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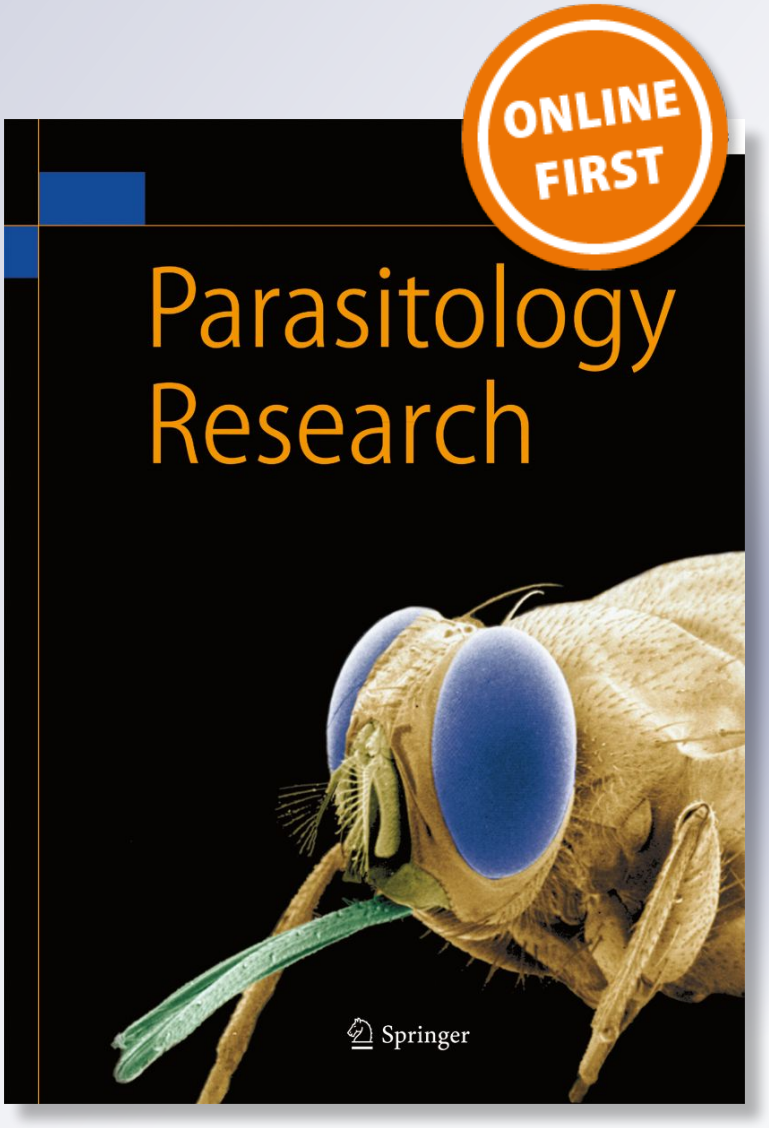

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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Insights into the impact of Ivermectin on some protein aspects linked to *Culex pipiens* digestion and immunity

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Abstract

In developing countries, low-cost control and treatment programs that offer combined approaches against diseases and their vectors are certainly needed. Ivermectin (IVM) has been well known for its role in the treatment of parasitic diseases, due to its effect on glutamate-gated chloride channels. These same channels are also present in the mosquito vector, and thus, research has focused on the insecticidal effects of this drug. Possible alternative mechanisms of IVM on the physiology of mosquitoes, however, have not been sufficiently elaborated. We assessed the protease activity, lipid peroxidation, and local expression of STAT, p53, caspase-3, and Bax markers to study the effect of this antibiotic on digestion and immunity in *Culex pipiens*. Sugar- and blood-feeding assays were employed to investigate the potential influence of blood feeding on the dynamics of these parameters. IVM was found to have an effect on protease activity, lipid peroxidation as well as the expression of different markers investigated in this work. The focus on the detailed effect of this drug certainly opens the gate to broadening the spectrum of IVM and expanding its health and economic benefit, especially that it is relatively more affordable than other antibiotics on the market.

Keywords Ivermectin · Digestion · Immunity · Enzyme activity · Proteins

Introduction

The discovery of Ivermectin (IVM) has provided a breakthrough in medicine and offered new choices for the treatment and control of chronic disabling parasitic diseases (Crump and Ōmura 2011). Among these diseases is filariasis, which is transmitted by *Culex* spp., in addition to some *Anopheles* spp. The most famous vector for this disease is *Culex pipiens*, which has a wide geographical range (Manguin and Boëte 2011) and is also implicated in the transmission of several arboviruses (Brugman et al. 2018) and avian malaria (Martínez-de la Puente et al. 2016). IVM is not only one of the agents included in the antifilariasis regimen, but its endocticidal effect has made it a potential contributor to the control of the arthropod vector. The action of IVM is mediated mainly through targeting glutamate-gated chloride (GluCl) channels. Other channels such as gamma aminobutyric acid- (GABA), histamine, and pH-sensitive chloride channels are also subject to the action of this antibiotic (Laing et al. 2017).

Alternative effects of IVM on various physiological processes inside the mosquito vector remain under-investigated. One of the processes detrimental for mosquito survival and reproduction is the feeding process. During feeding, many protein dynamics and enzyme activities orchestrate to gain maximum benefit and prevent potential harm related to blood acquisition (Manguin and Boëte 2011). Trypsin is the most important protease as regards blood digestion in mosquitoes. Its potent proteolytic activity is under meticulous control to avoid the deleterious effect of its over-activity on mosquito

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tissue (Noriega and Wells 1999). Another important aspect of mosquito physiology is the immune response and its various mechanisms. In addition to oxidative stress, immune signaling pathways such as the Toll pathway, the Imd (immunodeficiency) pathway, and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways are induced by cytokines such as interferon-gamma. These pathways play an important role in mosquito antiviral and anti-plasmodial immune mechanisms. Most studies are focusing on Toll and Imd pathways, while researches on the role of STAT signaling in mosquitoes are relatively few (Pakpour et al. 2014). The Jak/STAT pathway is a conserved immune signaling pathway that plays a crucial role in insects and mammals (Harrison 2012). Another biological process that can be observed in response to feeding is apoptosis. Apoptosis in insects is regulated by special signaling pathways and effector proteins such as the p53 pathway, insulin-like signaling, and ecdysone (regulates oogenesis) signaling. The actions mediated by the p53 pathway are achieved by various effector proteins such as Bax proteins and caspases (Gasco et al. 2002). The study of analogous pathways has witnessed great advances in vertebrates, and as regards insects, *Drosophila melanogaster* remains the best-studied model (Hurd et al. 2006). In *Drosophila melanogaster*, p53-mediated apoptosis has been found to confer antiviral immunity (Liu et al. 2013). In this study, we aimed at exploring the potential effect of ivermectin on certain aspects of mosquito digestion and immunity, in an attempt to further elucidate the endocticidal effect of this drug. Also, the revelation of unknown mechanisms of action of IVM suggests the expansion of its spectrum of use.

Materials and methods

Mosquito rearing and maintenance of mosquito colony

The colony of *Culex pipiens* (obtained from the faculty of Science, Cairo University and originally bred from larvae collected from Al-Wahat region, Giza governorate, Egypt) was maintained according to the international guidelines for animal researches. Mosquitoes were bred at a temperature of 27 ± 2 °C and a 12:12 photoperiod. After a period of 24-h starvation, blood feeding of 3–7-day-old female mosquitoes was performed on anesthetized Wistar rats, weighing 200–250 g each and aged 6–7 weeks. Fully fed mosquitoes were collected by an aspirator and stored at − 20 °CC. All samples were collected after 24 h of each feeding assay. Female mosquitoes were divided into 6 study groups, 30–50/each: group 1 (no feeding): which was collected 24 h after eclosion before receiving any feeding; group 2 (sugar feeding): fed only on sugar; group 3 (blood feeding on Wistar rat): which fed on living untreated Wistar rat. Ivermectin was investigated in the remaining 5 groups: group 4 (IVM 400 μg in blood meal) and group 5 (IVM 800 μg in blood meal): where mosquitoes were fed on Wistar rats receiving IVM 400 and 800 μg daily for 5 days, respectively. Groups 6, 7, and 8 (IVM in water): mosquitoes were supplied with tab water containing 25 ppm, 50 ppm, and 100 ppm IVM. For groups 4 and 5, control groups of mosquitoes fed on Wistar rats receiving daily intraperitoneal injections of 1 cc saline were included (Coleman et al. 2007).

Drug preparations and dosage calculation

The dose of IVM was adapted from Bastiaens et al. (2012) with certain modifications. Tectin 1% injectable solution (Arab Pesticides and Veterinary Manufacturing Co.), where 1 ml of solution contained 10 mg drug, was used. For the administration of IVM in water, the calculation of the concentration was based on the following equation: 1% = 1/100 = 0.01 × 1,000,000 = 10,000 ppm. One milliliter of Tectin solution was diluted in 100 ml water to achieve a concentration of 100 ppm (Figs. 1 and 2). To achieve concentrations of 25 ppm and 50 ppm, 0.2 ml and 0.5 ml of Tectin were diluted in 100 ml water, respectively. All IVM dilutions had to be freshly prepared for every dose. Wistar rats received the drug injections for 5 days and were anesthetized 24 h after the last dose to allow *Culex pipiens* females to feed on them.

Biochemical assays

Regarding colorimetric enzyme analysis for protease activity, the amount of azoprotein digested by the proteases within the sample was analyzed spectrophotometrically at 420–450 nm, where the degree of absorbance indicated the amount of proteases. The reading for each sample was converted to enzyme units (EU), where 1 EU is defined as the amount of enzyme activity that increases absorbance by 0.01 at a given wavelength. Chemicals used for this assay were purchased from Sigma, Aldrich (USA) (Castro and Cantera 1995). Lipid peroxidation assay (MDA-malondialdehyde-assay kit Sigma-Aldrich) was also performed. This assay is based on the reaction of the sample with thiobarbituric acid (TBA), which yields a colorimetric product that can be read by a spectrophotometer at 532 nm (Magalhaes et al. 2008).

Immunohistochemichal analysis

Immunohistochemistry (IHC) kits were purchased from ThermoFischer Scientific for the detection of the four tested protein markers (Bax, caspase-3, p53, and STAT). Epitope retrieval was performed using the Tris-EDTA buffer epitope retrieval method. The slides were incubated in Thermo Scientific Ultra Vision hydrogen peroxide block for 10 min. They were then washed twice in a buffer. Ultravision Protein

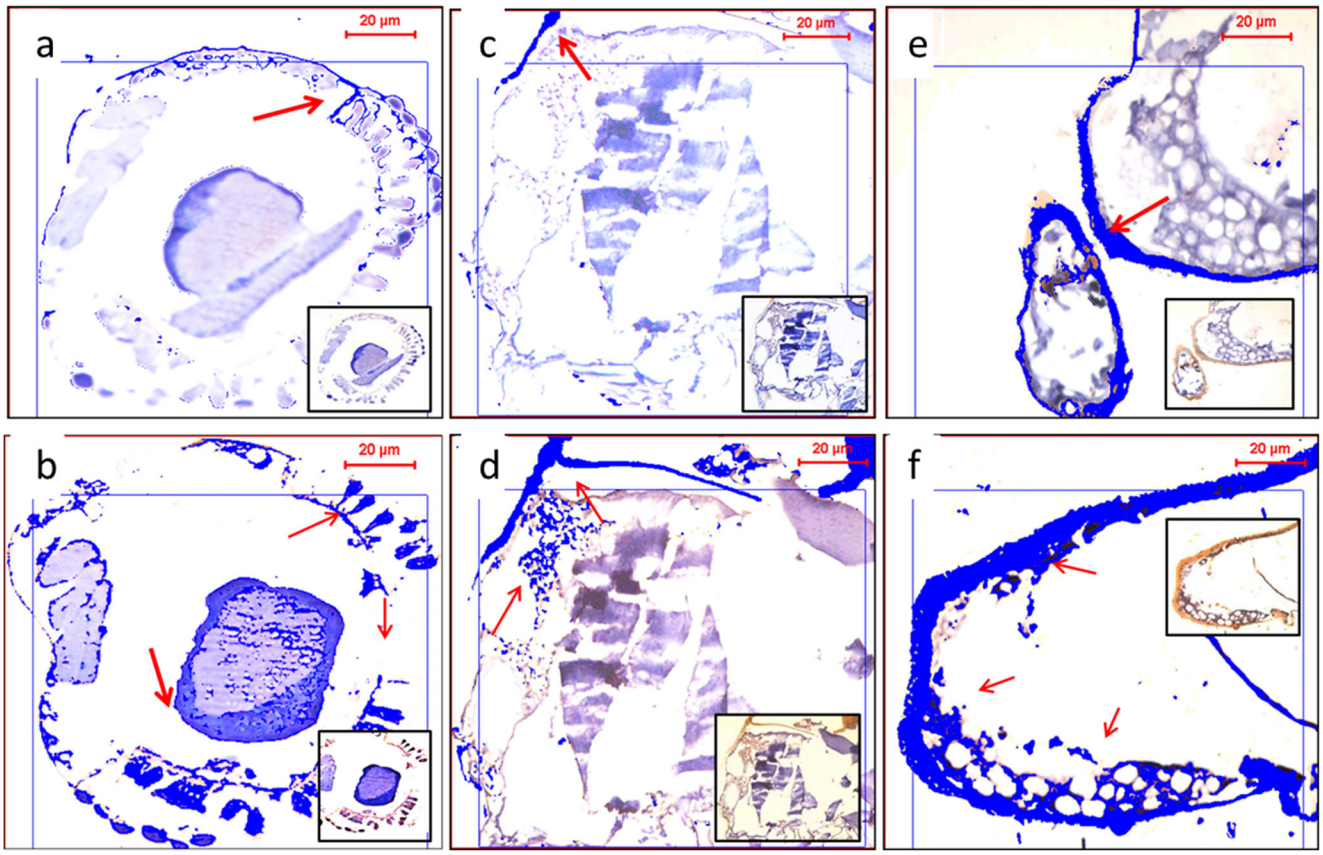

Fig. 1 Quantitative real-time image analysis for the expressed markers in head (**a**, **b**), abdomen (**c**, **d**), and fat body (**e**, **f**), revealing variable expression. Dark bluish coloration represents the area with positive protein expression in larger squares (red arrows). Brownish coloration in the smaller squares represents the rich areas in particular protein before software analysis

Block (TA-xxx-PBQ) was applied and incubated for 10 min to block non-specific background staining. Primary anti-mouse antibody for Bax and caspase-3 and anti-rabbit monoclonal antibodies (IgG) for p53 and STAT were used. Individual immunogens for these markers were synthetic peptides corresponding to a sequence at the N-terminal of the human Bax protein, recombinant protein derived from the N-terminus of human caspase-3 protein, recombinant human full-length wild type p53 protein, and a peptide corresponding to amino acids 636-645 of P42226 of STAT protein. Dilutions were prepared at a ratio of 1:100. Pathological and morphometric analysis was performed using Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd., Cambridge, England). Slides were examined with 100× and 200× power lenses. Optical density (OD) was automatically measured in 10 fields. Area percentage of each marker was measured in 10 fields and also

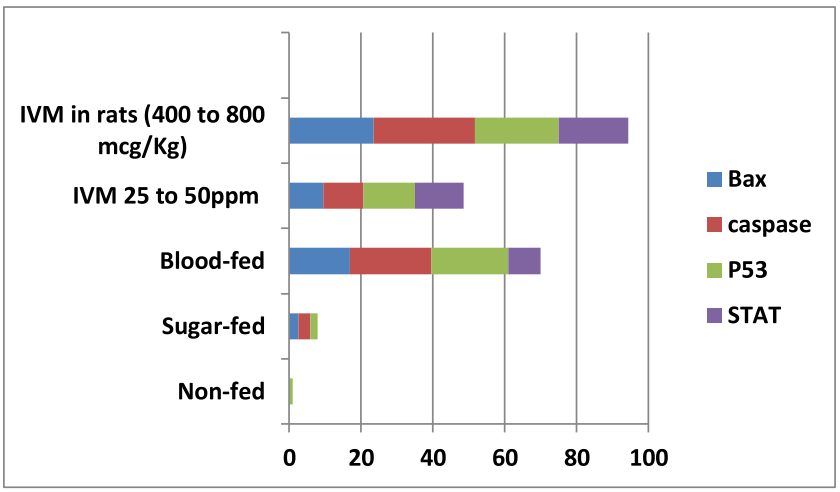

Fig. 2 Bar chart showing summary of different protein expression levels in the various study groups

in a real-time image from a microscope connected to a video monitor.

Statistical analysis

Data were tabulated and statistically analyzed using SPSS 17.0 software system in addition to Graphpad software version 6.0 (Prism). Data were analyzed using one-way ANOVA followed by Tukey Kramer multiple comparison test. Significance was tested at $P < 0.05$.

Results

Protease activity

Protease activity was found to be significantly increased with sugar feeding and even more with blood feeding, while IVM was found to significantly decrease the activity of this enzyme ($P < 0.05$). The mean value of protease activity in non-fed mosquitoes was 29.275 ± 0.66 EU. Sugar feeding significantly increased protease activity by 38.6% compared to non-feeding. Feeding mosquitoes on rat blood markedly elevated protease activity by 408.97% compared to non-feeding. Regarding the effect of small doses of IVM, sugar-fed mosquitoes were taken as control with a mean value of protease activity of 40.576 ± 0.435 EU. Feeding on IVM 25 ppm significantly decreased protease activity by 50.90% compared to sugar feeding only. Feeding on IVM 50 ppm significantly decreased protease activity by 74.99% compared to sugar feeding only. For the higher doses of IVM, blood-fed mosquitoes receiving 5 daily injections of 1 cc saline were taken as control and showed a mean value of 149 ± 1.826 EU. Feeding on rats receiving IVM 400 μg/kg significantly decreased protease activity by 15.86% compared to blood feeding only. Feeding on rats receiving IVM 800 μg/kg significantly decreased protease activity by 5.12% compared to blood feeding only (Table 1).

Lipid peroxidation

Concerning lipid peroxidation, the mean value of MDA in non-fed mosquitoes was 520.825 ± 1.26 nmol/gm. The mean value of MDA in sugar-fed mosquitoes was 875.8 ± 2.75 nmol/gm. The mean value of MDA in rat blood-fed mosquitoes was 6166.9 ± 0.91 nmol/gm. Significant difference was detected in the MDA content of the three groups, the highest being that of blood-fed mosquitoes and the lowest being detected in non-fed mosquitoes. Sugar feeding was found to increase the MDA level by 68.12% as compared to no feeding at all, while blood feeding was found to increase the MDA content by 1084.1% as compared to no feeding. The mean value of MDA in control blood-fed mosquitoes was 6166.9 ± 0.91 nmol/gm. The mean value of MDA in mosquitoes feeding on rats receiving IVM injections at a dose of 400 and 800 μg/kg was 2267.225 ± 51.85 and 1549.675[a] ± 900.65 nmol/gm, respectively. In samples fed on rats receiving IVM 400 μg/kg, MDA was found to decrease by 63.84% relative to the blood-fed control, while the level of decline was 74.87% in samples from mosquitoes that had fed on rats injected with IVM 800 μg/kg. No significant statistical difference was found between the MDA values of both doses of IVM injections, where $p = 0.87$ (Table 2).

Immunohistochemical detection of Bax protein, caspase-3, p53 protein, and STAT protein in mosquito tissue

As for the localizations of protein expression, the highest expressions were detected in the regions of the head, fat body, and gut for all tested markers (Figure 1). Non-fed mosquitoes showed negative expression for Bax, caspase-3, and STAT, while p53 showed a significantly low level (0.89 ± 0.43). Sugar-fed mosquitoes showed a very mild expression of apoptotic markers (2.56 ± 0.76, 3.24 ± 0.3, and 2.06 ± 0.09 for Bax, caspase-3, and p53, respectively), while negative expression (0%) was reported in STAT. Blood-fed mosquitoes

Table 1 Effect of different feeding assays on protease activity in the various study groups

Parameter/treatments	Protease activity (EU/mg) X ± SD	% deviation from control	
Non-fed mosquitoes	29.275 ± 0.66	___	
Sugar-fed mosquitoes	40.575[a] ± 0.435	38.6	Control = non-fed
Rat blood-fed mosquitoes	149[a] ± 1.826	409	
IVM 25 ppm	19.925[a] ± 0.499	− 50.90	Control = sugar-fed mosquitoes
IVM 50 ppm	10.15[ab] ± 0.129	− 74.99	
Rat blood (IVM 400 μ/kg)	125.375[a] ± 0.375	− 15.86	
Rat blood (IVM 800 μ/kg)	141.375[ab*] ± 1.109	− 5.12	

[a] Significantly different from normal control value at $p < 0.05$

[b] Significantly different from IVM 25 ppm value at $p < 0.05$

[a] Significantly different from saline value at $p < 0.05$

[b*] Significantly different from IVM 400 μ/kg value at $p < 0.05$

Table 2 Effect of different feeding assays on lipid peroxidation in the various study groups

Parameter/treatments	MDA in nmol/gm X ± SD	% deviation from control	
Non-fed mosquitoes	520.825 ± 1.26	–	
Sugar-fed mosquitoes	875.8[a] ± 2.75	68.12	Control = non-fed
Rat Blood-fed mosquitoes	6166.9[ab] ± 0.91	1084.1	
Rat blood (IVM 400 μ/kg)	2267.225[a] ± 51.85	− 63.24	Control = sugar-fed mosquitoes
Rat blood (IVM 800 μ/kg)	1549.675[a] ± 900.65	− 74.87	

[a] Significantly different from normal control value at $p < 0.05$

[b] Significantly different from sugar feeding value at $p < 0.05$

showed a significantly higher ($P = 0.0000$) expression level in all markers than non-fed and sugar-fed mosquitoes (16.9 ± 5.9, 22.6 ± 6.2, 21.5 ± 8.4, and 8.9 ± 4.3 within bax, caspase-3, p53, and STAT, respectively). *Culex* mosquitoes fed on rats receiving IVM showed significantly higher levels in all markers than groups receiving the drugs in water (Table 3; Figure 2).

Discussion

In the current study, the significant increase in proteolytic activity after sugar feeding might be the result of the early phase of trypsin secretion, as mosquitoes secrete trypsin in a biphasic manner (Noriega and Wells 1999). During this early phase of trypsin production, mosquitoes secrete a small amount of the enzyme as a test sample to estimate the amount of protein within the ingested meal. In cases of protein-rich meals such as blood, the degradation products formed by the early trypsin phase signal the need for a late trypsin response (Noriega and Wells 1999). Borges-Veloso et al. (2015) have identified eight trypsin-like serine peptidases in the midgut by mass spectrometry, which are responsible for digestion during sugar feeding in *Culex quinquefasciatus*. Graf et al. (1986) observed an increase in immunoreactivity of female *Aedes aegypti* to trypsin, 8 h after a blood meal, while later after 24 h, maximum activity was recorded. These results are supported by the present study on *Culex pipiens*, where blood-fed mosquitoes showed a higher protease activity than non-fed ones, even after the administration of IVM. The decrease in proteolytic activity observed after blood feeding of mosquitoes on IVM-treated rats suggests a potential role of this antibiotic in decreasing the mosquito benefit from the acquired blood meal, which forms the cornerstone of parasite entry into the mosquito vector. Further studies are therefore recommended to elaborate on possible unexplored mechanisms of action of this drug on mosquitoes. Moreover, the possibility of a similar effect on the parasite itself should be taken into consideration.

In the mosquito vector, the high boost in oxidative stress in response to blood feeding is, in part, attributed to naturally occurring blood components. A good example is the iron released from hemoglobin during the process of blood meal digestion, which can cause oxidative damage to mosquito tissues. This oxidative hazard is kept in check by mosquito antioxidant mechanisms. Saeaue et al. (2011) studied the effect of blood feeding on antioxidant enzymes and compounds in the mosquito *Aedes aegypti*. Mosquitoes were collected 48 and 72 h after acquiring the blood meal, and ferritin, glutathione peroxidase, glutathione S-transferase, catalase, and glutathione were measured. After the initial blood meal, only ferritin increased, while all tested enzymes increased after repeated blood meals. This observation of increased antioxidant defense in response to blood meal is in line with the results of the current study. Although IVM is well known for its endocticidal activity, the exact effect of this drug on insect physiology is still not sufficiently explored. It is evident that IVM acts on glutamate-gated chloride channels specifically found in invertebrates, leading to hyperpolarization and paralysis. This mechanism mediates both its insecticidal and antihelminthic activity. The action of this drug on the oxidative status in invertebrates, however, is not sufficiently elaborated. Even in mammals, researches on this spectrum of activity yield contradictory results. MDA levels in samples from mosquitoes fed on IVM-treated rats were significantly lower than those in samples from mosquitoes feeding on untreated animals, which certainly raise questions on whether this can be attributed to a potential antioxidant role of IVM. Behera et al. (2011) studied the change in oxidative damage in scabitic dogs before and after treatment with 0.2 mg/kg of 1% IVM. The level of lipid peroxidation was found to decrease after therapy, an effect that was further enhanced by the co-administration of vitamin E and selenium. On the other hand, Qureshi (2013) investigated the effect of high doses of IVM (5 mg/kg, 10 mg/kg, and 15 mg/kg) on lipid peroxidation, among other parameters, in Wistar rats. An increase in the MDA content was noticed in all doses, although this increase was statistically significant only in the two higher doses of the drug.

Regarding apoptosis, which is another mechanism of immunity, the majority of studies focused on apoptosis as a defense mechanism against various infections, while studies on apoptosis as part of the normal physiology in adult mosquitoes are few. In addition, immune pathways in mosquitoes have

Table 3 Expression levels of Bax, caspase-3, p53, and STAT proteins in different groups of mosquitoes included in the study

Assay sample	Bax	Caspase-3	p53	STAT
Non-fed	0	0	0.89 ± 0.43	0
Sugar-fed	2.56 ± 0.76	3.24 ± 0.3	2.06 ± 0.09	0
Blood-fed	16.9 ± 5.9	22.6 ± 6.2	21.5 ± 8.4	8.9 ± 4.3
IVM 25 to 50 ppm	9.4 ± 2.4	11.3 ± 5.6	14.3 ± 6.3	13.5 ± 4.8
IVM in rats (400 to 800 μ/kg)	23.5 ± 09.2	28.1 ± 4.5	23.4 ± 11.2	19.3 ± 7.1

been traditionally studied in the context of infection, whereas the dynamics of these pathways in relation to other physiological processes such as feeding and their manipulation by insecticides still needs more elaboration. In this study, apoptosis and immune signaling were assessed by analyzing the expression of p53, Bax, caspase-3, and STAT proteins in dependence of various feeding assays and in absence of infection. The choice of protein apoptosis markers in this study was based on the aim of exploring different pathways of mosquito apoptosis (intrinsic and extrinsic) and their regulation (Nikoletopoulou et al. 2013). The results of the current study clearly demonstrate dynamic changes in some proteins involved in these pathways; sufficient literature on these proteins, however, was not found. The detailed study of such pathways could disclose valuable targets for insect control. Expression of various markers was especially high in the mosquito head, fat body, and midgut. These tissues are important sites of immune-regulation in the insect vector. Arrese and Soulages (2010) stated that energy production and membrane biosynthesis in hemocytes were among the roles of the fat body during the immune response. The high expression in the mosquito head might be explained by the role of the insect brain in the regulation of phagocytosis by hemocytes through the secretion of serotonin (Qi et al. 2016). As for the midgut, apoptosis has been associated commonly with anti-plasmodial immunity in *Anopheles* (Kakani et al. 2016) and antiviral immunity in *Culex* (Vaidyanathan and Scott 2006; Wang et al. 2012). The high expression of apoptotic markers in IVM-treated mosquitoes may explain one of the mechanisms responsible for the high mortality rate caused by this drug. The apoptotic effect of this amazing drug was previously studied by Sharmeen et al. (2010) against leukemia. The authors reported that the drug successfully induced cell death in leukemia cells. The finding that caspase-3 became highly expressed after blood feeding is supported by Dong et al. (2016) who detected the enzyme by anti-caspase 3 antigen in addition to the demonstration of apoptotic cells in *Aedes aegypti*, concluding that the act of blood feeding induced apoptosis in mosquito tissues. Moreover, Dong et al. (2016) tested for apoptosis after infecting the mosquitoes with Chikungunya virus; however, no difference in caspase activity was detected.

In the current study, the STAT marker was significantly higher expressed in blood-fed samples, a finding that enforces the evidence for the boost in immunity induced by blood feeding. A study by Pakpour et al. (2014) supports this observation, since the study stated that the gamma interferon present in human blood activated the STAT signaling pathway. Souza-Neto et al. (2009) demonstrated the importance of the JAK-STAT pathway in the anti-Dengue defense mechanism of *Aedes aegypti*. In *Culex* mosquito, Paradkar et al. (2012) have demonstrated that a signaling molecule called Vago induced an antiviral state by the activation of the Janus kinase (Jak)-STAT pathway, thus restricting the infection by West Nile Virus in infected mosquitoes.

Methods for the application of IVM in mosquito control were demonstrated in several studies. The principal of insecticide zooprophylaxis is one of the strategies considered for vector control. It implies the administration of insecticides to animals in order to deliver a lethal dose to the vector biting them (Saul 2003). Additionally, IVM was also applied to toxic sugar baits for mosquitoes (Allan 2011). Moreover, studies on human volunteers receiving IVM have demonstrated that mosquitoes feeding on the volunteers' blood showed an increased mortality rate (Nasr et al. 1996; Chaccour et al. 2010).

IVM has been incorporated in various disease control programs supported financially by local governments and international organizations. In Africa, for example, it is part of Mass drug administration programs applied for the control of soil-transmitted helminths and neglected tropical diseases such as lymphatic filariasis and onchocerciasis (Hooper et al. 2013). In addition, recent trends considering the application of IVM in malaria control programs could offer promising results especially in areas of co-endemicity for multiple parasitic diseases, which offers also an economic advantage (Alout and Foy 2017).

Conclusion

The spectrum of the mechanisms of action of IVM on vector physiology still harbors many unexplored aspects that deserve to be investigated, especially that the control of parasitic diseases continues to face increasing challenges such as global warming, expansion of areas of war zones, immigration, and increase in poverty and famines. These factors along with decreased government funding in the developing countries for innovating drug development urge scientists to seek

alternative low cost control and treatment programs that offer combined approaches against diseases and their vectors. In this study, the exploration of the effect of IVM on the proteolytic activity and lipid peroxidation in mosquito homogenates in response to feeding, in addition to the expression of Bax, caspase-3, p53, and STAT in mosquito tissues, was done to shed light on the impact of IVM on certain aspects of *Culex pipiens* physiology. The focus on the detailed effect of this drug certainly opens the gate to broadening the spectrum of IVM and expanding its health and economic benefit, especially that it is relatively more affordable than other antibiotics on the market.

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Compliance with ethical standards

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed and were in accordance with the ethical standards of the faculty of Medicine, Cairo University.

Conflict of interest The authors declare that they have no conflict of interest.

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