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Is digitalis compound-induced cardiotoxicity, mediated through guinea-pig cardiomyocytes apoptosis?☆

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Abstract

Our aim in performing this study was to analyze *in vivo* the cell death mechanism induced by toxic doses of digitalis compounds on guinea-pig cardiomyocytes. We analyzed three study groups of five male guinea pigs each. Guinea pigs were intoxicated under anesthesia with ouabain or digoxin (at a 50–60% lethal dose); the control group did not receive digitalis. A 5-hours period elapsed before guinea pig hearts were extracted to obtain left ventricle tissue. We carried out isolation of mitochondria and cytosol, cytochrome *c* and caspase-3 and -9 determination, and electrophoretic analysis of nuclear DNA. TdT-mediated DUTP-X nick end labeling (TUNEL) reaction was performed in histologic preparations to identify *in situ* apoptotic cell death. Ultrastructural analysis was performed by electron microscopy. Electrophoretic analysis of DNA showed degradation into fragments of 200–400 base pairs in digitalis-treated groups. TUNEL reaction demonstrated the following: in the control group, <10 positive nuclei per field; in the digoxin-treated group, 2–14 positive nuclei per field, while in the ouabain-treated group counts ranged from 9–30 positive nuclei per field. Extracts from ouabain-treated hearts had an elevation of cytochrome *c* in cytosol and a corresponding decrease in mitochondria; this release of cytochrome *c* provoked activation of caspase-9 and -3. Electron microscopy revealed presence of autophagic vesicles in cytoplasm of treated hearts. Toxic dosages of digitalis at 50–60% of the lethal dose are capable of inducing cytochrome *c* release from mitochondria, processing of procaspase-9 and -3, and DNA fragmentation; these observations are mainly indicative of apoptosis, although a mixed mechanism of cell death cannot be ruled out.

Keywords: Apoptosis; Myocytes; Ouabain; Digoxin; Caspase-3; Caspase-9; Cytochrome *c*

1. Introduction

The main pharmacologic effect of digitalis compounds is their property to increase the contractile strength of heart muscle in a dose-dependent way positive inotropic effect. This property has been the mainstay of treatment of heart failure during the last 200 years. In addition to its inotropic effect, digitalis produces severe toxicity in the gastrointestinal and nervous systems, as well as in the heart itself (Ooi and Colucci, 2001; Dec, 2003).

Both inotropic and toxic effects have been attributed principally to total or partial inhibition respectively, of the responsible enzyme for Na^+ and K^+ transport through the cell membrane, that is, Na^+–K^+-dependent adenosintriphosphatase (Na^+/K^+-ATPase) (Skou, 1990; Repke et al., 1995). It has been proposed that the toxicity mechanism is due to a decrease in K^+ and an overload of Ca^{2+} intracellularly due to persistent inhibition of the Na^+-pump, producing electrophysiologic changes such as reduction in resting potential and a rise in cell automatism that leads to homeostasis loss

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and cell death (Langer, 1981). In a previous study (Ramirez-Ortega et al, 2006) in which the digitalis effect on HeLa cells growth was evaluated, it was shown that at toxic concentrations (>10 nM) these compounds are able to induce morphologic (nuclear condensation and fragmentation) and biochemical (release of mytochondrial cytochrome *c*, caspase-9 and -3 activation, nuclear DNA degradation) changes related with apoptotic cell death.

The majority of studies related with apoptosis induction by drugs used in *in vitro* systems leave doubts with regard to reproducibility of results when applied in an *in vivo* setting; therefore, it is important to study *in vivo*, the nature of the damage produced on heart myocytes by acute digitalis intoxication.

There are several studies on the mechanisms of heart tissue damage after acute intoxication with digitalis (Tegtmeier et al., 1992; Khatter et al., 1989; Pilati and Paradise, 1982); however, there are no reports on the cell death mechanism. Because both Ca^{2+} and K^{+} are critical regulators of cellular life and death (Marks, 1997; McConkey and Orrenius, 1996, McConkey and Orrenius, 1997; Hughes and Cidlowski, 1999; Bortner et al., 1997; Dallaporta et al., 1998), there ocurrs in digitalis intoxication an accumulation of Ca^{2+} and a loss of intracellular K^{+} (Charlemagne, 1993; Tanz and Russell, 1983; Hanstein, 1986), events that could be inducers of apoptotic cell death. In this study, we explored the hypothesis that an electrophysiologic imbalance produced by toxic doses of digitalis could trigger cell death by apoptosis in myocytes.

Our main objective was to determine, in cardiomyocytes of digitalis-sensitive animals (guinea pigs), the cell death pathway activated as a consequence of acute digitalis intoxication.

2. Materials and methods

2.1. Chemicals

Ouabain, digoxin, ethidium bromide, dithiotreitol (DTT), IGEPAL CA-630, phenylmethylsulfonyl fluoride (PMSF), cytochrome *c* type VI, trypsin type III, nagarse, Sodium Dodecyl Sulfate (SDS), acrylamide, and bisacrylamide were obtained from Sigma Chemical Company, (St. Louis, MO, USA), while colagenase type IV, Ham's F10, bovine fetal serum (BFS), and agarose were obtained from Gibco, Life Technologies (Grand Island, N.Y. USA). Kits for apoptosis detection *in situ* and the mixture of protease inhibitors were obtained from Roche (Penzberg, Germany). All other chemicals of the highest available purity were purchased commercially.

2.2. Animals

We performed the study on three groups of male guinea pigs (*Cavis porcellus*) weighing 600–700 g with the following distribution: group I, no treatment (control) ($n=5$); group II, ouabain treatment ($n=5$) and group III, digoxin treatment ($n=5$).

2.3. Intoxication of guinea pigs with digitalis compounds

With regard to intoxication of guinea pigs with digitalis compounds, all procedures were approved by The Institutional Research Committee for Animal Use and Care, and this investigation was in agreement with the U.S. National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The design of the digitalic intoxication experiments was based on the studies of Mendez et al. (1953), Moe and Mendez (1951) and Lu et al. (1993). Administration of digitalis or infusion media (isotonic saline solution [ss]) was carried out intravenously (i.v.) (jugular vein) and under general anesthesia (sodium pentobarbital, 38 mg/kg); guinea pigs were maintained under controlled ventilation throughout the procedure. Ouabain and digoxin were continously perfused until signs of severe intoxication appeared (atrial-ventricular blockade and multifocal premature beats). For group II, dosage was 121.57±21.05 nmol of ouabain/kg (88.57±15.33 μg of ouabain/kg), while dosage for group III was 307.77±42.13 nmol of digoxin/kg (240.36±32.90 μg of digoxin/kg). Infusion rate was 5 μg/kg/min; to group I (control), 8 ml of ss were infused at the same rate as treated groups. The intoxication level was diagnosed via an electrocardiogram (ECG) (Lead II). Animals were maintained at intoxication levels for 5 h, with ECG registration every 20 min by means of a polygraph VR6 (Electronics for Medicine, Honeywell). After this period, the heart was perfused (5 min) with 2% paraformaldehyde and 1% glutaraldehyde in cold ss buffered with phosphates at pH 7.4 (PBS). The heart was extracted and left ventricle was obtained. The left ventricle was chosen to obtain a significant amount of tissue to perform the different measurements and determinations, as required by the design of our study. We made an axial cut and the fragment was preserved in 10% formaldehyde in PBS. The preserved tissues were embedded in paraffin, and 4 μm histologic sections were obtained to detect apoptotic *in situ* cell death.

2.4. Electron microscopy analysis

Samples (block <1 mm^3) were fixed in 3% glutaraldehyde–formaldehyde in PBS pH 7.4 for 1 h at 4 °C; after washing in the same buffer, they were post-fixed in 1% OsO_4 in PBS pH 7.4 for 1 h at 22 °C. Samples were then alcohol-dehydrated and embedded in Epon. Semi-thin sections were stained with 1% toluidine blue and observed by light microscopy. Ultra-thin sections were stained with 60% uranyl acetate in alcohol and lead citrate, and were observed with a JEOL 10-10 electron microscope. Printed images were obtained utilizing a Epson stylus photo 820 printer.

2.5. Detection of in situ cell death

TdT-mediated DUTP-X nick end labeling (TUNEL stain) was employed to detect apoptotic cell death in guinea-pig heart-tissue sections following manufacturer instructions (Roche, Germany, 1 684 817). Counterstaining was carried out with Harris hematoxylin, and sections were evaluated with an Olympus microscope under 400× amplification. A positive TUNEL staining was considered when a brown-colored precipitate was detected and when this was located exclusively in the nuclei of intact cardiomyocytes. Sections were divided into four quadrants and five fields (40×-objective field) per

Table 1
TUNEL reaction in histology sections from guinea pigs hearts

Group	Case	Nuclei (+)/field*
I	1	3±2.4
	2	2±2.4
	3	7±2
	4	5±2
	5	9±3.2
II	1	11.5±3.5
	2	15±9
	3	9.46±4.6
	4	30.45±12.79
	5	22±3.8
III	1	2.55±1.4
	2	9.92±6.5
	3	6.08±4.63
	4	11.06±2.35
	5	14±4

I. – Control group (non treated).
II. – Ouabain treated group.
III. – Digoxin treated group.
*Mean±S.E.M.
Differences among groups: ouabain vs. control, $P<0.001$; digoxin vs. control, $P=0.03$; ouabain vs. digoxin, $P=0.088$.

quadrant were analyzed, recording the number of positive cells per field. Results of cell counts in groups II and III were compared with those of group I (Table 1).

2.6. DNA electrophoresis

To detect internucleosomal DNA breakage induced by toxic concentrations of digitalis, the presence of low-molecular-weight DNA fragments in guinea pig myocytes, was determined. Three groups of Guinea pigs (ouabain, digoxin and control; $n=3$ in each group) were treated as described in Section 2.3. To confirm the DNA fragmentation pattern after 18 h of causing acute cardiac intoxication, another group ($n=3$ for each treatment) was studied. Subsequently, the heart was extracted and a left ventricle fragment was obtained to isolate myocytes by treatment with trypsin (0.1%) and collagenase (0.025%). Contaminant fibroblasts were eliminated by incubating the obtained cell population in a culture flask with Ham's F 10 media, supplemented with 10% BFS for 1 h at 37 °C. Thus, myocyte-rich supernatant was separated to extract DNA (Herrmann et al., 1994). Isolated DNA was subjected to electrophoresis in agarose gel at 1% with ethidium bromide. Bands were observed with a transilluminator with ultraviolet light (UV) and photographs were taken with a Polaroid camera.

2.7. Isolation of heart mitochondria

Mitochondria from guinea pig hearts were isolated by the method of Bhattacharya et al. (1991). Hearts from control and ouabain-treated guinea pigs were quickly removed as described for DNA analysis, minced on ice, and resuspended in 10 ml of cold buffer A [250 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl pH 7.3, 1.5 mM $MgCl_2$, 1.0 mM DTT, 0.05 mM PMSF, and a mixture of protease inhibitors (Complete, Roche)], fragments were rinsed, and 10 ml of buffer A plus nagarse (10 mg) were added. After 10 min on ice, tissues were centrifuged at 11,169 ×*g* for 3 min at 4 °C (Sorvall RC-28S, rotor SS34). Pellets were homogenized (five times) in 10 ml of buffer A with a Potter–Elvehjem homogenizer and centrifuged at 1370 ×*g* for 10 min at 4 °C. Pellets were discarded and supernatants were then centrifuged at 13,528 ×*g* for 10 min at 4 °C. Mitochondrial pellets were resuspended in buffer A (250 μl) and supernatants (cytosol) were used for cytochrome *c* and caspase-3 and -9 determination. Protein concentrations were determined by the Lowry method (Lowry et al., 1951). Two mg from mitochondrial fraction (control and ouabain-treated) were resuspended in 1 ml of buffer B (10 mM Tris–acetate pH 8.0, 0.5% IGEPAL, 5.0 mM $CaCl_2$, 1 mM DTT, 0.1 mM PMSF) for cytochrome *c* extraction (Yang et al., 1997); mitochondria in solution were left on ice for 15 min and centrifuged at 100,000 ×*g* (Sorvall-Ultra Pro 80, rotor T880) for 60 min at 4 °C. Supernatants were utilized for cytochrome *c* determination by Western blotting and spectrophotometric analysis.

2.8. Western blot of cytochrome c

Thirty μg of protein extracts from cytosol or mitochondria were analyzed on a 15% SDS-polyacrylamide electrophoresis gel (PAGE). Separated proteins were then electroblotted onto polyvinylidene difluoride (PVDF) membranes (Amersham-Pharmacia, UK) after blocking, cytochrome *c* was detected using a rabbit monoclonal antibody raised against cytochrome *c* [(H-104): sc-7159, (Santa Cruz Biotechnology, CA, USA)] at a dilution of 1:500; blots were developed using a peroxidase-conjugated secondary antibody (IgG-HRP) 1:8000 (Amersham-Pharmacia). Antibody binding was visualized using enhanced chemiluminiscence (Roche Molecular Biochemicals) with X-Omat AR films (Kodak, Mexico). Preparation of cytosolic fractions was performed as described in Section 2.7.

2.9. Immunoblot analysis of caspase-3 (CPP32) and cleaved caspase-9

Cytosolic proteins (20 μg) were resolved by SDS-PAGE (12% W/V) at 70 mA for 2 h and transferred onto PVDF membranes. CPP32/17 was detected through incubation with a primary antibody against human CPP32 (anti-rabbit caspase-3, Cell Signaling Technology, #9662), 1:1000 in Tris-buffered saline-Tween 20 (TBS-T) -5% milk. Caspase-9 was detected through a primary antibody against cleaved caspase-9 (9501, Cell Signaling Technology). Caspase-8 was detected through the antibody (anti-rabbit caspase-8 [MACH; FLICE], AB1879, Chemicon). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit IgG (Dako, 1:5000 in PBS-5% milk), and immunoreactive bands were visualized by enhanced chemiluminiscence detection (Amersham). α-tubulin (Amersham, N 356, 50 kDa) was used as proteic load control.

2.10. Cytochrome c spectrophotometric analysis

Cytochrome *c* measurements in extracts from cytosol or mitochondria solubilized with 0.3% Triton X-100 were performed

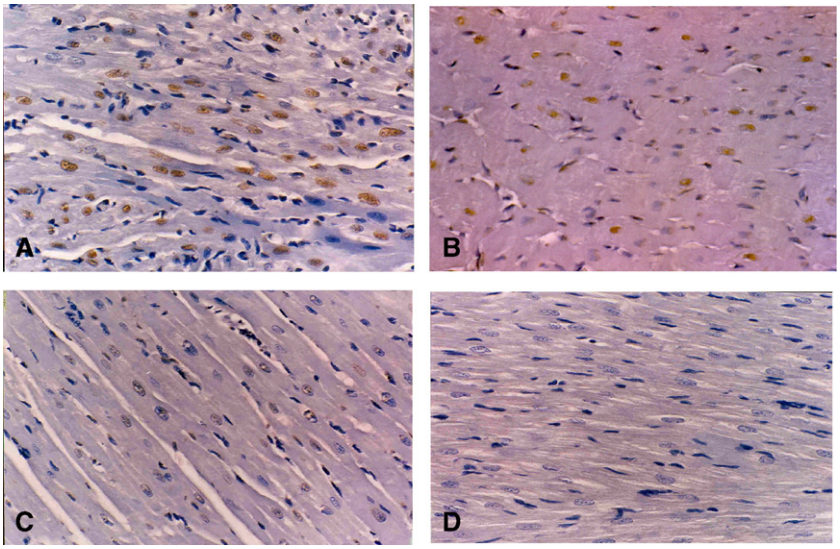

Fig. 1. Determination of myocardial cell apoptosis. *In situ* analysis of myocardial sections stained with TdT-mediated dUTP-X nick end labeling (TUNEL). Five h after treatment, 4-μm paraffin-embedded tissue sections were prepared from guinea-pig hearts. TUNEL staining (brown) of cardiac myocyte nuclei with counterstaining with hematoxylin. A) Positive TUNEL staining in a heart-tissue section of guinea pig intoxicated with ouabain. B) Histology section of guinea-pig heart tissue intoxicated with digoxin. Intact myocytes with positive nuclear staining for TUNEL. C) Histology section of non-treated guinea pig heart tissue, in which scarce cells with positive nuclear staining for TUNEL are shown. D) Negative control reaction in which the enzyme deoxynucleotidyl transferase was not added. Microphotographs were obtained as follows: A and D = 100× and B and C = 80×.

at 400–600 nm in a double-beam spectrophotometer (Shimadzu 2501 TC). Cytochrome *c* type VI purified from horse heart (Sigma) was used as reference.

2.11. Na^+/K^+-ATPase activity

In sarcolemmal vesicles obtained from the homogenate of intoxicated and non-intoxicated hearts, we measured Na^+, K^+-dependent hydrolisis of ATP as reported by Ramirez et al. (2005).

2.12. Statistical analysis

Data were expressed as means ± statistical error of the mean (S.E.M). Statistical evaluation was conducted by Student *t* test for unpaired data, and one-way analysis of variance (ANOVA). A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Digitalis intoxication

Non-treated guinea pigs showed a normal ECG trace that remained unchanged during 5 h under observation. Animals under digitalis intoxication presented ECG signs of severe intoxication (presence of premature ventricular beats and arrhythmia). Heart rate had a mean value of 300 beats/min (normal range = 230–380 cycles/min) during the 5 h that guinea pigs underwent intoxication. Na^+/K^+-ATPase activity determined in the sarcolemmal vesicles of intoxicated heart homogenates was 22.47 ± 8 μmol Pi/mg Protein/h while in non-treated it was 42.34 ± 5 μmol Pi/mg Protein/h.

3.2. In situ cell death detection

No myocardial sections from intoxicated hearts had areas of necrosis, hemorrhage or inflammatory tissue; tissue from ouabain-intoxicated guinea pigs and processed for apoptotic cell detection *in situ* (TUNEL staining) showed myocytes with an intense brownish stain located exclusively in cell nuclei (Fig. 1A); the same reaction was present in tissue preparations from digoxin-treated hearts with differences in nuclear-staining intensity as shown in Fig. 1B. It is noteworthy that the majority of nuclei with positive staining did not present condensation, while tissue sections corresponding to control guinea-pig hearts presented scarce cells with a weakly positive nuclear stain (Fig. 1C). Negative control assay showed no staining (Fig. 1D).

In ouabain-treated hearts, we found a greater number of cells with positive nuclear staining for TUNEL reaction (Table 1); counts ranged from 9–30 positive nuclei per field. In the digoxin-treated group, counts ranged from 2–11 positive nuclei per field, and in the control group the cell count with positive nuclear staining was always lower than that of digitalis compound-treated groups (2–7 nuclei positive per power field).

3.3. Nuclear fragmented DNA detection

When nuclear DNA from guinea-pig hearts intoxicated for 5 and 18 h with a 50% of LD_{50} was extracted, we observed that electrophoretic analysis of DNA in agarose gels (Fig. 2) demonstrated DNA fragmentation with similar images for digoxin-treated guinea-pig hearts (Fig. 2A, lane 2) and for ouabain-treated guinea pig hearts (Fig. 2B, lane 1). The size calculated by electrophoretic mobility of three bands observed

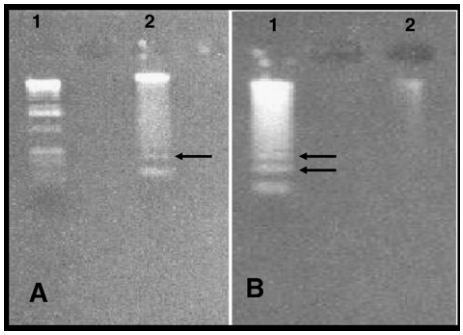

Fig. 2. Genomic DNA fragmentation of guinea pigs cardiomyocytes treated with digoxin or ouabain. Genomic DNA was isolated from heart myocytes with or without treatment and subjected to electrophoresis in agarose gel at 1%. Oligonucleosomal length DNA fragmentation was visualized under ultraviolet light. A) 1. DNA size marker, 2. DNA from guinea-pigs cardiomyocytes treated with digoxin; B) 1. DNA from cardiomyocytes treated with ouabain, 2. DNA from control cardiomyocytes. Arrows indicate fragmented DNA.

was of 220, 460, and 525 bp. DNA from non-treated guinea-pig hearts did not show fragmentation (Fig. 2B, lane 2).

3.4. Electronic microscopy

Five h after ouabain administration, heart tissue had a fixation process for ultramicroscopic analysis focused on mitochondrial status and nuclear chromatin organization. Electromicroscopic observation revealed that dosages at 50% of lethal ouabain dose produced mitochondrial damage and changes in nuclear chromatin arrangement. Electronic micrographies of left-ventricle tissue of control guinea pigs and ouabain-treated hearts in Fig. 3 A and B, respectively, demonstrated that in control tissue mitochondria are denser and their cristae arrangement is parallel, while myofibrils remain normal. Nuclear chromatin arrangement does not show abnormalities. After 5 h of intoxication with ouabain, some mitochondria demonstrated distorted cristae, and the membrane appeared to be broken. Part of the nuclear chromatin appears compacted toward the nuclear border. Autophagic lysosomal vacuoles are present in cytoplasm (Fig. 3B), which are not observed in control tissue.

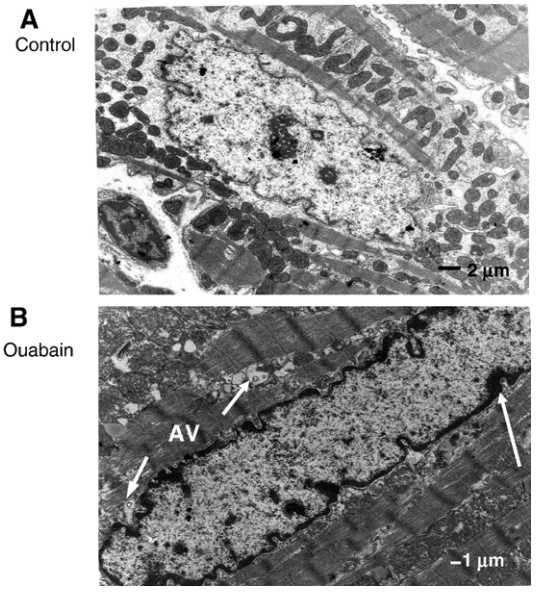

Fig. 3. Electron micrographs of guinea-pig ventricular muscle obtained 5 h after application of A) infusion medium and, B) ouabain (121 µg/kg). A representative cell shows that ouabain-induced alterations in mitochondrias and nucleus and autophagic vacuoles appear in the cytoplasm. AV = autophagic vacuoles.

3.5. Cytochrome c detection by Western blot

To investigate whether toxic doses of ouabain may induce cytochrome *c* release from mitochondria, we performed Western blot analysis in mitochondria and cytosolic fractions (Fig. 4A, and B). In Fig. 4A in the lane corresponding to treated heart, we can observe a band detected by an antibody raised against cytochrome *c*, which is not found in the lane corresponding to cytosol obtained from control heart tissue. When mitochondria were treated with the non-ionic detergent IGEPAL, their contents were released; this proteinic content was subjected to electrophoresis, and by Western blot methodology differences in cytochrome *c* content were detected between control heart tissue and ouabain-treated tissue (Fig. 4B). In the control mitochondrial extract, we detected a greater amount of cytochrome *c*.

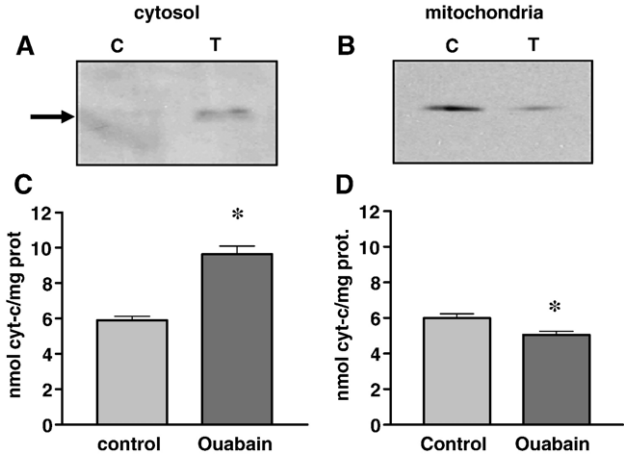

Fig. 4. Ouabain-induced cytochrome *c* release from mitochondria. A) Cytosolic protein (30 µg); B) mitochondrial protein (30 µg) were subjected to SDS-PAGE (15%), and immunoblotting analysis of presence of cytochrome *c* was performed with anti-cytochrome *c* antibodies, as described under Materials and methods section. Labeling was detected by enhanced chemiluminiscence. When hearts were treated with cardiac glycosides, there appeared to be more cytochrome *c* release into the cytosol as compared with control (non-treated heart), with a corresponding reduction in mitochondria. C = control, T = ouabain-treated. The results shown are representative of three independent experiments. Spectrophotometric analysis of cytochrome *c* from C) cytosol, and D) mitochondrial extracts. Cytosolic fractions and mitochondrial extracts obtained as described in Materials and methods section from control and ouabain-treated hearts were used for spectrophotometric determination of released cytochrome *c* (analysis at 550 nm). A standard solution of purified cytochrome *c* (15 mg/ml) was used as control. Spectra were recorder in the range of 600–400 nm. Results were expressed as mean ± standard error of the mean (S.E.M.) from four independent experiments. *$P<0.05$ vs. control.

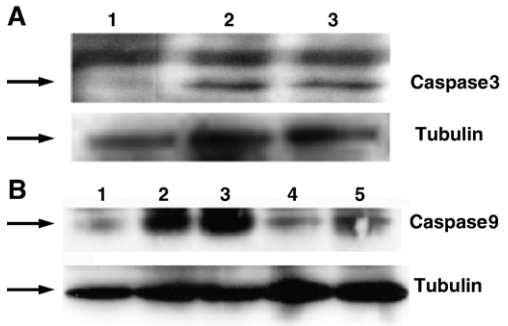

Fig. 5. Ouabain and digoxin induced processing of procaspases-3 and -9. Cytosolic protein extracts obtained from hearts treated and non-treated (control) with ouabain or digoxin were analyzed for presence and processing of caspase-3 and -9 by immunoblots as described in Materials and methods section. Labeling was detected by chemiluminiscence. A) Effect of ouabain and digoxin on caspase-3 cleavage. 1. Control, 2. Ouabain-treated, 3. Digoxin treated, B) effect of ouabain and digoxin on caspase-9 cleavage. 1. Control, 2. Ouabain-treated, 3. Digoxin-treated, 4. HeLa non-treated, 5. HeLa treated with cis-platin. Cytosolic extracts from HeLa cells treated with cis-platin 40 μM (18 h) were used as positive control of caspase-9 processing. The antibody does not recognize the uncleaved procaspase-9. It detects endogenous levels of the large fragment (37 kDa with prodomain/17 kDa) of caspase-9 following cleavage at aspartic acid 330.

3.6. Cytochrome c quantification in mitochondrial and cytosolic fraction

To support the previous observations, we performed spectrophotometric analysis of cytochrome *c* at 550 nm (band α) (Appaix et al., 2000) after reduction with ascorbate, in the mitochondrial extract obtained after treatment with 0.3% triton X-100 as well as in the cytosolic fraction from control heart tissue and from that treated with ouabain. Analysis of spectra at 550 nm showed differences in cytochrome *c* concentrations between the control cytosol (5.9±0.406 nmol/mg of protein, $n=4$) and those obtained from ouabain-treated heart tissue (9.64±0.804 nmol cytochrome *c*/mg protein, $n=4$) ($P=0.0004$) (Fig. 4C). Mitochondria from both tissues were treated with Triton X-100 to release cytochrome *c*, and absorbance was measured at 550 nm of the released proteinic content. The mitochondrial extract of control heart tissue contained more cytochrome *c* (5.983± 0.442 nmol/mg of protein, $n=4$) than that treated with ouabain (5.04±0.34 nmol/mg of protein, $n=4$) ($P=0.0273$) (Fig. 4D).

Cytochrome *c* normally resides in the space between mitochondrial internal and external membranes; its release is provoked by several cellular stress stimuli, such as the cytotoxic effect of drugs. This abnormal release of cytochrome *c* to cytosol promotes activation of the effector proteases (caspases) of cytochrome-dependent cell death (Wieckowski et al., 2001). Consistent with this mechanism, ouabain or digoxin treatment resulted in the processing of caspase-3 and -9 (Fig. 5A and B); thus, as control we used HeLa cells treated and non-treated with cis-platinum to observe the presence and degradation of caspase-9 induced by the toxic effect of this drug, as has been previously reported (Horky et al., 2001), and noticeable differences are shown between these (lanes 4 and 5 Fig. 5B). Analysis of caspase-8 did not show activation of this by the toxic action of the digitalics.

4. Discussion

In excitable cells (cultured brain cortex neurons) Xiao et al., in 2002 observed that ouabain at toxic concentrations (100 μM) induced cell death with apoptotic and necrotic characteristics. In heart myocytes, it is unknown whether cell damage produced after acute digitalis intoxication causes cell death, and if it this indeed does occur, its nature.

The main characteristic of apoptosis is DNA degradation, which in the initial phases is selective for the internucleosomal DNA bond regions (Xiao et al., 2002; Earnshaw, 1995). DNA rupture can be detected *in situ* by enzymatic labeling of the 3'OH terminal with modified nucleotides (biotin-DTUP, DIG-dUTP, fluorescein-dUTP) TUNEL stain (Khodarev et al., 1998).

With regard to the histochemical TUNEL stain, our results demonstrate that histology slides of ouabain-exposed heart tissues exhibit stronger nuclear staining as well as a greater number of stained nuclei than those treated with digoxin. Contrariwise, heart tissue obtained from control guinea pigs presented only some cells with weak positive staining. There are reports that cast doubt on the specificity of the TUNEL reaction; this test was performed initially, and after suggestive findings of apoptosis were observed with the staining, we extracted myocytes and analyzed whether DNA degradation was present. Afterwards, and to confirm our findings, we maintained our experimental subjects under intoxication for 18 h; final results confirmed our observations at 5 h.

DNA fragmentation was confirmed by the presence of characteristic bands on agarose gel electrophoresis. These bands with 220, 460 and 525 bp were observed only in lines corresponding to DNA extracted from ouabain- or digoxin-treated hearts; this degradation pattern was not observed in DNA from non-treated hearts. The smallest band presents a size similar to a nucleosome, and the 460 bp corresponds to a nucleosome dimmer. Deckwerth and Johnson, 1993, reported this observation in cell death studies of neurons grown in the absence of growth factors; in this study, oligonucleosomal DNA fragments are slightly larger than those observed in glucocorticoid-exposed thymocytes. This could be due to specific cell differences in the length of the internucleosomal region sensitive to the nucleases effect, or to different cell-specific nucleases activated by other mechanisms. We believe that the degradation of the cardiomyocytes DNA occurs in 200 bp fragments and dimmers with an approximate size of 400 bp. The larger sized band (525 bp) that appears persistently could be produced by a single-chain nuclease; however, this must be confirmed with a study designed to explore the effects of specific digitalis-activated nucleases.

In our study with histochemical TUNEL stain and agarose-gel electrophoresis of heart myocyte-isolated DNA obtained from digitalis-intoxicated guinea pigs, we found that these myocytes suffered a cell degradation process with internucleosomal DNA fragmentation. No tissue sections corresponding to digitalis-treated animals showed necrotic areas, indicating that digitalis-induced cell death is compatible with apoptosis. In the images observed under light microscopy, only scarce cells

showed the characteristic morphology of apoptosis (chromatin condensation and nuclear fragmentation). A possible explanation for this is that we are observing the first stage of apoptosis, and the condensation and posterior fragmentation process requires more than a 5-h period of the toxic digitalis effects to which the animals in our study were exposed. With electronic microscopy, we were able to observe that in some nuclei, chromatin condensed in the periphery of the nuclear membrane, a step prior to total chromatin condensation (Gavrieli et al., 1992; Messam et al., 1998; Susin et al., 2000), providing support for the previous consideration.

Cytoplasm of treated cardiomyocytes showed autophagic lysosomal vacuoles, which were not detected in control cells; these intracellular structures have been observed in pathological neurodegenerative disorders such as Parkinson's (Anglade et al., 1997) and Alzheimer's (Stadelmann et al., 1999) diseases and in myocytes from failing human hearts (Kostin et al., 2003). Thus, it is necessary to continue with a timely follow-up of these phenomena in order to describe the complete process of digitalis-induced morphologic changes.

The mechanism by means of which ouabain and digoxin induce apoptotic cell death remains under investigation. In *in vitro* studies with human (prostate adenocarcinoma, HeLa cells) and mouse (cortical neurons) cell cultures, it has been shown that after the toxic digitalis insult, intracellular concentrations of Ca^{2+} increase; this promotes cytochrome *c* release from mitochondria with consequent caspase activation and internucleosomal DNA degradation (Wieckowski et al., 2001; McConkey et al., 2000, Ramirez-Ortega et al., 2006). In our study, we provoked an electrophysiologic imbalance by administering toxic dosages (50–60% of the lethal dose) of digitalis compounds to a live animal and detected with two different methods that the effect of digitalis produces cytochrome *c* release from heart mitochondria in a similar way to that observed in *in vitro* experiments (McConkey et al., 2000; Slee et al., 2000; Pu et al., 2002). Cytochrome *c* release is one of the pathways that promotes activation of the caspase cascade (proteases that execute cell death through apoptosis) (Ekert et al., 2004; Chang and Yiang, 2000). Mitochondrial alterations leading to cytochrome *c* release are thought to mediate procaspase activation in many apoptosis models. In our study, we induced intracellular Ca^{2+} overload provoked by the toxic action of digitalis (Langer, 1981; Ishida et al., 2001) in guinea-pig cardiomyocytes. This favored caspase-9 and -3 activation as demonstrated by the presence of proteolytic degradation products from procaspase-9 and -3 in digitalis-intoxicated guinea pig-cytosols. Pelletier et al. (2005), previously reported that the addition of high Ca^{2+} concentrations to a cell extract is sufficient to induce caspase activation and to produce nuclear apoptosis.

We believe that the mechanism of ouabain and digoxin to induce apoptotic cell death either in cultured cells or in live organisms could follow a common pathway, in which Ca^{2+} plays a principal role. Experimentally exposing rat cardiomyocytes to the toxic action of ouabain, a Ca^{2+} overload is produced in the mitochondria. This intracellular Ca^{2+} overload has been related with a mitochondrial disfunction (Di Lisa et al., 1998). Among the later effects of a mitochondrial Ca^{2+} overload, the following have been reported: reduction of the oxidative phosphorylation with the consequent decrease of ATP availability and the sudden dissipation of the mitochondrial membrane potential; both Ca^{2+} overload as well as lowered availability of ATP which gets worse due to the lack of ADP and Pi supply to the mitochondria secondary to Na^+/K^+-ATPase inhibition provoke an opening of the mitochondrial permeability transition pore (MPT), with the consequent release of cytochrome *c* and Ca^{2+} into the extramitochondrial space, which triggers the cascade of events leading to cellular death

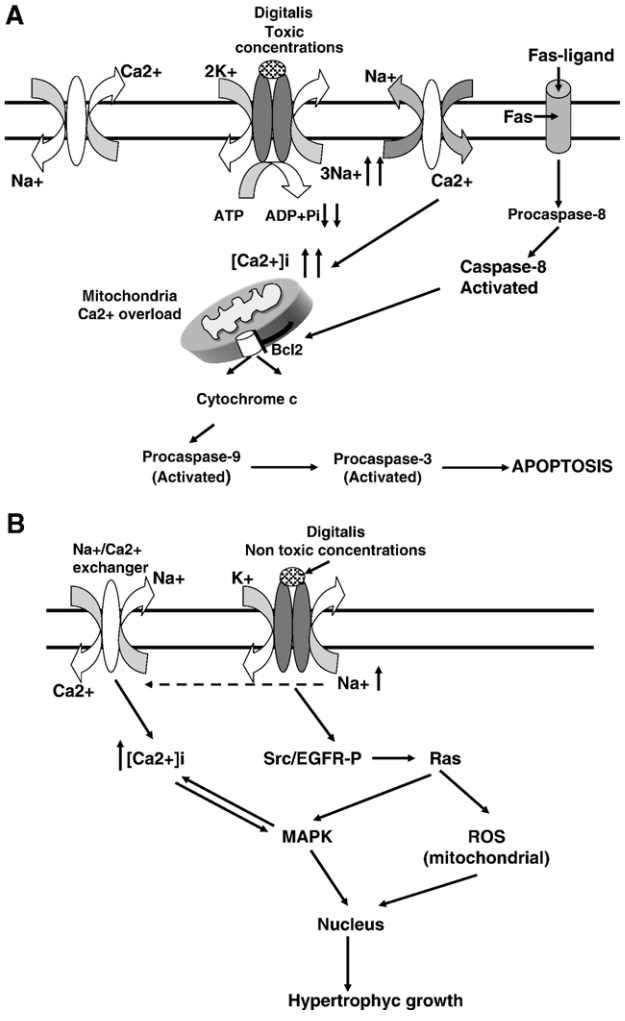

Fig. 6. Proposed mechanism for induction of cell death in cardiomyocites by acute digitalics intoxication. The persistent inhibition of cardiomyocytes Na^+/K^+ ATPase by digitalis provokes accumulation of [Na+]i which stimulates the Na^+/Ca^{2+} exchanger of the cytoplasmatic membrane to extract the accumulated Na^+; this produces a significant increase in $[Ca^{2+}]$i. The prolonged overload of Ca^{2+} and the lack of ADP and Pi secondary to pump inhibition provoke mytochondrial dysfunction with consequent release of cytochrome *c*. The caspases-9 and -3 cascade is triggered. The effector caspase-3 breaks multiple intracellular proteins producing cell death. A) Activated mechanism for toxic concentrations of digitalics B) activated mechanism for non toxic concentration of digitalis. Src (cytoplasmic tyrosine kinase), EGFR-P (phosphorilated receptor for epidermal growth factor), Ras (GTPase-protein), MAPK (mitogen-activated protein kinase). ROS (reactive oxygen species), Fas (Death receptor).

(Di Lisa et al., 1998). Finally, the results obtained allow us to propose a schematic mechanism in Fig. 6. In our model, we discarded a Fas-associated non-specific mechanism because we did not find an activated caspase-8 (Fig. 6A). On the other hand, a mechanism involving ATPase activation as part of a signal transducer system as proposed by Xie and Askari, 2002, Xie et al., 1999 (Fig. 6B) would be turned on under non-toxic digitalis-concentration conditions. In our experimental model, we provoked an acute intoxication which produces, aside of the effect of the Ca^{2+} overload, the decrease in ATP production by the mitochondria, and the affectation of the mitochondrial membrane potential, which causes the opening of the MPT and the consequent release of cytochrome *c*, activation of caspase-3 and -9, and DNA degradation.

In our work and unlike previous studies that employ *in vitro* systems to evaluate the apoptotic ability of digitalis, we used a living animal model, which possesses a greater resemblance to real conditions.

In conclusion, ouabain and digoxin at LD 50–60% induce cytochrome *c* release from mitochondria, procaspases-9 and -3 activation, peripheral nuclear chromatin condensation, and DNA fragmentation, all indicative of apoptosis. Although not all myocytes with DNA fragmentation display the typical morphologic features of apoptosis (total chromatin condensation, cell shrinkage), we believe that digitalis-induced cell death is mainly apoptotic, although a mixed mechanism of cell death cannot be ruled out.

Future experiments should be performed to explore these issues in a comprehensive manner.

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