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NEUROTOXIC EFFECT OF BAYLUSCIDE, SELECRON AND ETHANOLIC EXTRACT OF *ANAGALIS ARVENSIS* ON THE CEREBRAL GANGLIA OF *BIOMPHALARIA ALEXANDRINA* SNAIL

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ABSTRACT

The present study was designed to evaluate the neuropathological effect of Bayluscide, Selecron and ethanolic extract of *Anagalis* on the neurons of the cerebral ganglia in the freshwater snail *B. alexandrina*. The snails were subjected to lethal concentrtion (LC_{90}) of each compound (3.468 ppm for Selecron, 0.082 ppm for Bayluscide and 38.129 ppm for ethanolic extract of *A. arvensis*) till death of all snails after 90 minutes. Then the snails were dissected and the cerebral ganglia were removed. Electron microscopical examination of treated animals revealed severe ultrastructural alterations in the cerebral ganglia. These alterations included hyperchromatic, pyknotic or highly shrunken nuclei, extreme indentation of plasma membrane, atrophy of the perikarya of some neurons, margination of nucleoli, fragmentation or dilation of rough endoplasmic reticulum, damage of mitochondria and vacuolation and destruction of cytoplasm. In addition, degenerated synaptic vesicles and increased number of autophagosomes and myelin figures were frequently observed.

The present study was designed to evaluate the mode of action and neuropathological effect of Selecron, Bayluscide and ethanolic extract of *Anagalis arvensis* on the neurons of the cerebral ganglia in the freshwater snail *B. alexandrina*. The snails were subjected to lethal concentration (LC_{90}) of each compound (3.468 ppm for Selecron, 0.082 ppm for Bayluscide and 38.129 ppm for ethanolic extract of *A. arvensis*) till death of snails after 90 minutes. Then the snails were dissected and the cerebral ganglia were removed. Electron microscopical examination of treated animals revealed severe ultrastructural alterations in the cerebral ganglia. These alterations included hyperchromatic, pyknotic or highly shrunken nuclei, extreme indentation of plasma membrane, atrophy of the perikarya of some neurons, margination of nucleoli, fragmentation or dilation of rough endoplasmic reticulum, damage of mitochondria and vacuolation and destruction of cytoplasm. In addition, degenerated synaptic vesicles and increased number of autophagosomes and myelin figures were frequently observed

In the present study the acetylcholinesterase enzyme (AChE) activities was measured in *B. alexandrina* snails exposed to the same concentration of the tested compounds. The AChE activities in *B.alexandrina* showed wide variation along the treated snails and control snails, The AChE activities in *B. alexandrina* decreased significantly at bayluside bayluside (–55.3% reduction) followed by Selecron (– 49.2% reduction), and *A. arvensis* (–39.9% reduction).

INTRODUCTION

The use of molluscicides is one of the procedures recognized by the World Health Organization against schistosomiasis (WHO 1998). Selecron® 500 EC is an emulsifiable concentrate insecticide and acaricide with contact, stomach and translaminar action for the control of various insects on cotton, table and wine grapes, citrus, tomatoes, cruciferae, potatoes and onions. This insecticide finds its way to the habitat of freshwater snails through the drainage system (Abdel Kader and Sharaf El-Din, 2005). Bayluscide (5, 2'-dichloro-4'-nitrosalicylanilide) has been used to control several invasive and nuisance aquatic organisms. It was originally developed as a molluscicide in tropical regions to control the snail host of schistosomiasis and has been used as a synergist with other chemicals as a lampricide in the tributaries of the

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Great Lakes (Rye and King 1976). Bayluscide has also been used in the Florida aquaculture industry to control the freshwater snail host of the digenetic trematode responsible for metacercarial infections in fish (Francis-Floyd et al. 1997).

Anagallis arvensis (Family: Agavaceae) is a scarlet pimpernel annual plant. The aqueous and ethanolic extracts of this plant have been investigated for molluscicidal activity against vector snails of schistosomiasis and Facsioliasis (Mostafa *et al.*, 2005 and El-Kayat and Gawish, 2006).

Central nervous system of snails are suitable models for various types of fundamental neurobiological research because they contain relatively few, yet very large, readily identifiable neurons (Muller et al.1992). So, the aim of this study is to investigate the effect of selecron, bayluscide and ethanolic extract of *A. arvensis* leaves on nervous system of *B. alexandrina* snails.

MATERIALS AND METHODS

The experiment was conducted using *B. alexandrina* snails collected from different water courses at Qalubiya Governorate, Egypt, during spring season and transferred in plastic bags to the laboratory. They were kept in plastic aquaria (40x30x30 cm, with 100 snails per aquarium), allowed to feed on fresh leaves of lettuce and kept to acclimatize under laboratory conditions (24-26°C) over two weeks prior to the experiment.

The dry powder of *Anagallis arvensis* leaves were extracted by soaking at ethanol alcohol (0.5 kg/liter) for seven days. Then the solvent was filtered and distilled under vacuum and the crude extract residues were used in preparing series of concentrations in terms of weight/volume.

Experimental design

Preliminary experiments were carried out to determine the lethal concentration of three tested compounds. For each compound, a series of concentrations, that permits the computation of LC_{50} and LC_{90} values according to Litchfield and Wilcoxon (1949) and WHO (1965) procedures. The exposure and recovery periods were 24 hours each at room temperature (25±1^{o}C).

In order to study the toxic effect of the tested compounds on the cerebral ganglia of *B. alexandrina*, the snails were exposed to the lethal concentration of the tested compounds (LC_{90}) till death of all snails.

Group I: Snails of this group received no treatment and used as negative control

Group I : Snails of this group exposed to LC_{90} of Bayluscide.

Group III: Snails of this group exposed to LC_{90} of Selecron

Group IV: snails of this group exposed to LC_{90} of ethanolic extract of *A. arvensis*

Dissection and electron microscopical studies

After death of all snails, a definite number of snails from each group were randomly chosen and dissected for ultrastructural studies. Cerebral ganglia of tested snails were dissected out with the help of a Zeiss binocular microscope and immediately dropped in the appropriate fixative. The ganglia were fixed in formalin-glutaraldehyde fixative (4f1g) in phosphate buffer. Specimens were then postfixed in 2% oso4 in the same buffer at 4°C for 2 h. Samples were washed in the buffer and dehydrated at 4°C through a graded series of ethanol. Specimens were embedded in epon-araldite mixture. Lkb ultramicrotome was used to cut ultrathin sections which were picked upon 200 mesh naked copper grids and double stained with uranyl acetate and lead citrate. Scoping the grids was achieved by using jeol 100 cx tem.

Determination of AChE activity

After sonication of cerebral ganglia, samples were centrifuged at 1700g for 3 min at 4°C. The obtained supernatant was immediately assayed for AChE activity according to Ellman *et al.*(1961) technique adapted to the microplate (Guilhermino 1996). The enzyme activity is expressed as unit (U) per mg of protein. A unit corresponds to an n mol of substrate hydrolyzed per minute, using a molar extinction coefficient of 1.36×10^{-3} $M^{-1}cm^{-1}$.

RESULTS

Ultrastructural pattern of cerebral ganglia in control snails

At the ultrastructural level, there are two types of procerebral neurons. The cells of the first type are neurosecretory cells mainly found at the boundary of the perikaryal layer and appeared elongated with a large eccentric nucleus and a relatively small amount of cytoplasm containing abundant electron-dense neurosecretory granules. The second type of cells was smaller in size with large nucleus and thin cytoplasm containing few cytoplasmic organellae. The nuclei contained a thin layer of heterochromatin along the nuclear envelope and few large blocks of heterochromatin scattered in the central area.

Electron micrographs showed that, the mesocerebrum contained large-sized neurons with large centrally located nuclei containing one or more nucleoli and small patches of dispersed heterochromatin. In the metacerebrum the neurons were variable in size and shape. They have polymorphic nuclei with dense heterochromatin strip along the nuclear envelope and some patches of heterochromatin dispersing all over the euchromatin. The cytoplasm contains numerous cisternae of rough endoplasmic reticulum, Golgi complex, free ribosomes and secretory granules.

Ultrastructurally, the neuropile in the cerebral ganglia of *B. alexandrina* is composed of a complex network of nerve fibers, some of which contain in addition to mitochondria, dark and light synaptic vesicles.

Fig. 1 (a-f) showed groups of small procerebral neurons. Thin arrows indicate marginal neurosecretory cells, thick arrow points at perineurium, arrowheads indicate glial processes and asterisks point at extracellular space. (x4000),

Ultrastructural pattern of cerebral ganglia in treated snails

Electron microscopic examination of the cerebral ganglia post exposure to lethal concentration of Selecron, Bayluscide and ethanolic extract of *Anagalis arvensis* exhibited many interesting ultrastructural changes.
Fig. 2 (A-C) showed groups of altered (arrows) and completely degenerated (headarrows) procerebral neurons. Note degenerated glial cells (double arrows) and destructed extracellular tissue (*) (x4000),

Fig.1 Electron micrographs showing parts of horizontal sections of control cerebral ganglia of *Biomphalaria alexandrina* (a-f)

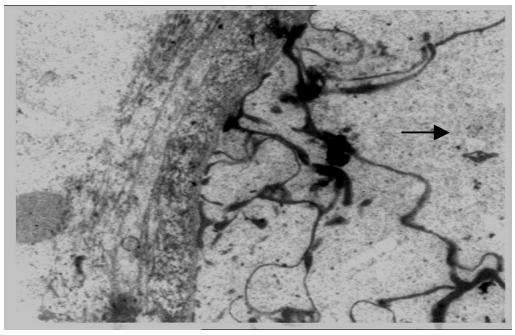

Fig. a Four different shaped metacerebral neurons. Arrows point at fat droplets, arrowheads indicate dense bodies and double arrows show glial processes extending in the extracellular space (asterisks). (x8000),

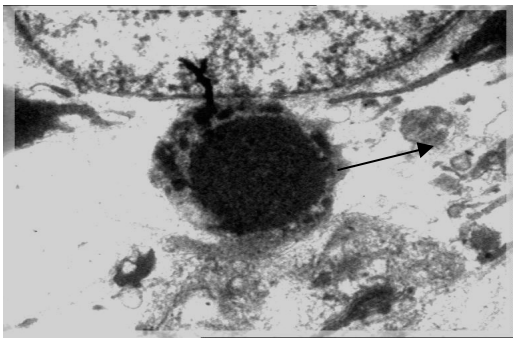

Fig. b Magnified procerebral neurosecretory cell with large nucleus, peripherally located nucleolus and large number of neurosecretory granules. (x15000),

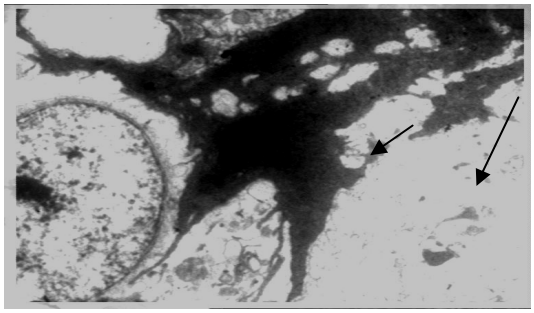

Fig. c Enlarged part of neurosecretory cell with part of the nucleus, Golgi complex, mitochondria, rough endoplasmic reticulum and secretory granules. Arrows indicate nuclear pores. (x26000),

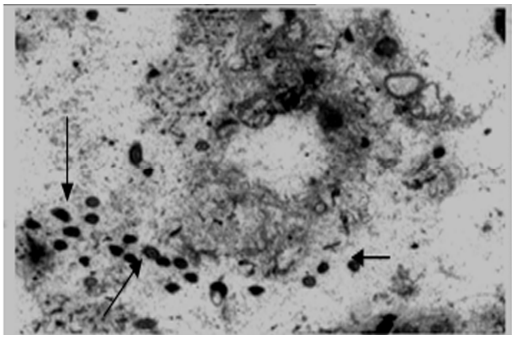

Fig. d Part of the neuropile containing axon profiles packed with large electron-dense vesicles (thick arrows) and clear vesicles (thin arrows). Double arrows pointed at synaptic membranes (x26000).

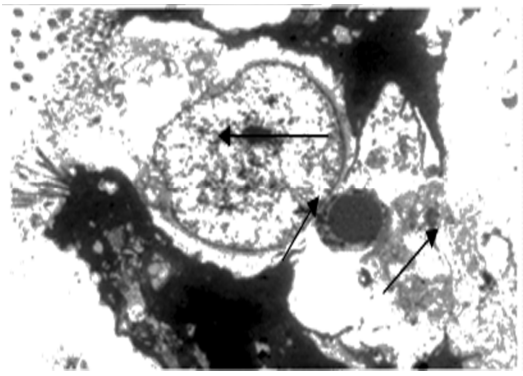

Fig. e a large sized mesocerebral neuron showing large nucleus with prominent nucleolus. (x3000),

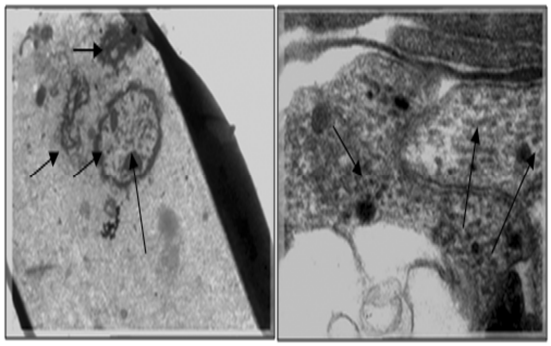

Fig. f magnified part of metacerebral neuron showing part of the nucleus and different cytoplasmic organellae. (x40000)

Table1 Actylecholine estrase activity in homogenates of *Biomphalaria alexandrina* exposed to bayluscide, Selecron and A. arvensis. Results are expressed as the mean value (U/mg protein).

Concentration (ppm)	Activity ±S.D.	% Reduction
Bayluscide (0.082)	56.8 ± 8.8	–55.3
Selecron (3.468)	64.6 ± 10.2	– 49.2
A. arvensis (38.129)	76.4 ± 12.8	–39.9
Control (0)	127.1 ± 23.4	

Fig.2 Electron micrographs showing parts of horizontal sections of cerebral ganglia of snails treated with Selecron (A), Bayluscide (B) and ethanolic extract of *Anagalis arvensis* (C). Showed groups of altered (arrows) and completely degenerated (headarrows) procerebral neurons. Note degenerated glial cells (double arrows) and destructed extracellular tissue (*) (x4000)

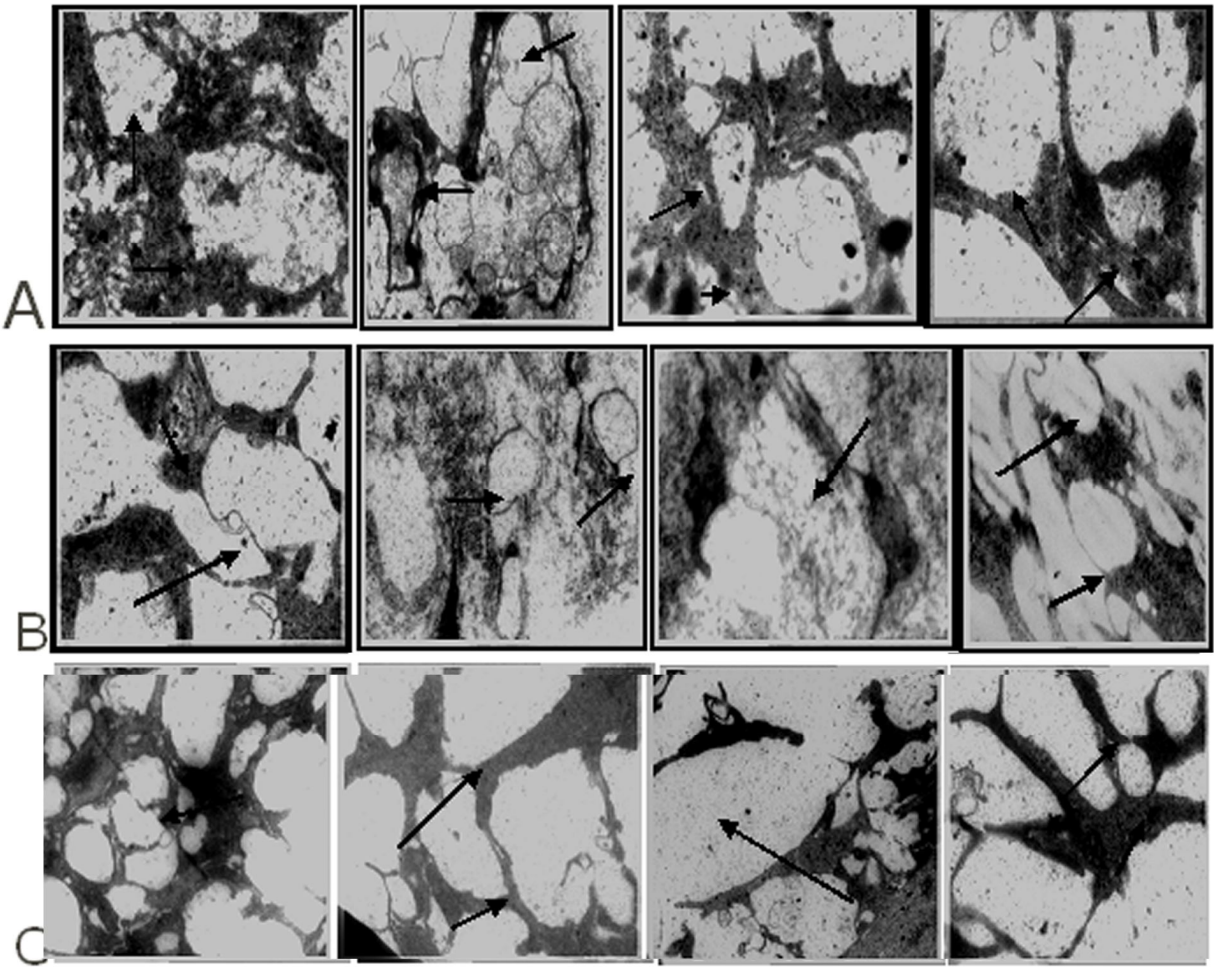

Fig.2 The measurement of acetylcholinesterase (AChE) activity is used worldwide as a biomarker of environmental contamination due to neurotoxic substances. The AChE activities in *B.alexandrina* showed wide variation along the treated snails and control snails, The AChE activities (Table 1) in *B. alexandrina* decreased significantly at bayluside (–55.3% reduction) followed by Selecron (– 49.2% reduction), and *A. arvensis* (–39.9% reduction).

DISCUSSION

The present investigation was designed to evaluate the toxic action of Selecron, Bayluscide and ethanolic extract of Anagalis arvensis on nervous system of the pulmonate snail *Biomphalaria alexandrina*, the intermediate host of *Schistosoma mansoni* in Egypt and to shed light on the possible use of this snail as a bioindicator for environmental perturbation.

The present study revealed impact of these compounds on the ultrastructure of neurons and neurosecretory cells in the cerebral ganglia of *B. alexandrina* and provided more precise information about the structural alterations induced in these cells. The nuclei of both ordinary neurons and neurosecretory cells revealed severe pathological changes. They appeared eccentric, hyperchromatic, pyknotic or highly shrunken with irregular contour and peripherally located nucleoli. Similar changes were reported in the cerebral ganglia neurosecretory cells of *Biomphalaria glabrata* treated with the herbicide Atrazine (Eissa and Omran, 2007) and in the neurons of buccal ganglia treated with methomyl and methiocarb (Essawy *et al*., 2009).

In their study on the cytotoxcity of TBT, Mizuhashi *et al*. (2000) observed dead or damaged neurons with chromatin condensation which is one of the morphological characteristics of apoptosis. The authors reported that the TBT provokes apoptosis-like neuronal cell death which might be mediated by intracellular Ca^{2+} and free radical generation via non-NMDA receptor activation. Also Reader *et al*. (1999) demonstrated a role of Ca^{2+}, protein kinase C and proteases in the induction of apoptosis in the hepatocytes of rainbow trout treated with TBT.

According to Mcllwain and Hoke (2005), changes in the size and position of the nucleus and nucleolus could be attributed to the effect of the neurotoxin on the cytoskeleton of the affected neurons. Moreover, segregation of nucleolar components was noticed in some

neurons of metacerebrum *of B. alexandrina* treated with these compounds. Nucleolar segregation could be due to the inhibition of RNA synthesis resulting from a decrease in the activity of RNA polymerase which catalyzes the synthesis of RNA (Cmarko *et al*., 2000).
The neurotoxic effect of these compounds on the cerebral neurons also appears from the observations on the cytoplasmic organelles. The primary change was the fragmentation and degranulation of rough endoplasmic reticulum accompanied with an increased number of free ribosomes. Similar changes were described in the buccal ganglia of *Eobania vermiculata* treated with carbamate molluscicides (Essawy *et al*., 2009). The degranulation and dilatation of rER are discussed as general changes of the cell in response to toxicants (Hamed *et al*., 2007). Most of these reactions are attributed to membrane destabilization and increased membrane permeability to ions under the influence of toxicants, followed by osmotic effect and finally cell death.

On the other hand, the present results clearly indicated that, treatment with selecrone, bayluscide or plant extract induced damage and loss of the cristae of mitochondria in the perikarya of the neurons as well as in the axons of the neuropile. These alterations resemble those described in snails, slugs and vertebrates as cellular stress symptoms after intoxication (Heiba *et al*., 2002; Prakash *et al*., 2009; Rawi *et al*., 2011).

In addition, the most obvious alterations observed in the present results were the presence of large dense lysosomes and autophagosomes in the cytoplasm of treated neurons. In *L. stagnalis*, lysosomes are prominent after experimental inactivation of neuronal secretory activity by incubation of cerebral ganglia in Vinca antitumor agents (Muller *et al*., 1990). Autophagy is normally a cellular degradation pathway particularly important during development stages and under certain environmental stress conditions (Klionsky and Emr, 2000). This phenomenon may be related to an autophagic degeneration of the cells or to a disruption of the regulatory mechanisms of autophagy described by Klionsky and Emr (2000).

The remarkable feature of the treated neurosecretory cells is the disappearance of neurosecretory granules and formation of large vacuoles in the cytoplasm that resulted in destruction of the cytoplasmic organelles. Vacuolation of neurosecretory cells was also noticed in the cerebral gangilia of *B. glabrata* treated with Atrazine (Eissa and Omran, 2007).

Degenerated synaptic vesicles, mitochondria and synaptic membranes were the most frequent changes in the neuropile of treated cerebral ganglia.. Degradation of the synaptic vesicles could be attributed to the interruption of axonal transport which may promote degradation of synaptic terminals. Similarly, Tsunoda *et al*. (2006) showed that, TBT-induced modulations of neurotransmitters and their metabolites in discrete brain regions of mice.

The measurement of acetylcholinesterase (AChE) activity is used worldwide as a biomarker of environmental contamination due to neurotoxic substances. The AChE activities in *B.alexandrina* showed wide variation along the treated snails and control snails, The AChE activities in *B. alexandrina* decreased significantly at Bayluside (55.3% reduction) followed by Selecron (49.2% reduction), and *A. arvensis* (39.9% reduction). Such a significant variation of AChE activities along the treated snails can be attributed to neurotoxic substances present in these compounds.

The inhibition of AChE by neurotoxic substances such as cadmium, copper, lead, organophosphorous, carbamate pesticides, polyaromatic hydrocarbons have been well established (Cajaraville et al.,2000, Sarkar,1992, Mmartinez et al.,1997, Matozzo et al., 2005, Sturm et al.,1999, Wells et al.,2001) at myoneutral junction of the nerve ending of muscle tissue. A ChE is the enzyme that is usually located in membranes of vertebrates and non-vertebrates animals (Bocquene et al., 1997). Cholinesterase enzymes (ChE) are often highly polymorphic enzymes in invertebrates.

According to the mechanism of action, the AChE is released at the myoneutral junction in organisms if an action potential is developed at the nerve ending and diffuses through the gap between the nerve and the muscle (the gap is about 100 A° wide). Anticholinetesterases such as organophosphate, carbamate pesticides, toxic elements (Cd, Pb, Cu etc) bind to the catalytic site of the AChE enzyme thus preventing the physiological inactivation of acetylcholine leading to an anomalous protraction of neurotransmission. The AChE activity was used as a biomarker of neurotoxic contaminants in copepods (Tigriopus brevicornis) from theVilaine River estuary of France (Forget et al.,2003). Methiocarb acts upon the central nervous system inhibiting acetylcholinesterase (AChE), which can cause overstimulation of the nervous system and ultimately the death of the animal (Stanek et al., 2003).

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