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Original Article

Evaluating the Coconut Water as a Replacement for the Fetal Calf Serum in Cultivation of Promastigotes of *Leishmania Infantum*

Vahid Nasiri[1*] Ph.D., Gholamreza Karimi[1] Ph.D., Habibollah Paykari[1] Ph.D., Gholamreza Motamedi[1] Ph.D., Shahla Rivaz[1] M.Sc., Mohammad Eslampanah[2] Ph.D.

[1]Department of Parasitology, Razi Vaccine and Serum Research Institute, Alborz, Iran.
[2]Department of Pathology, Razi Vaccine and Serum Research Institute, Alborz, Iran.

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A B S T R A C T

Backgrounds and Aims: The protozoan parasites of the genus *Leishmania* can be stated as the causative agents of the various clinical diseases. Fetal Calf Serum (FCS) is the major part of the *Leishmania* culture media, which is the most expensive ingredient in these media. The coconut water is packed with nutrients that yield an array of health benefits.

Materials and Methods: In the present study, the efficacy of the coconut water was evaluated in the cultivation of promastigotes of *Leishmania infantum*.

Results: The results indicated that, the coconut water enriched with culture medium could not support the growth of the parasites and cannot be used for cultivation of *Leishmania infantum*.

Conclusions: Leishmaniases are neglected tropical diseases that can cause human infections varying from self-healing cutaneous lesions to mucosal diffuse cutaneous and visceral forms. It is concluded that although the coconut water is an easy available and cheap replacement for FCS, it could not be used in cultivation process of *Leishmania infantum* promastigotes.

***Corresponding Author:** Department of Parasitology, Razi Vaccine and Serum Research Institute, Alborz, Iran. **Email:** v.nasiri@rvsri.ac.ir

Introduction

Leishmaniasis is regarded as a major neglected tropical disease of poverty acknowledged by World Health Organization (WHO) [1] as its victims are among the poorest. According to the ranking, it is a second most prevalent parasitic disease after malaria [2]. There are about 1.5-2 million new cases and 70 000 death reports per year. As a matter of fact, 350 million people are at risk of infection and disease with about 2.4 million disability-adjusted life-years [3]. As per WHO report indicates, nearly 200,000 to 400,000 new cases of visceral Leishmaniasis occur annually with 20,000 to 30,000 deaths per year [4]. Visceral leishmaniasis has been reported sporadically in Iran, though the disease is endemic in northwestern and southern areas of the country [5-7] with about 100–300 new cases of visceral Leishmaniasis reported annually [8].

The cell culture technique is an approach intending to prepare complex habitat conditions of living organisms in order to develop our knowledge in regard with their behavior and to find suitable ways (like effective vaccine) to prevent their negative side effects. Many different kinds of media that have been used for cultivation of genus *Leishmania* require expensive and batch to batch different qualities of fetal calf serum (FCS), that is not manufactured in many countries specifically most of the poor tropical countries , as one of their essential ingredients. FCS is a highly expensive reliable supply, which is extremely difficult to be obtained, especially in the developing countries [9]. As a matter of fact, its usage in the cell culture seems to be problematic for several reasons as complexity, high variability, as well as difficult characterizing [10]. Several attempts have been made to replace FCS in leishmania culture media with different kinds of sera, bovine serum albumin, a mixture of purine bases, vitamins, large concentrations of certain amino acids, hormones, hemins, hemoglobulins, human and animal urine and, more recently chicken serum [11–21] that the later introduced an alternative low- cost serum applied in the culture medium for primary isolation, routine cultivation and mass cultivation of *Leishmania* parasites [21, 22].

Coconut water is the liquid present naturally inside the young fresh coconuts that is rich in potassium, cytokinin, various minerals and antioxidants. Generally, plant tissue culture of coconut water is used as a natural source of cytokinins and other nutrients [23].

In the present study, the coconut water was assessed in regard with the preparation of medium for the cultivation and maintenance of *Leishmania infantum* promastigotes .

Materials and Methods

Medium preparation

The coconut water was extracted in sterile condition from fresh fruits without autoclaving to avoid degradation of the organic compounds by heat. Roswell Park Memorial Institute (RPMI)-1640 was used as a standard base medium and prepared by dissolving1.04 g of RPMI-1640 (sigma) in 90 ml of distilled water

and then 10 ml (10%) of the coconut water was added. The pH was adjusted to 7 and the total volume was adjusted to 100 milliliter. Four dilutions of the coconut water were prepared as follows: 1%, 2.5%, 5% and 10%. No antibiotics were used in the culture media. The media were sterilized by pressure passage through 0.22 µm membrane filter (Millipore, Germany). The above procedures were used for preparation of RPMI-1640 enriched with (10%) heat-inactivated fetal calf serum for positive control. The complete media were kept at 4°C.

Parasite cultivation

Mid-log phase promastigotes of *Leishmania infantum* (*Leishmania infantum*: MCAN/IR/07/Moheb-gh.) that previously had been grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), were concentrated by centrifugation at 3,000 g for 10 minutes which was washed twice with sterile phosphate-buffered saline solution (PBS) in order to remove any traces of FCS. The parasites were counted via invert microscopy in a Neubauer chamber (Haemocytometer) slide and diluted in PBS to a final concentration of 10^8 parasites/ml. Subcultures from each dilution of coconut water were prepared in 5 series, alongside of the positive control with 10% FCS enriched medium, and at each series, 10^6 mid-log phase promastigotes/ml was inoculated in 25 cm^2 plastic culture flasks that every flask totally contained 15 ml of parasites and complete media mixture. The flasks were placed in incubator at 26°C and in all cultures, the parasites growth pattern was assessed qualitatively and quantitatively by microscopic observations and Giemsa slide preparation. The number of parasites was counted every day using Haemocytometer slide. The study protocol was approved by Razi Institute Ethics Committee.

Statistical Analysis

SPSS-18 for windows® was used in order to analyze the study data. The differences between the means of the quantitative variables were assessed by Student's T-test and the value of $p<0.05$ was accepted as the statistically significant level.

Results

The study results indicated that the addition of the coconut water to the RPMI-1640 medium could not stimulate the growth of the promastigotes of *Leishmania infantum* in comparison to the growth observed in RPMI-1640 supplemented with FCS ($p<0.05$). It was also observed that the medium enriched with coconut water could not support the continuity of the parasites in successive passages. Fine grown parasites and typical morphology of the promastigotes were not observed in Giemsa-stained smears prepared from culture media of all the coconut water dilutions. The effect of various concentrations of the coconut water in the culture media on the growth quantity of the promastigotes is demonstrated in a growth curve (Fig. 1).

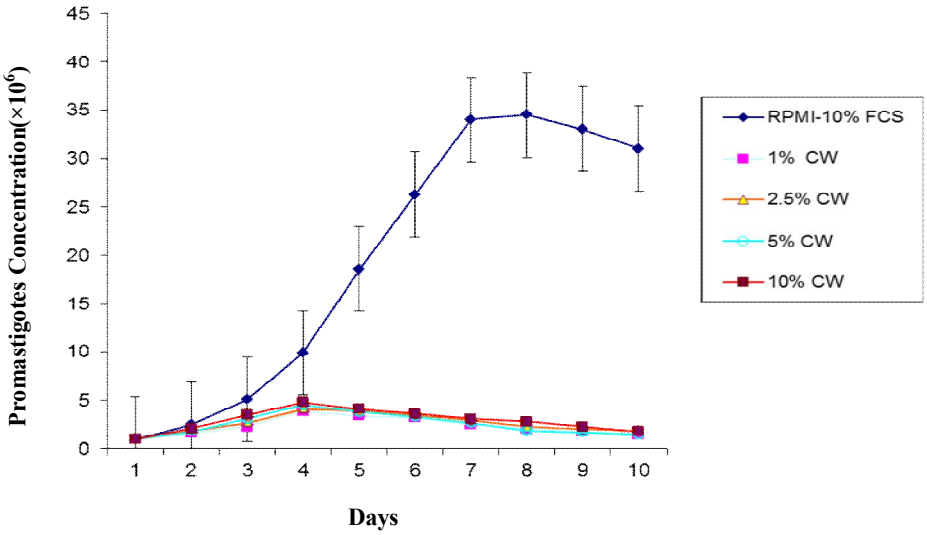

Fig. 1. Growth curve of promastigotes of *Leishmania infantum* in RPMI-10% FCS and RPMI-1640 with different concentrations of the coconut water (CW).

Discussion

Leishmaniasis is a typical example of zoonosis found on all the continents except for Australia and Antarctica, which is reported by the W HO as being one of the major tropical diseases. In fact, 20 million people in the world are infected per year with 400,000 additional phenomena [24].

In a study conducted on different aspects of Leishmanian parasites, the presence of liquid culture media able to produce a large amount of promastigotes is one of the essential needs. The routine commercially culture media like RPMI -1640, medium 199, and Schneider's *Drosophila* normally enriched with FCS or blood lysate [25, 26] are extremely expensive. In the mass cultivation of parasites, the major problem is that expensive and batch to batch different quality FCS needs to be used that is an importing material. It is not manufactured in many countries specially most of the poor tropical countries and thus, leishmaniasis is normally reported as one of the their important health problems [20]. Herman has studied the sera effect of rabbit, chicken, man, calf, hamster and cotton rat on the number and morphosis of *L. donovani* as well as cells in cultures of hamster-peritoneal macrophages that had been infected *in vivo* [17]. Moreover, a study carried on finding replacement for FCS in cultivation of promastigotes of *Leishmania major* and *Leishmania infantum* indicated that chicken serum is suitable for nutritional requirement of parasites. It is a comparatively available and inexpensive serum that can be replaceed in the media requiring FCS enhancement for promastigote forms. Therefore, a potentiality of the new medium is demonstrated to be used in long-term *in vitro* cultivation of *Leishmanian* promastigotes [21,

22]. The findings of other studies proposed that the serum and urine of many animals like hamster, rabbit and sheep seem to be suitable for cultivation of promastigotes, yet, collecting the serum of some like Hamster, is expensive and using that of some other, like sheep serum, are accompanied with many challenges in regard with adaptation problems of promastigotes to new serum (unpublished work). It needs to be implied that applying this new medium is also accompanied with some little adaptation problems of promastigotes to the new serum, though this problem takes place in any medium change. It is worth mentioning that the final adaptation of parasites to new medium should be prominently taken into consideration.

The coconut water contains several organic compounds and mineral nutrients important to plant development which plays a significant role as a physiological buffer. It is rich in magnesium, and phosphate containing high amounts of sugar around 2.5% (w/v). Besides, coconut water has high levels of nitrogen in the form of amino acids and phytohormones in an adequate balance for plant requirements [27]. The ability of coconut water to support plant growth in vitro is due to its ability to stimulate cell division and morphogenesis. Not only does coconut water support plant growth in vitro but also George (1993) has attributed the robustness and high survival rate of plants cultured on coconut water to the high carbohydrate content which could be used to meet the respiratory demands while surviving the physiological shocks of ex-vitro procedures [28].

Conclusion

Although the coconut water is an easy available and cheap replacement for FCS, the coconut water enriched with culture medium could not support the growth of parasites and thus, it cannot be used in cultivation process of *Leishmania infantum* promastigotes.

Conflict of Interests

We declare that we have no conflict of interest.

Acknowledgments

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