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ASSESSMENT OF POSSIBLE SYNERGISTIC EFFECT OF BISPHOSPHONATES AND STATINS IN MURINE EXPERIMENTAL TOXOPLASMOSIS GONDII

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Abstract

This open new horizons for drug repositioning to standard used drugs in treatment of acute toxoplasmosis. This study was conducted on 70 male Albino mice in Theodore Bilharz Research Institute (TBRI) to evaluate the possible synergistic effect of risedronate sodium (Bisphosphonates) combination with atorvastatin (statins) on acute *Toxoplasma gondii* (RH strain) infected mice versus the drug spiramycin (macrolides). Drug combination was used in two doses: the first combined dosage was 0.01mg/kg/day risedronate sodium and 1.25mg/kg/day atorvastatin (G B+S1) and the second one was 0.2mg/kg/day risedronate sodium and 20 mg/kg/day atorvastatin (G B+S2) and each drug of them was also used alone at a dose of 0.4 mg/kg/day for risedronate sodium (G B) and 40mg/kg/day for atorvastatin (G S) in comparison to spiramycin (G M) which was used at a dose of 200mg/kg/day.

Microscopy examination by Giemsa stained of peritoneal exudate from *T. gondii* infected mice showed reduction in number of *Toxoplasma* tachyzoites to 86.28% on the 5th day post infection (G B+S1), 98.35% in (G B+S2), 98.27% in (G B) and 85.03% in (G S) and spiramycin treated mice with reduction of 93.66%. Histopathological examination of liver sections collected from infected mice showed remarked significant improvement in mice treated with combined of risedronate sodium and atorvastatin.

Key words: Mice, *Toxoplasma gondii*, Risedronate sodium, Atorvastatin, Spiramycin.

Introduction

Toxoplasma gondii (*T. gondii*) is a worldwide distributed intracellular parasite that affected about 1 billion of people (Pagheh *et al*, 2020). The highest rates of infection with *T. gondii* were reported in Europe, Central America, Brazil, and Central Africa (Berger *et al,* 2009). In some Arabian Countries the toxoplasmosis among women ranged from 22.5 to 37.4% in Saudi Arabia (Shoura *et al*, 1973; Abbas *et al*, 1986), 37.5% in Libya (Kassem and Morsy, 1991), 37% in Jordan (Morsy and Michael, 1980), and 22.2% pregnant women and 20% non-pregnant ones in Egypt (Saleh *et al*, 2014). Al-Kappany *et al.* (2010) in Egypt isolated *T. gondii* from cats, Saleh *et al.* (2016) considered toxoplasmosis as an occupational disease, and Abass *et al.* (2020) reported toxoplasmosis in humans and edible and stray animals.

Toxoplasmosis causes serious manifestations, particularly in the immunocompromised persons as encephalitis, fatal pneumonia and/or disseminated infection. Acute toxoplasmosis in pregnant women may cause serious health problems to the fetus (congenital toxoplasmosis), including mental retardation, seizures, blindness, and death, or manifestations may not appear until the second or third decade of life (Dolores *et al*, 2015). Serologic tests are used to diagnose acute infection in pregnant women, but false-positive tests occur frequently, thus, serologic diagnosis must be confirmed at a reference laboratory before treatment with potentially toxic drugs should be considered (Jones *et al,* 2003). The commercial anti-toxoplasmosis drugs are Pyrimethamine, sulfadiazine (McAuley *et al,* 2009), Pyrimethamine-sulfadiazine with folic acid (Wishahy *et al,* 1971) and

Clindamycin-atovaquone combination (Azevedo Silva *et al,* 2019). But, the drugs may cause side-effects (D'angelo *et al*, 2008), or may be ineffective against cysts (Boyom *et al*, 2014). Toxoplasmosis was treated also with Spiramycin, which more or less not effective, and infection may develop resistance (Montazeri *et al*, 2017). Thus, development of new anti-toxoplasmosis with high efficacy and less side effects was a mandatory (Zhang *et al*, 2019).

To overcome the huge cost of the de novo drug development, the ongoing medications were evaluated as new drug repurposing (repositioning) that alternated alternative to de novo drug (Ashburn and Thor, 2004). Isoprenoids are compounds essential for all cells and most apicomplexans due to their active role in many biological processes, and enzymes, involved in this pathway by acting as molecular targets for drugs against parasites (Moreno and Li, 2008). *T. gondii* was unable to make its own isoprenoids, but can do with specific intermediate host cells. Bisphosphonates (FDA) and Statins (FDA) inhibited isoprenoid pathway in parasitosis by blocking their growth in the host cell (Li *et al*, 2017).

This study aimed to evaluate double-hit strategy of combining inhibitors of host isoprenoid pathway for more potent as a safe anti-*Toxoplasma* drug.

Materials and Methods

A total number of 70 laboratory bred male Swiss albino mice, aged 6-8 weeks and weighed 20-30gm, C57BL/6 strain were purchased from The European Country Farms in Egypt. They were housed in Theodor Bilharz Research Institute (TBRI) during the study period (June 2019 to August 2019). Mice were kept on a standard diet of 24% protein, 4% fat, 4-5% fiber and water ad-libitumin in plastic cages at room temperature of 21ºC and 60% humidity.

Ethical consideration: Protocol was approved by Faculty of Medicine, Cairo University and, TBRI Ethical Committee. All procedures related to experimented-with mice were treated due to the ethical standards approved after Declaration of Helsinki (1964).

Drugs: 1- Spiramycin: 3 million international units (M.I.U) was used in a tablet form provided by Pharaonia Pharmaceuticals (analytical standard code: J01FA02). Tablets were smashed into powder which was dissolved in phosphate buffered saline (PBS) and each tablet dissolved in 12ml PBS in labeled drug containers that were stored in refrigerator at 4±2°C (Garcia, 2007). Drug was given orally to mice by an esophageal tube. Spiramycin was given at a dose of 200mg/ kg/day for 5 consecutive days (Abdel Hamed *et al*, 2019). 2- Risedronate sodium pure crystalline powder was kindly provided by Future Pharmaceutical Industries (analytical standard code: UT/STS/API/054-03/A). It was prepared as stock solutions of different doses by dissolving the powder in PBS (4mg risedronate sodium in 100ml BPS, 2mg risedronate sodium in 100ml BPS and 1mg risedronate sodium in 1000ml BPS). It was given to mice by intraperitoneal (IP) by disposable insulin syringe (Erhirhie *et al*, 2014). Also, solutions were stored in the refrigerator at 4±2°C, in labeled containers (London and East, 2001). Three different doses of risedronate sodium were prepared 0.4mg/kg/day; 0.2mg/kg/day, &0.01mg/kg/day, each dose was given to infected mice for 5 consecutive days (Li *et al*, 2017). 3- Atorvastatin, in pure powder was provided by DELTA PHARMA (analytical standard code: AVN1ATA11B), and prepared as stock solutions of different doses by dissolving the atorvastatin powder in PBS as 400mg in 100ml BPS, 200mg in 100ml BPS & 12.5mg in 100ml BPS and given to mice by IP by insulin syringe (Erhirhie *et al*, 2014), and labeled prepared solutions were also stored in the refrigerator at 4±2°C. Atorvastatin in a dose of 40mg/kg/day (G S) was given alone to infected mice. Besides, Atorvastatin in a dose of 1.25mg/kg/day combined with risedronate sodium in a dose 0.01mg/kg/day (G B+S1) and atorvastatin in a dose of 20mg/kg/day combined with risedronate sodium in a dose of 0.2mg/kg/day (G B+S2) each dose for 5 consecuti-

ve days (Li *et al*, 2017).

Tachyzoites was obtained from peritoneal exudate on 3rd day post passage (Michael *et al,* 1979). Peritoneal exudate was diluted with PBS (Eissa *et al*, 2012). Each mouse was infected by IP with 20µl of 10-20 tachyzoites in PBS (Li *et al*, 2017). Drugs evaluation was shown in the following table.

Table 1: Study design for acute experimental toxoplasmosis in mice groups.

Group	Mice (ten each)
(N)	Non-infected non treated naïve mice (negative control)
(C)	Infected non treated mice (Infection control)
(M)	Infected mice received Spiramycin® in a dose of 200mg/kg/day (Grujić *et al,* 2005).
(B)	Infected mice received Risedronate sodium in a dose of 0.4mg/kg/day (Li *et al,* 2017).
(S)	Infected mice received Atorvastatin® in a dose of 40mg/kg/day (Li *et al,* 2017)
(B+S1)	1st combined dose 0.01mg/kg/day Risedronate sodium &1.25mg/kg/day Atorvastatin infected mice (Li *et al*, 2017).
(B+S2)	2nd combined dose 0.2mg/kg/day Risedronate sodium & 20mg/kg/day atorvastatin infected mice (Li *et al*, 2017).

(N: naïve group, C: infection control, M (macrolides): spiramycin 200mg/kg, B (bisphosphonates): risedronate sodium 0.4mg/kg, S: atorvastatin 40mg/kg, B+S1: (bisphosphonates & statins): Risedronate sodium 0.01mg/kg & atorvastatin 1.25mg/kg, B+S2 (bisphosphonates & statins): risedronate sodium0.2mg/kg + atorvastatin20mg/kg).

Drugs were given for 5 days from day zero post infection (PI) and Ketoprofen was allowed orally in dose of 2mg/kg/body/mouse to manage pain (Dobromylskyj *et al,* 2000). On 5th day post-infection mice were anesthetized with thiopental (Liang *et al,* 1987) and sacrificed. Drug efficacy was evaluated parasitological by tachyzoites number in peritoneal exudate (Al-Dakhil and Morsy, 1996), and histopathological by liver tissue for inflammations degree (Drury and Wallington, 1980). Liver inflammation and injury were assessed by using the Ishak modification of Knodell B hepatic activity index to measures intensity of inflammation and detection of architectural alteration (Theise, 2007).

Statistical analysis: Data were coded and entered using the statistical package for the Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA). Data was summarized using mean± standard deviation in quantitative data. Comparisons between variables were done using nonparametric Kruskal-Wallis and Mann-Whitney tests. For comparison, Chi square ($\chi 2$) test was used. Exact test was used instead when expected frequency < 5. P value less than 0.05 were considered as statistically significant.

Results

Infected mice showed that tachyzoites/ 20 µl exudate was 42511±3926, but in spiramycin treated mice number was reduced to 2695±396 with significant reduction 93.66% (P =0.003). Risedronate sodium mice treated with 0.4mg/kg/day showed a significant reduction of 736±102 with 98.27% (P < 0.001). Tachyzoites in mice treated with atorvastatin 40mg/kg/day was reduced to 6363±1491 with insignificant reduction to 85.03% (P = 0.179). Tisedronate sodium mice treated with 0.01mg/kg/day combined with 1.25mg/kg /day atorvastatin was reduced to 5832± 1169 with insignificant reduction of 86.28% (P =0.106), but mice treated with 0.2mg/kg/day risedronate sodium combined with 20 mg/kg/day atorvastatin number was reduced to 703±525 with significant reduction of 98.35 % (P =zero).

The histopathological evaluation depended on the degree of portal, lobular and interface inflammation. The intensity of inflammation was interpreted into specific score (Ishak score) according to heaviness of the cellular infiltration and presence of lymphocytic aggregation (Theise, 2007). These results were compared to the histopathological results of the naïve group that showed normal liver tissue with normal architecture of hepatic lobules and normally arranged polygonal hepatocytes. Histopathological examination in the infection control group showed that 16.7% of acute infected mice had score 1 in portal inflammation, 66.6% had score 2 and 16.7% had score 3. 16.7% had score 1 in lobular (focal) inflammation, 66.7% had score 4 and 16.6% had score 3. Interface inflammation was score 1 in 100% of mice. All infected treated groups didn't show interface inflammation and the portal and lobular inflammation was reduced

Details were given in tables (2 & 3) and figures (1, 2, 3, 4, & 5).

Table 2: *T. gondii* tachyzoites/20µl IP fluid in infection control and infected treated groups

Group	Mean ±SEM	% Reduction	P value
C	42511±3926	--	--
M	2695±396	93.66 %	0.003*
B	736±102	98.27%	< 0.001*
S	6363±1491	85.03%	0.179
B+S1	5832±1169	86.28 %	0.106
B+S2	703±525	98.35 %	0*

*(P value significant <0.05)

(C: infection control, M (macrolides): spiramycin 200mg/kg), B (bisphosphonates): risedronate sodium 0.4mg/kg, S (statins): atorvastatin 40mg/kg, B+S1 (bisphosphonates + statins 1st combination): risedronate sodium 0.01mg/kg + atorvastatin 1.25mg/kg, B+S2 (bisphosphonates + statins 2nd combination): risedronate sodium 0.2mg/kg + atorvastatin20mg/kg)

Table 3: Inflammation of collected liver tissues from infection control and infected treated groups

Pathology	Score	C %	M %	B %	S %	B+S1 %	B+S2 %
Portal inflammation	0	0%	50%	33.3%	0%	0%	0%
	1	16.7%	50%	66.7%	16.7%	33.3%	50%
	2	66.6%	0%	0%	66.7%	50%	33.3%
	3	16.7%	0%	0%	16.6%	16.7%	16.7%
Lobular inflammation	0	0%	50%	66.7%	0%	0%	0%
	1	0%	50%	33.3%	33.3%	53.3%	66.6%
	2	0%	0%	0%	50%	16.7%	33.3%
	3	16.7%	0%	0%	0%	0%	0%
	4	66.7%	0%	0%	16.7%	0%	0%

Discussion

In this study, in the acute *Toxoplasma* RH strain infected mice treated with the spiramycin alone at a dose of 200mg/kg/day for 5 days showed significant reduction of *T. gondii* tachyzoites number with a reduction of 93.66 % (P =0.003). This result agreed with Etewa *et al.* (2018) in Egypt who reported significant reduction in tachyzoites number in spiramycin treated RH strain infected mice with a rate of 94.59%. However, both results disagreed with Grujić *et al.* (2005) in Be lgrade who used type-1 (RH) or type-2 (Me49) strain of *T. gondii* and found that mice treated with spiramycin at 100 &20 mg/kg for a week showed a limited effect without ability to prevent the infected mice death. Nevertheless, Hagras *et al.* (2019) in Egypt who found highest efficiency reduction in tachyzoites number in spiramycin-loaded chitosan nanoparticles treated RH strain infected mice. They concluded that the non-toxic nature and the anti-parasitic effect of both spiramycin-metronidazole and spiramycin-loaded chitosan (CS) and spiramycin recommended the use of spiramycin-loaded CS NPs a potential drug for the treatment of human toxoplasmosis.

In the present study, mice treated with risedronate sodium at a dose of 0.4mg/kg/day for 5 days showed a significant reduction of *T. gondii* tachyzoites number to 98.27% (P < 0.001). Yardley *et al.* (2002) in the United Kingdom studied the in-vivo activities of 3 bisphosphonates against *Leishmania donovani* and *Toxoplasma gondii* and alendronate was essentially inactive against both parasites. They concluded that pamidronate was active against *L. donovani* by IV administration, and risedronate had a 50% effective dosage of five 2.6-mg/kg/body weight intraperitoneal doses, but was less effective against *T. gondii*-infected mice. Also, Shubar *et al.* (2008) in Germany used newly synthesized bisphosphonates 2F, 3B, 91A & 282A reported excellent therapeutic activity and low toxicity. They added that the anti-parasitic drugs may be promising agents for patients with acute and reactivated toxoplasmosis. Also, Szajnman *et al.* (2017) in Argentina reported that the most potent compound 22 (11,1-bisphosphonic acid) is a sulfone-containing compound, which had a 50% effective concentration (EC_{50}) of 0.11± 0.02µM against intracellular tachyzoites, and with low toxicity when tested in tissue culture with a selectivity index of >2,000. Compound 22 also showed high activity *in vivo* in

a toxoplasmosis mouse model. The compound inhibited the *Toxoplasma* farnesyl diphosphate synthase (*Tg*FPPS), but the concentration must be 50% of the enzymatic activity (IC_{50}) was higher than the concentration that inhibited 50% of growth. They concluded that it is an excellent novel compound that could lead to the development of potent agents against apicomplexan parasites. Besides, Li *et al.* (2017) in USA reported that bisphosphonates are widely used for bone disorders treatment. They studied the synergism of several bisphosphonates with statins both *in vitro* and *in vivo*, and concluded that it was possible to develop drug combinations that act synergistically by inhibiting host and *T. gondii* enzymes *in vitro* and in *vivo*. But, this disagreed with Nair *et al.* (2011) in USA who reported that in spite of the fact that apicoplast is home to a 1-deoxy-D-xylulose-5-phosphate pathway for isoprenoid precursor synthesis, which was believed to be the most conserved function of apicoplast, and fosmidomycin, a specific pathway inhibitor as an effective antimalarial. But, fosmidomycin didn't affect most of the other apicomplexans, especially *T. gondii* the para site plasma membrane is a critical barrier to drug uptake fosmidomycin.

In the present study, acute infected mice treated with atorvastatin at a dose of 40mg/kg/day for 5 days showed insignificant reduction of *T. gondii* tachyzoites number to 85.03% (P =0.179). This agreed with Cortez *et al*. (2009) found that the in vitro activity of statins stopped *T. gondii* multiplication in Swiss mice macrophages. Also, Nishikawa *et al*. (2011) in Japan found that *T. gondii* growth was suppressed by squalestatin, despite mevalonate producing isoprenoid intermediates in host cells. They concluded that lovastatin, compactin and squalestatin have anti-*Toxoplasma* activities and that the host cholesterol synthesis contributed to parasite growth in macrophages. Li *et al*. (2013) reported that in vivo Atorvastatin in Swiss mice infected with *T. gondii* strain RH increased their survival for < 30 days. Also, the present result agreed with Li *et al.* (2017) they treated mice with atorvastatin (10 & 20mg/kg/body/day) found that infection led to only a marginal survival difference between control and treated groups 24hr after infection with 20 parasites of type I strain. They added that atorvastatin (20mg/kg/day) protected 80% of *T. gondii* mice infected with 20 parasites of ΔTgFPPS strain, but same dose was ineffective against 100 parasites infection.

In the present study, combination between risedronate sodium and atorvastatin at a dose of 0.01mg/kg/day &1.25mg/kg/day respectively for 5 days showed an insignificant reduction of *T. gondii* tachyzoites to 86.28% (P =0.106), but combination between risedronate sodium and atorvastatin at a dose of 0.2mg/kg/day and 20mg/kg/day respectively for 5 days showed a significant reduction in number of tachyzoites to 98.35% (P =0.000) denoting synergistic effect between combined dose was better than that obtained from spiramycin 200mg/kg/day treated mice. This agreed with Li *et al.* (2017) who found that bisphosphonates and statins combination has a more powerful effect rather than usage of each drug alone, but they found more synergism at low dose combination.

In the present study, histopathological examination of acute infected mice groups treated with spiramycin 200mg/kg/day for 5 days, liver tissue showed much less inflammatory reactions compared to corresponding infection controls. This agreed with Etewa *et al.* (2018) who found that liver sections from RH strain acute infected mice and treated with spiramycin showed an obvious reduction of the inflammatory response with mild hepatocytes inflammation and necrosis.

In the present study, mice treated with risedronate sodium at a dose of 0.4mg/kg/day for 5 days, liver tissue showed the least inflammatory reactions in acute toxoplasmosis. This agreed with Garzoni *et al.* (2004) in Brazil who found that heart tissues of mice infected with *Trypanosoma cruzi* (same isoprenoid pathway as *T. gondii*) and treated with risedronate showed normal cardiac tissue,

with very rare amastigote nests and small mononuclear cell infiltrates. They added that risedronate could be a useful lead compound for the development of new drugs effective against Chagas' disease.

Conclusion

Spiramycin, the routinely used drug in treatment of toxoplasmosis, showed significant reduction of the number of tachyzoites, but its therapeutic effect was less than that obtained by the drug combination in acute toxoplasmosis infected mice. Thus, combination of bisphosphonates and statins may represent a good alternative treatment.

Moreover, the infected mice treated with risedronate sodium alone, showed a significant histopathological improvement in the pathology of the examined liver tissue. Accordingly, risedronate sodium alone may represent a promising anti-*Toxoplasma* treatment.

Recommendations

Conduct extensive studies using other Bisphosphonates and statins with different doses and prolonged regimens may highly increase its efficacy against *Toxoplasma* infection. In vivo studies on the efficacy of risedronate sodium alone and/or combined risedronate sodium and atorvastatin treating acute *Toxoplasma* infected patients are ongoing and will be published in due time elsewhere.

Toxoplasmosis health education must be tailored to women of childbearing age that may help to prevent infection.

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Explanation of figures

Fig. 1: *T. gondii* tachyzoites stained with Giemsa in IP fluid sample from IC group (x100 objective lens).

Fig. 2: Normal liver with preserved architecture showed hepatocytes (black arrow), arranged in thin plates with patent central vein (red arrow), sinusoids (blue arrow) and normal portal tract (Yellow arrow), (x10, H&E stain).

Fig. 3: Histopathological examination of liver tissue from infection control mice showed a single portal tract with marked lymphocytic cellular infiltrate (black arrow) (Score 3), (x10, H&E stain).

Fig. 4: Histopathological examination of liver tissue from acute infected mice treated with spiramycin 200mg/kg/day showed 2 foci of lobular (focal) inflammation (Score 1), (x40, H&E stain).

Fig. 5: Histopathological examination of liver tissue from acute infected mice treated with risedronate sodium 0.4mg/kg/day showed a single focus of lobular (focal) inflammation (Score 1) with preserved architecture (x40, H&E stain).