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Effects of chronic amiodarone treatment on rat testis☆

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ABSTRACT

Amiodarone is a potent agent used to treat tachyarrhythmias, which are especially refractory to other medications, in both adults and children. Although widely used as an antiarrhythmic drug, amiodarone causes many serious adverse effects that limit its use. This study investigated the possible morphological and apoptotic effects of amiodarone on rat testes. Amiodarone was administered to male Sprague-Dawley rats at doses of 20 or 200 mg/kg/day for 14 days. A histopathological examination of testicular tissue revealed the presence of inflammatory cells in the seminiferous tubule lumen together with swelling and vacuolization in the cytoplasm of some spermatogonia; these effects occured in a dose-dependent manner. Immunohistochemical staining showed evidence of apoptosis, including caspase-3, caspase-9, Bax and increased DNA fragmentation was detected via a terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. In conclusion, the results show that chronic amiodarone treatment causes dose-dependent degenerative and apoptotic effects on rat testes.

1. Introduction

Amiodarone is a potent antiarrhythmic agent that is used to treat ventricular and supraventricular tachycardia, which cannot be controlled using other antiarrhythmic agents, in adults and children (Connolly, 1999). Adverse effects and toxicity associated with amiodarone treatment have been reported in several tissues, including liver, heart, eye, skin, lung, nerve, and the hematopoietic system (Lane et al., 2010). The toxicity associated with amiodarone is usually dose-dependent, and cumulative effects may result in even greater toxicity. However, the mechanisms by which the toxicity of amiodarone occurs have not been fully elucidated for some tissues, despite numerous animal and human studies (Connolly, 1999; Lane et al., 2010; Van Erven and Schalij, 2010; Sakr et al., 2013; Martino et al., 2001). Phospholipidosis, apoptosis, membrane destabilization, and direct and indirect cytotoxic effects may lead to toxicity (Sakr et al., 2013). Phospholipids accumulate in cells exposed to amiodarone, and cell proliferation pauses in S phase of the cell cycle, but DNA replication continues in a significant portion of the cells. Apoptosis occurs via two main pathways: the participation of mitochondria and the interaction of death receptors with their specific ligands. Mitochondrial damage leads to activation of caspase-9 and ligation of death receptors activates caspase-8. Both activation of caspase-9 and caspase-8 leads to propagate the cascade of downstream effector caspases, including caspase-3 (Chipuk and Green, 2005; Isomoto et al., 2006). Amiodarone causes apoptosis by acting through the mitochondrial apoptotic pathway via caspase-2, 3, and 9 in H9c2 cells (Piccotti et al., 2005; Isomoto et al., 2006). Moreover, caspase-8 expression in human lung alveolar epithelium increases and certain antiapoptotic proteins decrease following amiodarone treatment (Kapatou et al., 2010). Its toxic effects on liver con-

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sist of mitochondrial toxicity, apoptosis, and necrosis (Kaufmann et al., 2005). As apoptotic effects are observed at therapeutic doses, supratherapeutic doses also likely cause apoptosis. Despite the physiological barriers to the brain and testes, amiodarone can accumulate in these tissues. Amiodarone-related sterile epididymitis and sexual dysfunction (e.g., impotence) are clinically observed (Gasparich et al., 1985; Dobs et al., 1991). Amiodarone accumulates 25–400 fold in the epididymides in a dose-dependent manner (Nikolaou et al., 2007). In spite of these studies and reports, the mechanisms underlying its toxicity on reproductive system are unclear and there is no adequate information on the effects of amiodarone induced testicular injury, especially in terms of apoptotic aspects.

This study was conducted to determine the morphological and apoptotic effects of chronic amiodarone exposure on testes. We investigated the possible effects of amiodarone on the male reproductive system by assessing the morphological features of and apoptosis in rat testes.

2. Materials and methods

2.1. Animals, experimental procedure, and dosing

In this experimental study, 21 male eight-week-old Sprague-Dawley rats (weighing between 292 and 355 g each) from the Karadeniz Technical University Animal Laboratory (Trabzon, Turkey) were used. All animal experiments were carried out in accordance with Karadeniz Technical University Animal Care and Ethic Committee directives and were approved by that committee (decision number 17.01.2012/1). All animals were housed in groups in plastic cages at a constant temperature (21–22 °C) and were maintained on a 12 h light/dark cycle (lights on at 7:00 a.m.). The rats were divided into three experimental groups, with seven rats per group. They were bred on a laboratory diet, with distilled drinking water provided ad libitum. The veterinarians in the laboratory were responsible for feeding, which was performed under controlled conditions. The control group received a control solution (ordinary drinking water) while the low-dose group received 20 mg/kg/day of amiodarone and the high-dose group recieved 200 mg/kg/day of amiodarone. In previous studies, amiodarone was administered to different experimental animal models at various doses and drug administration durations (Sakr et al., 2013; Kapatou et al., 2010; Mesens et al., 2012; Sirajudeen et al., 2002; Kolettis et al., 2007; Agelaki et al., 2007). However, amiodarone is usually given to rats at a dose of 30 mg/kg body weight in previous studies. We have given the highest dose (200 mg/kg/day) in the literature to animals in the high dose group (Almeida et al., 2008). The dose, 20 mg/kg/day, which is similar to the lowest dose for rats in the literature (18 mg/kg), was given to low-dose group (Sakr et al., 2013). 20 mg/kg/day amiodarone in rats represents a dose of approximately 200 mg per day as human maintanence dose. In the present study, amiodarone was administered to rats at doses of 20 and 200 mg/kg/day to evaluate the dose-dependent effects of the drug according to data in the literature (Sakr et al., 2013; Kapatou et al., 2010; Mesens et al., 2012; Sirajudeen et al., 2002; Kolettis et al., 2007; Agelaki et al., 2007; Almeida et al., 2008; Paget and Barnes, 1964). The rats were weighed weekly and the drug doses were adjusted accordingly. The control solution and amiodarone were given orally via gavage in two doses at 12 h intervals for 14 days. The duration of the treatment period was defined according to data in the literature (Quinn, 2005; Kapatou et al., 2010). The rats were weighed and the last dose was given 12 h before euthanesia. The right and left testes were then removed, weighed, and fixed in Bouin solution for histological examination. Absolute testicular and epididymidis values were determined by organ weight using a sensitive scale and the relative organ weight index was calculated. The relative organ weight index is the ratio of absolute testicular weight to body weight. Tissue samples from each experimental group were fixed in Bouin solution for 24 h and processed for paraffin embedding. Tissue sections (4 μm thick) were stained with hematoxylin and eosin.

Indirect immunohistochemical staining (using primary antibodies against caspase-3, caspase-9, and Bax) and an assay for apoptosis (terminal deoxynucleotidyl transferase dUTP nick end labeling; TUNEL) were performed on 5 μm thick paraffin tissue sections after 24 h.

2.2. Analysis of testicular pathology

We analyzed the rate of testicular pathology with hematoxylin and eosinstained slides. Briefly, the seminiferous tubules were viewed at the magnification of ×100 or × 400. Then, three pathological view points (inflammatory cells, spermatogonial swelling, cell vacuolization) were estimated on a semiquantitative scale of − to +++ according to their degree. Ten fields, each 0.2 mm^2 in area and occurring anywhere from the wound surface to a depth of 500 mm, were randomly selected and analyzed using a ×40 objective lens (Olympus CX41). The measurements of cross-sections for all hematoxylin and eosin were used image-analyzing software (Leica Q Win V3 Plus Image).

2.3. Immunohistochemical evaluation

Tissue samples from each group were fixed in Bouin solution for 48 h and processed for paraffin embedding. Sections (4–5 μm thick) were processed and mounted on polylysine microscope slides. Immunohistochemical staining was performed as described previously (Cansu et al., 2011a,b). Stored slides were subjected to microwave treatment in 0.01 M citrate buffer (Lot: 90030610D, Cat: AP-9003-500; LabVision, Fremont, CA, USA). Endogenous peroxidase activity was blocked in 3% hydrogen peroxide (Lot: AHP40114, Cat: TA-125-HP; LabVision). Epitopes were stabilized using serum blocking solution (Lot: 41080981, Cat: 85-9043; Zymed, South San Francisco, CA, USA) and five groups were then prepared from each slide. The first group was incubated with antibodies against caspase-3 (1:100) (rabbit polyclonal) (Cat: RB-1197-P; LabVision), and the second group was incubated with antibodies against caspase-9 (1:100) (rabbit polyclonal) (Cat: RB-1205-P; LabVision) and Bax (1:50) (mouse monoclonal) (Cat: sc-7480; Santa Cruz Biotechnology), and then diluted in UltraAb Diluent (Lot: 50481612, Cat: 00-3118; Zymed) for 60 min at room temperature. As long as the same treatment was received by the negative control sections, instead of the primary antibodies, they were incubated with rabbit IgG or mouse IgG. After incubation, secondary antibodies (rabbit anti-polyvalent, Cat: TP-125-BN; LabVision) were applied. 3-Amino-9-ethylcarbazole (Cat: TA-125-HA; LabVision) was then used as a chromogen. The slides were subsequently counterstained with Mayer's hematoxylin and examined under an Olympus CX31 photo-light microscope (Tokyo, Japan). Two observers, blind to the samples, evaluated the immunolabeling scores. The labeling intensity was graded semi-quantitatively and the histochemical score (HSCORE) was calculated using the equation HSCORE = $\sum$Pi (i + 1), where i = the intensity of labeling with a value of 1, 2, or 3 (weak, moderate, or strong, respectively) and Pi is the percentage of labeled spermatogenic cells for each intensity, ranging from 0 to 100% (Cansu et al., 2011a).

2.4. Determination of apoptosis

TUNEL was employed to determine the apoptotic cell number (Huerta et al., 2007). Testis sections were stained using an