



In Vitro Genotoxic Effects of *Sarcocystis gigantea* Cystozoites Acetone Powder Extract on Sheep Lymphocytes

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A B S T R A C T

The toxin of the protozoan intracellular parasite of sheep *Sarcocystis gigantea* is associated with many clinical and pathological signs. The aim of the study was to investigate *In Vitro* various chromosomal aberrations due to sarcocystosis infection. Macrocysts of *Sarcocystis gigantea* were isolated from local karadi sheep, homogenized with glass Dounce homogenizer; acetone powder was prepared from it and used in various concentrations to investigate the chromosomal aberration *in vitro* against sheep lymphocytes. The direct effects of parasite cystozoites acetone powder revealed various genotoxicity effects. These effects included chromosomal aberration (Isogap, Breaks and Dicentric) and chromatids aberration (Gap and Deletion). It had also an effect on the mitotic index of the lymphocyte cells division. These genotoxicities were studied for the first time with *in vitro* technique using sheep lymphocytes. These results reflected that *Sarcocystis gigantea* parasite could cause structural and internal aberration in the chromosomes of their hosts.

Keywords: *Sarcocystis*, genotoxic, acetone powder, *in vitro*

INTRODUCTION

Sarcocystis is a protozoan parasite belongs to the family sarcocystiidae and have an obligatory two-host life cycle including sexual development and the formation of oocysts in the intestinal mucosa of their final hosts (carnivores and omnivores). The asexual multiplication is seen in the vascular endothelial layer and the striated muscle cells of various body regions of the intermediate host (herbivores, omnivores and carnivores). The multiplication within striated muscle cells of skeletal muscle, smooth muscle and heart muscle leads to the formation of cysts of the parasite which may be microscopic or macroscopic according to their size. These cysts have a

characteristic morphology (size, shape and surface structure) for each probable species, and by which, the final "or definitive hosts" become infected through ingestion of infected raw muscle tissue (1-3).

Previous studies highlighted the toxicity of this parasite on the host (4, 5) and eventually, its pathological and clinical effects (2, 6). Other researcher pointed out that *Sarcocystis* parasite had potential genotoxic effects on sheep lymphocyte, *In Vivo*, as an intermediate host through the infection course directly (7, 8). Various methods were used to investigate these effects (9, 10).

Current study targeted the effect of *Sarcocystis gigantea* cystozoites acetone powder extract for the first time on cultured sheep lymphocytes *In Vitro* to investigate the

probable direct preliminary chromosomal aberration effects.

MATERIALS AND METHODS

Sarcocystis gigantea Cystozoites Preparation

The procedures used in this study were reviewed and approved by the scientific committee of the University of Baghdad's College of Veterinary Medicine in compliance with animal welfare ethical standards.

Sarcocystis gigantea cystozoites were prepared from mature large macrocysts collected from esophagus of local breed sheep (*Ovis aries* var. Karadi) at local abattoir. Species identified according to previous studies (10, 11). Briefly, cysts were transferred to the laboratory and washed three times with sterile PBS solution. Inside a sterile cabinet, cysts were placed in glass petri dishes and burst using sterile forceps, scissors and scalpel. Afterward, cystozoites were filtered through double layers sterile gauze into 10 mL test tube and washed three times with PBS. In water ice bath, cystozoites were homogenized with sterile Dounce glass homogenizer (Sigma-Aldrich).

Acetone Powder Preparation

Acetone powder was prepared according to the method of Harlow and Lane (11). This method was applied in preparation of other parasite (on published research). Briefly, homogenized solution of washed *Sarcocystis gigantea* cystozoites were placed on a sterile filter paper (Whatman filter paper No. 42) in Buchner funnel. Inside a sterile fume hood, acetone at -18 °C add (1:10 v/v) was added and the infiltrates were sucked with aid of vacuum pump. The process repeated three times. The precipitate allowed to be dry at room temperature (25 °C) over-night and stored in Peugeot glass bottle in refrigerator (4 °C). Inside a sterile fume hood, series diluents of parasite acetone powder are prepared (5 mg, 10 mg, 20 mg, and 40 mg per one mL of deionized distilled water. Sterilization was adapted for every step of the procedure.

Sheep Lymphocytes Preparation

Sheep lymphocytes were prepared according to previous studies (12-15). Briefly, 10 mL of blood samples in heparinized tube were collected from jugular vein of three clinically healthy sheep. Heparinized blood (0.5 mL ≈ 5000 cells/mL) were placed in a sterile culture tubes containing 5 mL of RPMI 1640 medium (Sigma-Aldrich), mixed gently, and then incubated in a slanted position at 37 °C for 72 h. This was used as control to other steps.

The *Sarcocystis gigantea* cystozoites acetone powder were dissolved in analytical reagent grade DMSO (Sigma-Aldrich) at 100× the required concentrations and diluted with serum-free RPMI 1640. Fresh solutions were prepared immediately before use.

Different concentrations of parasite acetone powder extracts (5 mg/mL, 10 mg/mL, 20 mg/mL, and 40 mg/mL) were used for 3 h in serum-free medium, followed by a further 24 h incubation in complete medium (medium with serum). Then washed 3 times with agent free medium (without any addition). Colcemid (Biogen Laboratories Inc. USA) was added at 0.5 µg/ml for the last hour of incubation. The incubation period was completed after 72 h as in control. Cells were harvested and slides of metaphase spreads prepared as described by previous studies mentioned in above.

Chromosomal Analysis

Chromosomal analysis included chromosomal aberrations (Isogap, Break, and Dicentrics), chromatid's aberrations (Gap, and Deletion) and lately the mitotic index for triplicate samples of each treatment with the results of 500 cells on studied slide.

The method of Shubber (16) where sheep lung was used with the aid of other reference (17) modification, briefly: "A tube of 4ml RPMI-1640 (Sigma) prepared medium, brought to room temperature, was used for each individual. The additive components were including, Heparinized whole blood 0.5 mL, Human AB⁺ plasma 1.0 mL, and Phytohemagglutinin - PHA (Sigma-Aldrich) 0.2 mL. For mitotic index (MI) analysis, slides were stained with Giemsa stain (Sigma-Aldrich) or 2 min and examined with a light microscope (Olympus) under 40× and 100× objective lens. It was determined as a percent of the mitotic cells to interphase nuclei in 500 cells". Equation of mitotic index which applied here is:

$$\text{Mitotic Index (\%)} = \frac{\text{No. of divided cells}}{\text{Total count (500 cells)}} \times 100$$

Statistical Analysis

Statistical analysis was done according to recommendation of Mills (18) and other (14). ANOVA was applied with complete random design (CRD). SPSS v.21 was used for that.

RESULTS AND DISCUSSION

Acetone powder of *Sarcocystis gigantea* parasite showed various genotoxic effects against local Karadi sheep lymphocytes (Figure 1) which included chromosomal aberration such as deletion and Isogap. These effects were dose dependent, where the significance ($P < 0.01$) increased with the increase of powder doses (from zero in control to 40mg/ml). The increasing percentages were ranged in average from 0.67 ± 0.58 in control to 4.67 ± 1.15 , 6.67 ± 1.52 , 8.34 ± 2.08 , and 13.67 ± 1.52 , respectively (Table 1).

Acetone powder methods was used to purify proteins (glycoprotein and saccharide protein) as a procedure from

other cellular components (19). It was originally suggested in 1955 (20). This method kept out protein from denaturation and do not affect its activity, and it was suitable for laboratory poor in their supplies. However,

several studies used other methods for purification like chromatography and precipitation with Ammonium sulphate (10, 11 and 21).

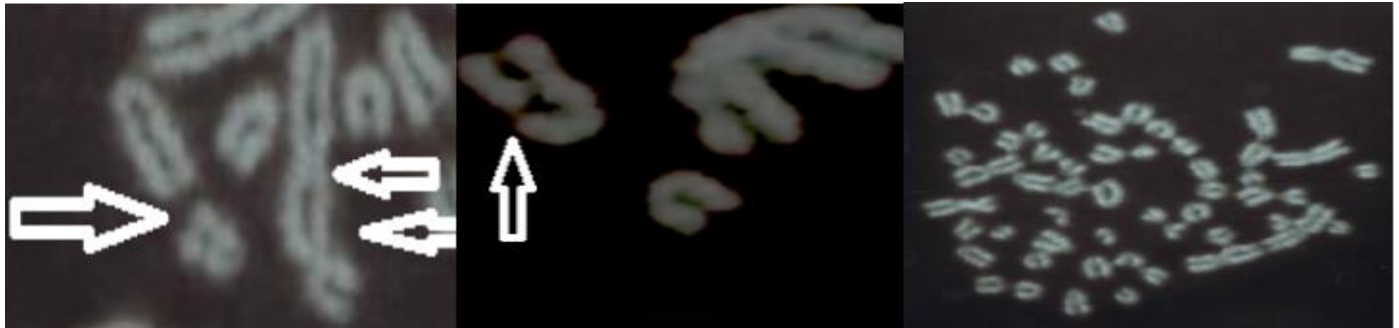


Figure 1. Chromosomal feature of local Karadi sheep lymphocyte. From right to left: Normal standard karyotyping, Deletion, break (one arrow) and dicentric (two arrows). 40 \times , 100 \times by inverted ark light technology microscope

Table 1. In vitro sheep lymphocytes chromosomal aberration by acetone powder of *Sarcocystis gigantea* parasite.

| Treatment ¹ | Chromosomal aberrations % \pm SD | | | Chromatid's aberrations % \pm SD | | Mitotic Index % \pm SD | No. of Analyzed cells |
|------------------------|---------------------------------------|-------------------|-------------------|---------------------------------------|-------------------|-----------------------------|-----------------------|
| | Isogap | Break(s) | Dicentrics | Gap | Deletion | | |
| Control | | | | | | | |
| a | 0 | 1 | 0 | 9 | 5 | 0.99 | |
| b | 1 | 1 | 1 | 8 | 2 | 1.15 | |
| c | 1 | 2 | 0 | 5 | 6 | 1.05 | 500 |
| Average | 0.67 \pm 0.58 | 1.33 \pm 0.57 | 0.34 \pm 0.57 | 7.33 \pm 2.08 | 4.34 \pm 2.08 | 1.06 \pm 0.08 | |
| 5 mg/mL | | | | | | | |
| a | 4 | 10 | 2 | 23 | 16 | 1.56 | |
| b | 6 | 8 | 3 | 19 | 18 | 1.28 | |
| c | 4 | 11 | 0 | 29 | 15 | 1.87 | 500 |
| Average | 4.67 \pm 1.15* | 9.67 \pm 1.52* | 1.67 \pm 1.52* | 23.67 \pm 2.51* | 16.33 \pm 1.52* | 1.57 \pm 0.29* | |
| 10 mg/mL | | | | | | | |
| a | 7 | 14 | 12 | 33 | 32 | 1.67 | |
| b | 5 | 19 | 19 | 31 | 19 | 1.09 | |
| c | 8 | 16 | 15 | 28 | 28 | 2.15 | 500 |
| Average | 6.67 \pm 1.52* | 16.34 \pm 2.51* | 15.34 \pm 3.51* | 30.67 \pm 2.51* | 26.34 \pm 6.65* | 1.64 \pm 0.53* | |
| 20 mg/mL | | | | | | | |
| a | 10 | 21 | 30 | 111 | 41 | 1.81 | |
| b | 9 | 18 | 19 | 134 | 35 | 1.96 | |
| c | 6 | 16 | 21 | 125 | 28 | 1.95 | 500 |
| Average | 8.34 \pm 2.08* | 18.34 \pm 2.51* | 23.33 \pm 5.85* | 123.33 \pm 11.59* | 34.67 \pm 6.50* | 1.90 \pm 0.08* | |
| 40 mg/mL | | | | | | | |
| a | 12 | 19 | 33 | 201 | 47 | 1.95 | |
| b | 14 | 18 | 28 | 166 | 51 | 1.99 | |
| c | 15 | 21 | 31 | 174 | 41 | 2.15 | 500 |
| Average | 13.67 \pm 1.52* | 19.33 \pm 1.52* | 30.67 \pm 2.51* | 180.33 \pm 18.33* | 46.33 \pm 5.03* | 2.03 \pm 0.10* | |

¹*Sarcocystis gigantea* acetone powder (mg/mL)

Previous studies described the mitogenicity of sarcocystosis parasite on various sheep types (4, 22, 23). In Tietz et al. (22); it was clear that extract of *Sarcocystis gigantea* protein had a powerful mitogenic effect. Current study revealed, for the first time, the direct effect of acetone

powder of *Sarcocystis gigantea* on the chromosomes of sheep lymphocytes, In Vitro culture technique.

Chromosomal aberrations were increased here resembling that of other intracellular parasite like *Toxoplasma* (24), in *Echinococcus* (25) and *Schistosoma*

(26). In sheep, chromosomal abnormalities were seen with other substances (12) as gap and break as well as a DNA fragmentation (27).

Chromosomal breaks were also increased respectively (1.33 ± 0.57 in control, then 9.67 ± 1.52 , 16.34 ± 2.51 , 18.34 ± 2.51 , and 19.33 ± 1.52 in other preparations).

Chromosomal breaks were observed in previous studies (29, 30) for other substances and material. It was clear that these effects work directly as a part of total DNA damages. Chromosomal dicentric aberration also increased respectively (0.34 ± 0.57 , 1.67 ± 1.52 , 15.34 ± 3.51 , 23.33 ± 5.85 , and 30.67 ± 2.51). Dicentric chromosomal aberrations pointed DNA double strands breaks (31). It could also be due to crosslinks and/or chromatids deletion (33).

On the other side of the study, it was clear that chromosomal chromatids aberrations (Table 1) were seen to be increased in significance ($P=0.001$) for chromatids gap (7.33 ± 2.08 , 23.67 ± 2.51 , 30.67 ± 2.51 , 123.33 ± 11.59 , and 180.33 ± 18.33 respectively). The same for chromatids deletion aberrations (4.34 ± 2.08 , 16.33 ± 1.52 , 26.34 ± 6.65 , 34.67 ± 6.50 , and 46.33 ± 5.03 respectively).

These results were seen previously (32) in mice due to the administration of the non-steroidal anti-inflammatory drug. Herein it could be explained how acetone powder of sheep *Sarcocystis* parasites made its clastogenic effects by causing these kinds of chromatids aberration as a suggested mechanism.

In the looking to these chromosomal aberrations here it could be concluded that there was no need for activation agents to be take place as in In Vitro (33). Chromosomal assay was also good to estimate the cytogenetic effects which assessed DNA damage at chromosomal level. The classical cytogenetic analysis without differential staining allowed a rapid overall estimation of defect of various chromosomal aberration, which was demonstrated previously (12, 14) and in the current study.

Lately, Mitotic index (MI) of In Vitro sheep lymphocytes culture showed to be increased (Table 1) in significant ($P=0.001$) from control (1.06 ± 0.08) to other treatments reaching 2.03 ± 0.10 in concentration of 40 mg/mL of acetone powder of *Sarcocystis gigantea* parasite. This MI increasing reflected the cytotoxicity of blood lymphocyte (33) and this was due to the parasite acetone powder in compared to control. MI was related to cellular proliferation and that could be associated with induction of chromosomal damages (14).

Acetone powder of *Sarcocystis gigantea* was related to many chromosomal aberrations *in vitro*. This relationship might need more to be investigate in intermediate host, final host, and laboratory animal for other *Sarcocystis* parasite species.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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السمية الجينية لباودر الاسبستون المحضر من حويئات اكياس الصنوبريات العملاقة في الخلايا اللمفاوية للاغنام في الزجاج

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الخلاصة

ان سم اكياس الحويصلات الصنوبريات العملاقة التي تعيش داخل الخلايا في الاغنام له الكثير من العلامات السريرية و الامراضية. هدفت الدراسة الحالية التحري عن انواع التشوهات الكروموسومية في المختبر بسبب الاصابة بداء الحويصلات الصنوبرية. الاكياس العيانية للطفيلي عزلت من الاغنام الكرادية المحلية، تم تكسيها بالة داونس وحضر منها باودر الاسبستون الذي استخدم بتركيز مختلفة للتحري عن التشوهات الكروموسومية في المختبر (في الزجاج) ضد الخلايا اللمفية للاغنام. ان التأثير المباشر لباودر الاسبستون المحضر من حويئات كيس الطفيلي اظهر العديد من التأثيرات السمية الجينية. وشملت هذه السمية الجينية العديد من التشوهات الكروموسومية (الفراغات المتناظرة ، الكسور وازدواج الجسم المركزي) والتشوهات الكروموتيدية (الفراغات والحذف) فضلا عن التغيير في مؤشر الانقسام الخلوي. ان هذه التشوهات الكروموسومية تم دراستها في الزجاج للمرة الاولى في المختبر على الخلايا اللمفية للاغنام. ووضحت هذه الدراسة ان اكياس الحويصلات الصنوبريات العملاقة قد تحدث تغييرات بنائية وداخلية في كروموسومات المضيف.

الكلمات المفتاحية: الحويصلات الصنوبرية، السمية الجينية ، باودر الاسبستون ، في المختبر (الزجاج)