



Comparative evaluation of amniotic fluid as an alternative to fetal bovine serum in the maintenance of *Leishmania major* and *Toxoplasma gondii*

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Received: 26 June 2020 / Accepted: 17 January 2021 / Published online: 25 January 2021
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Abstract

This study aimed to evaluate the efficacy of amniotic fluid (AF) as an alternative to fetal bovine serum (FBS) in the maintenance of *Leishmania major* promastigotes and *Toxoplasma gondii* tachyzoites. AF was collected by an obstetrician using sterile syringes during a cesarean section. The culture medium was supplemented with 5 different concentrations of FBS or AF including 2, 5, 10, 20, and 30%. These concentrations were used to maintain both mentioned parasites. *L. major* was maintained at temperatures 4 and 24 °C and examined once a week for 4 weeks, while *T. gondii* was maintained at temperatures 4, 24, and 37 °C and examined at hours 24, 48, 72, and 96. For *L. major*, at both 4 and 24 °C, we observed no significant difference between FBS and AF on day 7. However, on days 14, 21, and 28, the difference between FBS and AF was significant at both temperatures. For *T. gondii*, no significant difference was observed between FBS and AF at hour 24 and all temperatures. However, this difference was significant at hours 48, 72, and 96 and all temperatures. According to our results, although FBS had a greater efficacy than AF in the growth of *L. major* and the survival of *T. gondii*, the number of promastigotes increased over time in AF-containing medium and the number of tachyzoites reduced slowly with a mild slope. Therefore, AF can be a potential alternative to FBS.

Keywords Amniotic fluid · Culture · Fetal bovine serum · *Leishmania major* · *Toxoplasma gondii*

Introduction

Leishmania major (*L. major*) and *Toxoplasma gondii* (*T. gondii*) are parasitic protozoa, which infect human (Walker et al. 2014). These parasites have always been an interesting issue for many researchers. Therefore, maintenance

of these parasites in the laboratory is essential and important for research.

The parasite cultivation techniques are used to isolate parasites from clinical samples for diagnosis and to study their various aspects such as physiological, biochemical, and antigenic features. In addition, cultivation has numerous values in

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vaccine design and evaluation of its effectiveness, specific antigen extraction, drug-resistance assessment, and screening of potential therapeutic agents (Ahmed 2014; Visvesvara and Garcia 2002).

RPMI-1640, one of the basic liquid culture media, is a growth medium used in cell culture. Fetal bovine serum (FBS) with a final concentration of 10–30% is commonly used as a supplementary medium for better cell growth and to enrich the cell and tissue culture media (Helgason and Miller 2005; Limoncu et al. 2004). This culture medium contains many growth-supporting compounds, nutrients, and energy sources, and it is the most important source of proteins (Ali et al. 1998; Sandle 2014). The main disadvantages of FBS include high cost and toxic effects on cells when used in high amounts in the culture media (Becker et al. 1996; Newman 2003).

Amniotic fluid (AF) surrounds the embryo and fetus during growth. AF has many functions, and it protects the fetus from infectious agents due to innate antibacterial properties. Moreover, it serves as a reservoir of fluid and nutrients, containing proteins, electrolytes, immunoglobulins, and vitamins (Tong et al. 2009). During childbirth, the

amniotic sac ruptures and the fluid inside it comes out. Therefore, AF can be easily collected and is available for free (Kamath-Rayne et al. 2014), and these are the reasons why we focused on it.

Accordingly, in the present study, for the first time, AF was used as an alternative to FBS in the growth and maintenance of *L. major* and *T. gondii*.

Materials and methods

Amniotic fluid collection

In this experimental study, AFs were collected by an obstetrician using sterile syringes during a cesarean section at the Imam Khomeini Hospital, Sari, Iran. The AF was transferred to the parasitology laboratory of the Mazandaran University of Medical Sciences (MAZUMS), Sari, Iran, and after centrifuging at $3000\times g$ for 10 min, the supernatant was filter-sterilized using a $0.22\text{-}\mu\text{m}$ pore size syringe filter (Corning, Oneonta, NY, USA) and stored at $-20\text{ }^{\circ}\text{C}$ until used. This study was reviewed and approved by the

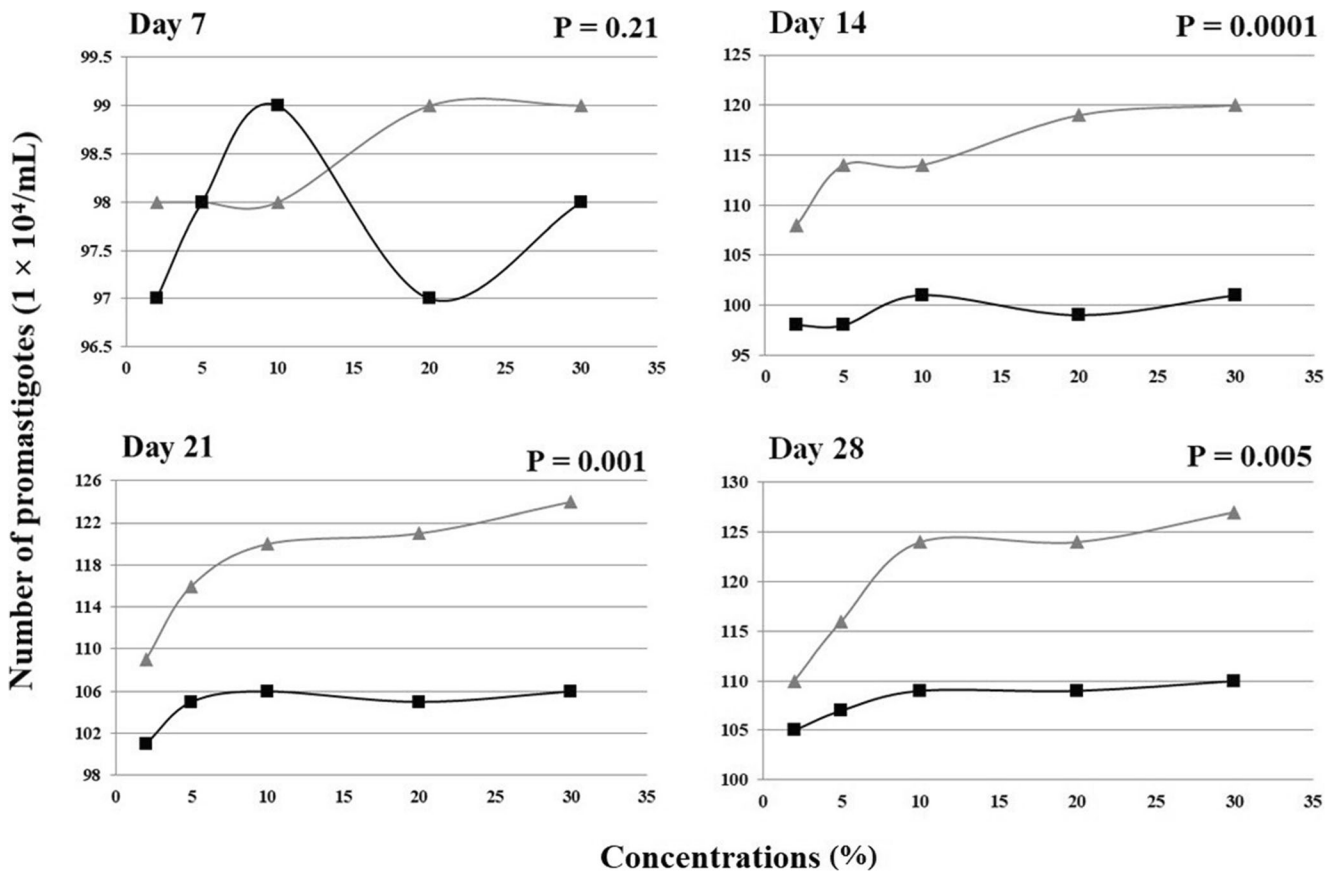


Fig. 1 The number of promastigotes in both media, fetal bovine serum (FBS) and amniotic fluid (AF), at $4\text{ }^{\circ}\text{C}$ in different days. The number of promastigotes was calculated using a hemocytometer under a light microscope with $\times 400$ magnification. Triangle, FBS; square, AF

Research Ethics Committee of MAZUMS (Ethical Code: IR.MAZUMS.REC.1399.361). An informed consent was obtained from all participants.

Assessment of AF or FBS on *L. major* maintenance

For this purpose, *L. major* promastigotes (MRHO/IR/75/ER), maintained in laboratory conditions, were used (Faridnia et al. 2018). RPMI-1640 medium (Gibco, Grand Island, NY, USA) was supplemented with 5 different concentrations of FBS (Gibco, Grand Island, NY, USA) or AF including 2, 5, 10, 20, and 30% in a volume of 4.5 mL each. Two sets of these 5 concentrations were prepared for each of FBS or AF to which 2.5×10^6 *L. major* promastigotes in a volume of 0.5 mL was added. The final volume was 5 mL for each tube. The total number of tubes was 20, of which 10 tubes were considered for FBS and 10 other ones for AF. Out of 10 tubes for each of FBS or AF, 5 were incubated at 4 °C and 5 other ones at 24 °C. The tubes were examined once a week for 4 weeks. Accordingly,

Table 1 Comparison of the number of promastigotes in FBS and AF during times (4 weeks) and at each of 4 and 24 °C by repeated-measures ANOVA

Medium	Degree centigrade	F ^a	df ^b	p value
FBS ^c	4	53.1	3	< 0.00001
	24	30.79	3	< 0.00001
AF ^d	4	91.5	3	< 0.00001
	24	50.4	3	< 0.00001

^aF-table

^bDegrees of freedom

^cFetal bovine serum

^dAmniotic fluid

20 µL of each tube contents was added to 20 µL of 2% formaldehyde in phosphate-buffered saline (PBS; pH = 7.2). The number of promastigotes was then counted using a hemocytometer under a light microscope with $\times 400$ magnification. The examination was performed in triplicate for each tube.

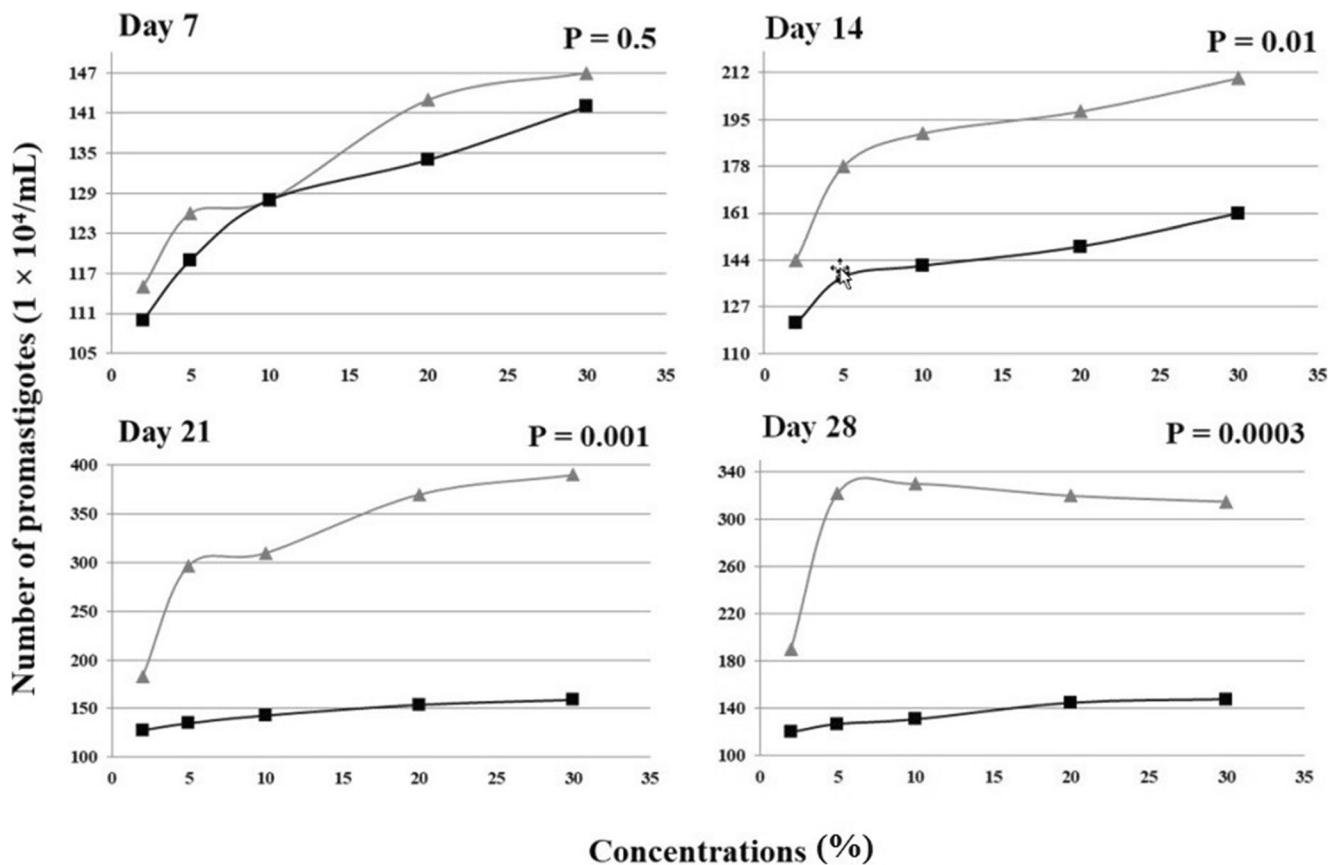


Fig. 2 The number of promastigotes in both media, fetal bovine serum (FBS) and amniotic fluid (AF), at 24 °C in different days. The number of promastigotes was calculated using a hemocytometer under a light microscope with $\times 400$ magnification. Triangle, FBS; square, AF

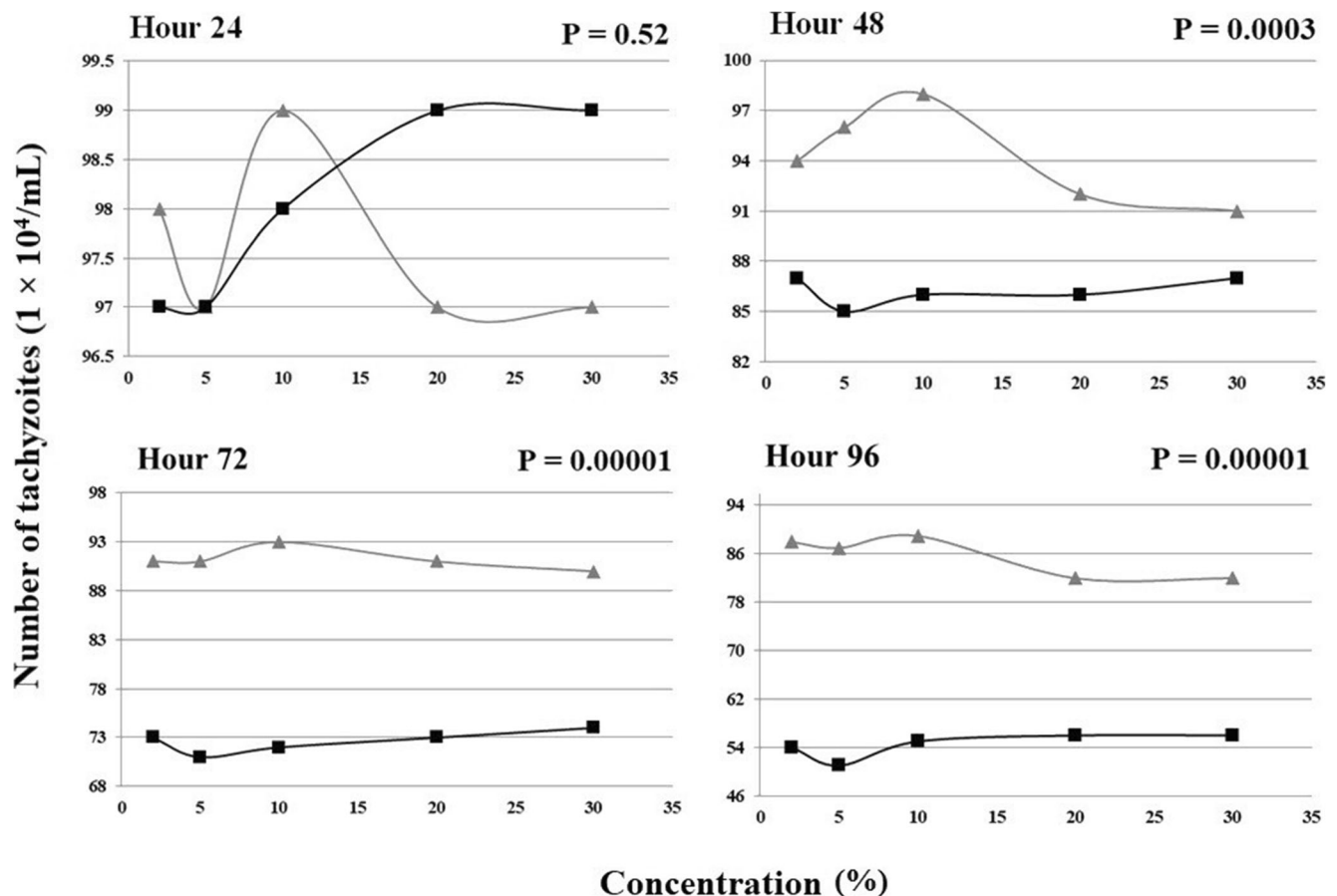


Fig. 3 The number of tachyzoites in both media, fetal bovine serum (FBS) and amniotic fluid (AF), at 4 °C in different days. The number of tachyzoites was calculated using a hemocytometer under a light microscope with $\times 400$ magnification. Triangle, FBS; square, AF

Assessment of AF or FBS on *T. gondii* maintenance

Freshly harvested tachyzoites of *T. gondii* RH strain from cell culture medium were used for this purpose (Kalani et al. 2016b). RPMI-1640 medium was supplemented with 5 different concentrations of FBS or AF including 2, 5, 10, 20, and 30% in a volume of 4.5 mL each. Three sets of these 5 concentrations were prepared for each of FBS or AF to which 5×10^6 *T. gondii* tachyzoites in a volume of 0.5 mL was added. The final volume was 5 mL for each tube. The total number of tubes was 30, of which 15 tubes were considered for FBS and 15 other ones for AF. Out of 15 tubes for each of FBS or AF, 5 were incubated at 4 °C, 5 at 24 °C, and 5 other ones at 37 °C. The tubes were examined at hours 48, 72, and 96. Accordingly, the number of tachyzoites was counted in 20 μL of each tube contents using a hemocytometer under a light microscope with $\times 400$ magnification. The examination was performed in triplicate for each tube.

Statistical analysis

The IBM SPSS v16 software (IBM SPSS Inc., Chicago, IL, USA) was utilized for statistical analysis. The repeated

measures analysis of variance (ANOVA) and two-tailed *t* test was used for analysis and the results were significant where *p* value was < 0.05 .

Results

L. major maintenance in AF and FBS media

The results showed that the number of promastigotes at 4 °C on day 7 was not significantly different between the FBS and AF groups ($p = 0.21$). However, on days 14, 21, and 28, this difference was significant and the highest difference was observed on day 14 ($p = 0.0001$) (Fig. 1). Moreover, at 24 °C on day 7, there was not a significant difference between these two groups ($p = 0.5$). However, on days 14, 21, and 28, the difference was significant and the highest difference was observed on day 28 ($p = 0.0003$) (Fig. 2).

The results of comparing the number of promastigotes in each of the two media (FBS and AF) separately at different times and temperatures 4 and 24 °C showed that there was a significant difference in the number of promastigotes ($p < 0.05$) (Table 1).

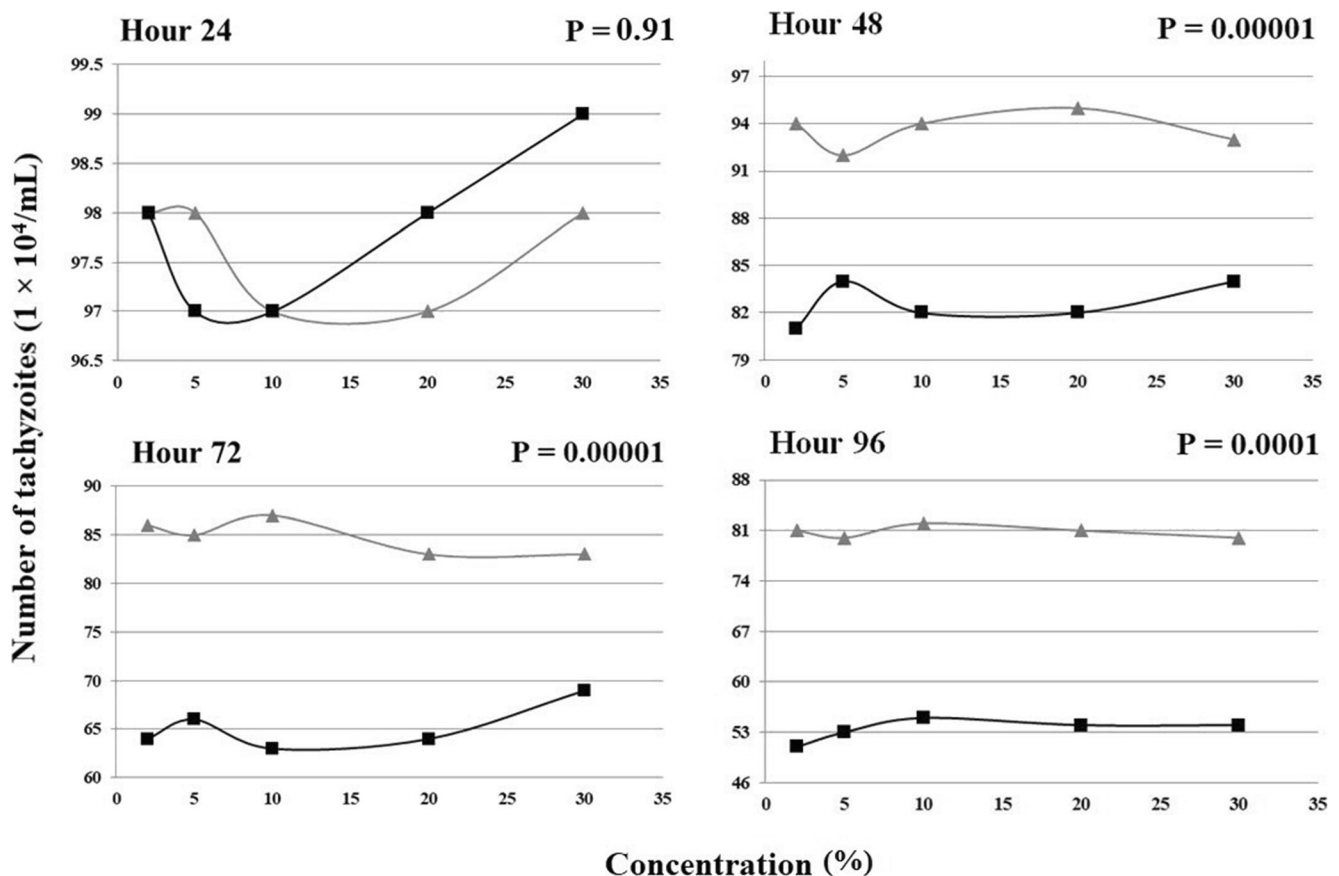


Fig. 4 The number of tachyzoites in both media, fetal bovine serum (FBS) and amniotic fluid (AF), at 24 °C in different days. The number of tachyzoites was calculated using a hemocytometer under a light microscope with $\times 400$ magnification. Triangle, FBS; square, AF

T. gondii maintenance in AF and FBS media

The results showed that the number of tachyzoites at 4 °C and hour 24 was not significantly different between the FBS and AF groups ($p = 0.52$). However, at hours 48, 72, and 96, this difference was significant and the highest difference was observed at hours 72 and 96 ($p = 0.00001$) (Fig. 3). Furthermore, at hour 24 and temperatures of 24 °C ($p = 0.91$) and 37 °C ($p = 0.37$), there was not a significant difference between these two groups (Figs. 4 and 5). However, at hours 48, 72, and 96, this difference was significant and the highest difference was observed at hours 48 and 72 ($p = 0.00001$) (Fig. 4). At 37 °C and hour 24, no significant difference was observed between the FBS and AF groups ($p = 0.37$). However, at hours 48, 72, and 96, this difference was significant and the highest difference was observed at hour 72 ($p = 0.00001$) (Fig. 5).

The results of comparing the number of tachyzoites in each of the two media (FBS and AF) separately at different times and temperatures 4, 24, and 37 °C showed that there was a significant difference in the number of tachyzoites ($p < 0.05$) (Table 2).

Discussion

In vitro techniques are important for cell and tissue culture. The most frequently used supplementary solution in the media is FBS, containing hormones, vitamins, salts, electrolytes, and other growth factors that should be free of blood cells, platelets, or other coagulation factors (Jochems et al. 2002).

Studies have showed that growth factors in AF play an important role in the growth and development of the human embryo (Hui and Bianchi 2011). The effect of AF on the growth of human corneal endothelial cells was compared with FBS, and the researchers concluded that these cells proliferated and grew more rapidly in DMEM-F12 supplemented with 20% AF than those supplemented with 20% FBS. Furthermore, these cells were appropriately established in DMEM-F12 containing 20% AF and their morphology was not changed. Therefore, the authors suggested the use of AF instead of FBS in the cell culture medium (Feizi et al. 2014). In the present study, we observed that there was a significant difference in the number of *L. major* promastigotes at both 4 and 24 °C on each of the days 14, 21, and 28 between AF and FBS groups. Moreover, there was a significant difference in the number of *T. gondii* tachyzoites at 4, 24, and 37 °C and

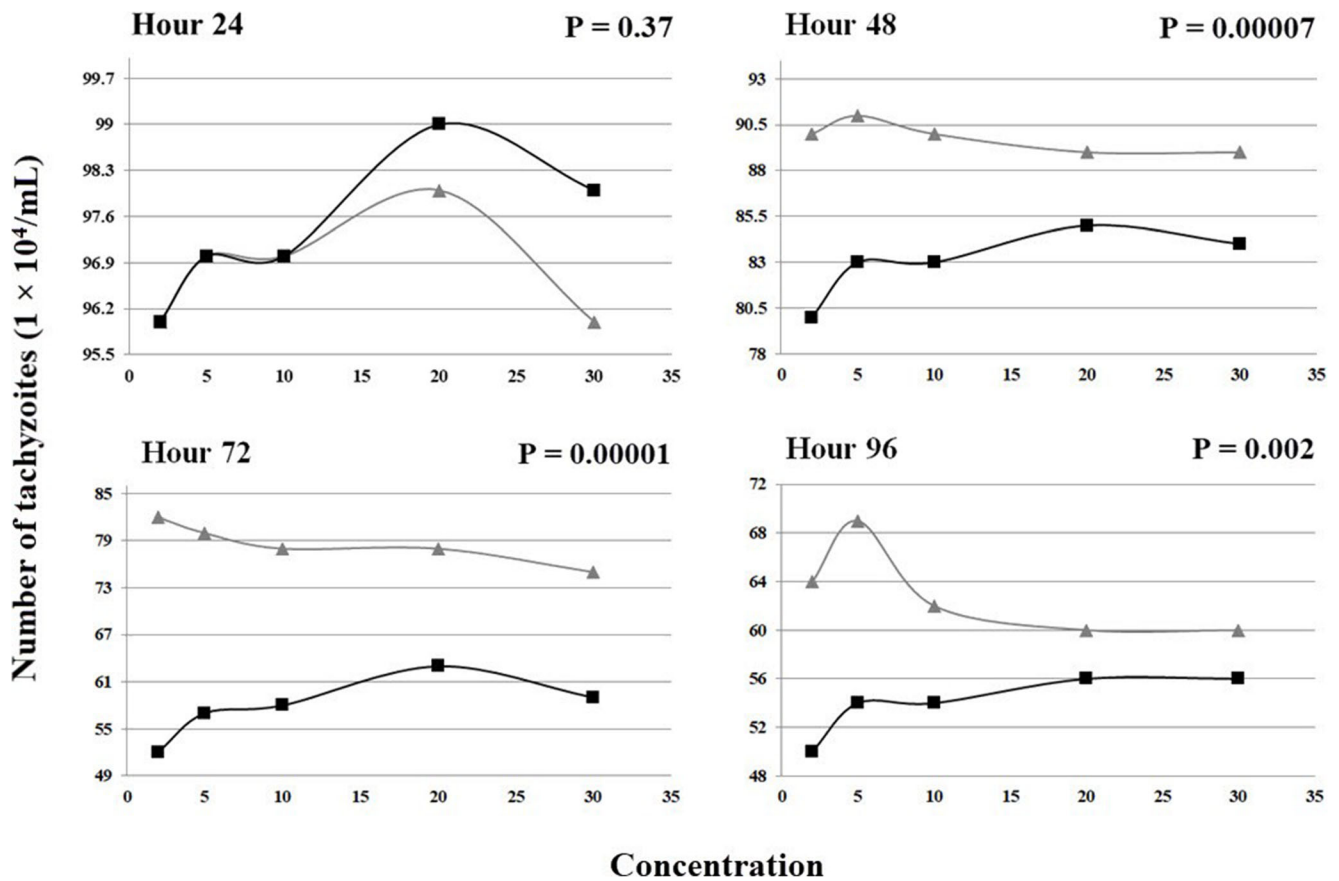


Fig. 5 The number of tachyzoites in both media, fetal bovine serum (FBS) and amniotic fluid (AF), at 37 °C in different days. The number of tachyzoites was calculated using a hemocytometer under a light microscope with $\times 400$ magnification. Triangle, FBS; square, AF

each of 48, 72, and 96 h between AF and FBS groups. Therefore, the effect of FBS was better in the maintenance of both the parasites than AF. It should also be noted that the latter study examined the effect of AF on corneal endothelial cells, but we did it on the two parasites.

The survival rate and viability of *T. gondii* tachyzoites in cow milk and ovine hydatid cyst fluid (HCF) in comparison

with RPMI-1640 supplemented with 10% and 20% FBS showed that tachyzoites were more notably survived in ovine HCF than the medium with 20% FBS. Survival time in milk and FBS-supplemented media was almost the same and there was no significant difference. The authors in the recent study concluded that the best condition for *T. gondii* tachyzoite maintenance was PBS at 4 °C, in which the parasite survived for 12 days (Kalani et al. 2016a). However, this finding needs further evaluation since other researchers have showed that RPMI-1640 is more successful than PBS in the maintenance of *T. gondii* tachyzoites (El-Bahy et al. 2018). In the present study, we observed that the FBS-supplemented medium is more appropriate than AF for *T. gondii* tachyzoite maintenance.

In another study, *L. major* promastigotes were maintained in different solutions, including brain heart infusion (BHI) broth, BHI with 5% FBS, BHI with 10% FBS, HCF, BHI with 5% HCF, and BHI with 10% HCF. The findings indicated that promastigotes were notably proliferated in BHI with 10% FBS as compared to other media, in which the highest number of promastigotes (448×10^5) was observed on day 6 and at 24 °C. This value at this time was 96×10^5 for HCF, 224×10^5 for BHI with 5% HCF, and 512×10^5 for BHI with 10% HCF, indicating a difference significantly between BHI

Table 2 Comparison of the number of tachyzoites in FBS and AF during times (96 h) and at each of 4, 24, and 37 °C by repeated-measures ANOVA

Medium	Degree centigrade	F ^a	df ^b	p value
FBS ^c	4	22.29	3	0.00003
	24	220.6	3	< 0.00001
	37	255.3	3	< 0.00001
AF ^d	4	957.4	3	< 0.00001
	24	911.9	3	< 0.00001
	37	1095.5	3	< 0.00001

^aF-table

^bDegrees of freedom

^cFetal bovine serum

^dAmniotic fluid

with 10% FBS and HCF alone (Fakhar et al. 2006). However, we observed that there was no significant difference in the number of promastigotes between AF and FBS media on day 7 at 24 °C ($p = 0.5$), suggesting that AF can be considered an alternative for FBS in the short-term. Most importantly, the number of promastigotes increased over time (days 7 to 28) in AF-containing medium, indicating that AF is even a reliable alternative for FBS for long-term period.

Researchers in different studies used various rich solutions for a possible alternative to FBS. In a study, the efficacy of 10% goat, cow, and buffalo milk in medium 199 was examined and compared to medium 199 with 10% FBS for the proliferation of *Leishmania donovani* promastigotes. The results showed that the number of promastigotes was not statistically different between the medium supplemented with FBS and the media containing various types of milk, indicating that milk can be used as an appropriate alternative to FBS (Muniaraj et al. 2007).

Conclusion

Our results showed that FBS has a greater efficacy than AF in the growth of *L. major* and the survival of *T. gondii*. However, the number of *L. major* promastigotes increased over time in AF-containing medium; therefore, AF can be a potential alternative to FBS. Although FBS was much better than AF in *T. gondii* maintenance, the number of tachyzoites reduced slowly with a mild slope in AF-containing medium as compared to FBS. Accordingly, further studies are recommended on AF and its efficacy on the maintenance of the protozoan parasites as an inexpensive, reliable, and available supplementary solution instead of FBS.

Acknowledgements The authors would like to thank the participants for their contribution in this study. We are very grateful to Dr. Masoud Keighobadi for all his efforts. He did not respond to treatment and expired on January 21, 2021, due to COVID-19. God rest his soul.

Funding This study was supported by the Vice Chancellor of Research at the Mazandaran University of Medical Sciences (Code: 7049).

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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