Original Article

Immunomodulating effect of *Schistosoma mansoni* soluble egg antigen on course of induced diabetes mellitus in experimental mice

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ABSTRACT

Background: Helminth infections, particularly *S. mansoni*, are known to induce a protective role against various forms of autoimmune diseases, including type 1 diabetes (TID). The observed *S. mansoni* significant inhibition or delay of diabetes development in non-obese diabetic mice (NOD), appeared to be due to a modulation of the diabetes-associated Th1 response towards protective Th2 responses through IL-10 production.
Objective: To study the effect of *S. mansoni* SEA on the immune response in induced TID mouse model.
Material and Methods: In this study, 90 male Swiss Albino mice of 6 weeks old, weighing between 90 and 100 g were divided into 5 groups; control group (I); Streptozotocin (STZ)-treated group (II); soluble egg antigen (SEA) -immunized group (III); (STZ+SEA) group (IV); (SEA+STZ) group(V). Mice were subjected to measurement of blood glucose levels at two and four weeks by colorimetric method, and measurement of IL-10 by enzyme linked immunosorbent assay (ELISA). Histopathological examinations of pancreatic sections of the five groups investigated signs suggesting presence or absence of pancreatic inflammation.
Results: Significant lowering of blood glucose level occurred at 2- weeks in groups III and V compared to group II, and at 4- weeks in groups III, IV and V compared to group II, and in group V compared to group IV. Significant higher IL-10 level occurred at 2- weeks in groups IV and V compared to group II, and in groups IV and V compared to group III and in group V compared to group IV. In 4- weeks, significant increase in IL-10 level occurred in groups II, IV, V compared to group I, and in group V compared to group IV. No significant difference between groups III and I was recorded. Histopathological changes of pancreatic sections of groups I and III showed normal architecture of pancreatic cells; while groups II and IV coinciding with STZ treatment showed vacuolation and necrosis of islets of Langerhans at 2- weeks. At 4- weeks the inflammation subsided in group IV. In group V, there was dilatation of blood vessels with inflammatory cells at both weeks.
Conclusion: *S. mansoni* derived SEA proved to be protective against T1D leading to improvement of blood sugar control and indicating the protective role of *S. mansoni* infection.

Keywords: Interleukin-10, NOD mice, soluble egg antigen, streptozotocin, type 1 diabetes.

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INTRODUCTION

Helminth infections are common parasites that infect about 1.5 billion people worldwide, especially in developing countries, causing chronic infection leading to malnutrition, anemia, impaired growth, and mortality[1]. These infections have been shown to affect the outcome of other diseases including autoimmune diseases[2]. Helminth parasites are strong inducers of Th2 associated responses such as eosinophilia, IL-4, IL-5, IL-13, and IgE production[2-4]. *S. mansoni* is well-known to induce a clear protective role against various forms of auto-immunity, allergic airway inflammation, colitis[5-9], and to reduce the severity of experimental autoimmune encephalomyelitis[10] as well as diabetes in NOD mice[1,2,11,12].

T1D is a Th1-mediated autoimmune disease[2,13] with life-threatening complications and considerable cost to health care systems[14]. It affects approximately 1 out of 300-400 children in western countries[15]. Over the past 3 decades, the rate of T1D has increased by approximately 4% per year in both Europe and USA[16], while still relatively uncommon in the developing world[17]. T1D incidence in Egypt was reported in 16-17% of population from 2012 to 2016[18,19]. The medical and financial expenses due to diabetes are massive, because it presents as an irreparable illness, complicated by renal failure, heart and brain ischemic complications, amputations, and blindness. Blood glucose dysregulation manifests when about 70% of pancreatic islet cells are lost[20].

In T1D lymphocytes, natural killer cells and other plasma cells, together with poor immune regulation, are involved in apoptosis and necrosis of the pancreatic β cells[21-22]. During its pathogenesis, lymphocyte infiltration into the islets is not only an initial characteristic of pathology, but also a fundamental step in disease development[3]. Type 2 diabetes (T2D) is not

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an autoimmune disease, but immunological features are implicated in the disease pathogenesis with a low-grade chronic inflammation and infiltration of adipose tissue by activated macrophages that produce cytokines enhancing insulin resistance[1]. Helminth infections can modify host's immune reactions to live and multiply and complete their life cycles. It is noticed that helminth infections are associated with protection against many autoimmune diseases[9,10]. Helminths induction of IL-10 has a protective effect on T1D and is involved in T2D-related inflammation[8,23,24]. Mice engineered to over-express IL-10 (by gene transfer) recover insulin sensitivity and are moderately protected from high fat diet-induced obesity and glucose intolerance and reduce lymphoid infiltration in pancreatic islets that prevent their destruction[21-24]. The hygiene hypothesis was first described by Strachan[5]; it assumes that the higher incidence of autoimmune diseases in the industrialized countries is the consequence of low contact to infectious agents[25,26].

NOD mice have been amply used for verification of the interaction between autoimmune diseases and infections, providing a good animal model for T1D[27]. As in humans, the development of diabetes in NOD mice has been linked to the loss of self-tolerance to β-cell autoantigens[28]. It has been shown that *S. mansoni* can significantly inhibit or delay the development of diabetes in NOD mice[29]. This appeared to be due to an alteration of the diabetes-associated Th1 response towards protective Th2 responses including IL-4, IL-5, IL-10, and IL-13 production[2,28,29]. The aim of the present study is to show whether *S. mansoni* SEA could alter the immune responses in a diabetic mouse model.

MATERIAL AND METHODS

This case control experimental study was conducted from February to June 2018. Measurement of all parameters was conducted in Biochemistry and Biotechnology department, Faculty of Medicine, Cairo University.

Experimental animals: Enrolled in the study were 90 male Swiss Albino mice of 6 weeks old, weighing between 90 and 100 g. Mice were bred and maintained in an air-conditioned animal house with specific pathogen-free conditions and were subjected to a 12:12-h daylight/darkness, and allowed unlimited access to chow and water. The morphological and behavioral changes of mice were monitored every day.

Material: SEA was obtained from Tudor Bilharz Institute, Imbaba, Giza. STZ (Serva, Heidelberg, Germany) was imported and delivered through Beta Lab Company, Cairo.

Study design: According to Hayashi *et al.,*[30], the smallest single dose of STZ required to induce diabetes in BALB/c mice is 150 mg/kg. Mice were injected intraperitoneally. A pharmaceutical-grade formulation of STZ was used to avoid impurities that may have harmful biologic activities[30]. Animals were divided into five groups as described in the following table. Each group was subdivided into 2 subgroups (n=9) sacrificed at 2 and 4 weeks sequentially.

I	**Control**	Mice injected only with citrate buffer pH 4.5 (1.0 ml/100 g)
II	**STZ**	Mice injected with 1.0 ml/100 g STZ dissolved in citrate buffer pH 4.5 (150 mg/kg).
III	**SEA**	Mice injected once with 1.0 ml/100 g SEA dissolved in phosphate buffered saline (PBS).
IV	**Curative**	STZ injected and followed by three SEA doses at 1, 3 and 5 days sequentially after elevating blood glucose (induction of diabetes).
V	**Protective**	Mice received 2 doses of SEA, followed sequentially after 1 and 2 weeks by STZ.

Biochemical assays: On experimental day 5, all mice were fasted for 6 hours, followed by measurement of blood glucose *via* retro-orbital vein blood sample to ensure development of hyperglycemia in STZ treated mice. Estimations of blood glucose level (enzymatic colorimetric method, Biodiagnostic, www.bio-diagnostic.com. Cat. No. GL 13 20) and IL-10 level (mouse ELISA BioLegend, Inc. www.biolegend.com. Cat. No. 431411) were done in all studied groups according to experimental time schedule. IL-10, commonly used for evaluation of immune response, was commercially available and therefore was chosen as a measure for assessment in all study groups.

Histopathology: Pancreatic samples were collected and fixed overnight in 40 g/L paraformaldehyde in PBS at 4°C. They were embedded in paraffin and serial 5-µm sections of the pancreas were stained with H&E[1].

Statistical analysis: Data were coded and entered using the statistical package SPSS version 22. Data were analyzed using mean and standard deviation. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test when comparing more than 2 groups. Correlations between quantitative variables were done using Pearson correlation coefficient (r)[31], where r < 0.3: no correlation, r = 0.3 - < 0.5: weak correlation, r = 0.5: fair correlation, r = > 0.5 – 0.75: good correlation, r > 0.75: very good correlation. Significant levels were considered when $P \leq 0.05$.

Ethical consideration: Animals were handled and cared for in accordance with the guide of Laboratory Animals published by the US National Institute of Health (NIH publications No. 8023, revised 1996) and approved by the Ethics Committee for Animal Experimentation at Faculty of Medicine, Cairo University.

RESULTS

Blood glucose levels in the five experimental groups after 2- and 4- weeks duration are compared in table (1) and figures (1 and 2). At 2- weeks there was significant higher levels in groups II, IV and V compared to group I (control group) (*P*=0.000) but no significant difference between group III and group I (*P*=0.99); significant lower levels in groups III and V compared to group II (*P*=0.000 and 0.002, respectively), but no significant difference between group IV and group II (*P*=0.96); significant higher levels in groups IV and V compared to group III (*P*=0.000); no significant difference between group IV and group V (*P*=0.07). At 4- weeks there was significant higher levels in groups II, IV and V compared to group I (*P*=0.000) but no significant difference between group III and group I (*P*=1.0); significant lower levels in groups III, IV and V compared to group II (*P*=0.000); significant higher levels in groups IV and V compared to group III (*P*=0.000); significant lower levels in group V compared to group IV (*P*=0.001).

Comparison of blood glucose levels in each of the five experimental groups at 2- and 4- weeks showed no significant difference between 2- and 4-weeks duration

Table 1. Blood glucose levels at 2- and 4- weeks duration in the five experimental mice groups.

	Blood glucose	95% confidence	Statistical analysis (*P* values)	
	Mean ± SD	Lower-Upper bounds	Between groups	Between time
2 weeks				
I	80.6 ± 5.14	76.7 - 84.6	**Vs group I**: II, IV, V: 0.000* (Increase), III: 0.99 (NS)	**I:** 1.00 (NS)
II	212.6 ± 16.0	200.35 - 224.9		**II:** 0.00* (Increase)
III	86.6 ± 8.8	79.8 - 93.4	**Vs group II:** III: 0.000* (Decrease), IV: 0.96 (NS), V: 0.002* (Decrease)	**III:** 0.9 (NS)
IV	202.4 ± 17.0	189.0 - 215.6		**IV:** 0.01* (Decrease)
V	176 ± 13.6	165.5 - 186.6	**Vs group III:** IV, V: 0.000* (Increase)	**V:** 0.3 (NS)
4 weeks				
I	80.0 ± 6.5	75.0 - 84.9	**Vs group IV:** V: 0.07 (NS)	
II	315.0 ± 41.8	282.9 - 347.0		
III	78.0 ± 5.0	5.0 - 74.0		
IV	182.0 ± 19.8	19.8 - 166.8		
V	145.0 ± 12.0	12.0 - 135.7		

* Significant, NS: Non significant

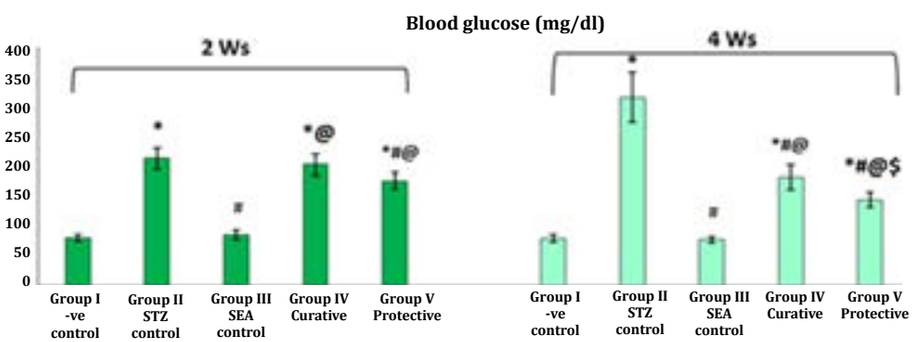


Fig 1. Blood glucose levels at 2- and 4- weeks duration in the five experimental mice groups. Data were expressed as Mean±SD, *P* value <0.05 was significant.
(*): Significant difference versus group I.
(#): Significant difference versus group II.
(@): Significant difference versus group III.
($): Significant difference versus group IV.

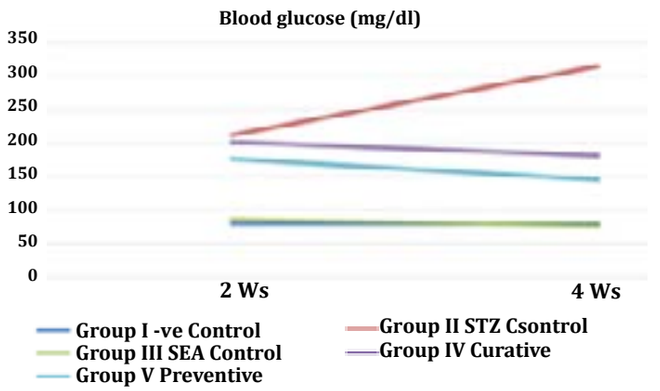


Fig. 2. Comparison of blood glucose levels at 2- and 4- weeks in the five experimental groups.

in groups I, III and IV (*P*=1, 0.9, and 0.3, respectively). While there was significant increased level at 4 weeks compared to 2 weeks (*P*=0.000) in group II, there was significant decreased in blood glucose level in 4 weeks compared to 2 weeks (*P*=0.01) in group V.

IL-10 levels at 2- and 4- weeks in the five experimental mice groups are compared in table (2) and figures (3 and 4). At 2 weeks, there was no significant difference in IL-10 levels between groups II and III compared to group I (*P*=0.2, and 1.0, respectively) while there was significant increase in IL-10 levels in groups IV and V compared to group I (*P*=0.000). In groups IV and V compared to group II there was a significant higher level (*P*=0.000), but no significant difference between group III and group II (*P*=0.5). There were significant higher levels in groups IV and V (*P*=0.000) compared to group III; as well as in group V compared to group IV (*P*=0.000). At 4 weeks there was significant increase in IL-10 levels in groups II, IV and V compared to group I (*P*=0.000) but no significant difference between group III and group I (*P*=0.9). Versus group II, there was a significant higher level in group III and V compared to group II (*P*=0.000); but no significant difference between group IV and group II (*P*=1.0). There were significant higher levels in groups IV and V compared to group III (*P*=0.000) as well as in group V compared to group IV (*P*=0.000). Comparison of IL-10 levels in each group at 2- and 4- weeks in the five experimental groups showed significant increased levels in groups 1V and V (*P*=0.000). Group III showed no significant difference except after 4 weeks (*P*=0.000) compared to group II.

Correlation between blood glucose and IL-10 levels: This correlation appeared to be fair (*P*=0.00, *r*=0.5), indicating the evident relation between higher levels of IL10 and lower levels of blood glucose. The significantly high blood glucose levels that were initially observed in all STZ injected groups were significantly lower in SEA treated groups after 4 weeks duration; at the same time this was associated with significantly higher levels of IL-10 in these groups. This was most evident in the protective group in which SEA treatment preceded STZ injection with significantly higher level of IL10 more than in the curative group in which STZ injections preceded SEA treatment (Figure 5).

Histopathological changes of pancreatic samples in the five groups of experimental mice are shown in figures (6 – 15). At 2- and 4- weeks respectively figures (6 and 7) showed pancreas section of group I (control group) presenting normal architecture of endocrine and exocrine glands; figures (8 and 9) showed pancreas of group II (STZ group) with vacuolation and necrosis of islets of Langerhans cells; figures (10 and 11)

Table 2. IL-10 levels at 2- and 4- weeks in the five experimental mice groups.

	IL-10 level	95% confidence	Statistical analysis (*P* values)	
	Mean ± SD	Lower-Upper bounds	Between groups	Between time
2 weeks				
I	28.7 ± 7.2	23.0 - 34.0		
II	72.8 ± 32.5	47.8 - 97.8	**Vs group I**: II=0.2 (NS), III=1.0 (NS), IV,V= 0.000* (Increase)	**I:** 1.00 (NS)
III	37.8 ± 10.0	30.0 - 45.5		
IV	207.0 ± 55.0	164.0 - 249.0	**Vs group II:** III=0.5 (NS), IV,V= 0.000* (Increase)	**II:** 0.000* (Increase)
V	327.0 ± 60.0	280.0 - 374.0		**III:** 1.0 (NS)
4 weeks				
I	26.7 ± 7.7	20.7 - 32.7	**Vs group III:** IV,V= 0.000* (Increase)	**IV:** 0.1 (NS)
II	165.6 ± 52.0	125.5 - 205.6		**V:** 0.1 (NS)
III	44.5 ± 11.0	35.7 – 53.0	**Vs group IV:** V= 0.000* (Increase)	
IV	158.0 ± 21.0	141.0 - 174.0		
V	278.0 ± 28.0	255.0 – 300.0		

* Significant, NS: Non significant

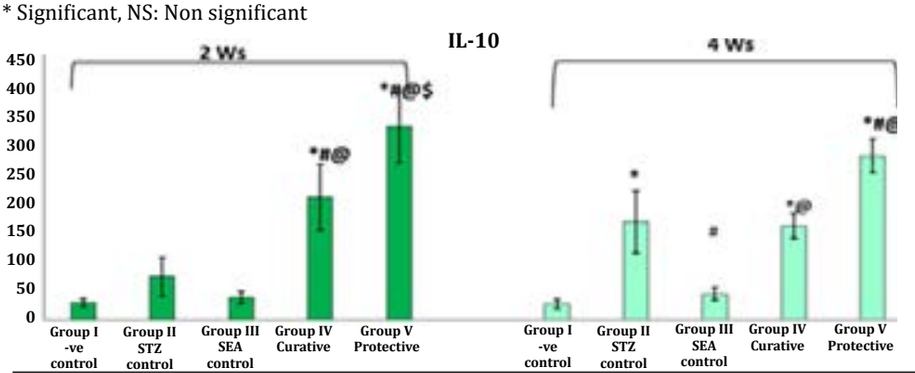


Fig 3. IL-10 levels at 2- and 4-weeks in the five experimental mice groups. Data were expressed as Mean±SD, *P* value <0.05 was significant.
(*): Significant difference versus group I.
(#): Significant difference versus group II.
(@): Significant difference versus group III.
($): Significant difference versus group IV.

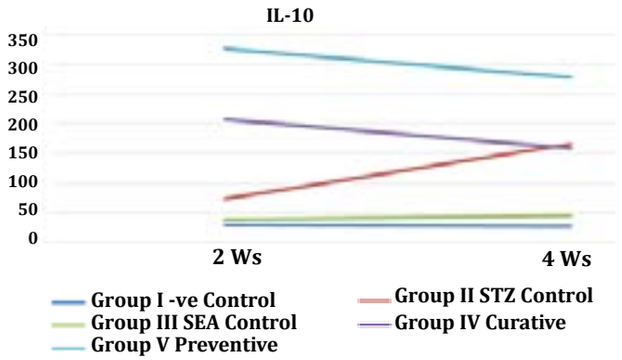


Fig. 4. comparison of blood glucose levels at 2- and 4-weeks in the five experimental groups.

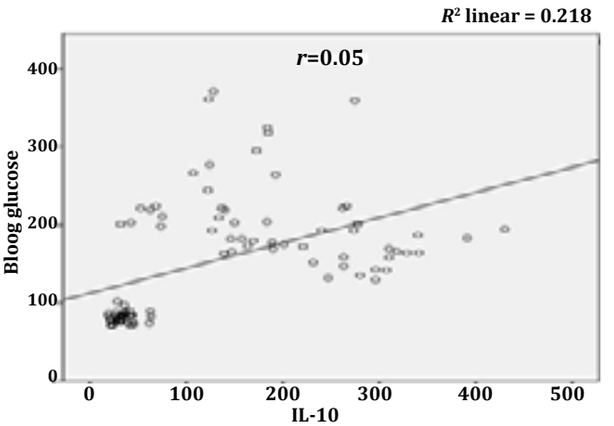
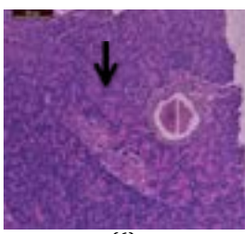


Fig. 5. Correlation between blood glucose and IL-10 levels. Fair correlation (P = 0.000, r = 0.5).

showed pancreas of group III (SEA group) with normal architecture of endocrine and exocrine pancreatic cells. Figures (12 and 13) showed pancreas of curative group IV (STZ+SEA) with vacuolation and necrosis of islets of Langerhans cells at 2 weeks then normal architecture of the islets at 4 weeks. Figures (14 and 15) of the protective group V (SEA+STZ) at 2- and 4-weeks respectively showed dilated blood vessels with inflammatory cells, in addition to normal appearance of islets of Langerhans at 4- weeks.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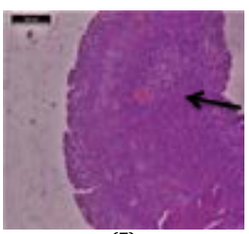
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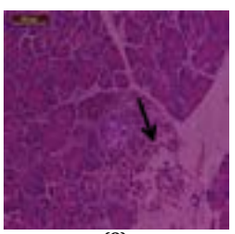
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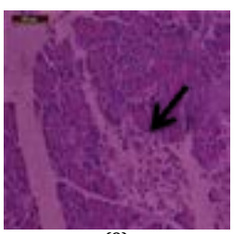
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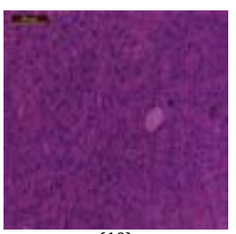
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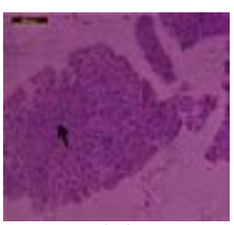
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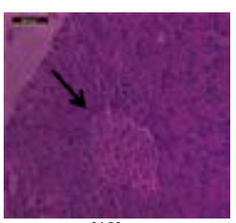
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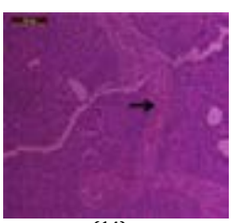
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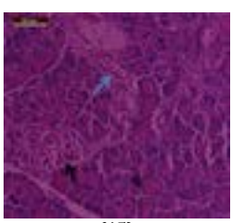
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Histopathological changes

Fig. 6. Pancreas of control mice (2 weeks) shows normal architecture of endocrine and exocrine glands. Black arrow indicated normal β cells of islets of Langerhans in control mice (100x).

Fig. 7. Pancreas of control mice (4 weeks) shows normal architecture of endocrine and exocrine glands. Black arrow indicated normal β cells of islets of Langerhans in control mice (100x).

Fig. 8. Pancreas of STZ mice (2 weeks) group showing vacuolation and necrosis of islets of Langerhans cells. Black arrow indicated destructive β cells in STZ induced mice (200x).

Fig. 9. Pancreas of STZ mice (4 weeks) group showing massive vacuolation and necrosis of islets of Langerhans cells. Black arrow indicated massive destructive β cells in STZ induced mice (200x).

Fig. 10. Pancreas of SEA mice (2 weeks) showing normal architecture of endocrine and exocrine pancreatic cells (200x).

Fig. 11. Pancreas of SEA mice (4 weeks) showing normal architecture of endocrine and exocrine pancreatic cells. Black arrow indicated normal β cells of islets of Langerhans (200x).

Fig. 12. Pancreas of STZ+ SEA mice (2 weeks) group showing vacuolation and necrosis of islets of Langerhans cells. Black arrow indicated destructive β cells in STZ induced mice (200x).

Fig. 13. Pancreas of STZ+ SEA mice (4 weeks) group showing normal architecture of islets of Langerhans cells. Black arrow indicated regenerative β cells in STZ induced mice (200x).

Fig. 14. Pancreas of SEA+STZ preventive mice group (2 weeks) showing at black arrow dilated blood vessels with inflammatory cells. (100x).

Fig. 15. Pancreas of SEA+STZ preventive mice group (4 weeks) showing blue arrow at dilated blood vessels with inflammatory cells. Black arrow indicated normal β cells of islets of Langerhans in control mice (200x).

DISCUSSION

T1D is an autoimmune disease resulting in the destruction of insulin-producing cells by a localized Th1-type inflammatory response[29]. During its pathogenesis, lymphocyte infiltration into the islets is not only an initial characteristic of pathology, but also a fundamental step in disease development[3]. The "hygiene hypothesis" postulates that the increased incidence of autoimmune diseases such as diabetes in the developed world is the result of decreased exposure to infectious agents[1,25,26]. Among the first researches to validate the protecting consequence of helminth infections on diabetes was done employing *S. mansoni* infection in NOD mice by Cooke *et al.*,[29], who prompted us to use in our study SEA as a protective agent. Several studies[2,33,34] found that soluble antigens from different helminths without live infection can also modulate the start and progression of autoimmune diabetes in the NOD mice. Later, the effect of other helminths (*T. spiralis, H. polygyrus,* and *L. sigmodontis*), was checked[1,28,32,] proving the preventive effect of such helminths on diabetes through their immuno-modulating function. Also, a study conducted on NOD mice showed that inoculation with *S. mansoni* markedly reduced the rate of T1D and suppressed lymphoid infiltration in the islets[29]. In addition, larval antigens from *S. mansoni* cercariae showed an ability to prevent diabetes in NOD mice[2]. These results supported that helminth-derived therapies could be submitted in the prophylaxis of glucose-intolerance patients or as tolerance-inducing co-therapies in the framework of β-cell replacement methods[36,37]. These experimental studies offer significant inferences for the use of many of the helminth-derivative involvements that have revealed usefulness in the NOD mice. Several studies acknowledged the ability to evade autoimmune diabetes before insulitis started, but not to modulate or halt pathology at an advanced phase[2,29,33]. Our present study is an attempt to determine the possible ability of SEA to alter the immune responses of NOD mouse models. Mean blood glucose and IL-10 levels were evaluated at 2- and 4- weeks.

Comparing our observations we found that STZ group showed a significant rise in blood glucose level in 2- and 4- weeks duration (P=0.000) indicating the establishment of the diabetic state. Control, SEA and curative groups showed no significant difference in mean blood glucose (P=1.0, 0.9 and 0.3, respectively). The protective group showed a significant lowering in blood glucose level in 4 weeks compared to 2 weeks durations (P=0.01). This means that elevation of blood sugar noticed in STZ group was lower in curative and protective groups after 4 weeks.

Immunological enhancement of IL-10, by SEA was apparent in both curative (STZ+SEA) and protective (SEA+STZ) groups after 2- and 4- weeks, and in group III (SEA), as compared to group II (STZ) in which SEA was absent. When the results were compared, we found that STZ group showed a significant rise in IL-10 level in 4 weeks than in 2 weeks (P=0.000). Comparison of IL-10 levels in each group at 2- and 4- weeks in the five experimental groups showed significant increased levels in groups 1V and V (P=0.000). Group III showed no significant difference except after 4 weeks (P=0.000) compared to group II. In the curative group IV the rise of the cytokine was rapid and sustained, while in protective group it was more rapid more marked and sustained.

Correlation between blood glucose and IL10 proved to be fair (P=0.000, r = 0.5). This displays the evident relationship between higher levels of IL-10 and lower levels of blood glucose. The significantly high blood glucose levels that were initially observed in all STZ injected groups were significantly lower in SEA treated groups in 4 weeks duration, at the same time this was associated with significantly high levels of IL-10 in these groups. This was most evident in protective group were SEA treatment preceded STZ injection with significantly high level of IL-10 more than in the curative group in which STZ injections preceded SEA treatment.

The anti-inflammatory cytokine, IL-10, has a paradoxical effect on T1D. A diabetes inducing role was observed when genetic expression of IL-10 on pancreatic islet cells was used[21,22], while other studies proved its protective effect[23,24]. A previous study[29] showed that schistosomiasis can significantly inhibit or delay the development of diabetes in NOD mice. This appeared to be due to an alteration of the protective Th2 responses including IL-4, IL-5, IL-10, and IL-13 production induced by the diabetes-associated Th1 response[28]. The breakdown of multiple tolerance pathways leads to destruction of the insulin producing β cells[3]. The development of diabetes in the NOD mouse specifies the role of CD4 and CD8 T-cell-mediated β cell destruction[1]. Zaccone *et al.,*[12] studied the effect of SEA of *S. mansoni* on NOD mice, they supported the results of prevention of diabetes through expansion of Th2 and Treg. producing IL-10. In a similar report[35], the mechanism of protection of SEA was shown to be due to its internalization into the acidic cell compartments of B cells inducing a 3-fold increase of IL-10 and reducing the allergic airway inflammation. This confirmed its high therapeutic effect. Using another helminth Liu *et al.,*[1] showed that infection with the intestinal nematode, *Heligmosomoides polygyrus*, at 5 weeks of age protected NOD mice from T1D until 40 weeks of age, while infection at 12 weeks of age reduced the onset of T1D in NOD mice. IL-4, IL-10, and IL-13 expression and the frequency of CD4, CD25, FoxP3 regulatory T cells were elevated in mesenteric and pancreatic lymph nodes.

In our study, while the pancreatic tissue sections confirmed that SEA had no inflammatory effect, the

destructive outcome of STZ causing vacuolation and necrosis of Langerhans cells was evident at 2 weeks coinciding with the elevation of blood glucose levels. At 4 weeks the inflammation subsided in group IV probably due to the protective role of SEA. In a similar study done by Liu *et al.,*[1] there was normal architecture of islets of Langerhans cells with no inflammatory cells in *H. polygyrus* treated mice.

In conclusion, pre-existing schistosomiasis proved protective against diabetes through *S. mansoni* derived SEA. Schistosomaiasis in diabetic individual may lead to better results of blood sugar control. *S. mansoni* and SEA modulates the immune response through improving inflammatory status, expansion of T helper cells with rising IL-10, downregulating pro-inflammatory cytokines and upregulating anti-inflammatory cytokines. *S. mansoni* antigens (in the absence of active infection) result in the migration of cytotoxic cells away from the pancreas thus modulating the destruction of pancreatic islets, reducing the onset and altering the course of diabetes in mice. The study of the mechanisms through which *S. mansoni* and SEA improve metabolic homeostasis might offer new insights into the development of novel therapeutics preventing diabetes and possibly developing schistosome-derived therapies for diabetes mellitus.

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