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Short Communication

Comparison of Eight Cell-Free Media for Maintenance of *Toxoplasma gondii* Tachyzoites

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Abstract

Background: Toxoplasmosis is considered as one of the most common infectious diseases caused by the protozoan parasite *Toxoplasma gondii*. Tachyzoite is the main form of *Toxoplasma* and continuously is maintained in cell culture or injected into the mice peritoneal cavity. This study was designed to evaluate the survival rate of RH strain of *T. gondii* tachyzoites in different cell free, nutrient and biological media at different temperatures.

Methods: This experimental study was performed at the Toxoplasmosis Research Center, Mazandaran University of Medical Sciences, Sari, Iran, in 2010. One ml of each solution including hypotonic saline (0.3%), normal saline (0.85%), RPMI-1640 (RPMI), RPMI with 10% fetal bovine serum (FBS), RPMI with 20% FBS, ovine hydatid cyst fluid, pasteurized milk of cow, and phosphate buffered saline (PBS) along with 4×10^4 *T. gondii* tachyzoites were added to plate wells and incubated in 4 °C, 22 °C, 37 °C, and 37 °C under 5% CO₂. The survival rate and viability assessment of parasites were performed daily and the results were analyzed using Univariate tests.

Result: Tachyzoites survival rate in PBS (4 °C) and normal saline (4 °C) were considerably high, compared to other solutions in different conditions ($P < 0.001$). The best temperature for *Toxoplasma* maintenance was 4 °C ($P < 0.001$).

Conclusion: This study introduces two available and economical solutions, PBS (4 °C) and normal saline (4 °C) media, for maintenance of *Toxoplasma* tachyzoites as appropriate choice media for a noticeable period of time (11 days) in vitro.

Introduction

Toxoplasma gondii, an obligate, intracellular protozoan parasite with widespread prevalence worldwide, is able to infect a wide range of vertebrates such as birds and human (1, 2). However, toxoplasmosis is an asymptomatic infection in healthy individuals, commonly causes severe systemic disease and remarkable fatality in immunocompromised especially in HIV-infected individuals. Furthermore, congenital disease leads to hydrocephalus and mental retardation in infants (3). Therefore, toxoplasmosis has been always an interesting issue for many researchers. Most of the previous experimental studies are being performed on tachyzoite, a form of *T. gondii* which causes acute toxoplasmosis and responsible for disease severity (1).

Owing to restriction of survival of tachyzoites in outside the host cell, the parasites must be maintained in animal models or cell cultures. Cryopreservation using liquid nitrogen at -196 °C is a reliable technique for long-term maintenance of live tachyzoites (4). Moreover, there are merely few experimental studies and protocols for maintaining tachyzoites of *T. gondii* in vitro (5-7). The routine methods of keeping alive and active tachyzoites are inoculation of *Toxoplasma* into mice peritoneal cavity, embryos egg as well as culturing in cell culture media. Nonetheless, each of these methods has complications and difficulties such as frequent passage every 2-3 d, high cost of cell cultures, personal permanent attendance, time consuming process, laboratory contamination (needle stick), need to advanced equipment for parasite culturing, preparing of mouse and last but not least, ethical aspect of work on lab animals particularly pathogenic strain such as *Toxoplasma* RH strain which is fatal for mice (5, 8).

Considering aforementioned facts, this investigation was conducted to propose a novel method for maintaining of *T. gondii* tachyzoites

by utilizing new available and biological materials and media in hope of raising the survival rate of tachyzoite in vitro.

Materials and Methods

Parasites

In this experimental study performed at the Toxoplasmosis Research Center, Mazandaran University of Medical Sciences, Sari, Iran, 2010, the RH strain of *T. gondii* tachyzoites were harvested by aspiration of the peritoneal cavity of Swiss-Webster mice injected 4 d previously with 0.5 ml of parasite suspension in sterile cold phosphate buffered saline (PBS; pH=7.4). Harvested fluid from the mice peritoneal cavity was washed twice in PBS, containing 100 IU/ml penicillin and 100 µg/ml streptomycin using centrifuge at 100×g for 10 min at 4 °C to remove peritoneal cells and debris. Then the number of parasites was determined by counting in a haemocytometer under phase-contrast microscopy (×400). The tachyzoites obtained from this procedure were used freshly in examinations.

The project was given approval by the Ethics Committee of Mazandaran University of Medical Sciences, Iran. The care and use of experimental animals complied with local animal welfare laws, guidelines and policies.

Media Preparation

We evaluated eight liquid media to maintain *T. gondii* tachyzoites, including hypotonic saline (0.3%), normal saline (0.85%), RPMI-1640 (RPMI), RPMI with 10% fetal bovine serum (FBS), RPMI with 20% FBS, ovine hydatid cyst fluid, pasteurized milk of cow, and PBS (pH=7.2). For affording the hydatid cyst liquid, after aspiration of cyst liquid, it was centrifuged at 2000×g for 5 min, and then upper liquid was used. For elimination and prevention of bacterial contamination in all media, penicillin (100 IU/ml) and streptomycin (100

$\mu\text{g/ml}$) were added, followed by filtration through a $0.22\ \mu\text{m}$ millipore filter and stored in $0-4\ ^\circ\text{C}$.

Assessment of parasites viability

Tachyzoites of *T. gondii* were counted in haemocytometer with a 40 objective of light microscopy, and the number was adjusted to 4×10^4 parasites/ml. One milliliter of the number adjusted tachyzoites was transferred to each of the 24-wells of cell culture plate along with 3 ml of mentioned different media. This test was done in triplicate. Plates covered by foil also incubated and sustained in diverse condition ($4\ ^\circ\text{C}$, $22\ ^\circ\text{C}$, $37\ ^\circ\text{C}$ and $37\ ^\circ\text{C}$ in 5% CO_2).

The viability of tachyzoites was monitored daily using trypan blue 1%. When the tachyzoites viability reduced to 50%, 0.5 ml of each medium containing tachyzoites was injected to the peritoneal cavity of one Swiss-Webster mouse for determining of infectivity of parasites.

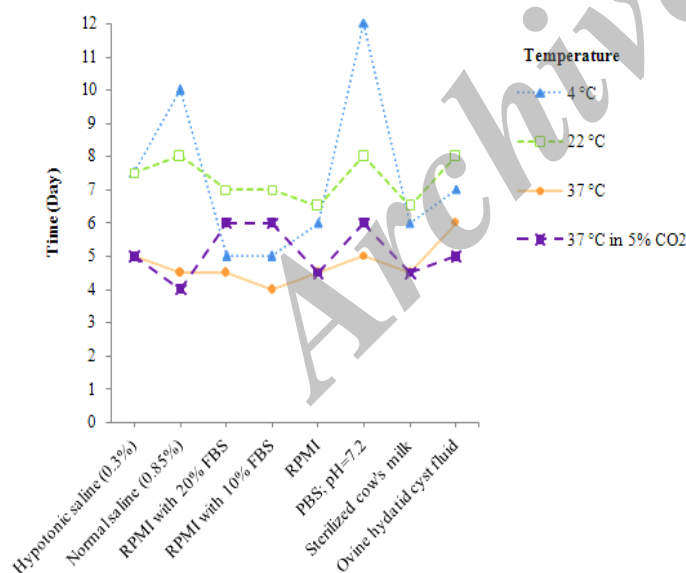


Fig. 1: Survival time of *T. gondii* tachyzoites at different temperature in the eight kinds of cell-free media

Data were analyzed using Univariate tests in SPSS 16 software (Chicago, IL, USA).

Results

The highest survival rate of tachyzoites was observed in PBS followed by normal saline ($P < 0.001$) (Fig. 1). Additionally, the tachyzoites survival rate was varied for each medium at various temperatures. In the other media, *Toxoplasma* remained stable for a short period and then slightly regressed (Fig. 2). In addition, *Toxoplasma* survival times in these media are influenced by the temperature. The best temperature for *Toxoplasma* preservation was $4\ ^\circ\text{C}$ ($P < 0.001$). In addition, interaction between temperature degrees and different media was statistically significant ($P < 0.001$) (Table 1). For survey of *Toxoplasma* infectivity, 0.5 ml of each solution containing tachyzoites was injected to one mouse peritoneum that resulted in mice death during 3-5 d.

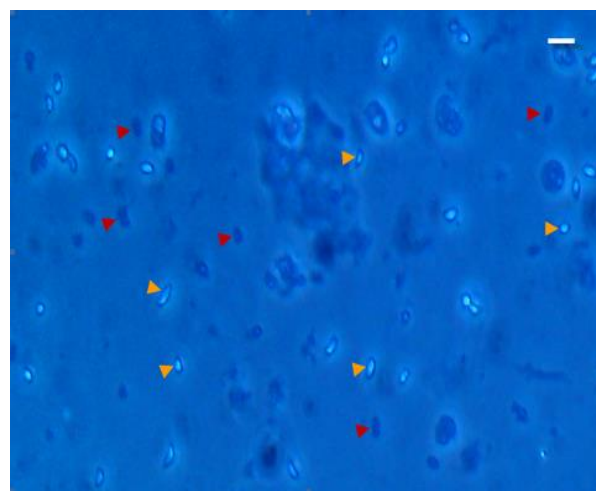


Fig. 2: The viability test using 1% trypan blue: viable tachyzoites excluded trypan blue dye and were seen transparent (orange arrowhead) and dead tachyzoites absorbed the dye and were seen dark blue (red arrowhead), under phase-contrast microscopy. Scale bar represent $10\ \mu\text{m}$

Table 1: Difference between mean of survival time of *T. gondii* tachyzoites in four temperatures with viability 50% in various media in pairwise comparisons

	Normal Saline %0.85	RPMI+FBS %20	RPMI+FBS %10	RPMI	PBS	Milk	Hydatid Liquid
Hypotonic Saline %0.3	-0.25	.83*	0.75	.83*	-1.50*	.83*	-0.25
Normal Saline %0.85	-	1.08*	1.00*	1.08*	-1.25*	1.08*	0
RPMI+FBS %20		-	-0.08	0	-2.33*	0	-1.08*
RPMI+FBS %10			-	0.08	-2.25*	0.08	0.08
RPMI				-	-2.33*	0	-1.08*
PBS					-	2.33*	1.25*
Milk						-	-1.08*

(*Statistically Significant in 5%)

Discussion

Our findings showed that the best medium and temperature for long-term maintenance of *T. gondii* tachyzoites are PBS and 4 °C. *T. gondii* is an obligatory intracellular parasite, which does not grow in cell-free media (9, 10). Usually tachyzoites of *T. gondii* are proliferated intraperitoneally in mice being used as the major source of antigen in serological diagnostic tests (Dye test, ELISA, IFA and DAT) in laboratories (5, 8, 11). Moreover, preserve and storage of viable tachyzoites are seemed to be necessary in other study fields such as immunization, therapeutic, biochemical, genetically and molecular researches (2).

Rats are considered as proper lab animals, which keeps the parasite as tissue cysts approximately 6-12 months. When a large numbers of organisms are needed, eggs and mice are applied regularly but mice cannot tolerate the pathogenic strain (RH) more than 3-4 d. Easy management and ethical values of cell culture make it different from using animal models in order to multiply tachyzoites (5, 7). The freezing technique using liquid nitrogen is another method for *Toxoplasma* tachyzoites and bradyzoites preserving, that more survival rate is achieved using Dimethyl sulphoxide (DMSO). In addition to unavailability of nitrogen tank in all laboratories, this method has unignorable disadvantages, which are hazardous for personal attendance (2, 12, 13).

Our results show that tachyzoites survival rate in PBS (4 °C) and normal saline (4 °C) has been higher than other solutions and tem-

peratures. Low temperatures increase the quantity and quality of *Toxoplasma* viability (9, 14). In all of eight media, higher viability of *Toxoplasma* was seen in lower temperature (4 °C). In addition, in the normal saline and PBS solutions (without nutrients, vitamins, proteins and fats), tachyzoites had maximum survival rate in comparison with media containing nutrients, carbohydrates, fats and vitamins.

Regarding to *T. gondii* intracellular living, outside of the cell is inappropriate for this parasite and because of its inability to proliferation and reproduction, tachyzoites have minimum metabolically activity (2).

Tachyzoites excessively absorb and store calcium (Ca^{2+}) in acidocalcisome (15). The function of Ca^{2+} , however, in the recent organelle is not clearly understood, but the organelle acts as a calcium reservoir to release it in a critical situation for homeostasis of the parasite (16). Therefore, it seems that Ca^{2+} may play a key role in the survival of the parasite but in milk that contains Ca^{2+} and various integrants such as proteins and vitamins, it was observed a low survival rate compared to PBS.

Amino acids are extremely important for many life processes such as metabolic pathways (17). Hence, RPMI medium was used as a medium rich in amino acids increase the parasite metabolic activities to evaluate its effect on the tachyzoites survival rate. Furthermore, the influence of RPMI medium containing FBS on the parasite survival was also evaluated; because FBS contains a high amount of albumin, acting as a micro carrier, to make vital macromolecules easily available for

tachyzoites, so it was possible to influence the parasite survival (18). In addition, hydatid cyst fluid consists of a variety types of lipids, carbohydrates, electrolytes, proteins, and as well as other nutrients, as likely to influence the parasite survival (19). Furthermore, hypotonic saline (0.3%) and normal saline (0.85%), as an isotonic solution were used for survey on osmotic pressure effect on tachyzoite survival. *T. gondii* tachyzoite is resistant to osmotic pressure and may stay alive outside the host long enough to transmit toxoplasmosis to a new host (20). The findings in the present study are parallel to the recent study in that tachyzoites appear to be able to withstand relatively the osmotic pressure changes.

Although proteins are crucial macromolecules in the intracellular survival of tachyzoites is well studied (21), our results showed that extracellular survival rate of tachyzoites in the media rich in protein including milk, hydatid cyst fluid, and RPMI with FBS was lower than that in the protein-free media, namely PBS and saline solutions.

Lack of FCS in the maintenance media such as Hep2, RDA and MDBK does not noticeably reduce multiplication of *T. gondii* tachyzoites (22). In these serum free media, tachyzoites survived for 14 days at room temperature or at 4 °C and in the dark.

In this survey, the nutrient media were not necessary for maintaining of *Toxoplasma* tachyzoites. Probably in the nutrient media such as RPMI, RPMI + FBS10% & 20%, hydatid cyst liquid and sterile milk, parasites excreted metabolic materials that were threat for parasite survive. Versus in simple media such as normal saline and PBS that have balanced osmosis pressure, the lowest degree of stress and hazard threats the parasite existence. Thus, in simple media, that parasite merely tries to survive and has the lowest metabolic activity, the most survival rate and viability was seen. However, for elimination of the harm metabolic materials in nutrient media, it is necessary to carry out serial weekly (23, 24). On the other hand, it seems that the parasite metabo-

lites in the nutrients media (milk, hydatid cyst fluid, and RPMI with/without FBS) have negative effects on the parasite survival, because the metabolites bring about change in pH of medium, leading to undesirable conditions for tachyzoite to survive. Therefore, it can be supposed that tachyzoite is extremely sensitive to pH changes as the obtained results confirm that the highest survival rate was observed in PBS, a strong ionic solution. It was also revealed that the best tachyzoite survival and viability rate occurred at 4°C that is likely related to reduce extremely the biological activities in this temperature.

In different studies, various media have been used for maintenance of intracellular parasites. Fluid of sheep hydatid cyst can be a suitable substituting for FBS in *Leishmania major* cultures (25). Moreover, Muniaraj et al. for primary isolation, cultivation and maintenance of *Leishmania donovani* promastigotes, introduced milk of cow, buffalo and goat as the alternative choice for FBS replacement (22).

Conclusion

Low temperature (4 °C) and non-nutrient solution are two important factors reducing the parasite metabolic activities, which lead to produce lower metabolites by tachyzoite and increase the parasite survival time. Consequently normal saline and PBS as available, economical and simple solutions could be useful for maintenance of *T. gondii* for 10-12 d.

Acknowledgments

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References

1. Petersen E. Toxoplasmosis. Semin Fetal Neonatal Med. 2007; 12(3):214-223.

2. Weiss LM, Kim K. *Toxoplasma gondii*: The model apicomplexan. Perspectives and methods. Academic Press; 2011.
3. Remington JS, McLeod R, Thulliez P, Desmonts G. Toxoplasmosis, in Infectious Diseases of the Fetus and Newborn Infant - Chapter 31 (Sixth Edition). W.B. Saunders: Philadelphia; 2006. p. 947-1091.
4. Zheng H, Chen Y, Lu F, Liu M, Yang X, Fu X, Zhao Y, Huang B, Huang S, Kasper LH. Cryopreservation of *Toxoplasma gondii* in infected murine tissues. Parasitol Res. 2012; 111(6):2449-2453.
5. Dubey JP, Frenkel JK. Toxoplasmosis of rats: a review, with considerations of their value as an animal model and their possible role in epidemiology. Vet Parasitol. 1998; 77(1):1-32.
6. Hofflin JM, Remington JS. Tissue culture isolation of *Toxoplasma* from blood of a patient with AIDS. Arch Intern Med. 1985; 145(5):925.
7. James GS, Sintchenko VG, Dickeson DJ, Gilbert GL. Comparison of cell culture, mouse inoculation, and PCR for detection of *Toxoplasma gondii*: effects of storage conditions on sensitivity. J Clin Microbiol. 1996; 34(6):1572-1575.
8. Blaker RG. Laboratory maintenance of *Toxoplasma gondii*. J Bacteriol. 1961; 82(2):315-316.
9. Diab MR, El-Bahy MM. *Toxoplasma gondii*. Virulence of tachyzoites in serum free media at different temperatures. Exp Parasitol. 2008; 118(1):75-79.
10. Weiss LM, Laplace D, Takvorian PM, Tanowitz HB, Cali ANN, Wittner M. A cell culture system for study of the development of *Toxoplasma gondii* bradyzoites. J Eukaryot Microbiol. 1995; 42(2):150-157.
11. Olisa EG, Herson J, Headings VE, Poindexter HA. *Toxoplasma gondii*. Survival time and variability in mouse host strains. Exp Parasitol. 1977; 41(2):307-313.
12. Eyles DE, Coleman N, Cavanaugh DJ. Preservation of *Toxoplasma gondii* by freezing. J Parasitol. 1956; 42(4):408-413.
13. Miyake Y, Karanis P, Uga S. Cryopreservation of protozoan parasites. Cryobiol. 2004; 48(1):1-7.
14. Chatterton JMW, Evans R, Ashburn D, Joss AWL, Ho-Yen DO. *Toxoplasma gondii* in vitro culture for experimentation. J Microbiol Meth. 2002; 51(3):331-335.
15. Rohloff P, Miranda K, Rodrigues JCF, Fang J, Galizzi M, Plattner H, Hentschel J, Moreno SNJ. Calcium uptake and proton transport by acidocalcisomes of *Toxoplasma gondii*. PloS One. 2011; 6(4): e18390.
16. Morlon-Guyot J, Berry L, Chen CT, Gubbels MJ, Lebrun M, Daher W. The *Toxoplasma gondii* calcium dependent protein kinase 7 is involved in early steps of parasite division and is crucial for parasite survival. Cell Microbiol. 2014; 16(1):95-114.
17. Wu L, Chen S-x, Jiang X-g, Fu X-l, Shen Y-j, Cao J-p. Separation and purification of *Toxoplasma gondii* tachyzoites from in vitro and in vivo culture systems. Exp Parasitol. 2012; 130(1):91-94.
18. Gebb C, Clark JM, Hirtenstein MD, Lindgren G, Lindskog U, Lundgren B, Vretblad P. Alternative surfaces for microcarrier culture of animal cells. Dev Biol Stand. 1981; 50:93.
19. Juyi L, Yan J, Xiufang W, Zhaoqing Z, Junliang L, Mingxing Z, Wei Z. Analysis of the chemical components of hydatid fluid from *Echinococcus granulosus*. Rev Soc Bras Med Trop. 2013; 46(5):605-610.
20. Räsänen S, Saari M. The survival of *Toxoplasma gondii* trophozoites in changes in osmotic pressure. Med Biol. 1976; 54(2):152.
21. Fentress SJ, Behnke MS, Dunay IR, Mashayekhi M, Rommereim LM, Fox BA, Bzik DJ, Taylor GA, Turk BE, Lichti CF, Townsend RR, Qiu W, Hui R, Beatty WL, Sibley LD. Phosphorylation of Immunity-Related GTPases by a *Toxoplasma gondii*-Secreted Kinase Promotes Macrophage Survival and Virulence. Cell Host & Microbe. 2010; 8(6):484-495.
22. Muniaraj M, Lal CS, Kumar S, Sinha PK, Das P. Milk of cow (*Bos taurus*), buffalo (*Bubalus bubalis*), and goat (*Capra hircus*): a better alternative than fetal bovine serum in media for primary isolation, in vitro cultivation, and maintenance of *Leishmania donovani* promastigotes. J Clin Microbiol. 2007; 45(4):1353-1356.
23. Marr J, Muller M. Biochemistry and molecular biology of parasites. Academic Press: Science; 1995.
24. McLeod R, Mack D, Brown C. *Toxoplasma gondii*-New advances in cellular and molecular biology. Exp Parasitol. 1991; 72(1):109-121.
25. Fakhari M, Habibi P, Motazedian MH. Evaluation of Hydatid cyst fluid as a substitute for fetal bovine serum (FBS) in culture of *Leishmania major*. Tabib-E-SHagh. 2006; 8 (1):47-52.