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Praziquantel induced oxidative stress and apoptosis-like cell death in *Raillietina echinobothrida*

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ABSTRACT

Praziquantel (PZQ) is an anthelmintic drug used against trematode and cestode parasites of humans and veterinary animals. Since praziquantel was introduced as a broadspectrum anthelmintic, numerous studies described its successful use against helminth parasites, but its exact mechanism of action is feebly understood. Therefore, the present study was carried out to evaluate the possible role of PZQ induced oxidative stress in apoptosis-like cell death in the poultry tapeworm *Raillietina echinobothrida*. Parasite viability assay revealed a time-dependent reduction in the worm viability compared to the control. Transmission electron microscopy showed typical apoptotic features like condensed nucleus, damaged nuclear envelope and altered mitochondrial membrane in PZQ exposed parasites. Results revealed chromatin condensation and DNA fragmentation in PZQ exposed parasites. There was a notable decline in the level of glutathione and glutathione-*s*-transferase activity leading to the augmented generation of reactive oxygen species. This led to the alterations in the mitochondrial membrane potential with increased active caspase-3/7, confirms the involvement of mitochondria in the event. The present study suggests that PZQ exerts oxidative stress leading to apoptosis-like events in the parasites resulting their death.

1. Introduction

Helminth parasites are one of the most common infectious agents, infecting more than one billion people and also causes extensive production losses in livestock (Charlier et al., 2014; Pullan et al., 2014). *Raillietina* is one of the most important avian cestode parasite prevalent throughout the Globe (59.525%) and the second most prevalent helminth recorded in northeast India (51.5%), responsible for malnutrition, growth retardation of young chicken, emaciation of the adult and decreased egg production of the hen (Yadav and Tandon, 1991; Permin and Hansen, 1998; Kumar et al., 2007). PZQ is an anthelmintic agent highly effective against a broad spectrum of cestodes and trematodes, with mild side effects and low cost (NdeffoMbah et al., 2013). Currently, PZQ is the drug of choice for all forms of schistosomiasis. Review of the literature reveals that PZQ exerts its antischistosomal activity by either altering the Ca^{2+} homeostasis, membrane fluidity, reducing glutathione (GSH) concentration or by compromising Na^+/K^+-ATPase activity, thereby making the parasites more susceptible to host immune attack (Pax et al., 1978; Lima et al., 1994; Ribeiro et al., 1998; Ferreira et al., 2015). Other notable morphological alterations caused by binding to surface protein (actin) of worms leading to disruption, vacuolization and blebbing of the tegument and subtegument (Tallima and El Ridi, 2007; Xiao et al., 2009). Despite a huge amount of data available regarding the clinical use of PZQ for more than three decades the exact mechanism of action still remains unsettled.

Apoptosis is an energy dependent, highly complex and sophisticated cascade of molecular events occurs during development, differentiation and homeostasis to maintain cell populations in tissues of higher animals. It also occurs as a defense mechanism during immune reactions and against infection caused by different infectious agents (Zitvogel et al., 2010). There are mainly two pathways, the extrinsic or death receptor-mediated and the intrinsic or mitochondria-mediated pathway of apoptosis and both pathways are connected and can influence each other (Igney and Krammer, 2002).

Reactive oxygen species (ROS) are short-lived diffusible entities such as hydroxyl ($^{\bullet}OH$) or alkoxyl ($RO^{\bullet}$) or peroxyl ($ROO^{\bullet}$) radicals, helps in modulating normal cellular functions by acting as a signaling molecule (Devasagayam et al., 2004). Excessive production of ROS can occur in response to toxicant exposure, radiation damage and also during parasitic infection (Circu and Aw, 2010). However, antioxidant systems of helminths act against ROS species

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generated by host's defense system like macrophages, neutrophils, eosinophils etc. Oliveira and Oliveira (2002) reported that ROS-induced cellular toxicity leads to shifting of metabolism in helminth parasites (*Schistosoma*, *Angiostrongylus*, and *Dirofilaria*) to minimize oxidative stress-induced damage. Thus, production of antioxidant enzymes is considered as an evasion mechanism used by the parasite to survive for a long time in the host (Chiumiento and Bruschi, 2009). The unique and highly restricted antioxidant system has been largely considered as a potential target for the development of anthelmintic since it is quite different from the mammalian host (Ross et al., 2012).

Here, we have analyzed several important apoptotic markers via morphological and biochemical methods in PZQ exposed parasites. Thus the present investigation describes a potential mechanism by which PZQ induced oxidative stress leads to apoptosis-like cell death in *Raillietina echinobothrida*.

2. Materials and methods

2.1. Drug and chemicals

Praziquantel (P4668), all enzymes, co-enzymes and fluorescent dye were obtained either from Sigma-Aldrich (St. Louis, USA), or Sisco Research Laboratories (Mumbai, India). Other chemicals were of analytical grade and obtained from Himedia (Mumbai, India). All reagents were prepared in Millipore water.

2.2. In vitro experiment

Live adult tapeworms (*R. echinobothrida*) were collected from the intestine of freshly slaughtered naturally infected domestic fowl and put in 0.9% phosphate buffered saline (PBS, pH 7.2). After washing in PBS, the test parasites were incubated at 37 ± 1 °C with 1 microgram of PZQ/ml of PBS containing 0.1% dimethyl sulfoxide (DMSO). Parasites without any drug served as control and were incubated in PBS having 0.1% DMSO only. Five replicates for each set of incubation medium were used (n = 6).

2.3. Parasite viability inhibition assay

Parasites exposed to PZQ were further screened for MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] reduction assay following Comley et al. (1989) with slight modification. At 0, 3, 6 and 9 h of treatment, parasite motility was checked and were suspended in 2 ml of PBS (having 1 mg of MTT/ml) and incubated for 2 h at 37 °C in the dark. The dark blue crystals of formazan formed were solubilized in 200 μl of DMSO. Then the optical density was quantified in a double beam UV–vis spectrophotometer (Cary 100, Varian) at 595 nm, using DMSO as a blank. Five replicates for each time point (0, 3, 6 and 9 h) were used (n = 4).

2.4. Ultrastructural studies

Worms exposed to PZQ (1 μg/ml) and control were fixed in modified Karnovsky's fixative, post-fixed in 1% OsO_4 (Osmium Tetroxide) buffered with 0.2 M sodium cacodylate for 1 h, dehydrated through graded acetone and embedded in Araldite. Ultrathin sections were stained with an alcoholic saturated solution of Uranyl acetate and Lead citrate (Reynolds, 1963). The stained sections were examined in a JEM 100CXII (Jeol) transmission electron microscope at an accelerating voltage of 120 kV. Three grids from each section of control and different treated groups were examined to ensure reproducibility of the results.

2.5. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) assay

Detection of DNA fragments in apoptotic cells generated by apoptotic-associated endonuclease was performed using a kit (APO-BrdU™ TUNEL Assay Kit, Invitrogen, USA), following the instruction given by the manufactuerer. More than five representative photographs from each group were analyzed and statistical analysis was done from three independent experiments. Apoptotic index (AI) was calculated as follows:

$$\text{Apoptotic index}\,(\%) = \frac{\text{Number of TUNEL positive cells}}{\text{Total number of cells in the field}} \times 100$$

2.6. DAPI (4, 6′-diamidine-2-phenylindole dihydrochloride) staining for chromatin condensation

PZQ exposed parasites at different intervals of incubation were taken out, washed and fixed in 4% paraformalde. For the evaluation of chromatin condensation, the sections were stained, using 1 μl/ml (Stock: 1 mg/ml) of DAPI in sterile PBS at room temperature for 25–30 min. The stained sections were examined under a confocal microscope (Leica, TCS SP5, Germany).

2.7. Detection of mitochondrial membrane potential (MMP) by rhodamine 123

Rhodamine 123 (Rh123) is a cell permeant, cationic fluorescence dye that is used to label mitochondria accoding to the negative membrane potential in the mitochondrial inner membrane. Healthy mitochondria with intact mitochondrial membrane accumulate higher Rh123, however damaged mitochondria accumulate lower Rh123, hence lower relative intensity. The MMP was detected following Emanus et al. (1986). Briefly, Rh123 (Sigma-Aldrich) was prepared in Millipore water stored at −20 °C as aliquots of 100 μg/ml solution. The stored solution was diluted to 10 μg/ml with PBS before using. After incubating in Rh123 for 30 min at 37 °C, parasite sections were washed thrice in pre-heated PBS. The sections were observed under confocal microscope at maximum excitation wavelength 511 nm and emission wavelength 534 nm. The relative fluorescence intensity of the Rh123 was calculated using ImageJ software (1.46s, NIH, USA) from five individual micrographs of each group and statistical analysis was done from three independent experiments.

2.8. Detection of active caspase 3/7

To test the involvement of caspases in PZQ induced apoptosis, we attempted to detect the active caspases 3/7 in both the control and treated parasites at different time intervals. Pro-apoptotic caspase 3/7 was detected following the manufacture's protocol from Invitrogen (Image-iT™ LIVE Green Caspase-3/7 Detection Kit, Life Technologies, CA, USA). Briefly at different time interval parasites were taken out and subjected to cryo-microtomy at a thickness of 7–10 μm. Sections were then labeled with FLICA (Fluorescent Labelled Inhibitor of caspases) reagent and incubated for 30–45 min at 37 °C in the dark. After incubation, the sections were washed in PBS, mounted in 1x wash buffer and observed under fluorescence microscope (Leica DM 4000 B, Germany).

2.9. Assessment of antioxidant (GSH) and its related enzymes (GST and SOD)

2.9.1. Preparation of worm homogenate

At different time intervals, homogenates of PZQ treated and control worms were prepared in lysis buffer containing 20 mM