



# Viability and infectivity of *Toxoplasma gondii* tachyzoites exposed to Butanedione monoxime

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**Abstract** The most important pathogenesis factor in the Apicomplexa parasites is invasion to the host cell. Given the inhibitory role of Butanedione Monoxime (BDM) on myosin–actin interaction, this study aimed to investigate the effects of this molecule on the vitality and infectivity of *Toxoplasma* tachyzoites in order to provide a new option for vaccine development. The tachyzoites of the RH strain of *Toxoplasma gondii* were exposed to different concentrations (1, 2, 4, 8, 16, 32, 64, and 128 µg/mL) of BDM, and mortality effect was assessed by flow cytometry. Then, the penetration ability of the tachyzoites was investigated in HeLa and macrophage cell lines. The infectivity of exposed tachyzoites to BDM were also investigated in mice through following up and detecting the etiological factor. The highest percentage of mortality (72.69%) was seen in the tachyzoites exposed to 128 µg/mL of the compound. The tachyzoites exposed to 32, 64, and 128 µg/mL of BDM began the proliferation in HeLa cells after 48 h, while this proliferation was initiated within 24 h in macrophage cells. All the mice inoculated with the BDM-treated tachyzoites died after 13 days. The mean survival time of the mice receiving tachyzoites exposed to 128 µg/mL of BDM was 12.4 days, which was significantly different from the negative control group ( $p = 0.001$ ). BDM, as the inhibitor of myosin–actin interaction, and other substances that block the entry of parasites into cells may be suitable candidates for vaccine production against

*Toxoplasma*. Yet, future studies are required to be conducted on the issue.

**Keywords** *Toxoplasma gondii* · Infectivity · Butanedione monoxime · Cell culture

## Abbreviations

LAV	Live attenuated vaccines
BDM	Butanedione monoxime
PBS	Phosphate buffered saline
FCS	Fetal calf serum
PMN	Polymorphonuclear neutrophil

## Introduction

Toxoplasmosis, a parasitic infection caused by *Toxoplasma gondii*, has been considered to be one of the most common parasitic infections among warm-blooded vertebrates, including humans. Based on serological findings, one-third of adult populations have been estimated to be infected with this parasite worldwide (Hill and Dubey 2018; Zhou et al. 2011). Toxoplasmosis in pregnant women precisely clarifies medical importance. In the first time of infection in pregnant women, the parasite may pass through the placenta, infect the fetus, and result in congenital abnormalities in the central nervous system and eyes and abortion in some cases (Arefkhah et al. 2019b). In addition, chronic or acute *Toxoplasma* infections in immunocompromised patients, without therapy, may cause life-threatening consequences (Arefkhah et al. 2019a; Carruthers and Suzuki 2007; Gatkowska et al. 2006).

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Despite the medical importance of this infection, few medicines have been shown to be effective. Pyrimethamine along with sulfadiazine as common drugs have side effects, such as teratogenic effects on the fetus, bone marrow suppression, kidney disorders, and hematological toxicity (Ben-Harari et al. 2017; Mandelbrot et al. 2018). Toxovax is the only vaccine produced for the prevention of *Toxoplasma* infection, which is only applicable to veterinary medicines. This vaccine acts as an inhibitor of tissue cyst formation and transmission to the fetus and, unfortunately, has not shown efficacy in humans (Li and Zhou 2018; Verma and Khanna 2013).

In Apicomplexa phylum, the most important factor in the pathogenesis of parasites is invasion to the host cell. The active entrance and movement mechanisms are based on gliding on solid materials. The external part serves as a ligand and attaches to the external surfaces. Its intracellular portion is located in the space between the plasma membrane and inner membrane complex and interacts with the actin of actomyosin motor via the tetrameric aldolase enzyme (Jewett and Sibley 2003; Meissner et al. 2013). The anterior myosin of the parasite interacts with the short actin filaments and the distal part attaches to the inner membrane complex through Myo A tail. The anterior myosin part moves on the actin filaments. The terminal part of myosin is fixed on the membrane complex and, consequently, the movement of the parasite occurs from the anterior towards the posterior surface (Meissner et al. 2013; Münter et al. 2009). Harnessing the entrance of the parasite into the host cell by some substances would be promising for therapeutic strategies.

Butanedione monoxime (BDM) is an inhibitory organic pharmaceutical molecule (Del Carmen et al. 2009; Ostap 2002), which interacts with myosin ATPase and hampers various myosin functions. However, it does not affect the actin filaments. As BDM can also block the entrance of *Toxoplasma* into the host cell, it reduces the number of intracellular parasites at a specific dose (Delbac et al. 2001; Wetzel et al. 2005). Penetration of *Toxoplasma* tachyzoites into the host cells can be inhibited by treatment of tachyzoites with different concentrations of BDM, an inhibitor of myosin-actin interaction, as a Live Attenuated Vaccines (LAV), therefore the present study was conducted to determine the mortality and infectivity effects of this compound on *Toxoplasma* in order to provide a new vaccine.

## Materials and methods

### Animals

In this study, fifty 6–8-week-inbred BALB/c mice (weight 22–25 g) were provided by Pasteur Institute, Tehran, Iran. The animals were kept at 22°C and 40–50% humidity and had access to standard food, water, and ventilation at the Laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran. During the experiments from March 2017 to February 2018, the animals were embedded in cages and maintained under controlled conditions. The experiments were undertaken based on the guidelines for laboratory animals and Ethics Committee of Shiraz University of Medical Sciences.

### Parasites

The virulent RH strain of *T. gondii* was obtained from Tehran University of Medical Sciences, Tehran, Iran. Tachyzoites of the RH strain of *T. gondii* were maintained by serial intraperitoneal passages in BALB/c inbred mice. Tachyzoites were collected 96 h after inoculation of  $2 \times 10^5$  parasites in the mice by repeated flushing in the peritoneal cavity using Phosphate Buffered Saline (PBS) at pH = 7.2. Then, the tachyzoites were collected and centrifuged at 200 g for 5 min in order to remove peritoneal cells and cellular debris. The supernatant was collected and centrifuged at 800 g for 10 min. The pellet, enriched with parasite tachyzoites, was rewashed with PBS and used in all experiments (Asgari et al. 2015).

### The direct effect of Butanedione monoxime on tachyzoites assessed by the flow cytometry method

$2 \times 10^5$  of tachyzoites were exposed to different concentrations (1, 2, 4, 8, 16, 32, 64, and 128 µg/mL) of BDM and then, the tubes were incubated at 4 °C for 3 h. After incubation, propidium iodide (Sigma Company, USA) was added at the final concentration of 50 µg/mL to each tube and the staining procedure was done in dark environment at 4 °C for 30 min.

Saponin 0.2% and intact tachyzoites were used for positive and negative controls, respectively. The mechanism of this staining is based on the acceptance of the dye by the DNA of the nucleus, which is due to cell death and apoptosis. Then, the mixtures were transferred to flowcytometric polystyrene tubes. In order to set up the flow cytometer, intact tachyzoites were used and gating was performed regarding the specific size of the parasites and their distribution range. These tubes were placed in the

FACS Calibur flow cytometer to assess the effects of different concentrations of the molecules on the vitality of the tachyzoites. The device was set to the PI color and 10–30 thousand cells were counted from each tube.

### Cell culture

HeLa cells and J774 macrophage cell line were provided from the Department of Immunology, Shiraz University of Medical Sciences. A monolayer of the cells was cultivated in 5 mL of the RPMI 1640 medium (Sigma Company, USA) enriched with 10% heat-inactivated Fetal Calf Serum (FCS; Gibco Company, USA) and 100 IU/mL penicillin–100 µg/mL streptomycin (Roche Company) using 25 cm<sup>2</sup> flasks (Corning Costar UK, UK) and was incubated in 5% CO<sub>2</sub> at 37 °C and > 80% humidity. The cells were routinely subcultured every 3 days by trypsinization and washing with PBS (pH = 7.2).

### Cell culture of *Toxoplasma gondii* tachyzoites exposed to Butanedione monoxime

To determine the effects of different dosages of the molecule on the ability of the tachyzoites to attack the cells, the parasite was cultivated in a HeLa cell and J774 cell line culture. After 70% confluency of the cell line in a monolayer condition, the tachyzoites exposed to different concentrations were added to the cell culture at 3:1 ratio. The flasks were then incubated at 37 °C, > 80% humidity, and 5% CO<sub>2</sub>. The cultures were checked by an invert microscope with 24-h intervals and the results of growth and propagation of the parasite were recorded and compared to the control sample. The load of the parasite was calculated using the following formula:

Load of parasite = the mean number of tachyzoites in a host cell determined in 100 infected cells × the ratio of the infected cells to 100 cells in the cell culture.

### The exposed tachyzoites infectivity in animals

A total of 50 mice were divided into 10 groups. Then,  $2 \times 10^5$  tachyzoites treated with different concentrations (1, 2, 4, 8, 16, 32, 64, and 128 µg/mL) of the compound were subcutaneously injected into the mice. Also, five mice as the positive control group were infected by intact tachyzoites and then treated with sulfadiazine and for the negative control group, intact tachyzoites were injected into five mice without any treatment. The mice were followed up for one month. Once the mice died, the liver touch smears were prepared and stained by Giemsa staining to detect the etiological factor.

### Data analysis

The data were entered into the SPSS software (version 16, Chicago, USA) and were analyzed using Mann–Whitney non-parametric test.  $P < 0.05$  was considered to be statistically significant.

### Results

The flow cytometry results of *Toxoplasma* tachyzoites exposed to saponin, as positive control, and different doses of BDM have been shown in Figs. 1 and 2. Accordingly, the highest rate of mortality (72.69%) was seen at the 128 µg/mL concentration of BDM.

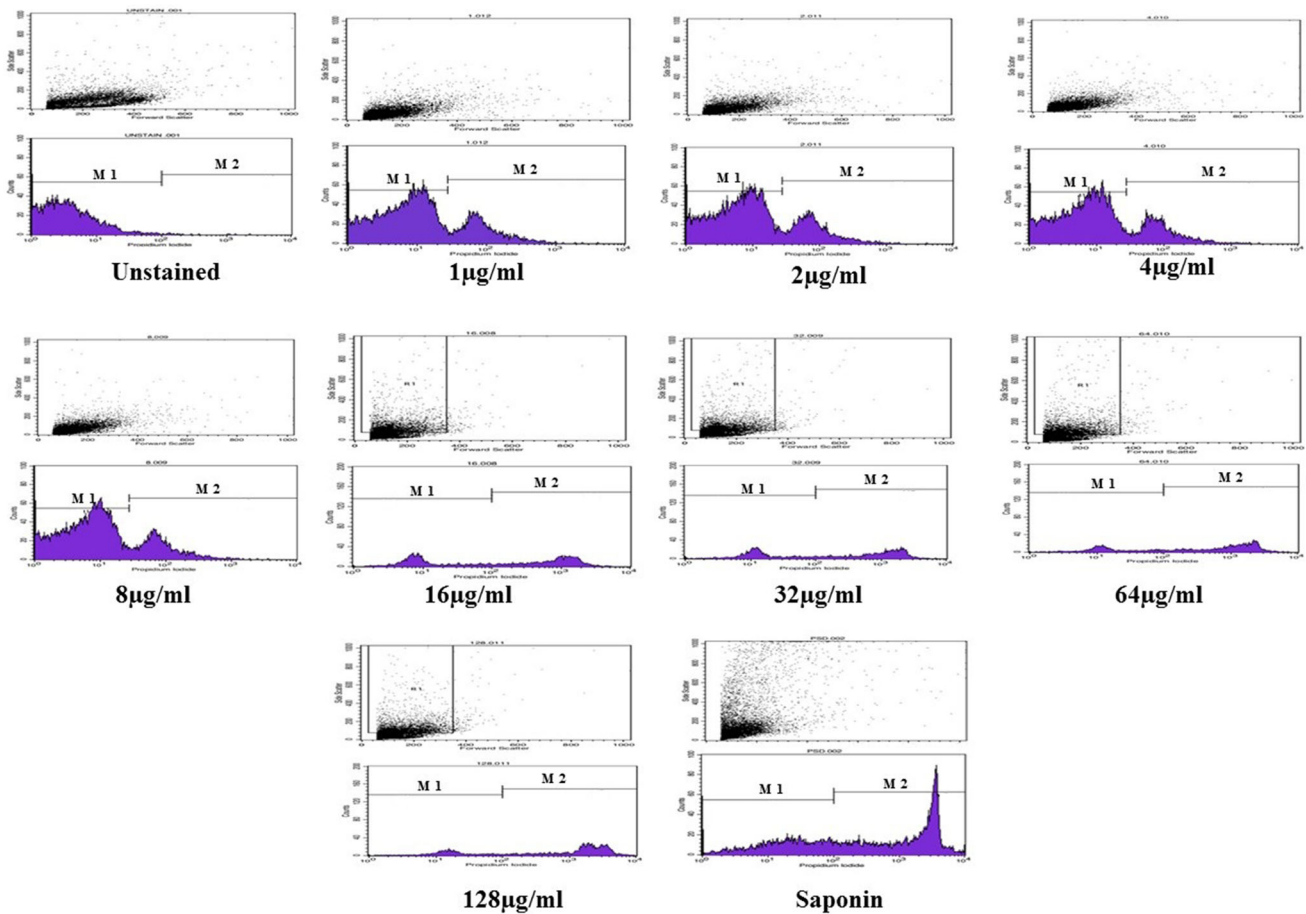
The tachyzoites exposed to different concentrations of BDM were cultured in the HeLa cell line and their infectivity and invasion ability were examined using invert microscopy. As fully illustrated in Fig. 3 and Table 1, the tachyzoites proliferation was observed after 48 h in the exposed parasites to 32, 64, and 128 µg/mL of BDM. However, the proliferation of the exposed tachyzoites to different concentrations of the molecule was seen after 24 h in the macrophage cells. The load of the tachyzoites exposed to different dosages of BDM in the HeLa cell culture for 48 h has been presented in Fig. 3.

The results indicated that all groups of mice died after 13 days. Hence, the infectivity in the tachyzoites of *Toxoplasma gondii* exposed to different dosages of this compound was maintained.

Regarding the results of the Log-Rank test, the mice receiving the tachyzoites exposed to 128 µg/mL of BDM had significant longevity (12.4 days) compared to the control group ( $p < 0.001$ ) (Fig. 4).

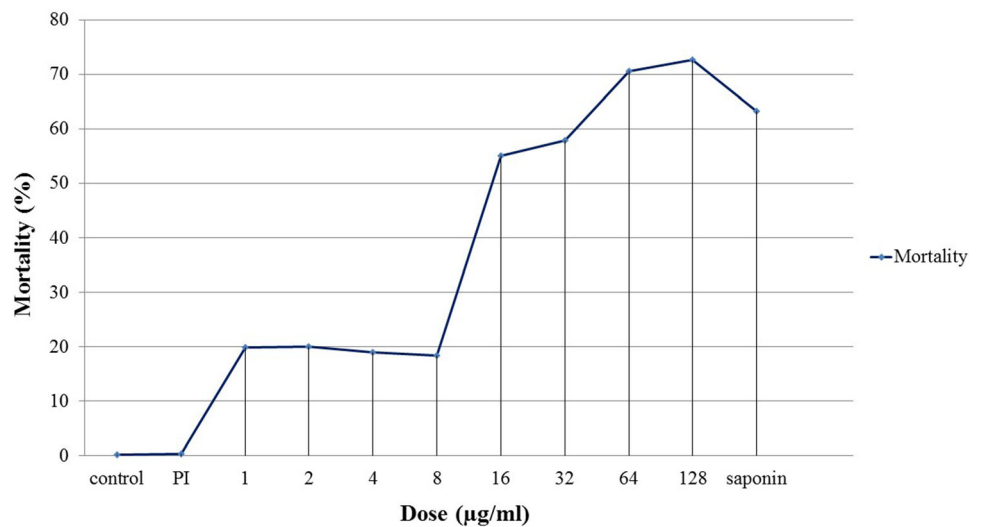
### Discussion

This study aimed to evaluate the effect of BDM on the entrance of *Toxoplasma gondii* tachyzoites. In this protocol, tachyzoites were exposed to different concentrations of BDM and then, the invasive power of these tachyzoites was assessed. In the flow cytometry analysis of different concentrations of the molecule on *Toxoplasma gondii* tachyzoites using PI staining, the highest mortality rate was observed in 128 µg/mL. In vivo results showed a significant difference between the case and negative control groups regarding the 12.4-day mean survival rate at the 128 µg/mL concentration of the molecule. The in vitro and in vivo results were correlated. Moreover, the flow cytometry results showed that the mortality of tachyzoites exposed to 128 µg/mL of BDM was 72%. Thus, the high



**Fig. 1** The flow cytometry results of *Toxoplasma* tachyzoites exposed to different doses of Butanedione monoxime

**Fig. 2** Relationship between different doses of Butanedione monoxime and percentage of mortality of *Toxoplasma* tachyzoites with flow cytometry

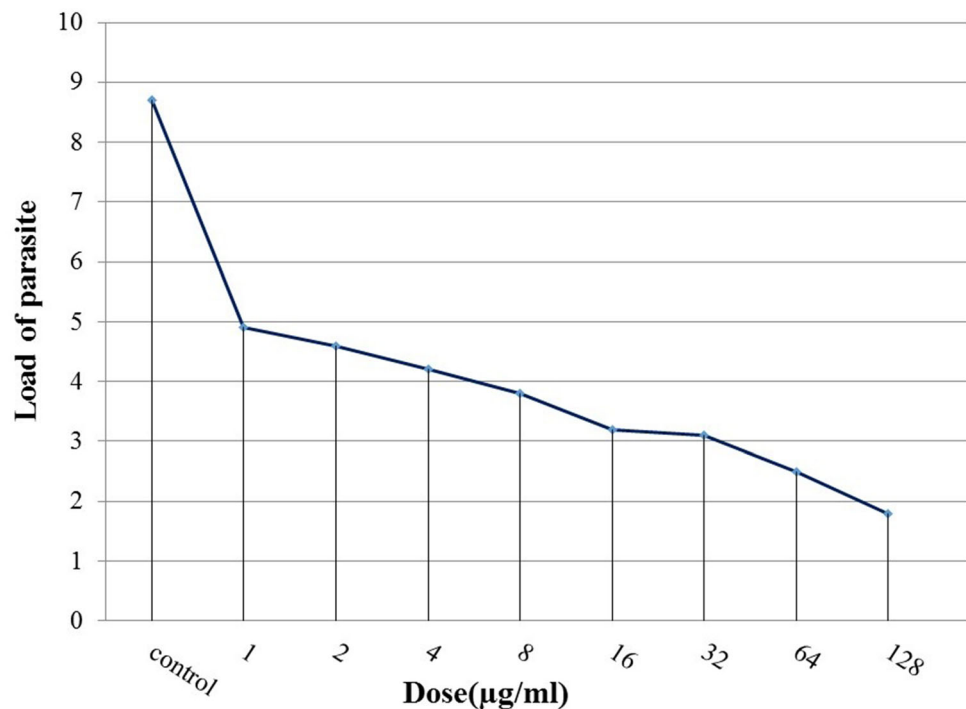


longevity of the mice belonged to the lower vitality of the tachyzoites.

The most important factor in Apicomplexan parasites is their ability to penetrate into host cells (Bargieri et al. 2014). BDM is an inhibitory organic pharmaceutical

molecule (Del Carmen et al. 2009; Ostap 2002), which interacts with myosin ATPase and hampers various functions of myosin. However, it does not affect the actin filaments. In addition to an inhibitory role, these therapeutic agents inhibit the interaction between myoA and actin. It

**Fig. 3** The load of tachyzoites cells exposed to different doses of Butanedione monoxime in HeLa cell culture after 48 h



**Table 1** The results of the proliferation of tachyzoites exposed to Butanedione monoxime in HeLa and macrophage cell line culture at different times; positive control: intact tachyzoites, negative control: not infected cells

	Cell lines					
	HeLa			Macrophage (J774)		
	Tachyzoites exposed to BDM	Positive control	Negative control	Tachyzoites exposed to BDM	Positive control	Negative control
<i>Proliferation time after infection</i>						
8 h	–	+	–	–	+	–
24 h	–	+	–	+	+	–
48 h	+	+	–	+	+	–
72 h	+	+	–	+	+	–

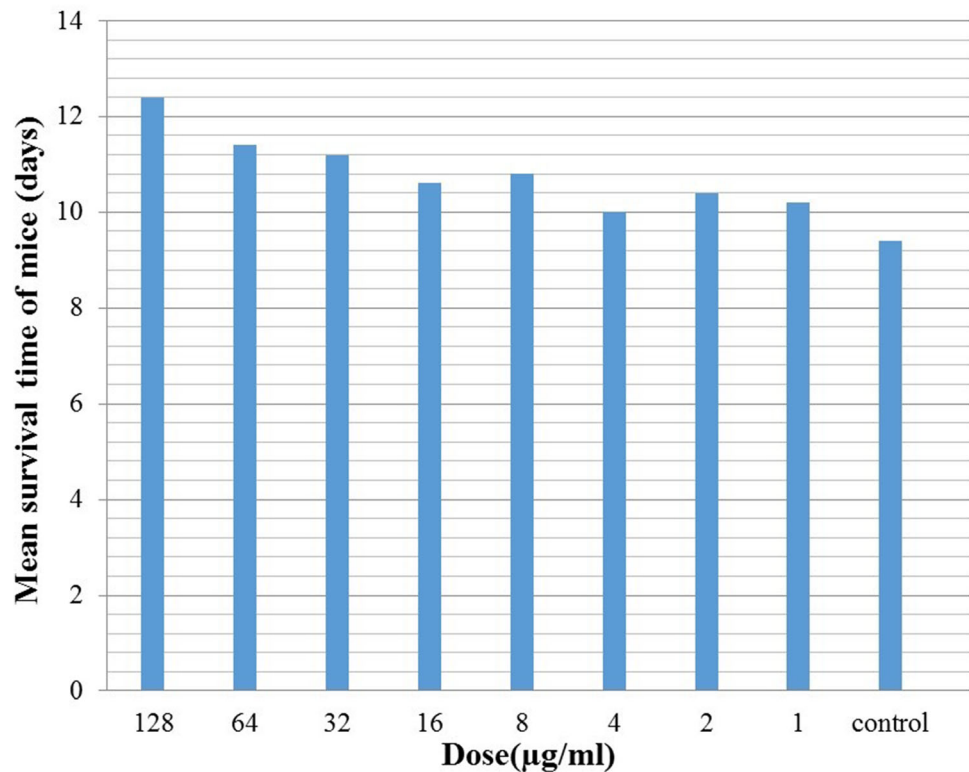
seems, BMD blocks the entrance of *Toxoplasma* into the host cell due to above inhibitory path (Delbac et al. 2001; Wetzel et al. 2005). Furthermore, it has been shown that, BDM as a myosin ATPase inhibitor impedes the invasion of *Plasmodium* species (Campino et al. 2018; Siden-Kiamos et al. 2006). However, a lower concentration of BDM can inhibit the invasion of *Plasmodium* species (Morales et al. 2018).

In this study, parasite proliferation was observed at 32, 64, and 128 µg/mL concentrations of BDM after 48 h in the HeLa cell culture, while the entrance of the parasite into the macrophages did not need time. The reason for the delayed invasion to HeLa cells compared to macrophages was related to their different cell entrance mechanisms. The entrance of the parasite into HeLa cells occurs in an

active form, but macrophages have a phagocytosis system for cell entrance.

A couple of studies have been conducted recently to evaluate the effects of BDM on a range of parasitic infections. In the research carried out by Dabrowolski et al., BDM was suggested as an inhibitor of *Toxoplasma gondii* motility and was introduced as an entrance/motility-limiting agent for *Toxoplasma gondii*. This agent counteracted the microneme secretions and, consequently, impeded the entrance of the parasite, which was consistent with the current study findings (Dobrowolski et al. 1997). Ostap et al. measured the activities of myosin 2 ATPase in skeletal muscle, human myosin, *Acanthamoeba* myosin, and avian isoform of myosin at 0–40 Mm concentrations. BDM represented inhibitory effects merely on the skeletal muscle myosin-2 (Ostap 2002).

**Fig. 4** The mean survival time of mice receiving tachyzoites exposed to the different doses of Butanedione monoxime



Steinberg et al. evaluated the effects of BDM on the morphological characteristics of *Saccharomyces* species and indicated a gradual reduction in the formation and growth of colonies on Agar medium at 1–30 µM BDM concentrations (Steinberg and McIntosh 1998). In another study by Esnault et al., BDM decreased inflation and budding capability of *Aspergillus Fumigatus* species and led to impaired cell wall disruption (Esnault et al. 1999). Furthermore, Schmidt and Wetzel et al. showed that the motility and invasion of *Cryptosporidium Parvum* were hindered by Cytochalasin D and BDM, respectively (Wetzel et al. 2005). In the study by Kiamus and Pinder also, BDM was used as the inhibitor of myosin dysfunction and decreased the motility of *Plasmodium berghei* ookinetes (Siden-Kiamos et al. 2006). Moreover, Kivistö et al. reported that 2,3-BDM, as a myosin II ATPase, could inhibit cardiac contraction in murine ventricular cardiomyocyte media culture (Kirschner Peretz et al. 2017; Kivistö et al. 1995). However, the results of several studies showed that BDM had additional effects, such as altering L-type  $Ca^{2+}$  channel activity, SR  $Ca^{2+}$  load (Gwathmey et al. 1991), cardiac action potential characteristics and conduction (Kettlewell et al. 2004; Lou et al. 2011), and induction of Polymorphonuclear Neutrophils (PMNs) polarity by chemotactic agents (Seveau et al. 1997).

By comparing the results of these studies with the studies mentioned above, it could be concluded that using the parasite paralysis method is a suitable candidate for a

*toxoplasma* vaccine and create a protective response. Also, BDM molecule could be useful in advancing this method due to its effect on inhibiting the *toxoplasma*'s motor system.

The limitations of this study are that protective response generated by the tachyzoites exposed to different concentrations of BDM was only evaluated by examining the Survival time of the mice, which can be done more accurately by examining immune factors such as cytokines and interleukins in future research.

## Conclusions

The tachyzoites exposed to high doses of BDM showed a low infectivity compared to the control group. Nonetheless, obtaining a more appropriate dose as well as a more effective material to paralyze the parasite as a vaccine target could be a good subject for future studies in order to introduce an effective vaccine.

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**Authors' contributions** QA and FM coordinated in designing the project and providing financial supports. SB and MSB Performed the experiments and analyzed the data. QA, SB and MSB carried out

drafted the original manuscript and all authors read and approved the final version of the manuscript.

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**Availability of data and materials** The dataset used and/or analyzed during the current study is available from the corresponding author upon reasonable request.

**Compliance with ethical standards**

**Competing interests** The authors declare that they have no competing interests.

**Ethics approval and consent to participate** This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S653). The guideline of the Institutional Animal Care and Ethics Committee of Animal Experimentation, Shiraz University of Medical Sciences was used to work on animal models in this study.

**Consent for publication** Not applicable.

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