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In vitro assessment of praziquantel and a novel nanomaterial against protoscoleces of *Echinococcus granulosus*

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Abstract

The present study describes the activity of a nanomaterial on protoscoleces of *Echinococcus granulosus*, which exhibited morphological changes and apoptosis. Apoptotic changes were deduced on the basis of effector caspase activation and nucleosomal laddering. Invaginated protoscoleces maintained *in vitro* became evaginated and had hooks, presumptive suckers and stalks. Degenerative changes of protoscoleces were evidenced after treatment with praziquantel and nano-combination. Protoscoleces treated with praziquantel had distinct attestation of necrosis and nano-combination-treated protoscoleces had signatures of apoptosis.

Introduction

Cystic echinococcosis (CE) is caused by the larval stage of the tapeworm *Echinococcus granulosus*. This species has a wide spectrum of intermediate hosts, including ungulates and humans, where unilocular cysts occur in the liver and lungs. The most common treatment is surgical removal of cysts and, in inoperable cases, chemotherapy remains the only choice (Naguleswaran *et al.*, 2006). Compounds used for chemotherapy are albendazole, mebendazole and praziquantel (PTZ) for their protoscolicidal activity. The latter compound is used to treat, but a direct action of PTZ on hydatid cysts is yet to be explored (Morris *et al.*, 1986, Morris & Taylor, 1988; Urrea-París *et al.*, 2001).

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The protoscolicidal activity of polyisohexylcyanoacrylate-bound acrylate-bound doxorubicin and poly (DL-lactide) nano-particle-loaded albendazole has been tested in the past (Liance *et al.*, 1993; Rodrigues *et al.*, 1995). In the present study the effect of the nanomaterial's protoscolicidal activity on the apoptotic pathway in drug-induced protoscoleces was evaluated by nucleosomal fragmentation and evaluation of the caspase cascade pathway. Apoptosis is a fundamental complex process which helps multicellular organisms to kill and remove unwanted cells during development, normal homeostasis and disease events (Porter & Janicke, 1999). This process can be monitored by chromatin condensation, nuclear pyknosis, nucleosomal fragmentation, activation of the caspase cascade and formation of apoptotic bodies (Cryns & Yuan, 1998).

Therefore, the present study describes protoscolicidal activity of nanomaterial appraised on the basis of the apoptotic pathway.

Materials and methods

Sources and in vitro *screening of chemotherapeutic agents*

The nano-combination used in the present study was kindly provided by M/S Amazon Global Inc. (California, USA). Pure PTZ was supplied by M/S Alembic Ltd. (Mumbai, India) and the pure compound was reconstituted as 10 mg/ml in dimethyl sulphoxide (DMSO). Viability of isolated protoscoleces of buffalo origin was assessed in triplicate by dye exclusion test and flame-cell activity (Walker *et al.*, 2004). Subsequently, protoscoleces were washed three times in phosphate-buffered saline (PBS) (pH 7.2) containing gentamicin sulphate (80 mg/l). Finally, protoscoleces were harvested in M199 medium supplemented with the required concentration of penicillin (100 U) and streptomycin (100 μg/ml) and incubated at 37°C and 5% CO_2. Finally protoscoleces were harvested separately with PTZ (0.1 mg/ml), nanoparticles at three different concentrations in DMSO (50, 25 and 10 ppm), DMSO and without anthelmintics. Organisms were collected at 24 h intervals for conducting microscopy and deduction of the apoptotic pathway.

Scanning electron microscopy

The collected organisms were washed three times in PBS (pH 7.2). Organisms were further fixed in 3% glutaraldehyde for 12 h at room temperature. Following fixation, dehydration of the organisms was done in ascending grades of alcohol. Finally, processed samples were sputter-coated with gold and examined by scanning electron microscopy at the Bose Institute, Kolkata (Quanta 200 MK2, FEI, The Netherlands).

Caspase assay

The caspase assay was done using the caspase3/CPP32 colorimetric assay kit (Bio Vision, San Francisco, USA). A total of 500 protoscoleces were taken from the treated and control groups and washed three times in PBS. Subsequently, protoscoleces were suspended in 50 μl chilled lysis buffer and triturated in a pestle and mortar, maintaining the low temperature. Further suspension was centrifuged at 10,000 *g* for 1 min. Finally, the supernatant fraction was taken into a tube and snap-cooled for the colorimetric assay. The protein content of each supernatant fraction was estimated by the Bradford assay (Bradford, 1976). The supernatant containing 150 μg protein was diluted in 50 μl of 2 × reaction buffer (containing 10 mM dithiothreitol). Finally, 5 μl of 4 mM Asp–Glu–Val–Asp-peptide nucleic acid substrate (200 μM final concentration) was mixed and incubated at 37°C for 2 h. Optical density values were taken at 405 nm (Beckman Coulter, California, USA).

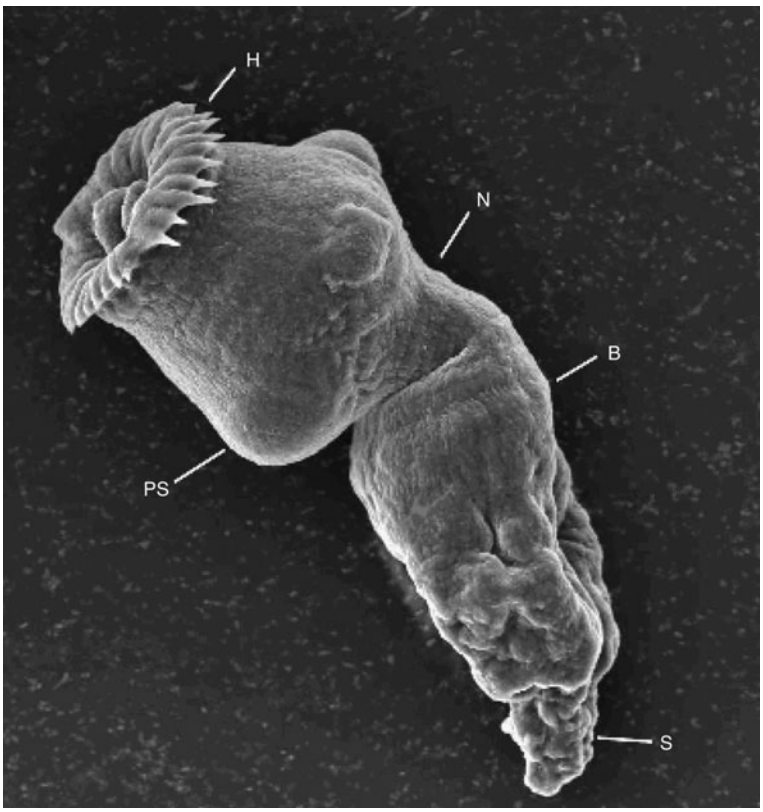

Fig. 1. The protoscolex of *Echinococcus granulosus* with hooks (H), presumptive suckers (PS), neck (N), body (B) and stalk (S) (magnification × 800).

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Table 1. Caspase-3 activity in the protoscolex of *Echinococcus granulosus* treated with the nanomaterial, praziquantel (PTZ), dimethyl sulphoxide (DMSO) and the control group.

Hours after treatment	Caspase-3 activity					
	Nanomaterial (ppm)					
	10	25	50	PTZ	DMSO	Control
24 h	0.059 ± 0.001	0.066 ± 0.001[b]	0.073 ± 0.001[b]	0.058 ± 0.0005	0.057 ± 0.0005	0.057 ± 0.0005
48 h	0.064 ± 0.001[a]	0.072 ± 0.001[b]	0.078 ± 0.000[b]	0.060 ± 0.0005	0.058 ± 0.001	0.057 ± 0.0005
72 h	0.071 ± 0.001[b]	0.094 ± 0.001[b]	0.105 ± 0.0005[b]	0.062 ± 0.000	0.060 ± 0.000	0.058 ± 0.0005

[a] and [b] denote significant difference at $P < 0.05$ and $P < 0.001$, respectively, in comparison with the control group.

Nucleosomal fragmentation

Genomic DNA was extracted from protoscoleces of the treated and control groups using a Q-BIO Gene kit (New York, USA). The concentration of genomic DNA was measured at 260 nm and samples containing 20 μg DNA were subjected to electrophoresis and visualized after ethidium bromide staining.

Data analysis

Numerical values were analysed by two-way ANOVA using GraphPad Prism® version 5, (GraphPad Software, Inc., San Diego, California, USA).

Results and discussion

Protoscoleces showing more than 90% viability were selected for the study. Isolated protoscoleces in the control group underwent evagination after 72 h of incubation and exhibited well-defined hooks, presumptive suckers, body and stalk (fig. 1). Degenerative changes in the PTZ-treated group were observed from 48 h onward, characterized by morphological and structural alterations. Protoscoleces treated with the nano-combination exhibited morphological changes which were characterized by sloughing of tegument and distortion of distinct body parts. Tegument of the protoscoleces became more porous after treatment with the nano-combination.

There was more caspase-3 activity in the nano-combination treated group in comparison with other groups. Further, protoscoleces treated with 50 ppm nano-combination had the highest caspase-3 activity ($P < 0.001$) compared with protoscoleces treated either with 25 or 10 ppm nano-combination. However, there was no significant difference in caspase-3 activity in the PTZ- and DMSO-treated groups compared with the control group (table 1).

The present *in vitro* model has been suggested as an ideal first-round test system for the screening of anti-cestodal activity of the nano-combination. Distorted ultrastructural changes indicate protoscolicidal activity of both PTZ and the nano-combination (Morris *et al.*, 1988; Urrea-París *et al.*, 2001). In mammalian cells and helminths, activation of caspase is a central element of apoptosis, which can be assayed by exposing cytosolic extracts from apoptotic and pre-apoptotic cells to substrates prepared from normal cells (Lazebnik *et al.*, 1993; Martin *et al.*, 1995). Characteristic DNA laddering in the 50 ppm and 25 ppm nano-combination-treated groups indicated apoptosis. However, DNA smearing in the rest of the groups suggested necrosis of the cells (Zhivotovsky & Orrenius, 2001). Therefore, from the present investigation, it may be concluded that the nano-combination has potential as a protoscolicidal agent, which could be deduced on the basis of ultrastructural changes, nucleosomal fragmentation and activation of the caspase cascade.

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