

Research article

Lens epithelial cell apoptosis and intracellular Ca^{2+} increase in the presence of xanthurenic acid

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Abstract

Background: Xanthurenic acid is an endogenous product of tryptophan degradation by indoleamine 2,3-dioxygenase (IDO). We have previously reported that IDO is present in mammalian lenses, and xanthurenic acid is accumulated in the lenses with aging. Here, we studied the involvement of xanthurenic acid in the human lens epithelial cell physiology.

Methods: Human lens epithelial cells primary cultures were used. Control cells, and cells in the presence of xanthurenic acid grow in the dark. Western blot analysis and immunofluorescence studies were performed.

Results: In the presence of xanthurenic acid human lens epithelial cells undergo apoptosis-like cell death. In the control cells gelsolin stained the perinuclear region, whereas in the presence of 10 μM xanthurenic acid gelsolin is translocated to the cytoskeleton, but does not lead to cytoskeleton breakdown. In the same condition caspase-3 activation, and DNA fragmentation was observed. At low (5 to 10 μM) of xanthurenic acid concentration, the elongation of the cytoskeleton was associated with migration of mitochondria and cytochrome c release. At higher concentrations xanthurenic acid (20 μM and 40 μM) damaged mitochondria were observed in the perinuclear region, and nuclear DNA cleavage was observed. We observed an induction of calpain Lp 82 and an increase of free Ca^{2+} in the cells in a xanthurenic acid concentration-dependent manner.

Conclusions: The results show that xanthurenic acid accumulation in human lens epithelial cells disturbs the normal cell physiology and leads to a cascade of pathological events. Xanthurenic acid induces calpain Lp82 and caspases in the cells growing in the dark and can be involved in senile cataract development.

Background

The primary cause of senile cataract development is still unclear. To date the involvement of α -crystallin, a molecular chaperone for β - and γ -crystallin, has principally been

considered in senile cataract development [1,2], the decrease in the chaperone ability of α -crystallin with age being implicated. Xanthurenic acid is produced from a metabolite of tryptophan (3-hydroxy-kynurenine) [3] in

the presence of 2,3-dioxygenase [4–7]. While 3-hydroxykynurenine [3] is photochemically inert [8] and serves as a protective UV filter of retina, xanthurenic acid is a photosensitizer [9,10]. Xanthurenic acid accumulates with aging in mammalian lenses [11,12] and is involved in the increased fluorescence of the lens with aging [13]. The glucoside of xanthurenic acid is also present in aged lenses [14]. Xanthurenic acid is an example of an endogenous ER stressor provoking an accumulation of unfolded proteins which in turn leads to an overexpression of Grp 94 and calreticulin in the lens epithelial cells of young mammals [15]. We have previously reported that porcine lens epithelial cells in culture respond to xanthurenic acid exposure by an overexpression of stress chaperone proteins, Grp 94 and calreticulin, in [15]. Here, we report that xanthurenic acid leads to human lens epithelial cells (HuLEC) death associated with caspase-3 activation, intracellular Ca^{2+} increase and calpain Lp82 induction. Previously, lens epithelial cell apoptosis was observed in an *in vitro* model of the cataract [16–18].

Materials and Methods

Reagents

We used the following polyclonal antibodies from Santa Cruz Biotechnology Inc. CA, USA: antibody against cytochrome c, secondary antibodies IgG-fluoresceine (FITC)-conjugated. Primary antibody against active caspase-3 p17 was from Promega, Madison, USA. Secondary IgG-Texas Red-conjugated antibodies and Mitotracker CMXRos, DiOC₁₈, Calcium Orange™, Hoechst 33342, propidium iodide were from Molecular Probes, Leiden, The Netherlands. Antibody against GPIP1 peptide of gelsolin was prepared as described previously [24]. Other reagents were from Sigma if not specified. Antibody against calpain Lp82 was from Dr. T. R. Shearer (University of Oregon, Oregon, USA).

Preparation of human lens epithelial cells primary culture

Lenses were obtained after transplantation of cornea from 58, 59, and 63 years old donors from Eye Bank University Hospital, Bern. The primary cells cultures were prepared separately from every donor using two lens capsules. The lens capsules from one donor were treated with 1.5 mg/ml of collagenase 1A and 4 overnight at 37°C. Thereafter, 1 ml of MEM medium with 10% FCS was added, and cells were centrifuged for 10 min at 300 g. The supernatant was discarded and the cells were re-suspended in 1.5 ml the growth medium, as described below, for 14 days in one well of 12-well plate. The cells were cultivated in the dark, in Minimal Essential Medium (MEM) with Earle's salts (Gibco BRL). Cells were grown under a humidified atmosphere of 5% CO₂ in air at 37°C in MEM supplemented with 10% fetal bovine serum, penicillin (10 U/ml), streptomycin (10 µg/ml) and fungizone (250 ng/ml). When confluent, they were incubated in MEM or MEM supple-

mented with xanthurenic acid. A 20 mM stock solution of xanthurenic acid was prepared in 0.5 M NaHCO₃, and diluted in 0.01 M PBS pH 7.4.

Cytotoxicity and apoptosis assay

Cells were observed with differential interference contrast and phase contrast optics on a Zeiss Avionert 405 M inverted microscope, and images recorded with a Matsumoto 3-chip CCD cooled camera with images stored using Adobe Photoshop 4. Cell viability was determined by staining the cells with Hoechst 33342 and propidium iodide (PI) (Juro, Switzerland) using 50 µg/ml of each dye. Fragmented, apoptotic, nuclei were observed with excitation at 350 nm, and necrotic nuclei at 530 nm.

Cell lysis and immunoblotting

Cells were washed twice with cold 0.01 M PBS, pH 7.4. For Western blotting, cells were lysed in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1 % Triton X-100, and the following protease inhibitors: 1 mM phenyl-methylsulfonyl fluoride, and leupeptin, aprotinin, and pepstatin, each at 1 µg/ml. The concentration of proteins was calculated from the absorption maximum at 280 nm, as described previously [15], and the concentration of xanthurenic acid from its absorption maximum at 342 nm (ϵ_M 6500). The lysate was centrifuged for 10 min at 14 000 g, and the supernatant was boiled in loading-buffer for 5 min. Proteins (50 µg per lane) were separated by SDS-PAGE containing 10 or 12.5% acrylamide. After transfer to Hybond ECL membrane (Amersham Pharmacia Biotech AB, Uppsala, Sweden) the proteins were probed with the appropriate antibodies. Chemiluminescence ECL system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was used for the detection of peroxidase-conjugated secondary antibody.

Immunofluorescence studies

Cells grown on glass coverslips were fixed for 10 min at room temperature in 4% paraformaldehyde in 0.1 M PIPES, pH 6.8, washed in PBS and permeabilized for 5 min in PIPES containing 0.05% saponin (65 µl per coverslip), washed in PBS, incubated for 10 min in cold acetone for additional fixing and permeabilisation, and again washed in PBS. Cells were incubated for 1.5 hour with the first antibody diluted in PBS containing 1% bovine serum albumine, and after washing incubated for 1.5 hour with the secondary antibody. The coverslips were then washed in PBS and incubated for 10 min with 65 µl of solution containing 1 µl of Hoechst 33342 dye (1 mg/ml), washed in PBS, and incubated with Antifade Kits (Molecular Probes, Leiden, The Netherlands) according to the supplier's instruction. Staining of mitochondria was performed using Mitotracker CMXRos, as follows: confluent cells cultures were pre-incubated without or with xanthurenic acid in MEM medium for 72 hours. The medium was removed

and replace with medium containing 100 nM Mitotracker CMXRos. After an incubation for 1 hour Mitotracker CMXRos was removed, coverslips were washed twice with PBS, and mounted on the slides using as antioxidative solution 9% w/v of Mowiol (Calbiochem) in 22% glycerol buffered with 0.2 mM Tris/HCl to pH containing 3.5% (w/v) of 1,4-diazabicyclo (2.2.2) octane (Sigma). Membranes were stained using overnight incubation in DiOC₁₈ at concentration of 12 μM in MEM medium.

Results

Xanthurenic acid activates caspase-3 and translocates gelsolin from the mitochondrial region to the cytoskeleton

We observed that 10 μM xanthurenic acid in the HuLEC cell culture medium activate caspase-3 (Fig. 1B). In the same cells the translocation of gelsolin from perinuclear region to cytoskeleton was observed (Fig. 1C, 1D). Previously it was reported that caspase-3 and -9 are associated with mitochondrial membranes [19]. Gelsolin is the cytoskeletal protein responsible for the disintegration of F actin during apoptosis induced by Fas [20]. It was also suggested that gelsolin keeps caspases in the inactive state [21]. We reported previously that gelsolin is abnormally cleaved during apoptosis induced by xanthurenic acid. This abnormal gelsolin cleavage could be a reason of absence of cytoskeleton breakdown in the presence of active caspase-3 (Fig. 1B, 1D).

Xanthurenic acid leads to mitochondrial migration, cytochrome c release, and destruction of mitochondrial structure

In the control cells mitochondria occupy the perinuclear region (Fig. 2A). In the presence of 10 μM xanthurenic acid mitochondrial migration was observed (Fig. 2B). However, at higher concentrations (20 and 40 μM) xanthurenic acid led to the destruction of mitochondria. An intrinsic apoptotic pathway is activated by cytochrome c release and apoptosome formation with APAF-1 and ATP [22]. The apoptosome leads to activation of caspase-9, which activates caspase-3. We observed that in the presence of 10 μM xanthurenic acid cytochrome c was release from mitochondria (Fig. 3). APAF-1 is present in the HuLEC and its level is independent from xanthurenic acid concentration (not shown). Thus, release of cytochrome c is responsible for the observed caspase-3 activation, and nucleus cleavage.

Mitochondrial damage in the presence of xanthurenic acid is associated with nuclear cleavage

In control cells about 5 percent are apoptotic (Fig. 4A, Fig. 5). In the presence of 10 μM xanthurenic acid condensed and/or cleaved nuclei were observed, which a mostly stained only with Hoechst 33342, and not with propidium iodide. This indicated that the observed cell death was apoptotic and not necrotic (Fig. 4B, 4C). With con-

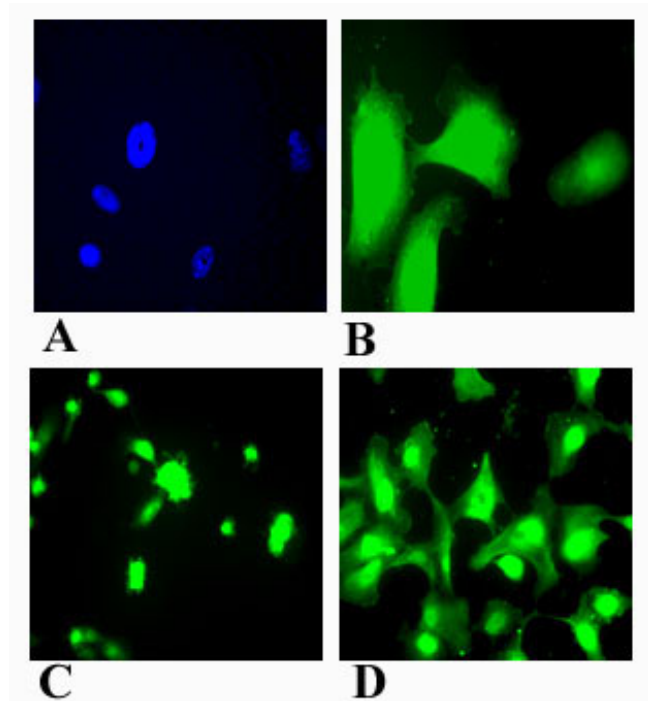


Figure 1

Caspase-3 activation and gelsolin translocation in HuLEC in the presence of xanthurenic acid for 96 hours. Cells stained for active caspase-3 (A, B), and gelsolin (C, D): (A) control cells stained with Hoechst 33342 and for cleaved caspase-3, (B) cells exposed to 10 μM xanthurenic acid and stained for cleaved caspase-3, (C) control cells, (D) in the presence of 10 μM xanthurenic acid.

centrations of xanthurenic acid of 20 μM (Fig. 4D,4E,4F) and 40 μM (Fig. 4G,4H,4I) the destruction of the mitochondrial structure, was associated with nuclear cleavage. Cell death depended on the xanthurenic acid concentration (Fig. 5). In the presence of 10 μM xanthurenic acid about 40 percent of cells were dead, and an increase of xanthurenic acid to 20 μM provoked about 70 percent of cell death.

Xanthurenic acid leads to damage of the cell membrane

The cell membranes were stained with DiOC₁₈ and co-stained with Mito-Tracker Red CMXRos, and the nucleus was stained with Hoechst 33342 (Fig. 6). In the control cell mitochondria were in the perinuclear region and the cell membranes were uniformly stained with DiOC₁₈ (Fig. 6A,6B,6C) In the presence of 10 μM xanthurenic acid the mitochondria migrated to the cell periphery and the cell membranes were not uniformly stained (Fig. 6D,6E,6F). In the presence of 20 μM of xanthurenic acid the mitochondrial structure was destroyed, nuclear DNA was degraded and membranes were not stained with DiOC₁₈ (Fig. 6G,6H,6I).

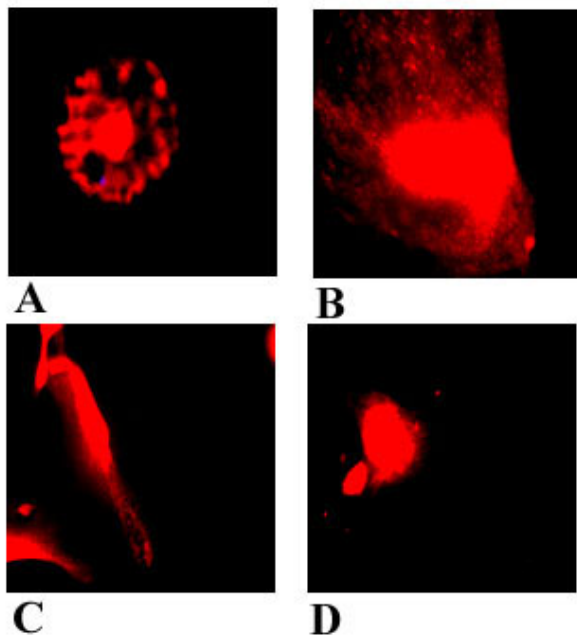


Figure 2
HuLEC, grown in the presence of xanthurenic acid for 96 hours, stained with Mito-Tracker Red CMXRos: (A) control cells; (B-D) in the presence of xanthurenic acid: (B) 10 μ M, (C) 20 μ M, (D) 40 μ M.

Xanthurenic acid causes an increase of free intracellular Ca^{2+} and an induction of the lens calpain Lp82

Ca^{2+} increases are associated with cataract development. We investigated intracellular Ca^{2+} by loading the cells with acetomethyl ester of Calcium Orange™. This dye becomes fluorescent when hydrolysed in the cell by esterases and conjugated with free Ca^{2+} . Cells were incubated without xanthurenic acid or with xanthurenic acid at concentration of 0.125; 0.25, 0.5; 1; 2; 5, 10, and 20 μ M. A presence of xanthurenic acid in the cell culture medium higher than 2 μ M provokes an increase of intracellular Ca^{2+} in comparison with control. In the presence of xanthurenic acid at concentration of 10 μ M and 20 μ M an intensive staining with Calcium Orange™ was observed indicating an increase of free Ca^{2+} in the cell in a xanthurenic acid concentration-dependent manner (Fig. 7).

The lens specific calpain Lp82 [23] was not detectable using the Western blot analysis in the lens epithelial cell culture cultivated in the absence of xanthurenic acid (Fig. 8, lane 1). In the presence of xanthurenic acid the calpain Lp82 was induced (Fig. 8, lanes 2-4).

Discussion

Our previous studies in cell culture showed that xanthurenic acid is a potent endogenous pathological substance in retinal pigment epithelium and smooth muscle

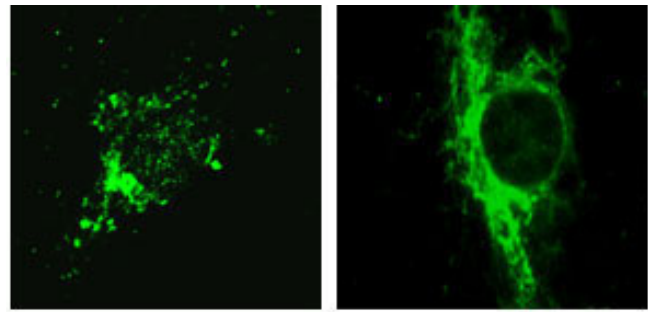


Figure 3
Cytochrome c release in HuLEC grown in the presence of xanthurenic acid for 72 hours. (A) control cells, (B) cells grown in the presence of 10 μ M xanthurenic acid.

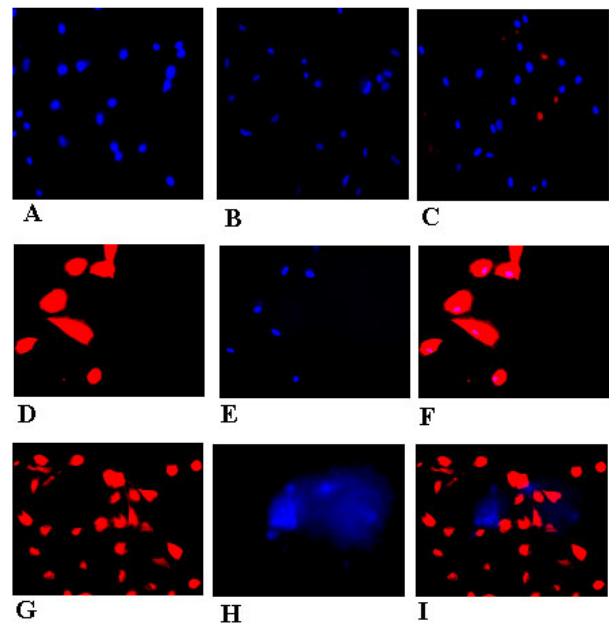


Figure 4
Nuclear DNA degradation in HuLEC in the presence of xanthurenic acid. (A) control cells stained with Hoechst; (B) cells grown in the presence of 10 μ M xanthurenic acid for 96 h and stained with Hoechst; (C) as (B) but stained with Hoechst and propidium iodide; (D-F) cells in the presence of 20 μ M xanthurenic acid: (D) stained with Mitotracker, (E) stained with Hoechst, (F) merge of (D and E); (G-I) cells in the presence of 40 μ M xanthurenic acid: (G) stained with Mitotracker, (H) stained with Hoechst, (I) merge of (G) and (H).

cells [24]. In this study, we observed that xanthurenic acid leads to lens epithelial cells death associated with an induction of caspase-3 and calpain Lp82. Apoptosis was observed in models of cataract upon lens treatment with

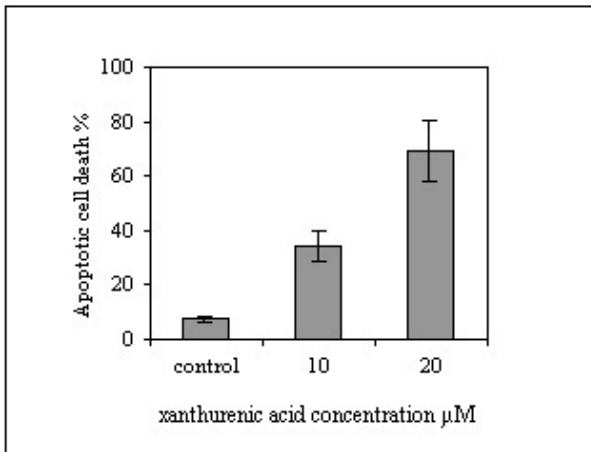


Figure 5
Apoptotic death of HuLEC in the presence of xanthurenic acid.

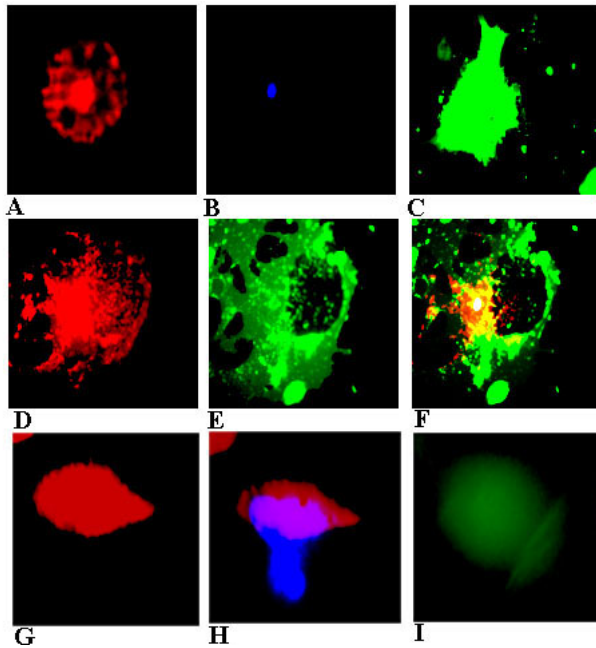


Figure 6
Staining of the mitochondria (Mito-Tracker Red CMXRos), nucleus (Hoechst) and cell membranes (DiOC₁₈) in HuLEC grown in the presence of xanthurenic acid for 96 hours: (A-C), control cells: (A) mitochondria, (B) nucleus, (C) membranes; (D-F) with 10 μM xanthurenic acid: (D) mitochondria, (E) membranes, (F) merge of D, E, and nucleus stained by Hoechst; (G-I) with 20 μM xanthurenic acid: (G) mitochondria, (H) merge of (G) and Hoechst staining, (I) membranes

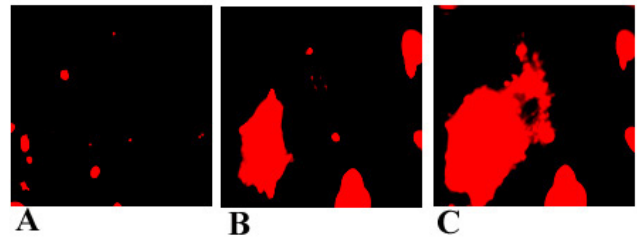


Figure 7
Increase of free Ca^{2+} in HuLEC after growth in the presence of xanthurenic acid for 96 hours. HuLEC were stained with Calcium Orange™: (A) control cells, (B) in the presence of 10 μM xanthurenic acid, (C) in the presence of 20 μM xanthurenic acid.

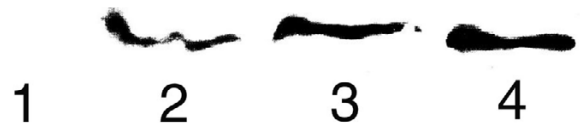


Figure 8
Induction of calpain Lp82 in HuLEC growing 24 hours in controlled cells (lane-1) and in the presence of 1,2,4 mM xanthurenic acid (lanes-2,3,4, respectively).

staurosporine, diamide, and ionophore [16–18]. In the selenite cataract model caspase-3 and calpain were induced [25]. In the normal apoptosis, such as observed with development, cells disappear because of caspase-3-dependent cleavage of DNA and the cytoskeleton [26,27]. The caspase remodeling of the cytoskeleton was indicated as a possible mechanism leading to the aging of lenses [28,29]. Apoptosis is considered as a common cellular basis for non-congenital cataract in mammals [30]. Recent data indicate that senile cataract is a result of proteolysis of crystalins by calpains [31]. Calpains are activated by Ca^{2+} [32].

Previously, it was reported that ER Ca^{2+} homeostasis affects the cells' sensitivity to apoptosis [33]. Lens epithelial cells overloaded by Ca^{2+} showed vimentin cleavage and opacification [34] Thapsigargin, a plant alkaloid, which depletes Ca^{2+} from the ER, was used to stop lens epithelial cell growth [35]. Here, we show that xanthurenic acid, an endogenous molecule, lead to induction of calpain Lp82 and caspase-3 activation. The simultaneous activation of caspase and calpain may lead to an abnormality of apoptosis because calpain cleaves caspases [36]. The calpain Lp82 is involved in cataract formation in connexin α -3 knockout mice [37]. The induction of calpain Lp82 leads to the cleavage of crystalins in the lenses and is involved in the senile cataract development [38]. Xanthurenic acid leads to formation of unfolded proteins [15].

An accumulation of unfolded protein can lead to Ca^{2+} release from intracellular stores as well to caspase induction [39,40]. The observed death of the lens epithelial cells has apoptotic characteristics because release of cytochrome c and caspase-3 activation were observed. However, the apoptosis-like process does not lead to a collapse of the cytoskeleton driven by caspase-3 cleaved gelsolin in the presence of Fas-induced apoptosis [20]. In our study, in the presence of xanthurenic acid cells look normal when observed by the light microscopy. However, when visualized by fluorescence microscopy with Hoechst and propidium iodide a nuclear dysfunction is evident. We have previously reported that xanthurenic acid leads to an abnormal cleavage of gelsolin. The gelsolin stains cytoskeleton of the dead cells. Ca^{2+} and bis-phosphatidylinositol (PPI2) regulate, respectively, association and dissociation of gelsolin to actin [41]. Thus, also the increase in Ca^{2+} may play a role in the association of gelsolin to cytoskeleton.

The mitochondrial damage observed in the presence of xanthurenic acid associated with cytochrome c release could lower energy necessary for the lens enzymes activation observed in senile cataract development.

In this study we observed that xanthurenic acid accumulation can be an upstream event leading to an induction of the lens proteases: caspase and calpain. An accumulation of xanthurenic acid in the human lenses with aging can change the intracellular Ca^{2+} homeostasis. In summary, xanthurenic acid can induce the pathology of the lens epithelial cells without participation of light.

Author contributions

All authors contributed equally to realize this work

Competing interests

None declared

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