exposed to the two higher concentrations of EGCG (25 and 50 µg/ml). In addition, treatment with EGCG 25µg/ml showed a greater number of Caspase 3-activated cells compared to EGCG 50µg/ml treatment (Fig. **2E**). Apoptotic cells were observed only in the ORN-Z of the OE. Moreover, some Caspase 3-activated positive cells preserved the typical morphology of the olfactory neurons (Fig. **2**). It is reasonable to discard unspecific cytotoxic effects of EGCG as there were no changes in the histological and morphological characteristics of the tissue independently of the treatment done. The increase in apopototic figures in EGCG treated tadpoles was confirmed by TUNEL (control: 1.5±0.5 vs EGCG 25: 6.5±0.5 (Fig. **3**).

Since the effect of EGCG on proliferation and apoptosis were observed with 25µg/ml and it was not different with

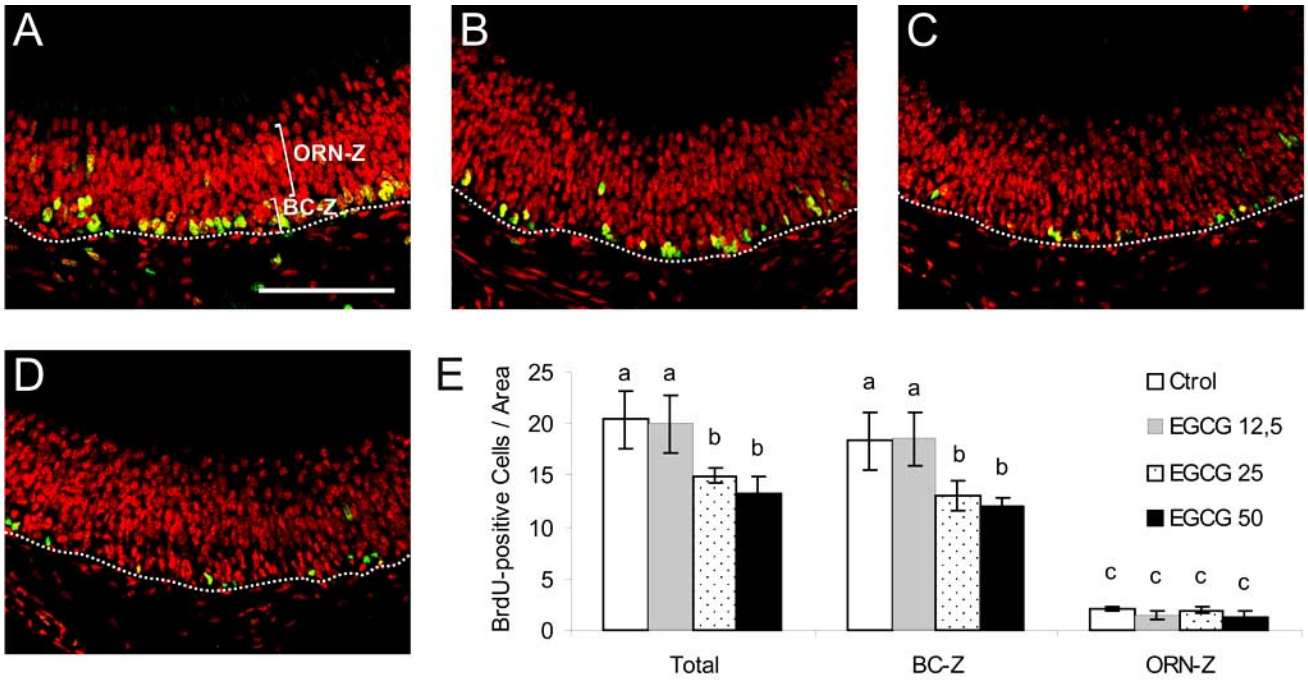

Fig. (1). Effects of EGCG exposure on the cell proliferation in the OE. Images are representative of animals exposed to 0 (control) (**A**), 12.5 (**B**) 25 (**C**) and 50 µg / ml EGCG (**D**). Note BrdU positive-nuclei. Tissue was counterstained with propidium iodide. The dotted line indicates the basal lamina. (**E**) Quantification of the number of BrdU + cells counted in 18 fields per treatment. Different letters indicate significant differences $p<0.001$. BC-Z: basal cell zone, ORN-Z: olfactory receptor neuron-zone. Bars: 100 µm.

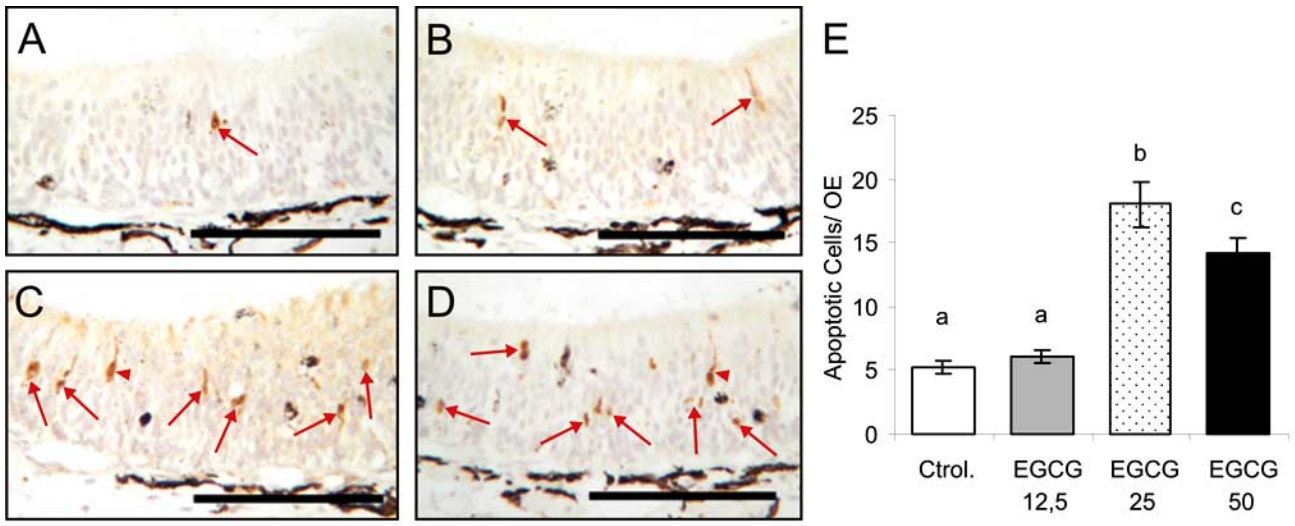

Fig. (2). Effects of EGCG exposure on the apoptosis in the OE. The microphotographs show representative images of animals exposed to 0 (control) (**A**), 12.5 (**B**) 25 (**C**) and 50 µg/ml of EGCG (**D**). The arrows and arrow heads show apoptotic cells, immunoreactivity for caspase-3 activated (aCasp-3-ir), some of these cells still retain the typical neuronal morphology (arrow heads). (**E**) Quantification of the number of cells aCasp-3-ir in 9 fields counted per treatment. Different letters indicate significant differences between groups $p<0.0002$ (between control and EGCG 25 and EGCG 50 µg/ml); $p<0.004$ (between EGCG 25 µg/ml and EGCG 50 µg/ml). Bars: 100 µm.

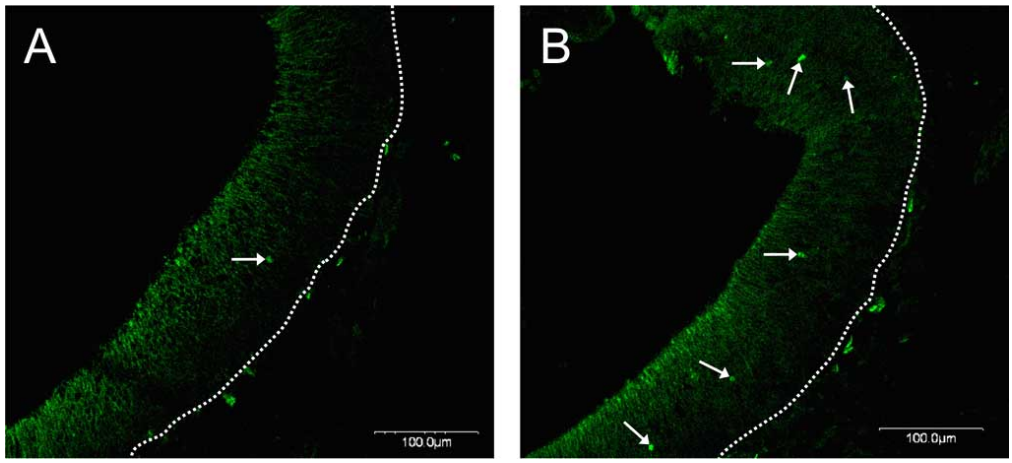

Fig. (3). Apoptosis in the OE measured by TUNEL technique. Images are representative of control animals (**A**) and animals exposed to EGCG (**B**). Arrows indicate TUNEL positive cells (apoptotic cells) within the OE. The dotted line indicates the basal layer. Bars: 100 µm.

50 µg/ml, the semiquantitative studies by Western blot of phospho-ERK1/2 and phosphor-AKT were made between samples from control animals and 25µg/ml ECGC exposed tadpoles. The relative levels of phospho-ERK 1/2 were significantly smaller in the samples of EGCG exposed animals (Fig. **4A**). On the other hand, there were no differences in phospho-AKT levels independently of the treatment received (Fig. **4B**). These results indicate that the effect of EGCG on proliferation and apoptosis could be related to a general fall in the activation of phospho-ERK route, pathway involved in signal transduction of different growth factors like VEGF.

There is extensive information about the beneficial effects of EGCG in cancer therapy. It has been demonstrated that EGCG, in this case, produces a fall in the cell proliferation and an increase in the apoptosis; however, little is known about the EGCG effects in normal cells. Proliferation and apoptosis are essential for normal development, turnover, and replacement of cells in the living systems. Cell signaling pathways, responsible for maintaining a balance between cell proliferation and death, have emerged as rational targets for the management of development.

The present study provides the first evidence that EGCG, the most abundant flavonoid found in green tea, can disrupt the proliferation/apoptosis balance in the OE. We have shown, using tadpole OE as model, that non-toxic concentrations of EGCG reduce significantly cell proliferation as well as produce an increase in apoptosis of olfactory neurons. The treatment with EGCG produced a decrease in the phospho-ERK 1/2 levels indicating a down-regulation of the MAP-Kinases route by this compound. On the other hand, the PI3-kinase pathway does not appear to be affected by EGCG, since the phospho-AKT level was not modified by the treatment. These results differ with those obtained in many cancer cells. Indeed, in tumor cells, it has been demonstrated

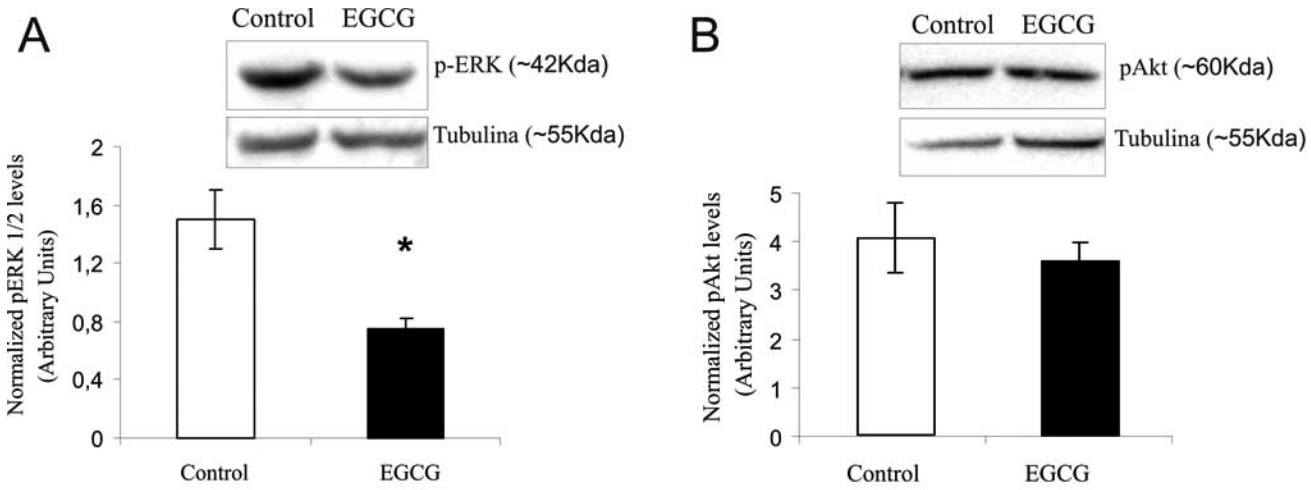

Fig. (4). Representative immunoblots of phospho-ERK1/2 (**A**) and phospho-AKT (**B**) levels from animals exposed without or with EGCG (0 or 25 μg/ml) for 24 hrs. The graphs show the quantification made from 4 different experiments. Values are relativized to β-tubulin and expressed in arbitrary units as means ± SEM. Asterisk indicate significant differences between groups $p<0.015$.

that EGCG caused a fall in the MAPKs pathway [26, 27] and the PI3K pathway [28]. It is known that these signaling pathways have been implicated in many growth factors-mediated physiological processes, including cell proliferation, differentiation, and apoptosis. The effect of EGCG on these pathways depends on the type of receptors for growth factors present in the cell. It is proposed that EGCG acts as a scavenger of growth factors such as PDGF, EGF, FGF and VEGF causing that they are not available for binding with their respective receptors blocking its biological effects [6, 13].

We have previously demonstrated by immunohistochemistry, the presence of VEGF and its receptor, Flk-1 in the olfactory system of *Rhinella (Bufo) arenarum* tadpoles [29]. These results suggest that VEGF, among others, may play a role during neurogenesis as it occurs in other neuronal tissues. For example, in mouse embryos cortical neurons, VEGF would act like a neuronal survival factor preventing the death by apoptosis without producing an apparent modification in cell proliferation [30]. Moreover, it has been suggested that the ERK 1/2 MAPKinases would be the cell signaling pathway mainly involved in this neuroprotective effect [30]. Even, in other models, in which neurotrophic functions of VEGF are described, MAPKinases pathway seems to be the main route for its functions [31, 32]. These results are in accordance to our results showing a decrease of phospho-ERK 1/2 level in EGCG-treated tadpoles.

Hence, we can correlate the decrease in basal proliferation and the increase in the apoptosis observed in EGCG-treated tadpoles with a specific down-regulation in the cell signaling route as a result of EGCG trapping VEGF. However, we should not ignore that EGCG is able to bind with other growth factors [6, 33], so, it is important to assess the state of Flk-1 phosphorylation to correlate unmistakably the effect of EGCG over VEGF.

The OE shows exceptional characteristics for the study of the proliferation processes, differentiation and neuronal survival. The presence of neuronal progenitor cells, neurons in different stages from differentiation, represents an appropriate model for the study of the factors involved in neurogenesis [34]. In this way, the tadpoles OE have unique characteristics for these studies because is a sensory structure fully functional, involved in the detection of food, conspecific and potential predators [35]; which implies the maintenance of a functional neuronal population throughout the larval life. The neuroregenerative capacity of the OE provides us a unique opportunity to study the factors involved in this process.

In summary, our results shows the disruptive effect of EGCG in proliferation/apoptosis balance in the OE and postulate it as a good experimental model for the study of the factors that regulate the neurogenic process. Moreover, it may help to better understand the effects of catechins on cell signaling pathways in normal cells.

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