Role of alpha-tocopherol in counteracting DNA damage induced by Ochratoxin A in primary porcine fibroblasts

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ABSTRACT - Ochratoxin A is a mycotoxin responsible for disease states in both humans and animals. OTA mechanisms of action are numerous, including lipid peroxidation. Oxidative damage results in the modification of macromolecules (i.e. DNA), cell death and tissue injure. Several strategies, such as the use of antioxidants, have been used to reduce OTA cytotoxicity. The aim of this study was to evaluate the role of alpha-tocopherol in counteracting DNA damage induced by OTA in cell cultures. Primary porcine fibroblasts, isolated from embryo and from ear, were incubated for 24h with several concentrations of OTA in order to detect DNA fragmentation. OTA produced DNA fragmentation in a concentration dependent manner in both primary cell cultures. The pre-treatment with alpha-tocopherol caused the reduction of DNA fragmentation in both primary cell cultures, after 24h of incubation with OTA. In particular, when OTA was added at 10 μg/ml in embryo fibroblasts, alpha-tocopherol at the concentrations of 1 nM was significantly ($P<0.05$) able to reduce DNA fragmentation by 16%. In ear fibroblast cultures, alpha-tocopherol at the 1nM concentration was significantly ($P<0.05$) able to reduce DNA fragmentation by 15.23% in the presence of 5 μg/ml of OTA.

Key words: Alpha-tocopherol, Ochratoxin A, Fibroblasts.

Introduction – Ochratoxin A (OTA) is a metabolite produced by several fungi strains and it is a common contaminant of food and feed. This mycotoxin is nephrotoxic, hepatotoxic, teratogenic and immunotoxic in humans and farm animals (O'Brien and Dietrich, 2005). Several mechanisms of action are involved in OTA toxicity, including cellular membrane peroxidation, DNA damage, inhibition of protein synthesis, disruption of calcium homeostasis and inhibition of mitochondrial respiration (Ringot *et al.*, 2006). Scientific literature (Baudrimont *et al.*, 1997; Schaaf *et al.* 2002) indicates that the OTA toxicity as well as the DNA damage, measured in various *in vivo* and *in vitro* studies, are most likely related to cellular oxidative damage mediated by lipid peroxidation. The molecular mechanisms involved in cell death or in antiproliferative effects induced by OTA are still unclear. Cell death is a process involving a great number of factors such as substance, dose/time exposure and cellular in vitro models investigated. In this regard, OTA could be responsible for apoptotic or necrotic process, as underlined by O'Brien and Dietrich (2005). Previous studies (Baldi *et al.*, 2004) showed that some antioxidant compounds were able to counteract the Reactive Oxygen Species (ROS) formation induced by OTA in cell lines. The aim of this study was to evaluate the DNA damage induced by OTA and the role of alpha-tocopherol in counteracting this damage in primary porcine fibroblast cell cultures.

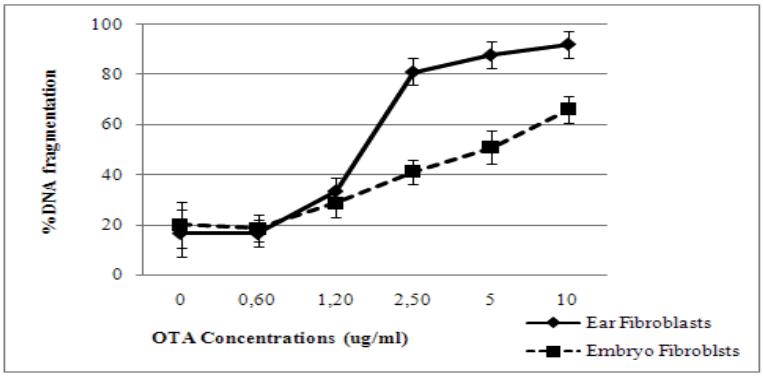
Material and methods – Primary porcine fibroblasts, isolated from embryo and from ear, were cultivated in Dulbecco's minimum essential medium DMEM (Invitrogen), supplemented with 1% glutaMax (Invitrogen), 2500 I.U./ml penicillin and 2.5 mg/ml streptomycin (Sigma) and 10% of FBS (Bio Whittaker). Cells were grown in a humidified atmosphere with 5% CO_2 in air at 37°C. Fibroblasts were seeded in flask

(density: 0.55×10^5 cells/ml) and, at sub-confluence, incubated with several concentrations of OTA (Sigma) (0-0.6-1.2-2.5-5-10 μg/ml) diluted in DMEM without red phenol and 0.6% FCS for 24 hours at 37°C. In a first experiment, DNA damage induced by OTA was evaluated with the diphenylamine method, as described by Sandau *et al.* (1997). After incubation, media were removed and centrifuged at 1,800 *g* for 20 min, to collect detached cells. The remaining adherent cells were scraped off the plastic and lysed in an appropriate volume of ice-cold lysis buffer [10 mM Tris, 1 mM EDTA (pH 8.0), 0.5% Triton X-100] for 30 min at 4°C. After lysis, the intact chromatin (pellet with high-molecular-weight DNA) was separated from DNA fragments (supernatant with low-molecular-weight DNA) by centrifugation for 20 min at 13,000 g. Samples were treated with the same volume of trichloracetic acid (TCA) 25% and precipitated for 16h at 4°C, and then centrifuged 20 min at 13,000 *g* at 4°C and the supernatants were removed. DNA was hydrolyzed by adding the same volume of TCA 5% to each pellet and heating 15 min at 90°C in a heating block. DNA contents were quantitated using the diphenylamine reagent. OD_{600} of the several fractions were determined. The percentage of fragmented DNA was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet, considering also the quantity released by cells undergoing apoptosis and lysis during the experiment. In order to evaluate the protective effect of alpha-tocopherol against DNA fragmentation induced by OTA a second experiment was set up. Cell monolayers were incubated for 3h with alpha-tocopherol solution (1nM and 1 μM), selected from previous studies. At the end of this period, according to the sensitivity of each cell model, selected concentrations of OTA (embryo fibroblast: 2.5-5-10 μg/ml; ear fibroblast: 1.2-2.5-5 μg/ml) were added for the following 24 hours at 37°C. After incubation, media were removed, cells were collected and DNA damage was quantified by diphenylamine method as describe above. Values are expressed as means ± SE (SAS, 1999).

Results and conclusion – OTA was able to induce DNA fragmentation in primary porcine fibroblasts as measured by diphenylamine assay 24 hours post stimulation. As showed in the Figure 1, controls revealed 20% fragmentation in both considered cell cultures, whereas at 1.2 μg/ml of OTA, DNA cleavage became more evident (30%) and increased with greater concentrations of mycotoxin. At the highest concentration used (10 μg/ml), the percentage of DNA fragmentation was 92.18% in ear fibroblast cultures, but 66% in the embryo's one.

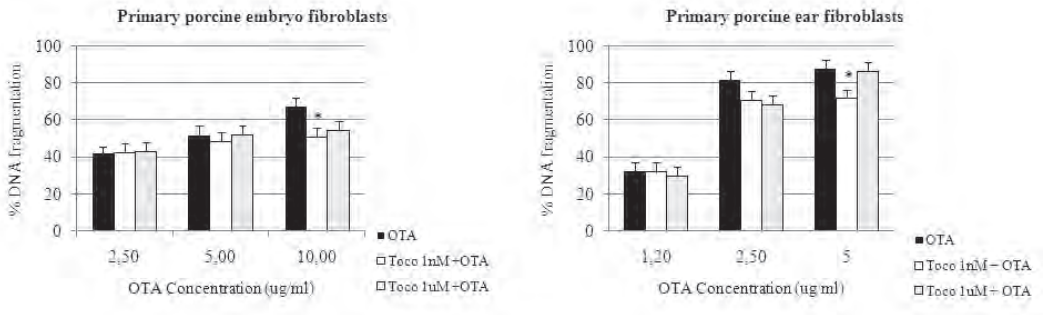
Using the quantitative analysis of DNA fragmentation described above, an interesting effect was observed after pre treatment with alpha-tocopherol and 24h of incubation of OTA in embryo fibroblast cultures. At the highest OTA concentration used (10 μg/ml), the pre treatment with alpha-tocopherol at 1 nM concentration was significantly ($P<0.05$) able to reduce DNA fragmentation. In the same cells the 1 μM alpha tocopherol pre treatment showed a reduction of DNA fragmentation about 12%. In ear fibroblast cultures, only 1 nM solution of alpha-tocopherol was significantly (*$P<0.05$) able to reduce the DNA fragmentation of 15.23% in the presence of 5 μg/ml of OTA.

Figure 1. DNA fragmentation in primary porcine fibroblast cultures after 24 hours of OTA stimulation.



At the other OTA concentrations used both pre treatment of alpha tocopherol decreased DNA fragmentation, but not in a significant manner. Oxidative damage resulted in

Figure 2. Effect of pre treatment of alpha-tocopherol (1nM and 1mM) in primary porcine fibroblasts on DNA fragmentation.



the modification of macromolecules, cell death and tissue injure. In particular, as observed by Schaaf *et al.* (2002) OTA was able to induce the formation of 8-oxoguanine adducts, unequivocal expression of oxidative damage to DNA. In our studies in fibroblast cultures, OTA increased DNA fragmentation in a concentration dependent manner. DNA fragmentation can be quantitatively determined by using the diphenylamine reagent, but this method is not able to discriminate between apoptotic and necrotic chromatin cleavage. It has been reported that oxidative stress induced by OTA could be counteracted by the use of antioxidant compounds. Fusi *et al.* (2008) showed that alpha-tocopherol was able to counteract DNA damage in a cell line (BME-UV1). Our results showed the alpha-tocopherol ability of partially preventing DNA damage induced by OTA in primary porcine fibroblast cultures. As suggested by Traber and Atkinson (2007), alpha-tocopherol is involved in maintaining the integrity of long-chain polyunsatured fatty acids in the cell membranes, acting directly as a peroxyl radical scavenger that terminates chain reaction. The beneficial effect of alpha-tocopherol found out could be ascribed to its monitoring role in the oxidative environment, and through its concentration changes, transferring information to the cell by the regulation of signal pathways (Azzi, 2007). In conclusion, treatments with alpha-tocopherol were able to counteract the oxidative stress induced by OTA at DNA level in primary porcine fibroblasts.

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REFERENCES – Azzi, A. 2007 Molecular mechanism of α-tocopherol action. Free Rad. Biol. Med. 43: 16-21. **Baldi**, A., Losio, M.N., Cheli, F., Rebucci, R., Sangalli, L., Fusi, E., Bertasi, B., Pavoni, E., Carli, S., Politis I. 2004. Evaluation of the protective effects of α-tocopherol and retinol against ochratoxin A cytotoxicity. Brit. J. Nutr. 91: 507-512. **Fusi**, E., Rebucci, R., Pecorini, C., Rossi, L., D'Ambrosio, F., Baldi A. 2008. Evaluation of the damage induced by ochratoxin A and the protective role of α-tocopherol in cultured bovine mammary epithelial cells. Vet. Res. Comm. 32: 343-345. **O'Brien**, E., Dietrich, D.R. 2005. Ochratoxin A: the continuing enigma. Crit. Rev. Toxicol. 35: 33-60. **Ringot**, D., Chango, A., Schneider, Y-J., Larondelle, Y. 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chem.-Biol. Interac. 159: 18-46. **Sandau**, K., Pfeilschifter, J., and Brüne, B. 1997. Nitric oxide and superoxide induced p53 and Bax accumulation during mesangial cell apoptosis. Kidney Int. 52: 378-386. SAS, 1999. **SAS** User's Guide: Statistics, Version 8.2. SAS Inst. Inc., Cary, NC, USA. **Schaaf**, G.J., Nijmeijer, S.M., Maas, R.F.M., Roestenberg, P., de Groene, E.M., Fink-Gremmels, J. 2002. The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. Biochim. Acta 1588: 149-158. **Traber**, M.G., Atkinson, J. 2007. Vitamin E, antioxidant and nothing more. Free Rad. Biol. Med. 43: 4-15.