

Anthelmintic Activity of Goat's Milk: Transmission Electron Micrographic Evidence

Omaima Adil Najm, Faizul Helmi Addnan, Mohamed Adel Elkadi, Wan Omar Abdullah, Nur Fariha Mohd Manzor, Noor Amiza Zainal Abidin, Fadlul Azim Fauzi Mansur*

Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, Kuala Lumpur, Malaysia

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*Corresponding author
Fadlul Azim Fauzi Mansur

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Abstract: Using the canine hookworm *Ancylostoma caninum* as model we aimed to investigate the anthelmintic activity of goat's milk *in vitro* by describing in detail the nature of damage taking place cross sectionally in the worm cuticle after exposure to goat's milk through careful observation by transmission electron microscope (TEM). Adult worms were obtained from routine culling of unwanted dogs from Selayang Municipal Council (MPS) animal detention center, Rawang, Malaysia. Fresh raw goat's milk was used. Worms were cut into 1mm³ slices, fixed in 4% glutaraldehyde, washed with 0.1sodium cacodylate buffer, post fixed in 1% osmium tetroxide, washed again in 1% sodium cacodylate buffer, dehydrated in acetone, embedded in resin, polymerized, ultrathin cross section cut and stained with uranyl acetate before being viewed under transmission electron microscope. Goat's milk resulted in worm cuticular damage in the form of irregularity and significant thinning similarly seen with cysteine proteinases mediated damage. Internal hypodermic changes were also observed in the form of mitochondrial degeneration which appeared as large vacuoles similarly seen in albendazole affected worms. Understanding anthelmintic mechanism through transmission electron micrography has provided critical information in developing novel therapies from natural products. It also provides basis for the consumption of goat's milk as functional food for parasitic worm control.

Keywords: milk, goat's milk, *Ancylostoma caninum*, albendazole, anthelmintic, TEM.

INTRODUCTION

Infection with gastrointestinal helminths (GI) is a major global public health problem [1]. Some of the main classes of anthelmintic drugs available for control of gastrointestinal helminth infection include benzimidazoles, imidazothiazoles and macrocyclic lactones [2]. Resistance to conventional classes of anthelmintic is real. Hence the rise of interest for novel classes of anthelmintic. Plant [3] and animal [4] based products may become good sources for future novel classes of anthelmintic. Milk has been found to be the perfect food for newborn babies supplying essential nutritional requirement during the early critical period of development [5]. Milk also contains other bioactive compounds like enzymes and trace minerals essential for a good functioning immune system [6]. Goat's milk has been found to have superior nutraceutical properties for humans [5]. Goat's milk has been found to be suitable for those particularly sensitive to cow's milk and lactose intolerant persons [7]. Specific components of goat's milk like lactoferrin, lysozyme and lactoperoxidase have been shown to have antibacterial [8], antifungal and antiviral [9] properties. Systematic review [4] revealed that camel's

milk had superior anthelmintic activity and over other milks like goat and cow's milk [10]. The anthelmintic mechanism of goat's milk is not known. The project therefore aims to investigate the anthelmintic mechanism of goat's milk by describing in detail the nature of damage taking place in the worm cuticle after exposure to goat's milk through careful observation by Transmission electron microscopy (TEM).

MATERIALS AND METHODS

Chemicals

Glutaraldehyde (Sigma-Aldrich®, St Louise, Missouri, USA), Sodium Cacodylate Buffer (Sigma-Aldrich®, St Louise, Missouri, USA), Osmium Tetroxide Sigma-Aldrich®(St Louise, Missouri, USA), Agar 100 Resin Agar Scientific®Ltd (Parsonage Lane, Essex, UK), DDSA (dodecyl saccinic anhydride hardener) Agar Scientific® Ltd (Parsonage Lane, Essex, UK), MNA (methyl nedic anhydride) Agar Scientific® Ltd (Parsonage Lane, Essex, UK), BDMA (n-benzyl dimethyl amine) Agar Scientific® Ltd (Parsonage Lane, Essex, UK), Toluidine blue Sigma-Aldrich® (St Louise, Missouri, USA), Uranyl Acetate Agar Scientific®Ltd (Parsonage Lane, Essex, UK)

Hanks' saline Sigma-Aldrich® (St Louise, Missouri, USA), Albendazole, Zentel™ GlaxoSmithKline, (Brentford, United Kingdom).

Milk Sampling

Goat's milk (Saanen Boer cross) was collected from Berkat Jaya farm in Sungai Buloh, Selangor, Malaysia. The freshly collected milk samples were immediately pooled in sterile bottles and kept in a cooling box until transported to the parasitology Laboratory, Faculty of Medicine and Health Science, Universiti Sains Islam Malaysia (USIM). Samples were kept at 4°C until use within 1-3 days.

Adult Worm Collection

Adult *A. caninum* was sourced from culled stray dogs at the Selayang Municipal Council (SMC) animal detention center, Rawang, Malaysia. Culled dogs were autopsied on site for their small intestines. The rest of the carcasses were disposed according to MPS biohazard safety guidelines. The intact collected small intestines were placed in a plastic jar. The plastic jar containing still warm small intestines from several dogs was placed inside an airtight polystyrene box to maintain bodily temperature. The parcel was then transported immediately to Faculty of Medicine and Health Science, USIM for further use. The collected intestines were immediately opened to reveal the intestinal mucosa by using dissection scissors from one end to the other. Using precision tweezers adult *A. caninum* were picked one by one from their mucosal attachment and transferred into petri dishes containing pre-warmed Hanks' saline at 37°C and they were continuously incubated at 37°C until commencing experiment.

Observations of Changes and Structural Damage of Adult *A. caninum* by Transmission Electron Microscopy (TEM)

Fixation

Worms were incubated in BD Falcon® 24 wells culture plates (Corning, New York, USA) (1 worm per well) with different treatments and controls at 37°C using incubator Heidolph (Schwabach, Germany). At timely intervals, one worm was removed from the well and placed into vials containing 4% glutaraldehyde for 24hrs at 4°C.

Washing

After 24hrs of fixation the worms with glutaraldehyde, worms were removed and replaced in three changes of 0.1M Sodium cacodylate buffer with 10 minutes interval between each change.

Post-Fixation

Worms were replaced with 1% aqueous Osmium tetroxide (OsO₄) for 2hrs at 4°C.

Washing

Stained worms were washed in 3 changes of 0.1M Sodium cacodylate buffer with 10 minutes intervals between each change.

Dehydration

Worms were placed in ascending grades of acetone; 35%, 50%, 75%, 95% with 10 minute interval between each change. Worms were placed in three changes of absolute acetone with 15 minute interval between each change (Table 1).

Table-1: A series of placed worm in acetone

Concentrations	Time
35%	10 minutes
50%	10 minutes
75%	10 minutes
95%	10 minutes
100%	15 minutes (3 changes)

Infiltration and Embedding in resin

Worms were infiltrated with acetone (100%) and resin mixture (Table 2). After infiltration, worms were placed into beam capsules and filled up with resin.

Polymerization

Worms in beam capsules were polymerized in oven at 60°C for 24-48 hours.

Thick Sectioning

The resin block was cut to 1µm thick sections by glass knife using Knife Maker Leica, EM KMR3 (Wetzlar, Germany). The section was placed onto glass slide and stained with toluidine blue. The section was

dried on a hot plate Leica (Wetzlar, Germany) and washed the stain. The section was examined under light microscope to select interest area.

Ultrathin Sectioning

Ultrathin sections (1A) were obtained using the ultramicrotome (Leica EM UC6). The sectioning required the preparation of glass knives with a boat at the cutting edge which was filled with water to float the section. Initially, the part of the resin containing the samples was trimmed off using a razor blade Stereo Microscope (Leica, Auftria). The thickness of sections was determined by the color of sections floating in the boat; Thick sections if the color appeared blue and gold;

ultrathin sections if the color appeared silver. The silver sections were picked up onto the bright surface of a copper grid.

Staining

Sections on copper grids were stained in uranyl acetate and lead citrate staining solutions. The

copper grid was immersed in a drop of uranyl acetate and placed on a piece of dental wax for 15 minutes and washed in double distilled water. The copper grid was dried with a piece of filter paper. The copper grid was then immersed in lead stain for 10 minutes following the above procedure and washed and dried in a similar manner.

Table-2: Infiltration the worms with acetone and resin mixture

Acetone	: Resin	Time
1	: 1	1 hour
1	: 3	2 hour
100% Resin		Overnight
100% Resin		2 hour

TEM

The staining sections were viewed at Universiti Malaya (UM), Faculty of Medicine under transmission electron microscope (TEM) Libra®120 (Oberkochen, Germany).

Milk Experiment

Adult *A. caninum* were transferred into various treatments (1 worm per well) containing goat's milk as well as positive (albendazole) and negative (Hanks' saline) controls (3 replicates per treatment $n=3$ for backup purposes) and incubated at 37°C using the incubator Heidolph (Schwabach, Germany) for 6hrs in BD Falcon®24 wells culture plate. One worm was removed, fixed in 4% glutaraldehyde, washed with 0.1 Sodium Cacodylate Buffer, stained in osmium tetroxide, dehydrated in acetone, Infiltrated with acetone and resin, embedded with resin, polymerized,

ultrathin cross section cut, stained in uranyl acetate and lead stain and viewed under transmission electron microscopy (TEM) (as mentioned above).

RESULTS

The ultrastructural worms incubated in albendazole showed the rough cuticle, the hypodermis and muscular layers were regions with most damages; affecting especially the mitochondria (Figure 1A). The cuticle showed smooth surface in specimens incubated in Hanks' saline, many mitochondria were distributed throughout the circular and longitudinal muscle layers (Figure 1B). Damages to the cuticle in the form of irregularity and significant thinning were observed in worms incubated in goat's milk as well as internal hypodermic changes were also observed in the form of mitochondrial degeneration which appeared as large vacuoles (Figure 1C).

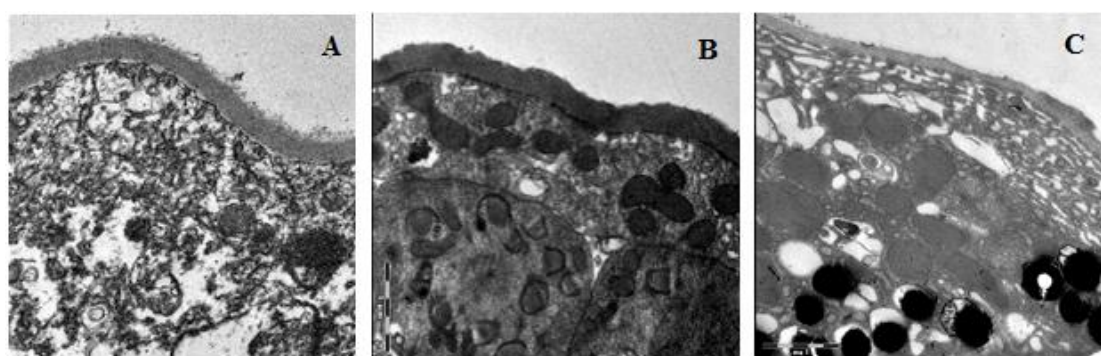


Fig-1: Transmission Electron Micrograph of Adult *A. caninum*. A; Incubated in Albendazole, B; Incubated in Hanks' Saline, C; Incubated in Goat's Milk Scale bars 1µm

DISCUSSION

Milk has already been shown to have anthelmintic activity against a range of adult intestinal nematodes as well as some effect against larval stages. Most studies focused on bovine milk [11-15], others concentrated on human milk [16] and camel milk [10; 17&18]. Systematic review [4] revealed that camel's milk had superior anthelmintic activity and over other milks like goat and cow's milk [10]. In studying the

anthelmintic mechanism of milk, electron micrographic studies will provide critical knowledge as to how the worm cuticular surface (SEM) and internal ultrastructures (TEM) are affected. By carefully observing such changes important deductions could be made as to how damages occur. For example, whether the damage process happens from the inside out [19] or externally exerted [20]. Transmission electron micrographic studies are important in the investigation

to outline possible anthelmintic mechanism of goat's milk against adult *A. caninum*. The present study observed damages to the cuticle in the form of irregularity and significant thinning. The irregular changes on worm cuticle have been described before in *O. dendatum* incubated in tannin rich hazelnut skin extract [21]. It can be expected that the TEM micrographic changes be similar to those of Cysteine proteinases mediated damage [3]. Other than cuticular damage, internal hypodermic changes were also observed in the form of mitochondrial degeneration which appeared as large vacuoles similarly seen in albendazole affected worms [22, 23].

CONCLUSION

Goat's milk demonstrated potent anthelmintic effects against adult stage of canine hookworm *A. caninum*. Goat's milk resulted in worm cuticular damage in the form of irregularity and significant thinning similarly seen with cysteine proteinases mediated damage. Internal hypodermic changes were also observed in the form of mitochondrial degeneration which appeared as large vacuoles similarly seen in albendazole affected worms. This information is critical in developing novel therapies and also provides basis for the consumption of milk as a functional food for parasitic worm control.

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