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Parasitological and Biochemicals studies on The Impact of Chitosan Nanoparticles on the course of Murine, Schistosomasis mansoni

Abdo N.A.1, GhobashyM.A.F.1, Mahmoud S.S.2, Ismail O. A.3, Hassan E.A.1
1-Zoology Department -Faculty of Science-Suez Canal University
2- Parasitology- Theodore Bilharz Research Institute
3- Faculty of Medicine- Suez Canal University
E-mail: noorhan.ahmed82@yahoo.com

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ABSTRACT
Schistosomiasis is a worldwide disease and affects more than 200 million individuals around the world but its treatment depending only on a single orally drug, praziquantel. Since praziquantel was developed in 1970, it has replaced other anti-schistosomal drugs to become only drug of choice for treatment of human schistosomiasis due to, high efficacy, excellent tolerability, few and transient side effects, simple administration and competitive cost, but many factors can affect on its efficacy (internal such as; ph, enzymes or external such as; temperature, humidity).

So the development of a vaccine would be essential for the control of schistosomiasis, which is recognized as the most important human helminths infection in terms of morbidity and mortality. A new approach of oral vaccination with chitosan nanoparticles appears interesting because of their great stability and the ease of target accessibility, besides chitosan immunostimulatory properties.

This work aimed to study the effect of chitosan loaded with praziquantel nanoparticles as a delivery drug system on parasitological parameters such as (worm burden, oogram, in addition to the effect of the drug on biochemical parameters.

The results showed that chitosan loaded with PZQ as nanoparticles have a great effect on worm burden reduction reached 97.12% and worm’s stages reached 99.6% dead ova. Also it improved host biochemical parameters performance. The present study suggests that drug delivery system represented in chitosan has more advantages rather than taking the treatment (PZQ) orally alone.

INTRODUCTION
Schistosomiasis affected about 250 million people worldwide and an estimated 4,400 to 200,000 people die from it each year (Thétiot-Laurent, et al., 2013). The disease is most commonly found in Africa, Asia, and South America but Schistosomiasis is listed as a neglected tropical disease (Lammie et al., 2006; Steinmann et al., 2006 and Hotez et al., 2009). Praziquantel is a drug available for the treatment of schistosomiasis, because of praziquantel lower cost per treatment, and for its safety (Cioli, 2000 and Danso-Appiah et al., 2013). A considerable portion of a drug is distributed nonspecifically over healthy tissues and organs in the body, thus leading to severe side effects.

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So, it is important to use a drug delivery to maintain the drug capsulated from many external and internal conditions such as gut(Ph. or enzymes) (Bell, 2001). Recently a nanoparticle as a delivery system to load PZQ to its target tissue are widely used (Kwon et al., 2005 and Reddy et al., 2007). Chitosan is a naturally occurring and abundantly available biocompatible polysaccharide (Shrestha et al., 2014). Collectively, as an attractive carrier and adjuvant, Chitosan has been used extensively in vaccine applications (Islam and Ferro, 2016).

MATERIALS AND METHODS

Study Materials:

1) Animals:
Eighty-four male Swiss albino pathogen-free mice, strain CD1, weighing (20-22 g) at the beginning of the experiment and had similar age (6-8 weeks) were used. The animals were provided by The Schistosoma Biology Supply Program (SBSP) of the Theodor Bilharz Research Institute (TBRI), Giza, and Egypt

2) Parasites:
-Schistosoma mansoni cercariae were obtained from laboratory-bred infected Biomphalaria alexandrina snails in SBSP at TBRI, Giza, Egypt.

3) Drugs and Dosage:
A. Praziquantel (PZQ) 500 mg/kg tablets (Praziquantel-Sedico Pharmaceutical Co.6th of October City, Egypt), was orally administered at a dose of 500 mg/kg body weight for two consecutive days (Piper et al., 1990). It was freshly prepared before use as a 2% suspension in Cremophor-E1 (Sigma Chemical Co., St. Louis, MO, USA).

B. Chitosan 500 mg/kg tablets (Degree of De-acetylation (93%) and sodium Tri-PolyPhosphate(PP) were purchased from Sigma Aldrich. Phosphate buffer saline (PBS) and acetic acid were obtained from Sigma–Aldrich, USA.

Study Design:
- Study type: an experimental randomized controlled study.

- Mice were grouped into five main groups; A, B, C and D, as the followings:
(A) A negative Control Group (G 1)
(B) A negative control group, which was divided into three sub-groups:
  - Subgroup I (G2): Received with Praziquantel in a dose of 500 mg/kg orally in two consecutive days (Gonnert and Andrews, 1977).
  - Subgroup II (G3): Received with Chitosan solution in a dose of 500 mg/kg orally (Schipper et al 1999) in two consecutive days.
  - Subgroup III (G4): Received with PZQ 500 mg/kg and Chitosan 500 mg/kg orally once.
(C) A positive Control Group, which was divided into one subgroup;
  - Subgroup IV (G5): infected untreated mice.
(D) A Group Harboring the Immature Stages Received Chitosan±Praziquantel Treatments 3 Weeks Post-Infection, And Divided Into Three Subgroups:
  - Subgroup V (G6): Infected and treated with PZQ alone in a dose of 500 mg/kg in two consecutive days.
  - Subgroup VI (G7): Infected and treated with Chitosan alone in a dose of 500 mg/kg in two consecutive days.
  - Subgroup VII (G8): Infected and treated by PZQ500 mg/kg loaded on Chitosan 500 mg/kg orally once.
(E) A Group Harboring the Mature Stages Received Chitosan ± Praziquantel Treatments after 6 Weeks from Infection, Which Was Divided Into Three Sub-Groups:
  - Subgroup VIII (G9): Infected and treated by PZQ alone in a dose of 500 mg/kg orally in two consecutive days.
  - Subgroup IX (G10): Infected and treated by Chitosan alone in a dose of 500 mg kg orally in two consecutive days.
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- **Subgroup X (G11):** Infected and treated by PZQ 500 mg\ kg loaded Schistosomiasis 500 mg\ kg orally once.

**Parasitological Parameters:**

A. **Worm Burden:** Infected animals were perfused to recover hepatic and Portomesenteric worms for subsequent counting (Smithers and Terry, 1965).

B. **Oogram Pattern:** by (Pellegrino et al., 1962).

C. **Tissue Egg Load:** The number of eggs per gram tissue (liver and intestine) was studied according to the procedure described by (Cheever, 1970).

**Statistical analysis**

The collected data will be tabulated and analyzed using IBM's personal computer using SPSS 16 microstate software package. ANOVA (analysis of variance) was used as the test of significance. P-value was considered significant if it was <0.05 (Field, 2000). T-test was used as the multiple comparison tests between all groups and the infected one to determine which pair was significantly different (Abdi and Sidak, 2007).

**RESULTS AND DISCUSSION**

1) **Worm Burden:**

In PZQ treated group 6 weeks PI (G9), in a Chitosan loaded with PZQ treated group 6 weeks post-infection (G11) and 3 weeks post-infection (G8) the results were showed highly significant from those recorded in infected control mice (P<0.001).

<table>
<thead>
<tr>
<th>Animal groups infected</th>
<th>Mean worm burden ± S.E. (Hepatic-portomesenteric)</th>
<th>Total worm burden</th>
<th>Worm % reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Couples</td>
</tr>
<tr>
<td>(G5) positive control</td>
<td>2.470±0.633</td>
<td>6.633±1.713</td>
<td>4.833±0.418</td>
</tr>
<tr>
<td>(G6) PZQ 5 weeks PL</td>
<td>2.001±0.443</td>
<td>4.333±0.700</td>
<td>0.250±0.23***</td>
</tr>
<tr>
<td>(G7) chitosan 3 weeks PL</td>
<td>0.00±0.00***</td>
<td>3.250±0.521*</td>
<td>3.772±0.462</td>
</tr>
<tr>
<td>(G8) PZQ- chitosan 3 weeks PL</td>
<td>0.00±0.00***</td>
<td>2.871±0.375**</td>
<td>0.563±0.359***</td>
</tr>
<tr>
<td>(G9) PZQ 6 weeks PL</td>
<td>0.69±0.43***</td>
<td>0.69±0.403***</td>
<td>0.69±0.403***</td>
</tr>
<tr>
<td>(G10) chitosan 6 weeks PL</td>
<td>2.00±0.443</td>
<td>4.752±0.567</td>
<td>0.401±0.730***</td>
</tr>
<tr>
<td>(G11) PZQ- chitosan 6 weeks PL</td>
<td>0.00±0.00***</td>
<td>0.401±0.527***</td>
<td>0.00±0.00***</td>
</tr>
</tbody>
</table>

*** Highly significant difference from the infected untreated group (P<0.001)

** Moderately significant difference from the infected untreated group (P<0.01)

* Significantly relative to the infected untreated group (P< 0.05)

2) **Oogram Pattern:**

In (G8,G9,G11) showed a highly significant difference in the dead ova mean number reached 77.397±5.163, 97.20±7.998 and 97.20±7.998 respectively from those recorded in negative control mice 18.30±4.020 (P<0.001).
**Table (2):** Oogram pattern in *Schistosomamansoni* infected mice treated with chitosan+praziquantel 3 and 6 weeks post-infection and sacrificed after 8 weeks from infection.

<table>
<thead>
<tr>
<th>Animal groups infected</th>
<th>Mean number of immature ova±S.E</th>
<th>Mean number of mature ova±S.E</th>
<th>Mean number of dead ova±S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>(G5) positive control.</td>
<td>54.50±4.647</td>
<td>27.20±1.840</td>
<td>18.30±4.020</td>
</tr>
<tr>
<td>(G6) PZQ 3 weeks PI.</td>
<td>24.40±4.862*</td>
<td>21.00±1.942</td>
<td>54.60±4.208**</td>
</tr>
<tr>
<td>(G7) chitosan 3 weeks PL.</td>
<td>29.00±4.832</td>
<td>23.40±2.028</td>
<td>47.80±3.590*</td>
</tr>
<tr>
<td>(G8) PZQ+ chitosan 3 weeks PL.</td>
<td>16.83±4.043**</td>
<td>5.77±1.536***</td>
<td>77.39±5.163***</td>
</tr>
<tr>
<td>(G9) PZQ 6 weeks PI.</td>
<td>0.8±0.396***</td>
<td>2.00±1.613***</td>
<td>97.20±7.999***</td>
</tr>
<tr>
<td>(G10) chitosan 6 weeks PL.</td>
<td>33.2±4.018</td>
<td>21.00±1.815</td>
<td>45.80±2.343*</td>
</tr>
<tr>
<td>(G11) PZQ+ chitosan 6 weeks PL</td>
<td>0.20±0.054***</td>
<td>0.20±0.109***</td>
<td>97.20±7.999***</td>
</tr>
</tbody>
</table>

- Values are expressed as mean ± S.E.
- The count of each stage represents its existence in a total of 100 ova.
- ** Highly significant difference from infected untreated group (P<0.001)
- * Significantly relative to the infected untreated group (P< 0.05)

3) **Biochemical Studies:**

In ALT results, collectively (G6, G8, G9, G11) showed a non-significant reduction where the mean number reached 51.50 ± 7.477U/L, 55.50 ± 7.880U/L, 59.00 ± 8.250 U/L, and 43.00 ± 6.710U/L respectively compared with negative control mice (G1)(P>0.05). Collectively in AST results (G 5, G 6, G 7, G 8, G 9, G 10, G 11) have a highly significant difference in the mean number 443.00 ± 64.000 U/L, 391.90 ± 56.777 U/L, 387.50 ± 56.000 U/L, 349.50 ± 50.333U/L, 214.43 ± 30.293 U/L, 498.00 ± 72.333 U/L and 211.50 ± 29.860 U/L in its comparison with negative control group (G 1) (P<0.001).

**Table (3):** The effect of chitosan+praziquantel 3 and 6 weeks post-infection on the liver functions in *Schistosoma mansoni* infected mice scarified after 8 weeks from infection.

<table>
<thead>
<tr>
<th>Animals groups</th>
<th>ALT</th>
<th>AST</th>
</tr>
</thead>
<tbody>
<tr>
<td>(G1) negative control.</td>
<td>43.52±6.837</td>
<td>23.85±6.687</td>
</tr>
<tr>
<td>(G 2) negative control+PZQ.</td>
<td>31.29±5.913</td>
<td>37.86±5.897</td>
</tr>
<tr>
<td>(G 3) negative control+chitosan.</td>
<td>52.50±7.577</td>
<td>35.85±4.693</td>
</tr>
<tr>
<td>(G 4) negative control+PZQ +chitosan.</td>
<td>58.00±8.143</td>
<td>30.45±4.100</td>
</tr>
<tr>
<td>(G 5) positive control.</td>
<td>62.50±8.633*</td>
<td>443.00±64.000***</td>
</tr>
<tr>
<td>(G 6)positive control+PZQ 3 weeks PL.</td>
<td>51.50±7.477</td>
<td>391.90±56.777***</td>
</tr>
<tr>
<td>(G 7)positive control+chitosan 3 weeks PL.</td>
<td>70.50±9.667**</td>
<td>387.50±56.000***</td>
</tr>
<tr>
<td>(G 8)positive control+PZQ+chitosan 3 weeks PL.</td>
<td>55.50±7.880</td>
<td>349.50±50.333***</td>
</tr>
<tr>
<td>(G 9)positive control+PZQ 5 weeks PL.</td>
<td>59.00±8.250</td>
<td>214.43±30.293***</td>
</tr>
<tr>
<td>(G 10)positive control+chitosan 6 weeks PL.</td>
<td>87.00±11.617***</td>
<td>498.00±72.333***</td>
</tr>
<tr>
<td>(G 11)positive control+PZQ+chitosan 6 weeks PL.</td>
<td>43.00±6.710</td>
<td>211.50±29.860***</td>
</tr>
</tbody>
</table>

- ** Highly significant difference from the negative control group (P<0.001)
- * Significantly relative to the negative control group (P< 0.05)

Schistosomiasis is an important parasitic disease caused by trematode worms of the genus *Schistosoma*. Schistosomiasis control strategies are predominantly based on the treatment of infected individuals with effective drugs. Recently, nanotechnology holds promise for medication and nutrition, because of materials at the nanometer dimension exhibit novel properties different from those of both isolated atoms and bulk material. Developed nanoparticles have great
potential to overcome the limitations associated with products currently available in the market for the treatment of schistosomiasis. Also, the use of nanotechnology can provide a novel diagnostic assay for Schistosomiasis (Mohamed et al., 2016).

Worm burden reduction is one of the most important assessments in drug efficacy (He XY et al., 1992). Concerning the PZQ effect on immature stage (juvenile stage) there was a mild significant reduction effect, it reached 52.76% which shows a deficiency in its activity spectrum. The low efficacy observed in immature stage when using PZQ as a treatment explained the treatment failure in a highly endemic area of schistosomiasis (Shu-hua et al., 1985; Botros et al., 2005 and Doenhoff et al., 2008). The present study showed that the efficacy of chitosan as a nanoparticle loaded with PZQ in juvenile stage has a highly significant reduction reached 77.22%, which means using nanoparticles chitosan in PZQ delivery has a high Percentage in schistosomiasis treatment even in immature stage. Concerning with PZQ treatment on mature stage found that, the reduction in the worm burden reached 91.36%, it means it still has high efficacy in this stage of the disease and that explained why PZQ has a wide range in its distribution (El-Bassiouni et al., 2016). On the other side the effect of chitosan on mature stage reached 48.67%, it showed low efficacy in this stage compared with its effect on juvenile stage, which means the complications of the disease were higher than its effect. The effect of chitosan loaded with PZQ after 6 weeks from infection (mature stage) reached 97.12% in worm burden reduction, this result was the highest percentage observed in the present study. This result refers to, chitosan success in delivering the PZQ to its target directly protecting the drug from any external or internal factors can affect the cure.

The chemotherapy is considered effective against S. mansoni when the eggs are mature stage (Pellegrino et al., 1962). Using Chitosan loaded with PZQ as nanoparticles showed increasing the dead ova reached 99.6% and approximately absence of mature and immature ova in the adult stage of S. mansoni. While using PZQ chemotherapy orally alone, its effect on mature stage reached 97.2% it approximately as the study reported by (Rabia et al., 2010) where PZQ treatment reached 100% dead ova; but it can’t be ignored that mass treatment has been proven to be insufficient to stop disease transmission, prevent re-infection, or reduce parasite-induced illness (King, 2009 and Matthews, 2011).

Determination of enzymes levels, such as ALT and AST is largely used during the assessment of liver damage by schistosomal infection (Al-sayed et al., 2014 and Aly and Mantawy, 2013). Due to membrane damage (necrosis) the liver releases the enzymes into circulation so they can be measured in the serum (Fahmy et al., 2014). The increment of such enzymes in serum may be due to destruction in hepatocytes by the action of parasite egg toxins which lead to their release into the circulation (Kadry et al., 2013 and Mahmoud and Elbessoumy, 2013). Membrane damage seems to be prim culprit for the marked increase in the serum marker enzymes such as ALT, AST following the infection of Schistosoma (Naik et al., 2011). In this study, the comparison between all groups with the negative control mice in, ALT level showed a high elevation in mice treated with Chitosan as a treatment for schistosomiasis in both immature and mature stages. The observed elevation in ALT reflects either acute active or chronic liver damage (Naik and Panda, 2007), this could be associated with mal-absorption due to damaged intestinal mucosa resulting from the extrusion of large numbers of eggs, or could be due to decreased synthesis, which may result from parasitic injury to hepatic cells (Oliveira et al., 2004 and Fahmy et al., 2014).

On the other hand, the effect of PZQ on both immature and mature stages showed no
elevation in ALT level. PZQ therapy has been associated with an elevation in serum ALT in up 27% of patients (Zimmerman, 1999).

In Chitosan loaded PZQ as nanoparticles found that, there was no elevation in ALT level which means it may not lead to cell membrane damage and convey the treatment directly to its target organ.

Despite extensive research into the biomedical and pharmaceutical applications of nanoparticles, and the liver being the main detoxifying organ in the human body, there are limited studies that delineate the hepatotoxicity of nanoparticles (Jing et al., 2010).

In the current study, AST level has a high elevation in all mice infected and treated with different treatments. PZQ increase AST level in serum (El-Lakkany, 2011) but still mice treated with Chitosan loaded PZQ have less chance in AST elevation from those recorded in negative control mice.

**REFERENCES**


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ARABIC SUMMARY

تأثير جزيئات الشيتوسان منتهية الصغر والمحملة بالبرازيكوانتيل على الفئران المصابة بالبلهارسيا المعوية

نوهران احمد عبده¹،ماهي عبد الفتاح غباشي¹،سهر سيد محمود²،علاء علي اسماعيل³،أحسان أحمد حسن³
¹قسم علم الحيوان-كلية العلوم-جامعة قناة السويس
²قسم الطفيليات-معهد تيودور بلهارس للابحاث
³كلية الطب-جامعة قناة السويس

بالرغم ان البلهارسيا مرض منتشر عالمياً، حيث ينتشر في أكثر من 200 مليون شخص حول العالم و لكنة يعتمد كلياً و فقط على علاج وحيد يكون البرازيكوانتيل.

منذ تطور البرازيكوانتيل عام 1970 فقد اختبر بديلاً عن جميع العلاجات الأخرى الخاصة بمرض البلهارسيا و ذلك لعدة أسباب منها: كفاءة العلاجات الجديدة بالإضافة إلى صغر المدة الممتازة و لكن هناك عدة عوامل أخرى تؤثر بشكل أو آخر على كفاءة علاجات الشيتوسان النانوية و نتائج هذا البحث تظهر أن جزيئات الشيتوسان النانوية والمحملة بالبرازيكوانتيل كدواء فعال للعلاج على العوامل الطفيلية مثل عدد الديدان، عدد البيضات الميتة، عدد البيضات في نسيج الكبد و الأمعاء الدقيقة و العوامل البيوكيميائية.

و جاتت النتائج أن جزيئات الشيتوسان النانوية و المحملة بالبرازيكوانتيل كان لها تأثير كبير حيث وصل نسبة الانخفاض في عدد الديدان الى 97.12% و وصل عدد البيضات الميتة الى 99.6% و زادت نسبة الانخفاض في عدد البيضات بنسبة 96.17% و نسبة الامعاء الدقيقة الى 97.39% مع تحسن في العوامل البيوكيميائية.