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Araştırma Makalesi ***Research Article***

Albendazole Sensitive vs. Resistant Nematodes – The Mitochondrial Ultra-Structural Changes

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Abstract

Studies on microtubule inhibitors have shown that the loss of equilibrium between tubulin and microtubules can generate a multitude of histochemical changes in mitochondria. This disruption of balance is also considered the basis of benzimidazole anthelmintic (BZ) activity. Studies have shown that BZ does not bond to the tubulin of the BZ-resistant *Haemonchus contortus,* as opposed to sensitive ones. This affinity alteration can be easily recognised by changes in the optical density and can help in the classification of *H. contortus* mitochondria, into sensitive (dark) and resistant (clear, unmodified zones). In order to confirm this hypothesis, we started our study from albendazole (ABZ) resistant and sensible *H. contortus* individuals, collected from the intestinal tract of sheep, aiming towards the identification of mitochondrial features, using the Electron Microscopy Transmission (EMT) technique. The EMT has confirmed that the structure of sensitive trichostrongilian populations was affected rapidly, only four hours after ABZ treatments. The main changes that appeared in the intestinal mitochondria of sensitive helminths were: *cristae* thickening and decreasing in number and cellular membrane thickening. Twelve hours after anthelmintic administration, a total blocking of metabolic functionality was observed, and finally, these changes completely altered the optical density of the mitochondria. In ABZ resistant populations, the optical density has remained normal; and the *cristae* number, size or functionality of resistant nematode mitochondria has remained unchanged.

Özet

Albendazol Duyarlıya Karşı Albendazol Dirençli Nematodlar - Mitokondrial Ultra-Strüktürel Değişiklikler

Mikrotübül inhibitörleri üzerine yapılan çalışmalar, tübülin ve mikrotübüller arasındaki denge kaybının mitokondriyada birçok histokimyasal değişikliğe neden olabileceğini göstermiştir. Bu denge bozulmasının benzimidazol anthelmentik (BZ) aktivitesinin de temeli olduğu düşünülmektedir. Çalışmalarda BZ'nin duyarlı olanların aksine BZ-dirençli *Haemonchus contortus* tübülinine bağlanmadığı gösterilmiştir. Bu eğilim değişikliği optik dansitedeki değişmeler ile kolaylıkla belirlenebilir ve *H.contortus* mitokondriyasının duyarlı (koyu) ve dirençli (berrak, değişmemiş) zonlar şeklinde sınıflandırılmasını kolaylaştırabilir. Bu hipotezi teyit edebilmek amacıyla, biz çalışmamıza koyun intestinal sisteminden toplanan albendazol (ABZ) dirençli ve duyarlı *H.contortus* bireyleri ile başladık ve Elektron Mikroskopisi Transmisyonu (EMT) tekniği kullanarak mitokondriyal özellikleri tanımlamayı hedefledik. EMT, ABZ uygulamalarından sadece dört saat gibi bir süre sonra duyarlı trikostrongilianların yapısının hızla etkilenmiş olduğunu doğruladı. Duyarlı helmintlerin intestinal mitokondriyalarında görülen başlıca değişiklikler; "krista kalınlaşması ve sayıda ve hücresel membran kalınlaşmasında azalma" olarak belirlendi. Antihelmentik uygulamadan on iki saat sonra metabolik işlevsellikte tam bir blokaj gözlemlendi ve son olarak da, bu değişiklikler mitokondriyanın optik dansitesini tamamen değiştirmiştir. ABZ dirençli popülasyonlarda, optik dansite normal kalmış; krista sayısı, dirençli nematod mitokondriya işlevselliği ya da boyutu değişmemiştir.

Introduction

Microtubules are tubular protein structures that have an external diameter of around 25 and an internal of 15 nanometres. Their length is variable because of the dynamic balance (equilibrium) between their length and the sub-units they are composed of (tubulin). Tubulin is a protein present in the cytosol and in the mitochondrial membrane of eukaryotic cells. Each tubulin unit is a compound formed by the combination of two very similar proteins (*alpha* and *beta*), both containing 450 amino acids and having the molecular weight of about 50.000 Da. The tubulin dimmers are permanently auto-associating and assembling available tubulin sub-units on one end of the microtubule, while detaching and dissolving sub-units on the other. Thus, the size of a microtubule is a result of the balance between the assembling and disassembling of tubulin sub-units (Lacey, 1990; Mandelkow and Mandelkow, 1989; Sant'anna et al., 2013; Schulze and Kirschner, 1988).

In vivo, the shifting of the balance between soluble tubulin and microtubule is regulated by an assemblage of endogenous factors: Guanosine Triphosphate (GTP) and Mg^{2+} are needed for the assembling process and conferring stability. The Microtubule Associated Proteins (MAP) is known for either interacting with the microtubules (or stabilizing their formation) or, with the tubulin sub-units (favouring the assembling in soluble oligomers). Conversely, the free or the complex calmodulin ions of calcium (Ca^{2+}) hinder the polymerisation and decrease the depolymerisation phase. Tubulin concentration is essential for this process because minimal concentrations are necessary to begin the assembling phase (Lacey, 1990; Mandelkow and Mandelkow, 1989; Sant'anna et al., 2013; Schulze and Kirschner, 1988).

Microtubules are involved in most cellular organelle activity (mitochondria, Golgi body, ribosomes, lysosomes, plasmalemma and nucleus) and play an important role in: forming the mitotic net, maintaining cell form and organelle mobility inside the cell (for the transport of vesicles and lysosomes; nutrient absorption by pinocytosis, excretion etc). In the same context, benzimidazolic products can inhibit the tubulin polymerising phase, and therefore, the forming of microtubules. Also BZ can compete with colchicines (which are "classical" microtubule inhibitors) (Lacey and Prichard, 1986; Lacey et al., 1987).

Studies, regarding other microtubule inhibitors, suggest that disrupting the equilibrium between tubulin and microtubules can cause a multitude of biochemical changes in the cell. This loss of stability (also considered to be the basis of the anthelmintic activity of BZ) brings about a multitude of major physiological changes in helminths like: anomalies in mitosis and meiosis, alteration of the acetylcholine esterase secretion, disintegration of helminth intestinal cells etc. (Kohler, 2001; Lacey, 1989; Roos, 1990; Roussel and Lacey, 1991; Waller and Prichard, 1985).

For example Borgers and De Nollin (1975), have demonstrated that a few hours after BZ (mebendazole) administration in the gastro-intestinal nematodes (GIN), the transport of secretory granules in the intestinal cells was blocked (obstructed), therefore the granules were accumulating and fusing at their place of synthesis. The inactivated enzymes that these granules were containing during this prolonged storing period were able to could develop their lytic action against the cell, leading to intestinal cell necrosis. At the same time, the microvillus border, characteristic of these cells, will become deprived of protection (the glycocalix) and of the necessary enzymes for regular nutrient digestion and absorption (Borgers and De Nollin, 1975).

Another dysfunction can occur in glucose absorption. Both, the absorption of 3-O-methyl-glucose and the activity of *fumarate-reductase* will be blocked. These alterations will lead to irreversible action against helminth homeostasys, leading to the death of the worm (Kohler, 2001; Öztop et al., 1999; Roos, 1990).

The few existing studies have shown that, BZ does not bond to the tubulin of BZ-resistant populations of *H. contortus*, as compared to sensitive ones. For example, Roussel and Lacey (1991), confirmed that there are two types of interactions between BZ-products and the trichostrongylid tubulin: an irreversible and a reversible bonding. An increase of the irreversible bonding has been observed, compared to reversible bonding, in the case of resistant populations of *H. contortus* and *T. colubriformis*. This affinity change was followed by a structural change of *beta*-tubulin from the resistant *H. contortus*. All these identification tests were based on the optical density changes that are characteristic for tubulin. The presence of these changes (the disintegration of intestinal cells, followed by necrosis), produced an optical differentiation of *H. contortus* mitochondria: in sensitive populations (dark zones) and in resistant ones (clear, unmodified zones).

These findings constitute *the aim* of this study, our goal is to that cell lysis and the disintegration and accumulation of secretory granules, will not take place in resistant helminths, confirming the resistance of these individuals to BZ.

Materials and Methods

Animals and the collecting of *H. contortus*

Sensible and resistant *Haemonchus contortus* individuals were collected from the intestinal tract of lambs in order to identify mitochondrial cells, with the Electron Microscopy Transmission (EMT) technique. The experimental protocol has been approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine Timișoara in the respect of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986) and conducted accordingly. *H. contortus* individuals were collected from euthanized lambs (six animals) used in our prior resistance studies (Cosoroabă et al., 1996; Cristina, 1997).

The resistance to ABZ of *H. contortus* populations has been established with certainty in a previous study, after 11 passage treatments with increasing doses of Albendazole (Valbazen, Pfizer) following well established methodologies (Coles et al., 1992; Hubert and Kerboeuf, 1992; Hunt and Taylor, 1989; MAFF 1986).

Also certain sensible *H. contortus* populations to ABZ treatments, (5mg.kgbw^{-1}) were identified and according to the scheme presented in Table 1, six sheep were chosen to be sacrificed. Albendazole was chosen because it is the most common methyl-benzimidazole dewormer in our country for using use in sheep, having a higher chance of finding resistance.

The sampling was performed twice: firstly after four hours and respectively after twelve hours after the anthelmintic administration. This sampling pattern was created taking into account that, after absorption in the liver, albendazole (ABZ) ($C_{12}H_{15}N_3O_2S$) is quickly transformed in its primary metabolites: first in albendazole sulphoxide (ABZSO) ($C_{12}H_{15}N_3O_3S$), mainly considered responsible for the therapeutic activity, (the peak being reached after three to six hours from the administration) and secondly in albendazole sulphone (ABZSO2) ($C_{12}H_{15}N_3O_4S$) (Behm and Bryant, 1979; Dayan, 2003).

The EMT technique

Fixating and washing

Freshly collected helminths were placed into a fixative solution of 2% (0.14 M) glutaraldehide, in an arsenate solvent, for one hour at 4 °C on ice (pH 7.4). After this, three successive washings were performed (five minutes each) in an arsenate buffer (pH 7.4).

The *post-fixation* was accomplished in 1% osmium tetra oxide for 30 minutes at 4 °C on ice, followed again by a washing in arsenate buffer (pH 7.4) having carried on the samples were pre-coloured with 1% tannic acid (0.1M), for 30 minutes at room temperature. Pre-coloured samples were washed with a solution of 1% 0.05M, natrium sulphite to the same pH (pH 7.4).

Sample dehydration and embedding

A typical dehydration was accomplished with different concentrations of ethanol as follows: initially with 70% ethanol for 10 minutes, then with 100% ethanol for two passages, for 10 and 15 minutes respectively, and finally with 100% propylene oxide for 15 minutes. The pre-embedding and embedding was done using a specific penetration embedding medium for electron microscopy: EMbed 812 Resin for Microscopy (EMS Catalogue No. 14120 - Electron Microscopy Sciences, Hatfield, USA) following the instructions described in Table 2.

To reduce viscosity, prior to mixing, the resin and the anhydride were warmed at 60°C and before using use, the two mixtures (A and B) were mixed, and the accelerator was added. The hardness of the block was achieved by a mixture of 1:1 between components A and B. The tissues were drained and put in contact with the embedding medium. The samples were included in EMS Flat Embedding Moulds (EMS Catalogue No. 70900), sitting for two hours at room temperature. After this the temperature was raised to 60 °C, for 48 hours (for a graduated polymerization).

Readings

The sections were fixated in 200 mesh formvar resin grids and coloured with 7.5% uranyl acetate and 0.4% lead citrate. The EMT readings were done with EMT-Tesla BS 500 (Czech Republic) at magnifications of x 7,000; x 20,000 and respectively x 50,000.

Results

The main ETM images of Sensitive vs. Resistant *H contortus* are presented in Figure 1. Beside the increased number of mitochondria we also observed the growth and abnormal shapes of this organelle. In image A mitochondria are displayed at x 20,000 magnification four hours after ABZ (5 mg.kgbw^{-1}) administration in sensitive *H. contortus*. External and especially internal membrane alterations, defined by thickening and decreased *cristae* number, followed by structural alterations of mitochondrial *cristae* (enlargement and abnormal shape) can be seen.

Table 1. The steps of experimental model accomplishing.
Tablo 1. Deneysel model elde ediş basamakları.

Resistance Establishing													**Sensitive**		**12 animals**	
												15 - Weaned lambs			**6S**	**6R**
												Days of life: Initial = 90; Final = 420	**15 – Young sheep**		**Treatment**	
												▼ ▼ ▼			**Albendazole (Valbazen) 5 mg.kgbw^{-1}**	
Passage treatment	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**Certain Resistant**	**Certain Sensitive**	**Sampling I**	
															3S	**3R**
Albendazole (Valbazen) Dose: mg/kgbw	▼ **3.5**	▼ **3.5**	▼ **3.5**	▼ **4.0**	▼ **4.0**	▼ **4.0**	▼ **4.5**	▼ **4.5**	▼ **4.5**	▼ **5.0**	▼ **5.0**	▼ **5.0**	***Haemoncus contortus Larvae Isolated***	***to ABZ at 5 mg/kgbw***	**E M T**	**I. Reading after four hours**
	FECRT/ day	**0**	**30**	**60**	**90**	**120**	**150**	**180**	**210**	**240**	**270**	**300**	**330**				**Sampling II**	
E.P.G.	**Initial X**	2250	-	3050	-	2400	-	2950	-	2950	-	2800	-	**New experiment E.P.G. in day 450 ►**			**3S**	**3R**
	Final X	-	950	-	950		1100	-	1350	-	1100	-	1500	**3250**	**2950**		**II. Reading after 12 hours**	
Organelle reading, Magnifications x 7,000; x 20,000; x 50,000																

E.P.G. = Eggs Per Gram, F.E.C.R.T. = Faecal Egg Count Reduction Test, E.M.T. = Electron Microscopy Transmission

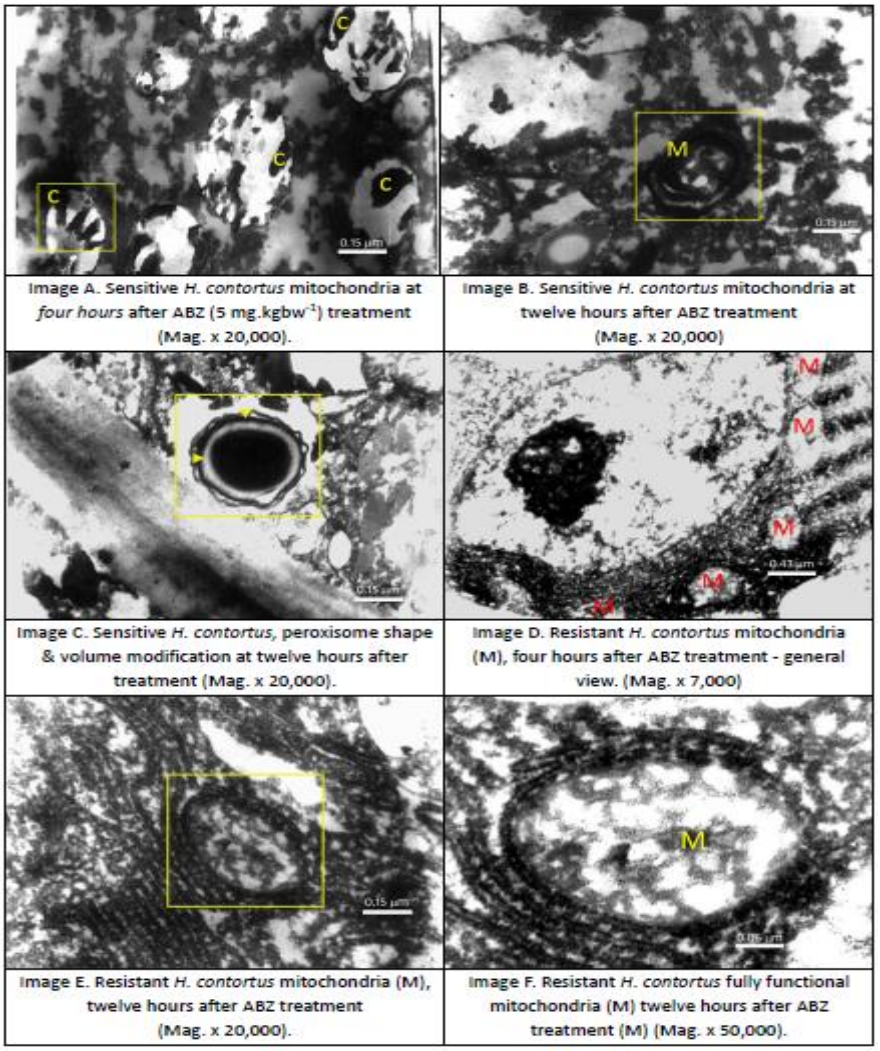

Figure 1. Sensitive and resistant *H. contortus* EMT images.
Şekil 1. Duyarlı ve dirençli *H. contortus* EMT görüntüleri.

Table 2. Description of EMbed Kit 812 used for EMT.
Tablo 2. EMT için kullanılan Embed Kit 812 tanımı.

Componence and phases	Quantities required
Mixture A	
Embed 812	62 ml
DDSA	100 ml
Mixture B	
Embed 812	100 ml
NMA	90 ml
Final Embedding Mixture	
Mixture A	162 ml
Mixture B	190 ml
BDMA	7.5-10 ml

DDSA: dodecenyl succinic anhydride; NMA: nadic methyl anhydride; BDMA: benzyldimethylamine (accelerator)

The progress of the destructive process, twelve hours after ABZ administration in sensitive *H contortus,* can be seen, in image B. The membrane becomes thicker and the inner alterations more pronounced. The mitochondrial *cristae* were altered dramatically because of these structural and functional changes and the presence of paracrystalline inclusions and concentric *cristae* was observed. It is also worth of mention, that the opacity increased dramatically and the membranes became impermeable. This was followed by biochemical alterations produced by the metabolites rapid accumulation of (proteinic enzymes, lipidic proteins, disintegration of secretory granules etc); the substance transportation will be blocked, causing the interruption of mitochondrial functionality and finally necrosis.

In image C, the deleterious activity in sensitive individuals of *H. contortus* it can be also proven by the modification of the peroxisomes, structures nearby mitochondria. Though these ubiquitous organelles are remarkably adaptable to the changing environments, under albendazole activity after twelve hours it has been noted that the number, volume, shape and function were modified, proving the efficiency of the anthelmintic attack.

Images D, E and F, depict the EMT images of ABZ resistant *H. contortus* of intestinal mitochondria. Here, the presence of all structural and numerical unmodified *cristae* is noticeable, justifying the preservation of the functionality and of the optical density, clearly showing that the mitochondria is not are not affected in individuals that survive after 5mg.kgbw^{-1} albendazole doses.

Discussion

In general, mitochondrial structural abnormalities were presented by some authors in muscle disorders (myopathies) (Lindal et al., 1992), central nervous system (Lewis and Dalakas, 1995), oncologic (Debatin et al., 2002) and also in drug toxicity situations (Barile et al., 1998; Lewis and Dalakas, 1995). Although these dysfunctions are so different, depending on their patho-mechanisms, the ultra-structural changes that can be observed in mitochondria are alike (Pavelka and Roth, 2005; Sant'anna et al., 2013).

Our findings support the efforts of other experimental models aimed at investigating BZ resistance, they have illustrated the mitochondrial alterations under the cellular stress due to the administration of anthelmintics and the releasing of the apoptotic process until the death of the cell, in the case of sensitive *H. contortus* individuals (Gilleard, 2013; Kwa et al., 1994; Kwa et al., 1995; Sant'anna et al., 2013).

This study has confirmed that the sensible *H. contortus* populations are structurally affected a short time after BZ treatment. The changes that appeared in the intestinal mitochondria after four hours in sensitive individuals were: decreasing and thickening of the mitochondrial *cristae*, thickening of the cellular membranes (especially the internal membrane) accompanied by modifications of the protein enzyme metabolism, alterations of the internal membrane, *cristae* disappearance and lack of mitochondrial functionality. It has been observed that all this was accompanied by the modification of mitochondrial optical density (darker shades).

Changes observed, twelve hours after the anthelmintic administration: total blocking of the metabolic functionality of mitochondria and other components (like peroxisomes), inhibited via the interruption of metabolite transport, the accumulation of metabolites and finally, necrosis.

In the albendazole resistant populations, *H contortus* mitochondria were not affected by any changes of number, size or organelle functionality. In these cases the optical density has remained normal.

The mitochondrial architecture ultramicroscopic study could be used as an alternative investigative method for benzimidazoles efficacy / resistance, or another method to test the efficiency of other nematodicide products.

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