Is L-buthionine sulfoximine reverse the benzimidazole resistance in *Haemonchus contortus* of small ruminants?

Om Prakash, S Gomathinayagam, Tirunelveli Jeyagopal Harikrishnan, Muthusamy Raman, V Pandiyam and P Azhahianambi

**Abstract**

Anthelmintic resistance (AR) in parasites of livestock is an emerging problem in many parts of the World. Hence, finding new classes of anthelmintic drugs or anthelmintic resistance reversal agents is the need of the hour to sustain the global livestock production. The aim of present study was to study the Benzimidazole (BZ) resistance reversal property of L-buthionine sulfoximine (L-BSO) using *in vitro* egg hatch assay (EHA). Thiabendazole (TBZ) alone and combination of TBZ and 200µM, 500µM and 700µML-BSO were used with proper control and the hatching percentage and ED₅₀ values were recorded. It was found that significant (P<0.001) reversal of BZ resistance occurred when *H. contortus* was treated with L-BSO (>500µM) and the effect was dose dependent. Hence, L-BSO might potentially be used as a BZ resistance reversal agent to treat BZ resistant nematode parasites of livestock.

**Keywords:** *Haemonchus*, benzimidazole, buthionine sulfoximine, small ruminants

**Introduction**

The *Haemonchus contortus*, commonly known as the barber’s pole worm, parasitic nematode, a major cause of loss of production in sheep and goat industry. This parasite can inflict a considerable amount of damage to a flock or herd in a short span of time. Treatment and control of this gastrointestinal parasite have been successful using various anthelmintics, such as benzimidazoles, levamisole, and ivermectin, which exert selection pressure resulting in the development of anthelmintic resistance (Torres-Acosta et al., 2012) [56]. Unraveling the anthelmintic resistance mechanisms employed by *H. contortus* will help us to find solutions to overcome the anthelmintic resistance and use better treatment and control methods. The effect of glutathione (GSH) on the eggs of *H. contortus* susceptible/resistance to anthelmintics was investigated using *in vitro* egg hatch assay (EHA). The modulators or GSH analogue like Diethylmaleate, D, L-buthionine sulfoximine and patulin induce an unexpected decrease in the susceptibility of egg to thiabendazole (Kerboeuf and Aycadi, 1999) [31]. L-BSO increases the toxicity of nifurtimox, benzidazole to the epimastigote, trypomastigate and amastigote stages of *Trypanosoma cruzi* (Faundez et al., 2005) [19]. The enzyme activity of *Burgia malayi*, the causative agent of lymphatic filariasis is irreversibly inhibited by the BSO ( Hussein and Walter, 2005) [29]. Buthionine sulfoximine is a potent and specific inhibitor of γ-glutamylcysteine synthetase and inhibits glutathione biosynthesis and causes depletion of cellular glutathione levels (Griffith and Meister, 1979) [25]. Glutathione S-transferase (GST) which is part of parasite detoxification system associated with the establishment of parasitic nematode infections within the gastrointestinal environment of the mammalian host (Rossum et al, 2004) [53]. On the above hypothesis the present study was conducted with the objective of is BSO potentiate the activity of TBZ in *H. contortus*.

**Material and Methods**

**Collection of Parasites and Harvesting of Eggs**

Abomasums of sheep and goat were collected in normal saline from the slaughter house, Perumbur, Chennai, India and transported to the laboratory. Abomasums were cut open along greater curvature and manual removal of adult *H. contortus* was carried out from the abomasums content and mucosal fold. The worms were washed two times with normal saline and female worms were separated and incubated in normal saline for an hour at 37 °C for release of eggs. After incubation, normal saline was collected in centrifuge tube and centrifuged (Eppendorf, 5810R, Germany) at 2000 rpm for 5 minutes to sediment the eggs. The supernatant was poured off and sediment was examined for the presence of eggs. The concentration of egg was adjusted to 50 eggs/µl.
Assessment of benzimidazole resistance by EHA

Assessment of benzimidazole resistance by egg hatch assay (EHA) was performed as using harvested eggs from the slaughter house, Perumbur, Chennai, India using pure thiabendazole -99 percent (Sigma Aldrich- T8904, USA) in five concentration of 0.05, 0.1, 0.3, 0.5, and 1.0 µg/ml as per the procedure of World Association for Advancement of Veterinary Parasitology (WAAVP) proposed by Coles et al., 1992 (12) with slight modification (Lourde Raj, et al., 2006; Laksmipriya, 2012) (41, 36).

Assessment of reversal effect of buthionine sulfoximine by EHA

The effect of BSO was performed as similar to egg hatch assay, in this test different concentrations (200µM, 500µM and 700µM) of BSO (B2515, Sigma Aldrich, USA) added along with thiabendazole.

Estimation of Intracellular glutathione concentration

The total intracellular glutathione (GSH) was determined by a calorimetric method described by Greech et al., 1999 (24). TBZ resistant eggs and larvae of H. contortus were used in this assay. One gram of eggs and larvae were exposed to 1.0 µg/ml of TBZ and 200µM, 500µM and 700µM of BSO at 2, 4, and 6 hours intervals. The observance was read at 413nm against a blank and the amount of glutathione is expressed as nano mole per gram of larvae by UV- VIS mini-1240, spectrophotometer (A109035i, Shimadzu Asia Pacific Pte Ltd, Singapore).

Table 1: Comparative egg hatching percentage (Mean ±SEM) after in vitro egg hatch assay (EHA) to see the anthelmintic resistance and reversal of anthelmintic resistant in Haemonchus contortus in sheep and goat treatment with thiabendazole ( Alone) and a combination of thiabendazole and 200, 500 and 700µM L- buthionine sulfoximine (L-BSO) compared with control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No L-BSO (Alone TBZ)</th>
<th>L-BSO 200µM</th>
<th>L-BSO 500µM</th>
<th>L-BSO 700µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (No TBZ)</td>
<td>82.44 ±1.59</td>
<td>78.02 ±1.31</td>
<td>77.31±1.29</td>
<td>79.75 ±1.14</td>
</tr>
<tr>
<td>TBZ 0.05 µg/ml</td>
<td>74.78 ±1.58</td>
<td>62.53 ±1.64</td>
<td>52.73±1.57</td>
<td>36.70 ±1.77</td>
</tr>
<tr>
<td>TBZ 0.1 µg/ml</td>
<td>62.04 ±1.90</td>
<td>54.34±1.96</td>
<td>40.04±1.88</td>
<td>26.29 ±1.18</td>
</tr>
<tr>
<td>TBZ 0.3 µg/ml</td>
<td>48.80 ±2.82</td>
<td>40.09±2.53</td>
<td>34.24±2.25</td>
<td>22.48±1.68</td>
</tr>
<tr>
<td>TBZ 0.5 µg/ml</td>
<td>37.56 ±2.16</td>
<td>24.80 ±1.93</td>
<td>16.39±1.39</td>
<td>4.53 ±0.86</td>
</tr>
<tr>
<td>TBZ 1.0 µg/ml</td>
<td>4.02±0.64</td>
<td>2.80 ±0.39</td>
<td>1.21 ±0.27</td>
<td>1.02 ± 0.23</td>
</tr>
<tr>
<td>ED50</td>
<td>0.193µg/ml</td>
<td>0.137 µg/ml</td>
<td>0.077 µg/ml</td>
<td>0.033µg/ml</td>
</tr>
<tr>
<td>Result</td>
<td>Resistant</td>
<td>Resistant</td>
<td>Susceptible (Reversed)</td>
<td>Susceptible (Reversed)</td>
</tr>
</tbody>
</table>

*Level of significance: P<0.001

Assessment of reversal effect of buthionine sulfoximine

The mean percentage of egg hatch at different concentration of TBZ i.e. 0.05, 0.1, 0.3 and 1.0µg/ml with combination of 200µg/ml, 500µ and 700µM were recorded and It was significantly (P<0.001) reduced in all concentrations. Using probit analysis the ED50 values after the combination of TBZ and 200 µM, 500 µM and 700 µM concentrations of reversal agent BSO, as 0.137, 0.077 and 0.033µg/ml TBZ were recorded respectively.

Statistical Analysis

Data analysis was carried out using SPSS 20 version software (SPSS 20.0; Inc., Chicago, Illinois, USA). To analysis the mean hatching percentage and intracellular glutathione level between resistant populations before and after treatment with BSO, data were subjected to a multivariate analysis (ANOVA) with Tukey’s post-host test. The differences were considered statistically significant when P<0.001. The ED50 were calculated using probit analysis.

Result

In the present study a total of 1003 abomasal samples of sheep and goat were examined at the slaughter house, Perambur, Chennai, India. Out of which 534 samples were found to contain male and female worms of Haemonchus contortus on dissection. Prevalence of H. contortus in small ruminants slaughtered in Perambur slaughter house, Chennai, Tamil Nadu, India was 53.2% recorded.

Assessment of resistance by Egg hatch assay (EHA)

The assessment of resistance and susceptible worms were based on the ability of eggs to hatch at concentration greater than 0.1µg/ml of thiabendazole (TBZ). In present study, samples were found to be resistant to TBZ by EHA, where larvae could be seen even at concentrations higher than 1.0 µg/ml of TBZ. The mean hatching percentage at different concentrations, i.e. control, 0.05, 0.1, 0.3, 0.5 and 1.0 µg/ml was recorded and analysed (Table 1). Using probit analysis ED50 value for benzimidazole resistance worm was 0.193µg/ml.

Fig 1: Thiabendazole Resistance and effect of BSO in Thiabendazole resistance
**Estimation of intracellular glutathione**

Intracellular glutathione has been estimated in eggs and larvae of TBZ resistant *H. contortus*, before and after exposure to different concentration of 200, 500, and 700µM of BSO and OD values were taken at 412nm in 2, 4, 6 hour intervals. It was found that the glutathione level was significantly reduced in all concentrations (Table 2). Regression coefficient (R square) between intracellular reduced glutathione (GSH) and egg hatching percentage at 1.0µg/ml of TBZ in 2, 4, and 6 hours intervals were 0.372, 0.615 and 0.354 calculated respectively.

**Table 2: Reduced intracellular glutathione (Mean±SEM) in *Haemonchus contortus* when treatment with thiabendazole (1.0 µg/ml) alone and combination of thiabendazole and L-BSO 200 µM, L-BSO 500 µM and L-BSO 700 µM, at two, four and six hours intervals**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intracellular glutathione (nmol/g) (Mean± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Two hours</td>
</tr>
<tr>
<td>TBZ (1.0 µg/ml)</td>
<td>39.09±1.08</td>
</tr>
<tr>
<td>TBZ+ L-BSO 200 µM</td>
<td>28.94±1.23</td>
</tr>
<tr>
<td>TBZ+ BSO 500 µM</td>
<td>18.02±0.69</td>
</tr>
<tr>
<td>TBZ+ BSO 700 µM</td>
<td>8.03±0.39</td>
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</table>

Level of significance: P < 0.001

**Fig 2:** Estimated intracellular reduced glutathione (nmol/g) in *H. contortus* of sheep/goat after treatment with thiabendazole alone and combination of thiabendazole 200µM L-BSO, 500µM L-BSO and 700 µM L-BSO at two, four and six hour interval

**Discussion**

The previous studied about prevalence of *H. contortus* reported by scientist time to time. Fakae (1990) [18] conducted survey in the Nigerian derived savanna (from August 1987 to July 1988) on the seasonal fluctuations in the composition of *Haemonchus contortus* burden of naturally infected African Dwarf sheep and goats and found that the incidence of *H. contortus* infection was high 77.8–100% with no definite seasonal distinction. *H. contortus* infection lowers in the summer season as compared to winter and rainy season Garg et al. (2003) [20], Yadav et al. (2006) [60] recorded that the seasonal variation throughout the year and was highest during the rainy season (88.54%) followed by summer (83.15%) and winter (76.01%). Shugufa et al. (2005) [54] studied the incidence of gastrointestinal nematodes in sheep of Kashmir valley. Five types of nematodes viz., strongyles, Trichostrongylus spp., *Haemonchus* spp., Nematodirus spp. and Marshallagia spp. were identified. The seasonal prevalence of infections indicated that the nematode infection (overall) was highest in summer (67.14%) and lowest in winter (44.31%). Al- shaibani et al. (2008) [1] observed for 12 months and *H. contortus* (24.6%) were found to be predominantly of gastrointestinal nematode parasites. Qamar et al. (2008) [50] found overall the highest (43.69%) seasonal prevalence in sheep and Goats was recorded during summer; followed by autumn (38.46%), spring (37.12%), while the lowest (28.79%) was recorded during winter. Lashari et al. (2015) [37] reported the prevalence of Fasciola hepatica, Avitellina centripunctata, *Haemonchus contortus* and Trichuris globulosa was 21.41, 12.23, 6.50 and 5.73%, respectively and suggest that the age, sex, body weight and breed are important factors which influence the prevalence of gastrointestinal parasites. Tasawar et al. (2010) [55] reported the overall prevalence of *Haemonchus contortus* was 77.7% in sheep at Government Research Centre for Conservation of Sahiwal Cattle (RCCSC) Jehangirabad, District Khanewal from February 2007 to June 2007. Mesele et al. (2014) [46] performed cross sectional was conducted to estimate the prevalence of haemonchosis in small ruminants through examination of 613 Abomasum of small ruminants, 355 sheep and 258 goats. The overall prevalence in this study was 38.6%, with a prevalence of 22.8%, and 15.8% were recorded for sheep and goats respectively, similarly number of studied about prevalence of haemonchosis in sheep and goat conducted time to time with 57.8%, 9.18% (Degheidy et al. 2014) [13], 40.9% (Gebresilassie and Tadele, 2015);sheep and goats 67.2% and 56.6% respectively(Bulbul et al. 2015), 12.1% (Boukhari et al. 2016) [7] and 38.0% (Castle, 2017) [11]. Coles et al. (1992) [12] given the procedure of egg hatch assay and eggs hatching with an ED₅₀ value in excess of 0.1 µg TBZ per ml were indicative of benzimidazole resistance. Varady and Corba (1999) [19] found egg hatch assay to be sensitive and accurate to determine benzimidazole resistance in *H. contortus*. They demonstrated that the ED₅₀ in resistant strain was different from the susceptible strain by resistant factors. Calvete et al. (2012) [10] also found 11 per cent of...
TBZ resistant nematodes in sheep farms in Northeast Spain using egg hatch assay. The mean percentage of egg hatch in 42 resistant samples was above the discriminating dose of 0.1 μg per ml of TBZ and larvae hatched even at higher concentration of TBZ. This is similar to the report of Le Jambre (1976) [38] who showed that TBZ resistant strains hatched in higher concentration of TBZ than non-resistant strains. Taylor et al. (2002) [57] reported eggs from susceptible individuals rarely hatch at concentrations greater than 0.1μg / ml of thiabendazole. In the previous studied many scientist and researchers reported the anthelmintic resistance by egg hatch assay with range of ED50 Value as 0.586 (Arunachalam et al. 2005) [23]:0.627, 0.678 and 0.388 μg / ml (Easwaran et al. 2009) [16, 17] and 0.8 and 0.6 μg/ml of benzimidazole (Lourde raj et al. 2006) [41]; 17.9% Lakshmipriya (2012) [36]; ED50 for egg hatch was 0.196 with lower and upper limit of ED50 was 0.051 and 0.329 (Dinesh, 2013) [14]; 0.059 μg/ml (Rialch et al. (2013) [31]; 0.299μg/ml of albendazole (Minakshisundram et al. 2014) [45]; The ED50 for egg hatch was 0.196, indicating suspected resistance to benzimidazole anthelmintics (Kumbhakar et al. 2013) [55]; Egg hatchability 19.66% (Goncalves et al., 2016) [53]. Buthionine sulfoximine (BSO) was used as reversal agent in the present study and found significant reversal effect which was dose depended. The reversal of resistance occurred when TBZ resistance eggs were treated with BSO and resulted in inhibition of egg hatching. Beugnet et al. (1997) [5] performed egg hatch assay (EHA) and demonstrated the relationship between P-glycoproteins (p-gp) and benzimidazole resistance through the use of the p-gp inhibitor verapamil, a calcium channel blocker. They had shown that, in the presence of verapamil, the toxicity of the drug increased and that benzimidazole resistance could be partially reversed. Kerboeuf et al. (1999) [31] examined membrane drug transport mechanism of resistance to anthelmintics by flow cytometry using rhodamine 123, a p-gp transport probe and found a higher level of green fluorescence in eggs of resistant H. contortus, indicating high levels of expression of pgp in resistant eggs. Riou et al. (2003) [52] confirmed that cholesterol could modulate p-gp activity on nematode eggs and change the level of resistance to anthelmintics. They suggested changes in membrane lipid contents could provide another way to improve the reversion of resistance and to increase the efficacy of anthelmintics. Kerboeuf et al. (2008) [32] suggested that the combination of anthelmintic targeting nematodes with an inhibitor of p-gp efflux pumps had a significant effect on both egg excretion and the number of worms coming from anthelmintics resistant nematodes. They opined that this discovery would open up new perspectives in nematode control by maintaining a good efficiency of the treatment, while reducing the doses of active compound. They analyzed the functional consequences of the localization for xenobiotic transport and drug resistance in nematodes and compared with results obtained in vertebrates. They suggested that understanding of such mechanisms was crucial in overcoming the failure of drug treatments due to the development of resistance. In this study addition of BSO to TBZ has inhibited hatching of resistant eggs by increasing toxicity of TBZ. Blackhall et al. (2008) [32] studied an association between a specific allele of p-gp and survival of benzimidazole treatment. They suggested that by reducing the amount of drug to reach its target, p-gp could act as a general defensive mechanism against xenobiotics. Bartley et al. (2009) [3] showed the influence of various p-gp interfering compounds on the efficacy of ivermectin sensitive and resistant nematode isolates. They also demonstrated that in the presence of p-gp interfering agents, the in vitro susceptibility to ivermectin of both sensitive and resistant isolates of T. circumcincta and H. contortus was increased. Bartley et al. (2009) [10] described the increased sensitivity of resistant larvae to ivermectin after the co-incubation with pluronics 85 but in vivo co-administration of ivermectin with this p-gp modulator to sheep did not show an improved efficacy against resistant H. contortus. Lifschitz et al. (2010) [39] showed, by field trial done in Argentina that the efficacy of both ivermectin and moxidectin against resistant Cooperia spp. in cattle tended to increase after their co-administration with loperamide (LPM) as a p-gp modulator. Lifschitz et al. (2010) [40] reported the effects of loperamide (LPM), a p-gp modulating agent, on both ivermectin kinetic behaviour and anthelmintic activity in infected lambs. Described that the raft-like structures (RLSs) in egg shell colocalized with a large proportion of the p-gp. They analyzed the functional consequences of the colocalization for xenobiotic transport and drug resistance in nematodes and compared with results obtained in vertebrates. They suggested that understanding of such mechanisms was crucial in overcoming the failure of drug treatments due to the development of resistance. Godoy (2010) [22] studied that the expression of HcPgps-A, in transfected LLC-PK1 cells, and to see the effect of ivermectin and moxidectin on inhibition of rhodamine 123 transport by the transfected cells. Rhodamine 123 was actively transported by HcPgp-A. Ivermectin was four fold more potent in inhibiting rhodamine 123 transport by HcPgp-A than was moxidectin. The result provided the first information that MLs can inhibit the transport of Pgp substrates by a parasitic nematode ABC transporter and may indicate an active role for H. contortus Pgps in ML resistance. Heckler et al. (2014) [126] evaluated the in vitro effect of eight P-gp modulating drugs to potentiate IVM efficacy against an IVM-resistant field isolate of H. placei. The association of IVM with cyclosporin-A, ceftriaxone, dexamethasone-sone, dimazene aceturate, quercetin, trifluoperazine, verapamil, or vinblastine resulted in increased IVM (10–4M) efficacy of 5.1, 49.06, 76.42, 3.31, 28.85, 13.74, 45.64% and 43.61%, respectively. Hussein et al. 1996 [28] reported that effect of DL- buthionine- S, R sulfoximine (BSO), a selective glutathione (GSH) depleting agent, on the GSH synthesis of Ascaris suum. The GSH concentration of reproductive and muscle tissues of A. suum was 8.5 ± 0.3 and 14.3 ± 1.3 nmol/mg protein, respectively and after treatment of parasites with BSO for 24 h, the GSH content of reproductive tissue was totally depleted as compared to untreated control. Faundez et al. (2005) [19] found that L - buthionine-S, R sulfoximine (BSO), increased the toxicity of nifurtimox and benzimidazole towards the epmastigote, trypomastigote and mastigate form of Trypanosoma cruzi. BSO at 500μm decreased the total glutathione-derived thiol by 70 to 80% in 48 hours. Hussein, et al. (2005) [29] the Treatment of the Brugia malayi with different concentrations of 20, 100, 200 and 500 μM BSO lowered the GSH content by 70%, 47%, 35% and 23%, respectively. The effect of BSO is dose dependent. Buthionine sulfoximine (S-(n-butyl) homocysteine sulfoximine) is a potent and specific inhibitor of y-glutamylcysteinesynthetase; administered to animals orincorporated into tissue culture media it inhibits glutathione biosynthesis and causes depletion of cellular glutathione levels. Glutathione, the most abundant intracellular nonproteinsulhydryl compound, has various cellular functions, mainly related to the thiolgroup of the cysteine.
residue. The reduced glutathione (GSH), as a cosubstrate of glutathione peroxidase, plays an essential protective role against oxygen-reactive species that may be generated under various conditions. This protective mechanism results in increased formation of oxidized glutathione (GSSG), which is actively transported across the cell membrane, so that its intracellular concentrations are kept low. Drug resistance has been correlated to increased GST and GSH level in certain nematodes like *Haemonchus contortus* ([Kwalek et al. 1984](#30)). [Ullah et al., 2017](#38) estimated reduced glutathione as total acid soluble sulfhydryl concentrations colorimetrically using Ellman's reagent ([5, 5′-dithiobis-(2-nitrobenzoic acid) or DTNB]) according to thymoquinone and curcumin effect on *Fasciola gigantica*. The level of reduced glutathione was significantly inhibited by both curcumin as well as thymoquinone at the highest concentration (60 µM) used. The present study showed the increase GSH level in resistance *H. contortus* and after treatment with GSH inhibitors BSO level of GSH was decreased. The our studied showed the relationship between egg hatching percentage and reduced glutathione with regression of coefficient at 2,4, and 6 hours interval after treating with thinbendazole and L-BSO. When reduced intracellular glutathione is responsible for is 61.5% hatching of egg when treated with 500µM L-BSO.

**Conclusion**

The present experiment was conducted to see the potentiation of thiabendazole efficacy by buthionine sulfoximine (BSO). BSO can be used as AR reversal agent along with BZ drugs. Enhances the TBZ toxicity by reducing the GSH concentration. Suppressing the antioxidant/redox potential of the worms.

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**References**

31. Kerboeuf D, Ayaecrd J. Unexpected increased thiabendazole tolerance in Haemonchus contortus resistant to anthelmintics by modulation of glutathione activity, Parasitological Research, 1999; 85(8-9):713-718
33. Kreeck RC, Waller PJ. Towards the implementation of the "basket of options" approach to helminth parasite control of livestock: Emphasis on the tropics/subtropics Vet. Parasitol, 2006; 139:270-282
34. Kripali Pant, Rajput MKS, Jitendra Kumar, Shivani Sahu, Vandna, Rajkumari et al., Prevalence of helminthes in small ruminants in Tarai region of Uttarakhand, Veterinary World, 7, 265-266
42. Loyacano AF. Effect of gastrointestinal nematode and liver fluke infections on weight gain and reproductive performance of beef heifers Veterinary. Parasitology. 2002; 107:227-234
43. Luersen K, Walter RD, Muller S. Plasmodium falciparum infected red blood cells depend on a functional glutathione de novo synthesis attributable to an enhanced loss of glutathione,. Biochemistry. 2000; 346:545-552
50. Qamar MF, Maqbool A, Khan MS, Ahmad N, Munee MA. Epidemiology of Haemonchosis in sheep and Goats under different managemental conditions, Veterinary World. 2008; 2(11):413-417


