Clastogenic Effects of the Fasciolicides Closantel and Nitroxylin on Mice Somatic and Germ Cells

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Summary Salicylanilide (Closantel) and halogenated phenol (Nitroxylin) have been reported to be an active fasciolocidal agents used in human and in farm animals. The clastogenic effects of Closantel at 5, 10, 15, 20 mg kg⁻¹ b.wt. and Nitroxylin at 10, 20, 30, 40 mg kg⁻¹ b.wt. were tested in mice somatic and germ cells through 3 cytogenetic parameters. Chromosomal aberrations, sister-chromatid exchanges and sperm-shape abnormalities. The results demonstrated that, after intraperitoneal (i.p.) injector with a single doses for 24 h. Closantel and Nitroxylin induced different types of structural chromosomal aberrations in bone-marrow cells which increased significantly with different doses. The frequencies of SCEs were increased significantly with Closantel compared to Nitroxylin induced non-significant frequencies of SCEs except at high dose (40 mg kg⁻¹ b.wt.). The percentage of chromosomal aberrations in diakinesis-metaphase I spermatocytes increased in a dose dependent manner and were found to be statistically significant with the 2 drugs. The 2 fasciolicides induced a signifycant percentage of sperm-shape abnormalities. The highest doses induced 12.74 ± 0.49 and 7.02 ± 0.13 ($p < 0.01$) for closantel and nitroxylin respectively, compared to the control. In conclusion, the 3 cytogenetic parameters used to evaluate the effect of Closantel and Nitroxylin revealed that the 2 fasciolicides have a clastogenic effect on mice somatic and germ cells *in vivo* studies.

Key words Closantel, Nitroxylin, Chromosomal aberrations, SCEs, Sperm-shape.

Salicylanilide (Closantel) and halogenated phenol (Nitroxylin) are the most widely used broad-spectrum anthelmintics. They used extensively for the control of *Fasciola* spp. infestations in sheep and cattle (Richards *et al.* 1990, Keyyu *et al.* 2003, Morsy *et al.* 2005), *Haemonchus* spp. (Jeannin *et al.* 1990, Van Wyk *et al.* 1997) and *Oestrus ovis* in sheep in many parts of the world (Swan 1999, Suarez *et al.* 2004). Infections with fascioliasis in human is an incleasingly recognised public health problem in different countries like Egypt (Hammouda *et al.* 1995), in Chile (Apt *et al.* 1995) and Australia (Laird *et al.* 1992).

These drugs are known uncoupling mitochondrial oxidative phosphorylation in mammalian systems, which act in similar way in flukes tissues. (Campbell and Montague 1981, Edwards *et al.* 1981). The initial effect of Closantel is on glycolysis and accumulation of glucose-6-phosphate (Rohrer *et al.* 1986). Toxicity studies in laboratory animals showed that Closantel has a non-mutagenic potential in Salmonella Ames test, and a dominant lethal test in male and female mice. Tolerance studies in sheep and cattle demonstrated that oral and parental clinical doses were very well tolerated and devoid of serious side-effects (Van cauteren *et al.* 1985).

In the present study we evaluate the clastogenic effect of Closantel and Nitroxylin on somatic and germ cells through 3 cytogenetic parameters: chromosomal aberrations in somatic and germ cells, sister chromatid exchanges and sperm shape abnormalities test.

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Material and methods

Chemical agents

Closantel, *N*-(5-chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl) 2-hydroxy-3,5-diiodobenzamide (Fig. 1). Purchased by Egypt M.O.H. reg. No.: 1538–2000.

Nitroxynil (Dovenix): 4-hydroxy-3-iodo-5-nitrobenzonitrile (Fig. 2) purchased by Homa Cairo. 69002 LYON.

Fig. 1. Closantel, *N*-(5-chloro-4-[(4-chlorophenyl)cyanomethyl]-2-methylphenyl) 2-hydroxy-3,5-diiodobenzamide (Fig. 1). Purchased by Egypt M.O.H. reg. No.: 1538–2000.

Fig. 2. Nitroxynil (Dovenix): 4-hydroxy-3-iodo-5-nitrobenzonitrile (Fig. 2). Purchased by Homa Cairo. 69002 LYON.

Animals

Mice were obtained from the animal house of the National Research Center. The experiments were carried out on male Swiss mice 12 weeks old, weighting 25–30 g. Food and water were provided *ad libitum*.

Dosage and treatment

Groups of mice (5/dose) were i.p. injected with single doses of closantel 5, 10, 15, 20 mg kg⁻¹ b.wt. and of nitroxynil 10, 20, 30, 40 mg kg⁻¹ b.wt. the therapeutic doses for sheep, goats and cattles. Mice were sacrificed 24 h after treatment.

Chromosomal aberration

Bone-marrow metaphases were prepared following the method of (Yosida and Amano 1965) and stained with phosphate buffer Giemsa.

For preparation of spermatocytes at diakinesis, metaphase I. Testes were dissected out and processed according to (Evans *et al.* 1964). 100 well spreaded metaphases were analyzed per animal.

Sister chromatid exchanges (SCEs)

The method described by MC Fee *et al.* (1983) was applied. Mice were injected with colchicine (0.6 mg kg⁻¹ b.wt.) 2 h prior to sacrificing. Bone-marrow cells were fixed and stained with fluorescence plus Giemsa stain method of (Perry and Wolff 1974). 40 well-spread metaphases per animal were examined microscopically for SCEs.

Sperm-shape abnormalities

Groups of animals (5/dose) were i.p. injected for 5 successive days with 5, 10, 15 and 20 mg Closantel kg⁻¹ b.wt. and 10, 20, 30, 40 mg Nitroxynil kg⁻¹ b.wt. Animals were sacrificed 35 d after administrating the first dose.

The epididymides excised and minced in isotonic sodium citrate solution (2.2%) dispersed and filtered to exclude large tissue fragments. Smears were prepared, after staining the sperms with Eosin y (Wyrobek and Bruce 1975). At least 1000 sperms/animal were assessed.

Table 1. Number and percentage (%) of chromosome aberrations in mouse bone-marrow cells after i.p. treatment with different doses of “Closantel” and “Nitroxylnil” for 24 h

Treatment and doses (mg kg ⁻¹ b.wt.)	Metaphases with chrom. aberr.		No. and % () of metaphases with					
	Including gaps mean %±S.E.	Excluding gaps mean %±S.E.	Chromat. gap	Frag. and/ or break	Deletions	Robert. trans.	More than one aberr.	Polyploidy
I. Control (non-treated)	4.8±0.37	2.6±0.40	11 (2.2)	4 (0.8)	2 (0.4)	—	—	7 (1.4)
II. MMC i.p. injection at 1 mg kg ⁻¹ (+ve control)	25.4±1.59**	21.4±1.91**	20 (4.0)	59 (11.08)	13 (2.6)	10 (2.0)	15 (3.0)	10 (2.0)
III. Closantel								
5	5.8±0.37	2.08±0.20	15 (3)	11 (2.2)	3 (0.6)	—	—	—
10	7.0±0.31*	3.8±0.37	16 (3.2)	12 (2.4)	4 (0.8)	—	—	3 (0.6)
15	10.6±0.68**	6.4±0.40*	21 (4.2)	13 (2.6)	8 (1.6)	2 (0.4)	4 (0.8)	5 (1.0)
20	10.8±0.86**	7.0±0.95**	19 (3.8)	15 (3)	10 (2.0)	1 (0.2)	5 (1.0)	4 (0.8)
IV. Nitroxylnil								
10	8.2±0.37*	4.8±0.49	18 (3.6)	11 (2.2)	3 (0.6)	1 (0.2)	—	9 (1.8)
20	10±0.55**	5.6±0.55*	22 (4.4)	13 (2.6)	1 (0.2)	1 (0.2)	—	13 (2.6)
30	10.8±0.58**	6.2±0.66**	23 (4.6)	13 (2.6)	3 (0.6)	2 (0.4)	—	13 (2.6)
40	11.4±0.51**	6.2±0.58**	26 (5.2)	20 (4.0)	3 (0.6)	1 (0.2)	—	7 (1.4)

* Significant at 0.05 level. ** Significant at 0.01 level.

Table 2. Frequency of SCEs/cell in mouse bone-marrow cells after i.p. treatments with different doses of “Closantel” and “Nitroxylnil” for 24 h

Treatment and doses (mg kg ⁻¹ b.wt.)	SCEs No.	SCEs/cell Mean %±S.E.
I. Control (non-treated)	784	3.92±0.11
II. MMC (+ve control) i.p. injected at 1 mg kg ⁻¹ b.wt.	2495	12.47±0.34
III. Closantel		
5	920	4.60±0.19
10	1426	7.13±0.32**
15	1610	8.05±0.66**
20	1990	9.95±0.41**
IV. Nitroxylnil		
10	778	3.90±0.44
20	874	4.37±0.22
30	1121	5.61±0.15
40	1157	5.79±0.20*

* Significant at 0.05 level. ** Significant at 0.01 level.

Control

In all experiments a concurrent negative control received only distilled water and “Mitomycin C” at a dose 1 mg kg⁻¹ b.wt. i.p. was used as a positive control were maintained.

Statistical analysis

The significance of experimental versus control data was calculated using the t-test.

Results

Chromosomal aberration in somatic cells

Single i.p. treatment of Closantel at 5, 10, 15, 20 mg kg⁻¹ b.wt. and of Nitroxylnil at 10, 20, 30,

Fig. 3. Sister-chromatid exchanges (a), fragment (b) in bone-marrow cells, a diakinesis-metaphase I cell with *x-y* univalent (c) and autosomal univalent (d) from mouse spermatocytes after i.p. treatment with Closantel and Nitroxylin.

Fig. 4. Types of sperm-shape abnormalities found in normal and fasciolicides treated mice. Normal sperm with a definite head by a marked hook (a), without hook (b), Amorphous shape (c), (d), Triangular (e), Banana shape (f), coiled tail (g), (h).

Table 3. Number and percentage (%) of chromosome aberrations in mouse spermatocyte after i.p. treatment with different doses of "Closantel" and "Nitroxylin" for 24 h

Treatment and doses (mg kg ⁻¹ b.wt.)	Mean %±S.E.	No. and % () of metaphases with					
		X-Y Univ.	Autosomal Univ.	X-Y Univ. + Auto. Univ	Gap in X-Y	Frag or break	Chain IV
I. Control (non-treated)	4.8±0.37	16 (3.2)	8 (1.6)	—	—	—	—
II. MMC i.p. injection at 1 mg kg ⁻¹ (+ve control)	19±0.55**	48 (9.6)	28 (5.6)	13 (2.6)	—	6 (1.2)	—
III. Closantel							
5	6.40±0.51	22 (4.4)	8 (1.6)	2 (0.4)	—	—	—
10	10.60±0.81**	35 (7.0)	15 (3.0)	2 (0.4)	1 (0.2)	—	—
15	12.00±0.83**	38 (7.6)	16 (3.2)	5 (1.0)	—	1 (0.2)	—
20	14.40±0.74**	48 (9.6)	14 (2.8)	7 (1.4)	1 (0.2)	—	2 (0.4)
IV. Nitroxylin							
10	9.2±0.58*	24 (4.8)	15 (3.0)	3 (0.6)	2 (0.4)	2 (0.4)	—
20	10.4±1.12**	28 (5.6)	17 (3.4)	3 (0.6)	1 (0.2)	3 (0.6)	—
30	12.2±0.78**	29 (5.8)	17 (3.4)	9 (1.8)	2 (0.4)	4 (0.8)	—
40	12.4±0.51**	28 (5.6)	13 (2.6)	13 (2.6)	2 (0.4)	6 (1.2)	—

* Significant at 0.05 level. ** Significant at 0.01 level.

40 mg kg⁻¹ b.wt. the therapeutic doses (Fairweather and Boray 1999a), increased the percentage of chromosomal aberrations in bone-marrow cells of mice. It reached 7.0 ± 0.95 and 6.2 ± 0.58 ($p < 0.01$) 24 h after treatment with the highest doses of the 2 drugs respectively compared with 2.6 ± 0.40 for the control (after excluding chromatid gaps).

Different types of chromosome aberrations in bone-marrow cells described with details in Table 1 and Fig. 3.

Table 4. Number and percentage (%) of sperm-shape abnormalities in mice after i.p. treatments with different doses of "Closantel" and "Nitroxylin"

Treatment and doses (mg kg ⁻¹ b.wt.)	Abnormal sperm No.	Mean %± S.E.	Amorphous	Without hook	Triangular
I. Control (non-treated)	146	2.92±0.18	37 (0.74)	25 (0.50)	25 (0.50)
II. MMC (+ve control) i.p. injection at 1 mg kg ⁻¹	972	19.44±0.52	21 (4.22)	169 (3.38)	156 (3.12)
III. Closantel					
5	270	5.40±0.35**	52 (1.04)	51 (1.02)	57 (1.14)
10	396	7.92±0.46**	51 (1.02)	82 (1.64)	93 (1.86)
15	462	9.24±0.43**	79 (1.58)	64 (1.28)	99 (1.98)
20					
IV. Nitroxylin					
10	148	2.96±0.06	32 (0.64)	21 (0.42)	16 (0.32)
20	213	4.26±0.20	52 (1.04)	39 (0.78)	23 (0.46)
30	329	6.58±0.08**	90 (1.80)	66 (1.32)	34 (0.68)
40	351	7.02±0.13 **	100 (2.0)	35 (0.70)	49 (0.98)

Treatment and doses (mg kg ⁻¹ b.wt.)	Banana shapa	Small head	Big head	Coiled tail	Double head
I. Control (non-treated)	26 (0.52)	12 (0.24)	11 (0.22)	10 (0.20)	—
II. MMC (+ve control) i.p. injection at 1 mg kg ⁻¹	125 (2.50)	79 (1.58)	88 (1.76)	80 (1.6)	64 (1.28)
III. Closantel					
5	34 (0.68)	10 (0.20)	8 (0.16)	58 (1.16)	—
10	57 (1.14)	25 (0.50)	16 (0.32)	72 (1.44)	—
15	71 (1.42)	36 (0.72)	23 (0.46)	90 (1.80)	—
20					
IV. Nitroxylin					
10	14 (0.28)	23 (0.46)	30 (0.60)	12 (0.24)	—
20	18 (0.36)	24 (0.48)	37 (0.74)	17 (0.34)	3 (0.06)
30	28 (0.56)	42 (0.84)	43 (0.86)	18 (0.36)	8 (0.16)
40	40 (0.80)	27 (0.54)	48 (0.96)	45 (0.90)	7 (0.14)

* Significant at 0.05 level. ** Significant at 0.01 level.

Sister chromatid exchanges (SCEs) in somatic cells

Single i.p. treatment of Closantel at 5, 10, 15, 20 mg kg⁻¹ b.wt. induced significant frequencies of SCEs. It reached $9.95 \pm 0.41/\text{cell}$ ($p < 0.01$) at 20 mg kg⁻¹ b.wt. compared with $3.92 \pm 0.11/\text{cell}$ for control. Nitroxylin at 10, 20, 30 mg kg⁻¹ b.wt. had no significant effect on SCEs induction. The mean frequency at 40 mg kg⁻¹ b.wt. reached $5.76 \pm 0.20/\text{cell}$ ($p < 0.05$) (Table 2), that compared with $12.47 \pm 0.34/\text{cell}$ for Mitomycin C (MMC) +ve control at 1 mg kg⁻¹ b.wt. (Fig. 3).

Chromosomal aberrations in germ cells

Mean proportion of chromosomal aberration in spermatocytes induced after i.p. treatment with previous doses of 2 drugs. were dose dependent. Both drugs caused a significant increase in the percentage of chromosomal aberrations over that of the control (Table 3). The most dominant aberrations were $x-y$ univalent followed by autosomal univalent, fragments and/or breaks, gaps in $x-y$ and translocations as chain IV were also observed (Fig. 3).

Sperm-shape abnormalities in germ cells

Various morphological sperm-shape abnormalities are listed in Table 4. The 2 drugs at the test-

ed doses induced high significant percentage of abnormal sperms. It reached 12.74 ± 0.49 and 7.02 ± 0.13 ($p < 0.01$) after treatment with the highest doses respectively (Fig. 4).

Discussion

The anthelmintics that have the longest half-life in the body are the salicylanilide Closantel and the halogenated phenols Nitroxylin.

This is explained by their strong plasma protein binding which is more than 99% for salicylanilides (Mohammed-Ali and Bogan 1987, Rothwell and Sangster 1997, Stove 1998, Rothwell *et al.* 2000) and 98% for nitroxylin (Alvinerie *et al.* 1995).

Closantel and Nitroxylin are lipophilic compounds that are capable of carrying protons (H^+) across membranes and therefore may act to uncouple oxidative phosphorylation in the flukes. And increased carbon flow along energy-producing pathways that indicated by increased glucose uptake, decreased glycogen content, increased end-product formation (especially succinate) changes in respiratory intermediates, increase in oxaloacetate: malat ratio, decreased ATP synthesis and changes in mitochondrial ATP ase activity. That is the mode of action for the 2 drugs on mature and immature flukes (Van den Bossche *et al.* 1979, Kane *et al.* 1980, Rohrer *et al.* 1986, Martin 1997, Fairweather and Boray 1999a).

Their unique pharmacokinetic behaviour appears to play an important role in the efficacy and safety of these compounds (Swan 1999).

The strategic use of single therapeutic doses of Closantel and Nitroxylin in the present study were investigated to evaluate their mutagenic potential through different cytogenetic parameters in mice somatic and germ cells.

The results indicated that the percentage of chromosomal aberration in somatic and germ cells increase linearly with doses. Both drugs induced a high percentage of chromosome aberrations in spermatocyte cells, that reflect the male reproductive toxicity specially of Closantel (Mantovani 1992). Toxicity studies for Van Cauteren 1985, showed that Closantel at repeated oral dose was without effects up to $40 \text{ mg kg}^{-1} \text{ b.wt.}$ in rats except for focal swelling of epididymis in male due to formation of spermatoc granuolomas. With Nitroxylin the testis appear to be most susceptible to fasciolicide action. The precise mechanism behind the disruption of spermatogenesis and other reproductive activities is not known, although for nitroxylin it has been linked to its potential uncoupling action leading to less energy being available for cell division (Stammers 1976, Fairweather and Boray 1999b).

Separation of chromosomes forming univalents was the common type of abnormalities in mouse spermatocyte cells after treatment with the 2 drugs. This phenomenon has been observed with other chemical agents *e.g.* Cephalosporines antibiotics (Donya 2002) and berenil antitryptanosomal drug (Donya 2006).

SCEs have been used as a sensitive markers for assessing chromosome instability to determine the clastogenic effect of chemical compounds (Anderson *et al.* 1990). Closantel at the tested doses induced a significant levels of SCEs frequencies this is indicative of genomic instability and de-ranged DNA repair, which may account for segregation of certain genes (McKinnon 1987). However, Nitroxylin have no significant effect on SCEs except for high dose with frequency at low level ($p < 0.05$).

The effects of Closantel and Nitroxylin, in the present study agreed with the results of previous study on the mutagenis effect of other fasciolicide (fasinex). It induced structural chromosomal aberrations, and increased SCEs frequencies, as well as micronucleus formation in river buffalo lymphocyte cultures *in vitro* (Ahmed and Othman 2003).

Sperm morphology assay is considered more sensitive in detection germ cell mutagens than other germinal mutagenicity assay (Wyrobek *et al.* 1983). The results of sperm abnormalities re-

vealed that all of tested doses of Closantel and 2 higher doses of Nitroxylin induced highly significant percentage ($p < 0.01$) of a variety number of abnormal sperms. Damage to the sperm cells by substances may occur by either physiological, cytotoxic or genetic mechanisms (Wyrobek 1978). The doses dependent increase in the percentage of chromosomal aberrations in spermatocytes and sperm abnormalities induced by Closantel and Nitroxylin emphasize the positive correlation between cytogenetic damage and sperm abnormalities.

In conclusion, the 3 cytogenetic parameters used to evaluate the effect of the salicylanilide (Closantel) and halogenated phenol (Nitroxylin) revealed that, both drugs induced a clastogenic effect on somatic cells (chromosome aberrations and SCEs) and germ cells (chromosome aberration and sperm-shape abnormalities) in mice *in vivo* study under the tested conditions.

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