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THE EFFECT OF GENISTEIN AGAINST *SCHISTOSOMA MANSONI* IN EXPERIMENTALLY INFECTED MICE

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Abstract

Schistosomiasis is one of the most significant parasites especially in tropics and subtropics. The only recognized drug used for treatment of all species of *Schistosoma* is praziquantel (PZQ). Worries about drug resistance motivated the quest for novel drug sources. Genistein, which is a natural product present in soybeans, was assessed for its antischistosomal effects in experimentally *Schistosoma mansoni (S. mansoni*) infected mice either solely or in association with PZQ. All groups that were infected subjected to portal and mesenteric veins perfusion, evaluation of worm burden, liver and intestinal ova count, and the oogram. Moreover, ultrastructural changes in the worm's tegument and interleukin IL-1β (IL-1β) levels in infected mice were also assessed. There were significant reductions in the total worm number and egg load of liver and intestine with significant decrease in immature and mature eggs in all groups received treatment in comparison with the infected non treated control one. Tegument of adult worms exposed to genistein exhibited distortion of suckers and erosions with loss of tubercles and spines. There was decrease in the IL-1β level in all treated groups. The best results, in all parameters, were given by combined (genistein & PZQ) treatment. Genistein possessed moderate activity against *S. mansoni* adults and considered as a good adjuvant to PZQ.

Key words: *Schistosoma mansoni, Genistein*, praziquantel, *in vivo*, tegumental alteration, IL-1β

Introduction

Regardless of continuous control measures, schistosomiasis was still a big health issue in several tropical and subtropical countries (Chitsulo *et al,* 2000). The importance comes from its wide endemic area, number of people infected and tsignificant morbidity (Van Nassauw *et al,* 2008). In Sub-Saharan Africa, where the disease is prevalent, the principle species causing human infections are *Schistosoma mansoni* and *S. haematobium* (Deol *et al,* 2019). *S. mansoni* is the most prevalent species endemic in 55 countries involving Egypt (Barakat, 2013).

The main blamed factor for schistosomiasis pathology is *Schistosoma* ova that are detained in the tissues of the host notably in the hepatic and intestinal tissues. The stuck ova generate a persistent antigenic stimulation, accompanied by the mobilization of cells of inflammation and immunity to the infection sites that led to development of granulomas and consequently chronic fibrosis (Chuah *et al,* 2014).

Treatment and control of schistosomiasis depend on praziquantel and thus, drug resistance was a fast-approaching risk (Caffrey, 2007; Doenhoff *et al,* 2008). Besides, PZQ has incomplete efficacy profile, as it loses action toward developing larval stages of the parasite (Utzinger *et al,* 2003), so finding a novel medication to replace or potentiate PZQ is presently a pressing need.

Genistein is an isoflavone which is a biologically active substance found in soy products. It is reported to have a several molecular effects, such as suppression of inflammation, apoptosis promotion, and steroidal hormone receptors modulation (Mukund *et al,* 2017). Salahshoor *et al.* (2018) reported that administrating of genistein ameliorated morphine induced liver damage in mice. Furthermore, genistein has been shown to safeguard against hepatic inflammation and fibrosis induced by diet lacking the methionine-choline in mice (Yoo *et al,* 2015).

This study aimed to evaluate the effect of genistein and in combination with PZQ on *S.*

mansoni in experimentally infected mice by measuring different parameters as worm burden, tissue egg load, oogram and assessment of changes in the morphology of adults' tegument by scanning electron microscopy and serum interleukin 1β levels.

Materials and Methods

Experimental animals and infection: The experimental research work was performed on 100 Pathogen-free CD-1 laboratory bred Swiss Albino male mice, obtained from Schistosome Biological Supply Center (SBSC) Theodor Bilharz Research Institute (TBRI), Giza, Egypt, aged 6-8 weeks and weighed 20±2g. Infection was done with 70±5 freshly shed cercariae for each mouse via tail immersion method **(**Liang *et al,* 1987**)**. Mice were kept in monitored conditions of temperature and humidity (25±2°C, 70%), with free access to standardized food and water.

Praziquantel: Discocide® 600mg (EIPICO, Egypt) was introduced orally to mice at a dosage of 500mg/kg/day for two consecutive days, started on six weeks post infection as an aqueous suspension in 2% Cremophor EL (Sigma-Aldrich, St. Louis**,** USA).

Genistein: Genistein powder was purchased (Abcam, ab120112, Chemical Cambridge, UK, Catalog No: G6649), dissolved immediately before use in 10% dimethylsulfoxide (DMSO) and 90% distilled water. The drug was administrated orally at a dosage of 100mg/kg bodywt in 4 divided doses per mouse for duration of two weeks, started at 4 weeks post infection (Nassef *et al,* 2014).

Experimental design: One hundred mice were divided as follows: forty non-infected mice were allocated to negative control group (GI) and sixty *S. mansoni*-infected mice were randomly distributed to four groups of fifteen mice each (n= 15), GII: infected mice received no treatment (infected control or positive control), GIII: received genistein alone, GIV: received praziquantel alone, and GV: received PZQ and genistein. The final doses of the drugs were given to mice and two weeks later.

Serum sampling: By ether anesthesia, blood samples were collected from mouse by a cardiac puncture. Sera were separated and stored at -80°C until used. Mice were sacrificed by cervical dislocation and dissected.

Schistosoma mansoni worm burden: Portal and mesenteric veins perfusion was performed with sterile physiological saline (0.9% NaCl, w/v), male, female and paired recovered worms were counted under a stereomicroscope and worm count was determined for each group, compared to untreated group and reductions of worm count were estimated (Smithers and Terry, 1965).

Tissue egg load: ova count/gm tissue in liver and ileum: Samples of perfused liver and ileum were taken from each mouse, weighed and digested in 5% KOH at 37°C for 16hr. Eggs were checked at an amplification of ×40 and mean number of hepatic and intestinal eggs/g of tissue was determined (Herbert *et al,* 2010).

Egg developmental stages (Oogram): Briefly,three parts of each animal's distal portion of ileum were washed in 0.9% saline solution and dried on filter paper. Each piece was squeezed between two slides and studied under a light microscope (Mati and Melo, 2013). Eggs were counted and sorted by developmental stage and morphological criteria as immature (Pellegrino *et al,* 1962).

Histopathological study: Liver samples of different groups were fixed in formalin 10%, paraffinized and stained with H & E (Harris, 1900). **N**umbers of granulomas in five serial low power fields was recorded, and examined by light microscope with an ocular micrometer with a digital camera (Olympus BX-41, and E420DC7, 4V, respectively Olympus Corporation, Tokyo, Japan). For each granuloma, mean measurements of 2 perpendicular diameters were recorded. Forty to fifty granulomas were measured for each mouse and reduction in granuloma diameter, compared with *S. mansoni* infected control was estimated (Mahmoud and Warren, 1974).

SEM: Adult males recovered from the treated groups and control positive group, were put in 4% glutaraldehyde for fixation, rinsed

in 0.1M sodium cacodylate buffer (pH 7.2) and exposed to 1% osmium tetroxide for an hr. Then, samples were exposed to ascending series of ethanol for dehydration, dried, put on metal plates, and gold coated. Samples were examined and captured using an electron scanning microscope (JEOL JSM 5200, Tokyo, Japan) at the Faculty of Medicine, Tanta University, Egypt.

Measurement of interleukin 1β concentration in serum samples: Sera from all groups were tested by ELISA (EIAab®, China, Catalog No: E0563m) to detect mouse Interleukin-1 beta (IL-1β) concentrations in serum according to manufacture instructions.

Statistical analysis: Data were analyzed using SPSS version 23 statistical package (Armonk, NY: IBM Corp, 2015). Descriptive statistics included number and percentage of non-numerical data. To detect significant difference among groups, One-way ANOVA test (F test) was used. Data without normally distributed, Kruskal-Wallis test (KW) was used, and Post hoc test to show any significant difference between groups. Z test compared two proportions. Data were considered significant if P value was equal to or less than 0.05. Reduction percentage in worm numbers, egg counts, granuloma parameters and levels of IL1β post treatment was calculated (Tendler *et al,* 1986) as follows, % reduction = (mean value of infected controls−mean value of treated mice /mean value of infected controls × 100.

Ethical considerations: This study was approval by the Institutional Ethical Committee of Faculty of Medicine, Menoufia University, following the Internationally Valid Animal Ethics Guidelines. Experimental procedures were done after endorsement by the TBRI Institutional Ethical Committee.

Results

In all treated groups, there were significant reductions in total worm burden and significant reductions in liver and intestinal egg load with a significant decrease in immature and mature eggs and increases in the numbers of dead eggs as compared with infected control group.

Genistein alone, showed a significant total worm reduction rate (33.58%) with a significant decline in egg count in liver (65.20%) and intestine (84.27%) coexisted with a significant decrease in immature and mature eggs (62.14% & 55.80% respectively) and a significant increase in numbers of dead ova as compared to untreated infected one.

PZQ resulted in much higher significant percentages with significant difference with genistein treated group in all parameters except intestinal egg count was without significant difference. The highest liver and intestinal egg load reduction rate and highest reduction in immature and mature eggs were recorded with combined genistein and PZQ treated group (95.49%, 96.53%, 97.74%, 100.0% & 93.4% respectively) with significa-nt difference between results of genistein and PZQ treated ones (Tab. 1).

Reduced tissue egg load by different treated groups was reflected histopathologically on the hepatic granulomas number. Highest percentage of reduction was recorded with combined treatment group (68.04%) followed by PZQ treated group (56.49%), and then genistein treated group (22.90%) as compared with control infected non treated one with significant difference. Not only granulomas numbers were reduced but also the diameters as same sequence in percentage reductions in different treated ones (Tab. 2).

Histopathological study: Liver sections of *S. mansoni* infected untreated mice revealed a large fibrocellular granuloma, typical for *Schistosoma,* with living ova in center and surrounded by lymphocytes, eosinophils, polymorphonuclear cells, epithelloid cells and fibrous tissues (Fig.1b). In infected group treated by either genistein or PZQ showed reduction in granuloma size as compared to control positive one. Fibrocellular granulomas were less characterized, with declined eggs and surrounded by lymphocytes, eosinophils, neutrophils, epithelloid cells & fibrous tissues (Fig.1c & d). In combined genistein & PZQ treated mice, liver section sho-

wed marked reduction in granuloma size with nearly restoration of liver architecture, scattered inflammatory cells with normal central vein and hepatocytes (Fig.1e).

In genistein treated group and PZQ treated group, SEM showed distorted suckers with tegumental maceration and erosion of some tubercles lacking spines (Fig. 2c, d & e). In combined genistein and PZQ treated group changes were more severe forming marked swelling of adults and most suckers internum were closed, marked edema and narrowed by gynecophoric canal, loss of normal tegument architecture with complete loss of all tubercles spines (Fig 2f, g & h).

Interleukin 1β serum levels: *S. mansoni* caused a significant increase in IL-1β cytokine levels as compared with control negative one. All treated groups gave significant reduction in IL-1β levels 31.58%, 37.85% & 42.11% respectively. Mean IL-1β levels in different treated groups returned to near normal levels without significant difference as compared with normal non-infected one (Fig. 3).

Table 1: Effect of Genistein, PZQ & both treatment on total worm burden and egg load/g tissue in infected mice.

Groups	Total worm burden	Egg load/g liver	Egg load/g intestine
GII (n=15)	13.40±2.41	27862.20±3501.31	24234.60±2121.91
GIII (n=15)	8.90±3.11*$ (33.58)	9695.40±2088.45*$ (65.20)	3811.40±1894.21*(84.27)
GIV (n=15)	2.90±1.27*# (79.64)	6492.30±3061.47*# (79.22)	4807.40±2300.59* (81.53)
GV (n=15)	0.80±0.05*#$ (95.49)	965.90±366.96*#$ (96.53)	547.20±298.88*#$ (97.74)

*Significance (P < 0.05) vs GII, #Significance vs GIII & $ significance vs GIV by Kruskall-Wallis or one-way ANOVA followed by a post-hoc test.

Table 2: Effect of Genistein, PZQ & combined treatment on oogram pattern in *S. mansoni* infected mice.

Groups	Oogram pattern		
	Immature	Mature	Dead
GII (n=15)	53.30±5.42	40.90±3.03	6.40±2.01
GIII (n=15)	21.10±3.57*$ (62.14)	18.10±3.07* (55.80)	22.30±3.20*
GIV (n=15)	10.80±2.39*# (82.20)	10.30±1.64*# (74.90)	91.90±3.57*#
GV (n=15)	0*#$ (100.00)	2.70±2.26*#$ (93.40)	96.60±2.84*#$

Table 3: Effect of Genistein, PZQ & combined treatment on numbers and diameters of hepatic granulomas in infected mice.

Groups	Granuloma number	Granuloma diameter
(GII) (n = 15)	13.10±1.20	365.02±12.61
(GIII) (n = 15)	10.10±1.52*$ (22.90)	321.10±6.22 *$ (12.03)
(GIV) (n=15)	5.70±1.57*# (56.49)	210.59±5.57*# (43.42)
(GV) (n=15)	4.20±0.15*# (68.04)	201.28±12.69*# (45.49)

Discussion

Due to their broad range of protective effects including antioxidant, antimutagenic, anticarcinogenic, antiproliferative properties, interest in potential health benefits of isoflavonoids was grown (Miadoková, 2009). Genistein, a soy-derived isoflavone was considered a central intermediate important in the development of more complex isoflavonoids (Dixon and Ferreira, 2002). Genistein is characterized by its natural source with low toxicity and high safety (Spagnuolo *et al,* 2015). All these properties inspired us to test its effects on *S. mansoni* in experimentally infected mice. Significant reductions in total worm burden, liver and intestinal egg load and immature & mature eggs resulted with genistein alone given to *S. mansoni* infected mice. PZQ was reference drug for *Schistosoma*, more effective than genistein alone, with highest reduction percentage ensued from combined treatment of both.

Effect of genistein on total worm burden and egg load was due to its direct effect on the adult worms and its effect on different worm systems including their reproductive system. Toner *et al.* (2008) studied the effect of genistein on the liver fluke, *Fasciola hepatica* and founded that the drug caused disruption of the flukes. Also, the drug affected the gut, reproductive organs and neuromuscular system. The effect of genistein on the vitelline follicles influenced the egg production by adult *Fasciola*.

In vitro exposure of *Fasciolopsis buski* to genistein increased the activity of nitric ox-

ide (NO) synthase, leading to overproduction of NO which is believed to cause oxidative stress, DNA damage, and disturbance of energy metabolism, calcium homeostasis, and mitochondrial function, all of these consequences may influence the embryogenesis (kar *et al,* 2002).

Reduced tissue egg load in different treated groups was reflected histopathologically on number of hepatic granulomas. Highest percentage of reduction was recorded in combined treatment group (68.04%) followed by PZQ treated one (56.49%) then genistein treated group (22.90%) with significant difference as compared with control infected non treated group, and granulomas diameters were reduced in different treated groups.

In the same context, Sobhy *et al.* (2018) reported that using genstein significantly reduced the mean hepatic granulomas diameter and numbers compared to the control in both acute and chronic stages, also when genistein used with PZQ, both exhibited a substantial decrease in dimensions and numbers of hepatic granulomas in *S mansoni* infected mice.

The cell mediated immune response of the host to the soluble *S. mansoni* egg antigen induces granuloma formation that progress to irreversible fibrosis and hence to serious portal hypertension (Elbaz and Esmat, 2013). First, a moderate type 1 helper (Th1) response to the egg antigen is produced that usually develops into powerful Th2 immune response with enrollment of eosinophils, monocytes, and lymphocytes which is the bases of granuloma formation and fibrogenesis of the liver (Wynn *et al*, 2004; Wilson *et al*, 2007). Decline in granuloma numbers and granuloma sizes after treatment were explained by reduced ova counts (Davydova *et al,* 2016).

Wan *et al.* (2017) disclosed that treatment with genistein significantly decreased the level of hepatic granuloma and fibrosis in *Schistosoma japonicum (S. japonicum)* infected mice. In this study genistein was shown to suppress the activation of NF-κB signaling pathways, and thus decrease the expression of MCP-1, TNF-α, & IL-10, a possible factor to secure against egg-induced liver granuloma and fibrosis caused by *S. japonicum* infection. Li *et al.* (2013) showed that genistein reduced fibrogenic genes expression to weaken fibrosis formation. Ganai and Husain (2017) found that genistein had protected activity to liver in chronic hepatic damage and fibrosis caused by D-Galactosamine. Ma *et al.* (2018) found that inhibitors of tyrosine kinase, included genistein as antifibrotic agents against schistosomiasis.

Tegument of *Schistosoma* is an important structure for survival of the parasite, it has a key role in formation and release of different nutrients, nutrients absorption and defending the worm against the immune response of the host and hence an effective target for drug interaction (Xiao *et al,* 2002; Shaohong *et al,* 2006; Zhang and Coultas, 2013). Focal damage occurred in tegument, by an anti-schistosomal drug, exposed *Schistosoma* antigens (epitopes) to the host immune response, disfigured the worm shape by inability of oral and ventral suckers to ingest food causing death (El-Sayad *et al,* 2017).

By SEM both PZQ and genistein induced variable tegumental damage degree, but the most severe affection was with combined usage of both drugs. Evident surface changes, by genistein on helminths were reported by Roy and Tandon (1996), Pal and Tandon (1998a, b) and Toner *et al.* (2008) on *Fasciolopsis buski*, *Raillietina echinobothrida* and *Fasciola hepatica* respectively. Toner *et al.* (2008) found that genistein induced changes in calcium concentration with rapid muscule contraction followed by tegument and suckers deformities. Also, Pal and Tandon (1998a) and Kar and Tandon (2004) clarified these as parasites exposed to genistein had a deficiency in tegumental enzyme activity (acid phosphatase, alkaline phosphatase and adenosine triphosphatase) that led to paralysis and deformities in the tegument with death of treated parasites. Changes in worm tegument blocked the female ability to

interact with male and decreased the efficacy of male in holding female (Haseeb *et al*, 2008) that reflected on infectivity and pathogenicity (Soliman, 2012).

IL-1β is mainly engaged as a crucial factor in human Th17 cell development, either individually or in cooperating with IL-23, IL-6, and TGF- β (Acosta-Rodriguez *et al,* 2007). IL-17 participated in development of *Schistosoma* egg induced granuloma (Shainheit *et al,* 2011). So, IL-1β has a role in the granuloma development and critical established chronic infections (Zaiss *et al,* 2013). Also, lower IL1β expression reduced granulomatous inflammation (Zhang *et al,* 2012).

The present results showed that *S. mansoni* caused a significant increase in the levels of IL-1β cytokine as compared with control negative group. Using different treatment drugs, genistein, PZQ and combined genistein and PZQ caused significant reduction in IL-1β levels by 31.58%, 37.85% & 42.11% respectively. Mean IL-1β levels in different treated groups had returned to near normal levels with no significant difference when compared with normal non infected group. Wan *et al*. (2017) and Sobhy *et al*. (2018) reported that genistein caused reduction in serum level of pro-inflammatory cytokines including IL1β. Moreover, El-Lakkany *et al*. (2011) and Metwally *et al*. (2018) found that PZQ caused reduction of serum levels of IL-1β, which agreed with the present study.

IL-1 is a polypeptide with two forms known as IL-1α & IL-1β implicated in the acute stage of inflammation (Paulus, 2000; Di Iorio *et al*, 2003). IL-1β is first formed as precursor that needed to be processed into its activated form by a protein complex, present in cytoplasm, known as an inflammasome (Kim *et al,* 2013).

Genistein inhibited the expression of messenger RNA (mRNA) responsible for synthesis of cytokines of inflammation (MCP1, TNFα, IL1β, IL4 & IL10) mediated by NFκ B signaling pathways (Wan *et al,* 2017), but PZQ inhibited the formation of NLRP3 inflammasome in macrophages responsible for IL-1β maturation and reduced macrophages number in spleen responsible for secretion of IL-1β (Kong *et al,* 2017). Highest reduction of IL-1β level resulted by combined genistein and PZQ by synergistic inhibitory effects of both drugs on IL-1β levels.

Conclusion

Combination of genistein and PZQ significantly reduced total worm burden, liver and intestinal egg load, immature and mature eggs and pathological changes in liver and hepatic granuloma in *S. mansoni* infected mice. Also, using both drugs induces severe tegumental changes in worms and significantly affected the level of IL-1β cytokine in improving the pathogenesis. Consequently, genistein as an adjuvant to PZQ gave better antischistosomal effect.

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Explanation of figures

Fig 1 a- Liver tissue of control normal mice (GI) showed normal hepatic architecture, central vein (Cv) at center of lobule surrounded by hepatocytes (Hc) with strongly eosinophilic granulated cytoplasm. (H & E, X100). b- Liver tissue of positive control mice (GII) showed a large irregular fibrocellular granuloma (G) chronic inflammatory cells (lymphocytes, eosinophils and epithelloid cells) surrounding *S. mansoni* ovum (yellow arrow) (H & E, X100). c- Liver tissue of genistein treated mice (GIII) showed moderate sized granuloma surrounding *S. mansoni* ovum (yellow arrow) (H & E stain X 100). d- Liver tissue of PZQ treated mice (GIV) showed moderate sized to small granuloma surrounding *S. mansoni* ovum (yellow arrow) (H & E, X100). e- Liver tissue of combined genistein & PZQ treated mice (GIV) showed restoration of liver lobules, central vein (Cv) at center of lobule surrounded by hepatocytes (Hc) and lymphocytic infiltration (H & E, X200).

Fig 2: a- SEM of adult *S. mansoni* male (control positive) showed intact oral sucker (OS), ventral sucker (VS) and gynecophoric canal (GC), dorsal surface with well-developed tubercles (X250). b- SEM of adult *S. mansoni* male tegument (control positive) (dorso-lateral region) showed tegumental wrinkles (yellow arrow) and regularly arranged coarse tubercles (T) with spines (S) (X1000). C- SEM of adult *S. mansoni* male from genistein treated (GIII) showed distortion of ventral sucker (green arrow) (X250). d- SEM of adult *S. mansoni* male from genistein treated group showed tegumental maceration (yellow arrow), with erosion of some tubercles (green arrow) and disappearance of some spines (blue arrow) (X1000). e- SEM of adult *S. mansoni* male exposed to PZQ (GIV) showed maceration of some areas of tegument (yellow arrow) with loss of some spines (blue arrow) (X 1000). f- SEM of adult *S. mansoni* male treated with combined genistein and PZQ (GV) showed marked swelling of adult worm (X 100). g- SEM of adult *S. mansoni* male worm treated with combined genistein and PZQ (G V) showed closed interim of oral suckers (blue arrow) (X450) and marked edema and narrowing of gynecophoric canal (yellow arrow) (x450). h- SEM of adult *S. mansoni* male treated with combined genistein and PZQ (GV) showed loss of normal tegumental architecture with complete loss of all tubercles (blue arrow) and complete loss of spines (yellow arrow) (X450).