The effect of ivermectin on secondary spermatocytes and serum total proteins

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Abstract: Ivermectin, a broad-spectrum anti-parasitic agent first used in veterinary medicine, is active against numerous species of helminths and arthropods. In this study, we aimed to demonstrate the effects of administration of ivermectin on secondary meiotic division and serum total proteins. Male mice treated with single injections of 200ug/kg b.w. IVM. Meiotic chromosomes were prepared after 6 hours, 2, 5, 10 and 12 days to cover the different phases of meiotic division. Blood samples were collected after 1, 7 and 14 days of the last injection to determine total protein content. Euploidy (haploid no which equal 20 chromosomes) was recorded in 8.6 of the scored cells of secondary spermatocytes. Hyperhaploid (metaphases that include more than 20 and less than 23 chromosomes) was also considered. A total of 46 hyperhaploid metaphases were registered for 2100 examined cells. The hyper-haploidy index was 2.49% versus 0.8% for the control. Acentric fragments were occasionally occurred. After 1 and 7 days, single injections of IVM led to elevate the total protein content than that resulted after double treatment. However, the data obtained after 14 days were closed together. In conclusion, IVM is produced a considerable signs of chromosomal damage to germ cells. So, the cytogenetic studies revealed high clastogenicity of the drug. On the other hand, the differences in total protein concentration obtained between treated and control samples indicate genotoxic potential for IVM.

Keywords: Ivermectin (IVM), secondary spermatocytes, euploidy, Hyperhaploid metaphases, serum protein content.

INTRODUCTION

In case of affording patients, ivermectin once a week for two weeks currently offers quick, better, and safe results, restricting further morbidity and secondary transmission (Bachewar *et al.,* 2009). The response from crusted scabies to oral ivermectin is variable and combination therapy with topical scabicides and keratolytics seems to be the best choice (Nofal, 2009).

Abedin *et al.* (2007) concluded that treatment of scabies with ivermectin in an endemic population is more efficacious as compared to topical permethrin application for reducing the baseline prevalence, decreasing the chain of transmission and chances across reinfection.

Recently, IVM 3.15% long-acting (IVM-LA) preparations to be administered at 630µg/kg to cattle were introduced into the veterinary pharmaceutical market (Lifschitz *et al.,* 2007).

Ivermectin treatment protocols were using for controlling *Trichinella spiralis* (Basyoni and El-Sabaa, 2013); increased the efficacy of worms count reduction of the multi-resistant isolates from *Haemonchus contortus* (Bartley *et al.,* 2012); seemed to be sufficiently effective and safe for practice use (Mellgren and Bergvall, 2008); shown highly efficacy and safety in a patient infected with human T cell lymphotropic virus type I with *Strongyloides stercoralis* hyper infection syndrome (Pacanowski *et al.,* 2005); represented an effective drug for severe forms of scabies occurring in patients affected by other dermatoses that prevent the use of topical treatments (Angelo *et al.,* 2004); is also affective in the treatment of ascariasis; cutaneous larva migrans and head lice (Develoux, 2004); was less effective than albendazole for treatment of cutaneous gnathostomiasis (Kraivichian *et al.,* 2004); are inexpensive, safe, requires very little labor and is very effective at eradicating pinworms from mice (Sueta *et al.,* 2002) and reduced adult infections with some rarer gastrointestinal nematode parasites of sheep (Rehbein *et al.,* 2000).

Moreover, Alvarez *et al*. (2008) indicated that the co-administration of albendazole and ivermectin did not induced an adverse kinetic interaction. However, it induced a small but significant effect on some sensitive behaviors (Davis *et al.,* 1999).

The present work searched for the potential mutagenicity of IVM in mice. This was achieved by using cytogenetic and total protein content.

MATERIALS AND METHODS

Experimental animals

Male albino mice (*Mus musculus*) between 9 and 15 wk of age with an average body weight of 30g were used. They were supplied by Abbasia Farm of the Egyptian Organization for Vaccine and Biological Preparations.

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Mice were supplied with standard laboratory chow and tap water. Mice were allowed to acclimate for at least one week prior to the study. All experiments were conducted according to the protocol approved by Institutional Animal Ethics Committee (IAEC) at Ain Shams University, Egypt.

Chemicals and solutions

Ivermectin was used in the form of Bomectin injection (Bomac-Laboratories LTD). The recommended dose is 200ug/kg b.w. (Chouela *et al.,* 2002 and Vaidhyanathan, 2001).

Treatment for meiotic chromosomes

Thirty sex male albino mice of the same strain and age were used for this assay. Mice were divided into two groups, first group served as control and injected intraperitoneally with sterile distilled water. The second group received single injections of 200ug/kg ivermectin. Cytogenetic analysis of MII spermatocytes was made six hours, two, five, ten and twelve days after injection with distilled water or ivermectin, corresponding to treated diakinesis, late pachytene, mid-pachytene, zygotene and preleptotene (Ciranni and Adler, 1991).

Preparation of mammalian meiotic chromosomes

Meiotic chromosomal preparation was performed according to Leopardi *et al.* (1993). Male albino mice were injected i.p. with 0.1ml of colchicine solution, 2 hours before sacrificing them by cervical dislocation.

Testes were removed and placed in isotonic sodium citrate solution (2.2% wt/vol) at room temperature. Tunica was pierced and testes were swirled in the solution to remove adherent fat. Then testes were transferred into fresh 2.2% sodium citrate solution and the tubules were gently pulled out. The mass of tubules was held with fine, straight forceps and their contents were teased out with similarly fine, but curved forceps. When the tubules appeared flat and opaque they have been allowed to settle, then the supernatant fluid was transferred into centrifuge tube.

Centrifuge the cell suspension obtained at 500 r.p.m. for 5 minutes. The supernatant is discarded, and the pellet is resuspended in 2ml of a 1% (w/v) sodium citrate solution added drop by drop with a Pasteur pipette while flicking the tube with a finger to mix well. After 10 minutes, the suspension is centrifuged for 5 minutes at 500 r.p.m. The supernatant is removed and 2ml of fixative (3 parts of methyl alcohol to 1 part glacial acetic acid) is added dropwise and flicking the tube vigorously after each drop.

After 10 minutes, cells were sedimented again by centrifugation and were resuspended in fresh fixative. The change of fixative was repeated after further 10 minutes. The final suspension was prepared in a 0.5ml of fixative. Few drops of this suspension were allowed to fall on a grease free slide at room temperature. After drying, slides were stained for 5 minutes in Giemsa.

Scoring criteria

For each animal 70 cell were microscopically analysed for chromosomal aberrations with 100 x magnifications in phase contrast.

Aneuploidy assay (MII): Hyperploid metaphases were those showing 20 chromosomes plus one or more single chromatid, as well as 21 or 22 chromosomes. A hyperploidy index was calculated by the ratio between the number of hyperploid metaphases and the sum of euploid and hyperploid cells. Hypoploidies were not considered in the estimation of induced aneuploidy, since their frequency was quite variable and totally unrelated to chemical treatment, no doubt because of an unavoidable component of technical artefacts (Leopardi *et al.,* 1993).

Blood collection for protein analysis

Thirty sex male mice were injected with single doses of 200ug/kg IVM. After two weeks 18 of the animals received another dose of IVM. Blood were collected from each group after 1, 7 and 14 days of the last injection. Blood serum of the treated animals and accompanied control was prepared.

Blood samples were collected from eye plexus by nonheparinized haematocrit tubes into eppendorf tubes. The Eppendorf tubes were put in the refrigerator for 1 hour of clotting, then centrifuged for 10 minutes at 3000 r.p.m. The supernatant serum was transferred into a new clean Eppendorf tube using Pasteur pipette. The tubes were kept in the deep freezer at ~20^0c till analysis.

Determination of serum total proteins

The concentration of total proteins was determined according to Biuret's method using Bio-Adwic Kits (El Nasr Pharmaceutical Chemicals Co.) (Holme and Peck, 1983).

Procedure: 1 ml of the biuret reagent (R1) was placed in a series of test tubes. 20ul from each sample of blood serum were added to the biuret reagent. The content of each tube was mixed well and allowed to stand for 10 minutes. This preparation was accompanied by another tube containing 20ul of the standard solution (R_2) and 1ml of biuret reagent (R1).The absorbance value (A) of the standard and the sample was measured against the reagent blanks at 545 nm.

Calculation

Serum total protein (gm/100 ml)=A sample/A standard x 5

STATISTICAL ANALYSIS

Statistical differences in the frequencies of hyperhaploid metaphase II, fragments and serum total proteins in treated and control animals were compared by Student-t-test.

Table 1: Variation of the hyper-haploidy index and of the frequency of cells with fragments scored at the second meiotic metaphase of the mouse spermatogenesis after single dose of ivermectin

Stage at treatment	Intervals	Number of examined cells	No. of haploid plus hyper haploid M_{11}	Hyper-haploid		MII with fragments	
				No.	Hy-index (%)	No.	%
Control	0	420	372	3	0.8	1	0.24
MI-diakinesis	6 hours	420	384	5^{ns}	1.3	0	-
Late pachytene	2 days	420	368	7*	1.9	6	1.4
Mid pachytene	5 days	420	380	10**	2.6	4	0.9
Zygotene	10 days	420	366	9*	2.4	2	0.4
Pre-leptotene	12 days	420	349	15****	4.3	8	1.9
Total	-	2520	1847	46	2.49	20	0.8

ns=Non significant *p≤0.05=significant **p≤0.02=highly significant ****p≤0.01=very highly significant

Table 2: The effect of single dose (200ug/kg ivermectin) on total protein concentration in serum of mice

Treatment period	Group	Mean ±S.D.	Range
1 day	Control	5.080 ± 0.306	4.774-5.386
	Treated	6.454 ± 0.521^{ns}	5.239-8.298
7 days	Control	5.714 ± 0.673	4.728±5.714
	Treated	8.681 ± 0.480***	7.224-10.187
14 days	Control	5.825 ± 0.055	5.77-5.881
	Treated	6.429 ± 0.2306*	5.475-6.916

M=Mean *p≤0.05=significant S.E.=Standard error ***p≤0.01=Highly significant n.s. = Non-significant

Table 3: The effect of repeated dose (200ug/kg ivermectin) on total protein concentration in serum of mice

Treatment period	Group	Mean ±S.D.	Range
1 day	Control	4.703 ± 0.061	4.601-4.814
	Treated	5.11 ± 0.345^{ns}	4.029-5.930
7 days	Control	5.6567 ± 0.2446	5.385-6.145
	Treated	7.077 ± 0.264***	6.101-8.0616
14 days	Control	5.6495 ± 0.2315	5.418-5.881
	Treated	6.622 ± 0.0743***	6.365-6.927

M=Mean S.E.=Standard error ***p≤0.01=Highly significant n.s.=Non significant

RESULTS

Chromosomal study in germ cells

Aneuploidy assay (MII)

For aneuploidy assay in MII, a total of 70 MII cells per animal were scored and classified as, euploidy that spread has 20 chromosome, haploid number, n=20, and hyperhaploid cells that MII with 20<n≤22 chromosome. The number of hyper-haploid spermatocytes reached a maximum at 12 days (15/420 cells). Samples prepared after 6hs has the lowest score which is not significantly different from the control. Significant results were obtained after 2 days and 10 days of the treatment (p≤0.05, table 1).

Also, the difference was significant (p≤0.02) after 5 days and after 12 days (p≤0.01). Consequently, the frequency of aneuploidy is calculated as the ratio of hyper-haploid MII spermatocyte (n>20) to the sum of of euploid and hyperploid cells.

The values of the hyper-haploid indices achieved through the last three durations were the most frequent. Acentric fragments were also observed in a low frequency (fig. 1). The last duration (12 d) showed the high score. Polyploidy was also observed, but it was not considered in this part.

Examine of total protein in blood serum

Single dose: It is clearly noticed from table (2) that protein content was increased after one day of ivermectin treatment. However, the difference was found to be non-significant as compared to the control level. On contrast, data obtained after 7 and 14 days post-treatment revealed significant increase (p≤ 0.01 and p≤0.05, respectively).

Double doses

Experiment carried out after 1 day of double doses injection showed non-significant increase (p≥ 0.05) in the serum total proteins. The treated samples showed 5.11± 0.345, while the accompanied control contained 4.703 ±

0.061. Ivermectin induced high significant elevation ($p \leq 0.01$) in the concentration of the total protein 7 days and 14 days after experimentation (table 3).

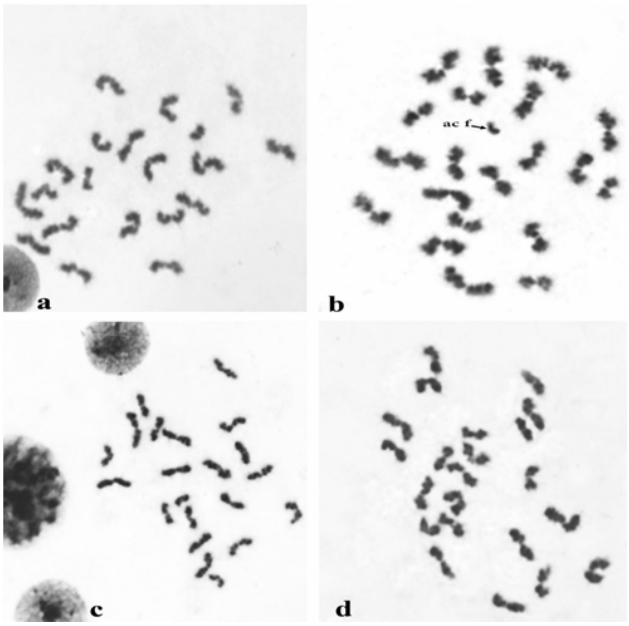

Fig. 1: Secondary spermatocytes at metaphase II of meiosis treated samples showing: a-Haploid MII spermatocytes n=20 b- ac f= Acentric fragments c =hyperhaploid with n= 21 d =hyperhaploid with n= 22.

DISCUSSION

Abamectin and ivermectin are 2 closely related members of the Avermectin family of 16-membered macrocyclic lactones derived from the actinomycete *Streptomyces avermectinius* which exhibit extraordinary anthelmintic activity (Molinari *et al.,* 2010). Ivermectin, is provided with no cost, is effective against microfilariae (blocking their transmission) and can be administered annually as a single oral dose with virtually no side-effects (Richard-Lenoble *et al.,* 2003). Gulbas *et al.* (2004) suggested that *Strongyloides stercoralis* could present with isolated focal hepatic lesions and severe eosinophilia, and resolved with ivermectin treatment.

Cytogenetic studies of mammalian germ cells is of great importance as it evaluates the genetic risk associated with exposure to mutagenic agents (Russo, 2000).

In this study, chromosomes were prepared for male mice according to the method of Leopardi *et al.* (1993). Using this method, many different stages of spermatogenesis have been recognized in small mammals and in man (Meredith, 1969).

Scoring the chromosome number in metaphases of secondary spermatocytes is a simple and direct approach to evaluate meiotic aneuploidy. Its advantage over the analysis of female germ cells is the availability of a larger number of analyzable metaphases (Russo *et al.,* 1983 and Leopardi *et al.,* 1993).

Concerning the aneuploidy assay in the current study, different time intervals between treatment and sacrifice were tested to follow the different stages of spermatogenesis. The hyper-haploid index for the control value was 0.8%. This is agreement with the results obtained by Leopardi *et al.* (1993). They found that the hyperploidy control frequency ranged between 0.4 and 1.0% in somatic cells and from 0.3 to 0.9% in germ cells. In both somatic and germ cells, the maximum yield of induced hyperploidy did not exceed 3.5%.

However, the actual incidence of non-disjunction during maternal and paternal germ-cell development is higher because most aneuploidies are not compatible with normal development, and embryos carrying such anomalies are aborted, contributing to fetal westage (Sankaranarayanan, 1979).

Hypoploid MII spermatocytes were not included in the calculation of aneuploidy frequency. It is important to mention that hypo-haploid and polyploid cells were not included as their frequencies are influenced by technical artifacts due to the existence of cytoplasmic bridges among synchronous meiotic cells (Russo, 2000).

Remarked elevation in hyperploid indices was observed following IVM treatment. After 6h of an injection, the data were found to be non-significant. The values were increased by increasing the time. Data obtained after 12 days of the treatment represent the high score, as it reached 4.3% versus 0.8% for the control. A number of published papers have well demonstrated increase in aneuploidy after chemical exposure to different premeiotic cell stages. It can be detected by chromosomal analysis of MII spermatocytes (Adler, 1993).

In the present work, the low value of hyperhaploid index detected after 6h, and the gradual increase obtained through the different sampling times for the author's opinion indicate, the need of long time until the drug can reach the target cells. As proposed by Liang *et al.* (1985) it may attribute to the existence of the blood testis barrier.

On the other hand, aneuploidy may arise from chromosomal loss rather than disjunction (Backer and Allen, 1987).

With respect to the relative contribution of maternal and paternal gametes, cumulated human data onto the origin of trisomy 21 indicate that 20-30% of the errors occur to spermatogenesis, almost equally during the first and the second meiotic divisions (Mikkelsen *et al.,* 1980).

In addition, Luippold *et al.* (1975) reported that the yield of chromatid aberrations was similar in bone marrow and

spermatogonia, suggested that spermatogonia are certainly no less sensitive aberration induction than in bone marrow. On the other hand, the genotoxic effects of ivermectin were reviewed by several investigators.

Accordingly, El-Nahas *et al. (*2008) concluded that ivermectin had slight effects on male fertility, but when taken with verapamil induced stronger effects on germ cells, increased frequency of meiotic structural chromosomal aberrations and increased x-y chromosomal dissociation, raising the attention, to the genetic quality of mature sperm. Molinari *et al.* (2009) mentioned that ivermectin (IVM) and its commercial formulation ivomec (IVM 1.0%) did not modify sister chromatid exchange frequencies, while they induced DNA-strand breaks revealed by single cell gel electrophoresis SCGE in Chinese hamster ovary (CHO (k1) cells. Moreover, reports indicated that both abamectin and ivermectin were able to induce single DNA-strand breaks *in vitro* (Molinari *et al.,* 2010).

Moreover, the current investigation aimed to study the serum protein pattern change following IVM treatment. Total protein content was elevated to IVM effect. Very high significant increase was detected after 7 days of single dose. Then, the value approached to the control level after 14 days in both single and double injections. The present result are in agreement with Stromberg and Guillot (1987). In their work, calves were treated with IVM for 4 weeks. The infected calves had moderate anemia and increased plasma proteins; within one week after IVM treatment, the value began to return to normal level.

To the author's opinion, these observations were mainly related to the action of the drug and to the food intake. Some drugs cause misreading of the genetic code and hence cause abnormal protein synthesis (Mycek *et al.,* 1997). On the other hand, plasma proteins are sensitive to nutritional influences. Severe dietary protein deficiency induces a hypoproteinemia (Weimer, 1961).

Data concerned the association with IVM and plasma or serum proteins are scanty.
Klotz *et al.* (1990) measured ivermectin in five healthy individuals and it averaged 93.2±4.4% (SD). Such strong binding should be taken into consideration, especially in patients with malnutrition or with diseases in which a decrease in plasma proteins and consequently a higher free fraction of ivermectin could be expected.

The detected dropping in the total protein concentration may also reflect individual variation in uptake and excretion of the drug. Marked individual variation was observed in the pharmacokinetics of IVM (Scott and McKellar, 1992).

The present data pointed to the decrease in total protein contents after 14 days of drug administration in both groups. This may be related to the half-life of the plasma proteins. The clearance half-life of most plasma proteins ranges between 1: 3 weeks. On the other hand, most drugs are bound to plasma proteins particularly albumin. This inhibits the passage of the drugs outside the circulation and prevents glomerular filtration and excretion in urine (Kumar and Clark, 2002).

As claimed by Ganong (2003) the rate of degradation of the plasma protein is expressed as their turnover, fractional clearance or as their half-lives.

The present results are in accordance with that of Pound *et al.* (2004). They stated that ivermectin concentrations decreased to from detectable within 21 days after an injection of blood serum and 14 days after ingestion in Penned White Tailed Deer, *Odocoileus virginianus* (Zimmermann).

Velebný *et al.* (2008) showed decreased in total protein concentration on mice treated with the anthelmintic drug praziquantel alone or combination with beta-glucan incorporated into liposomes.

Recently, Real *et al.* (2011) showed a greater inhibitory potency of the macro cyclic lactone ivermectin on the wild-type protein. Ardelli and Prichard (2007), Williamson *et al.* (2011), De Graef *et al.* (2013) suggested that P-glycoprotein might be involved in resistance to both ivermectin and moxidectin in some trichostrongyloid nematodes.

In the present work, IVM showed clastogenic and aneugenic effects. These results indicate that in the use of this drug, consideration should be given to the possibility that it might lead to genetic hazards.

CONCLUSIONS

IVM showed aneugenic property in the secondary spermatocytes. Such observations represent the extent of the hazardous effects of the tested drug. From the previous published work, loss of chromosome material at cell division leads to death of embryos.

The changes in the serum protein fractions add another warning for the mutagenicity of IVM. It is concluded from the present work that, the mutagenic effects fortunately diminished with time. Accordingly to the obtained results cautious use of IVM is advisable.

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