

# Experimental conversion of virulent RH *Toxoplasma gondii* tachyzoites *in vitro*

N.A. Hammouda,<sup>1</sup> I.R. Ibrahim,<sup>1</sup> E.D. Elkerdany,<sup>1</sup> A.Y. Negm<sup>1</sup> and S.R. Allam<sup>1</sup>

التحويل التجريبي للطور النشط من المقوسات القندية المفوعة، في المختبر

نيبال حمودة وإيمان إبراهيم وإيمان دري الكردي وعزة يحيى نجم وسونيا علام

خلاصة: إن آليات تحوّل الطور السريع (النشط) من المقوسات القندية إلى الطور البطيء (الحامل)، هي آليات معقدة وغير مفهومة على وجه الدقة حتى الآن. ولذلك أردنا بهذا البحث أن نحفز تحوّل الطور النشط من السلالة RH إلى الطور الخامل، عن طريق تغيير الأس الهيدروجيني للمستنسب. ومع زيادة قلوية الوسط إلى درجة pH8 حدثت تغييرات مورفولوجية في الأطوار النشيطة المزروعة. فقد زاد حجم معظم هذه الأطوار وتغيّرت من شكل هلالى منتظم إلى شكل مستدير أو بيضوي. كما تكوّنت بها أجسام شبه كيسية. ومع استعمال جهاز مُحوَسَّب لتحليل الصور لوحظت اختلافات جوهرية في الحجم الكلي لتلك الأطوار وفي نواها بالمقارنة بالمجموعة الشاهدة. ولقد أظهرت الأطوار المحوّلة كذلك اختلافات كبيرة عن المجموعة الشاهدة بالتحليل الكمي للDNA، ولم تُحدث عدوى في الفئران.

**ABSTRACT** We aimed to induce conversion of RH-strain tachyzoites to bradyzoites by changing the pH of the culture medium. Alkalkization of the medium to pH 8 induced morphological changes in the cultured tachyzoites. The majority of the organisms increased in size and changed from a regular crescent shape to a rounded or ovoid shape. Cyst-like structures were formed. Using a computerized image analyser, significant differences in the size of the whole organisms and in their nuclei were observed compared to the control group. The converted organisms also showed significant differences from the control group by quantitative DNA analysis, and did not infect mice.

## Conversion à titre expérimental de tachyzoïtes virulents de *Toxoplasma gondii* RH *in vitro*

**RESUME** Notre objectif était de provoquer la conversion de tachyzoïtes de souche RH en mérozoïtes en modifiant le pH du milieu de culture. L'alcalisation du milieu jusqu'à un pH de 8 a provoqué des changements morphologiques dans les tachyzoïtes mis en culture. Dans leur majorité, les organismes ont augmenté en taille et ont passés d'une forme de croissant régulier à une forme arrondie ou ovoïde. Des structures à forme de kystes se sont formées. A l'aide d'un analyseur d'images informatisé, des différences significatives dans la taille des organismes complets et dans leur noyau ont été observées par rapport au groupe témoin. Les organismes convertis ont également montré des différences significatives par rapport au groupe témoin par l'analyse quantitative de l'ADN, et ils n'ont pas infecté des souris.

<sup>1</sup>Department of Parasitology, Faculty of Medicine, University of Alexandria, Alexandria, Egypt.

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## Introduction

Toxoplasmosis is an important protozoal disease of humans and domestic animals [1]. In the intermediate host, *Toxoplasma gondii* exists in two stages: rapidly dividing tachyzoites, which are thought to be responsible for the acute infection, and bradyzoites, which are located within the cysts and are believed to persist for the life span of the host. These dormant stages are able to revert into tachyzoites, for example in immunocompromised patients [2]. The factors that influence interconversion between the bradyzoite and the tachyzoite stages are still unknown [3]. An understanding of the mechanism of interconversion would improve the control of toxoplasmic reactivation.

Studies have shown that immunological factors are not necessary for cyst formation [4]. Therefore, *in vitro* studies may be used to avoid the complexity of the host response. In the present study, our aim was to convert tachyzoites to bradyzoites in culture simply by changing the pH of the culture medium. Changes in the morphology, DNA content and cell cycle phases during transformation were assessed.

## Methods

The strain of *T. gondii* used was the virulent RH strain that is passed in Swiss albino mice every 3 days by intraperitoneal (ip) inoculation.

For the preparation of macrophages from mice, 8- to 10-week-old Swiss albino mice were infected ip with 3 mL of 2% sterile hydrolysed starch solution. Then, 6 days later peritoneal exudate cells were harvested by injecting 5 mL saline into the abdominal cavity and removing the cell suspension after 5 minutes. Macrophages were collected in culture medium [RPMI

1640 (Gibco) + 10% fetal calf serum, L-glutamine, penicillin 100 U/mL, streptomycin 100 U/mL] [5,6].

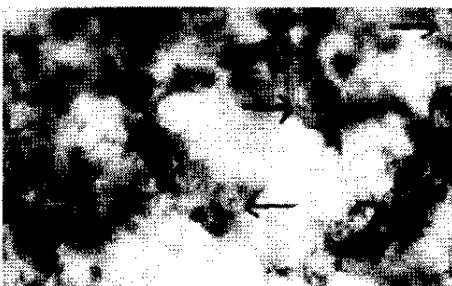
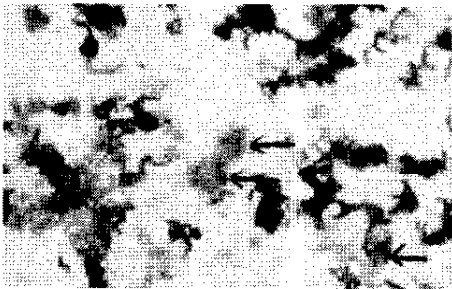
RH tachyzoites were harvested from the peritoneal cavity of the infected mice and washed in culture medium (RPMI 1640). After 6 hours of incubation, tachyzoites were then placed together with macrophages in a modified medium consisting of RPMI 1640 + 5% fetal calf serum + 50 mmol/L HEPES (Sigma) + 2 g/L sodium bicarbonate [7]. The pH of the medium was adjusted to 8 using sodium hydroxide. The culture tubes were maintained at 37 °C in air to avoid pH variation due to carbon dioxide. In the control tubes the medium was replaced by the standard culture medium (RPMI 1640 + 5% fetal calf serum) at pH 7.4. The infected macrophages were grown in 5% carbon dioxide/air [8].

For the experimental groups, cultured organisms were examined on day 0 (group 1), day 2 (group 2) and day 4 (group 3) from the start of incubation. For the control group, organisms were cultured *in vitro* at pH 7.4 and examined at the same time points as the experimental groups. The culture tubes were examined by light microscopic examination. Smears taken from each group were stained with Giemsa stain and examined by light microscope ( $\times 1000$ ). Organisms of groups 2 and 3 were compared with group 1 as well as with the control group, and any morphological changes were noted and photographed.

For further morphometric studies of cells and nuclei, another set of smears stained with Giemsa was analysed using a computerized image analyser (Leica) at a wavelength of 560 nm. The slides were then Feulgen-stained, which provided an accurate method for determining the relative DNA content for each individual para-



**Figure 1** Tachyzoites of *Toxoplasma gondii* after 2 days of incubation (group 2). The regular crescent shape has been lost and they are bigger, rounded or ovoid. Some organisms have diffuse outlines (arrow) with bigger nuclei (Giemsa  $\times$  1000).



**Figures 2 and 3** Cyst-like structure of *Toxoplasma gondii* tachyzoites incubated for 4 days (group 3). They are about 10–15  $\mu$ m in size with well-defined walls and few parasites (arrows) (Giemsa  $\times$  1000).

site. The cell cycle phases for each group were also traced. Readings were taken from 100 parasites in each group and statistically analysed using the *t*-test.

The infectivity of the incubated organisms was assessed. Organisms were collected from groups 2 and 3 on the second and fourth days and from the control group, washed and counted. To assess the infectivity of the parasite after *in vitro* incubation, 6 Swiss albino mice were infected with 3000 organisms from each group. Then, 2 to 3 months later the brains of the infected mice were homogenized in saline and examined to detect *Toxoplasma* cysts if present [9].

## Results

As regards light microscopic examination of Giemsa-stained smears, no changes were noticed in the morphological characters of *T. gondii* tachyzoites in the control tubes. In groups 2 and 3, most *Toxoplasma* organisms became distorted in shape and size compared to the control group and to group 1 (the baseline) (Figure 1). The regular crescent shape was lost and the organisms became bigger, rounded or ovoid. Some organisms had diffuse outlines. The nuclei were still clearly identifiable but enlarged (Figure 1). Cyst-like structures were noticed in group 3 only; they were about 10–15  $\mu$ m in size with well-defined walls and contained only a small number of parasites (Figures 2 and 3). These changes were not synchronized in all incubated organisms.

As regards morphometry, there were significant differences between the three groups in whole-cell size and size of nuclei (Tables 1 and 2). However, the mean quantity of DNA in 100 parasites was significantly different in the three studied groups

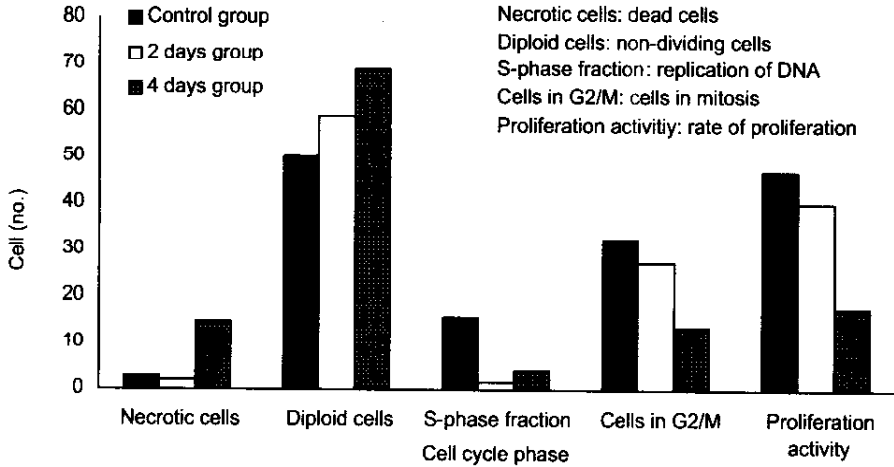


Figure 4 Cell cycle phases of the studied groups.

Table 1 Cellular area of 100 incubated *Toxoplasma* organisms in the groups studied

Comparison	Paired differences Mean $\pm$ s	t-value	P-value
Groups 1 and 2	0.4080 $\pm$ 0.139	29.35	0.000
Groups 1 and 3	3.1160 $\pm$ 0.280	111.19	0.000
Groups 2 and 3	-2.7080 $\pm$ 0.283	-95.54	0.000

s = standard deviation.

Table 2 Nuclear size of 100 incubated *Toxoplasma* organisms in the studied groups

Comparison	Paired differences Mean $\pm$ s	t-value	P-value
Groups 1 and 2	0.4000 $\pm$ 0.161	24.87	0.000
Groups 1 and 3	2.7280 $\pm$ 0.281	97.18	0.000
Groups 2 and 3	-2.3280 $\pm$ 0.292	-79.72	0.000

s = standard deviation.

(Table 3). Signs of cell activity and proliferation were significantly lower in groups 2 and 3 than in the control group (Figure 4).

As regards experimental infection of mice, after 3 months, examination of the brains of mice infected with organisms of group 3 showed no evidence of *Toxoplasma* cysts.

## Discussion

The mechanisms promoting tachyzoite to bradyzoite conversion in *T.gondii* are very complex and so far poorly understood. Tachyzoites are responsible for the acute phase of the infection, which is usually rapidly overcome by the immune defences of

**Table 3 DNA content of 100 incubated *Toxoplasma* organisms in the studied groups**

Comparison	Paired differences Mean $\pm$ s	t-value	P-value
Groups 1 and 2	-46.00 $\pm$ 99.919	-4.60	0.000
Groups 1 and 3	-186.00 $\pm$ 183.688	-10.13	0.000
Groups 2 and 3	140.00 $\pm$ 165.145	8.48	0.000

s = standard deviation.

the host. The parasite then develops the chronic phase by encystment of the bradyzoites. Knowing the factors responsible for interconversion between either of these *Toxoplasma* stages would help to control toxoplasmosis, particularly in immunosuppressed patients [10,11].

We therefore aimed to induce the *in vitro* conversion of RH *T. gondii* tachyzoites, which had never been shown to form cysts spontaneously, to bradyzoites by changing the pH of the medium in the absence of added immunological factors.

We found that alkalization of the culture medium to pH 8 induced morphological changes in the cultured tachyzoites in groups 2 and 3. Geimsa staining showed that the majority of the incubated organisms increased in size and lost their regular crescent shape to become rounded or ovoid, while some organisms retained certain tachyzoite characteristics. This could be attributed to the development of intermediate stages before complete conversion to bradyzoites. This has also been observed by Soete et al. who reported that an intermediate stage coexpressing bradyzoite- and

tachyzoite-specific proteins could be found because surface proteins are not immediately lost during conversion [11]. These authors also found that bradyzoites derived *in vitro* from peritoneal tachyzoites were different from those obtained from brain cysts since the former parasites did not react with bradyzoite-specific markers. Cyst-like, round or oval structures of about 10–15  $\mu$ m were observed in group 2 only. The wall was well defined but thinner and the parasite density was lower than in cysts observed *in vivo* from brain suspension.

These observations were supported by morphometric measurements using a computerized image analyser. There were significant differences in the size of the organisms and of their nuclei in both groups 2 and 3 compared to group 1 and to the control group.

Many trials have been carried out to induce tachyzoite to bradyzoite conversion *in vitro* and *in vivo*. *In vivo* production of cysts was reported in mice and rats after immunization [12,13]. Villard et al. developed a method for obtaining cysts from the RH strain of *T. gondii* by giving the injected mice sulfadiazine and sodium bicarbonate in the drinking water [14]. Cysts with an ultrastructure similar to that of bradyzoites were detectable in the brain. There are many reports describing the *in vitro* formation by the RH strain of structures looking like cysts, for example using anti-toxoplasma serum and complement [15] or gamma interferon to inhibit tachyzoite multiplication on long-term cultivation [10]. Suzuki et al. found that interferon was not effective in triggering differentiation [16]. However, it eliminated a large proportion of the intracellular parasites and selectively killed tachyzoites or inhibited their multiplication, thereby selecting differentiating or slowly

growing parasites. Bohne et al. described conversion in the presence of interferon, although the conversion was not synchronous for all the parasites inside the parasitophorous vacuole [17]. This confirms our finding that not all the parasites in group 2 and 3 showed signs of conversion as indicated by changes in their size and shape.

We found significant differences in the DNA content of the organisms in the different groups. This finding is supported by the results of El-Azzouni and El-Kaffash [18], who also found significant differences in the DNA content of the forms of *T.gondii*. Differences in DNA content may be related to the emergence of bradyzoite-specific antigens. This could also be the source of the low mitotic activity noticed in bradyzoites. Sibley et al. [19] suggested that it is possible to produce *Toxoplasma* mutants using ultraviolet irradiation or chemical treatment. However, the genomic characteristics of the RH strain at different stages and under different conditions need to be further investigated in order to correlate the virulence and genetic heterogeneity of the RH strain [19,20].

In our study the different cell cycle phases of the parasite were traced. In groups 2 and 3, most of the parasites were in the diploid phase (non-dividing). This indicated that the organisms had lost their ability to proliferate and had shifted to a slowly dividing stage. This was confirmed by measuring the rate of proliferation where the lowest rate was found in group 3. The increase in necrotic cells observed in group 3 may be due to the unfavourable pH of the culture. Suzuki et al. related this to the selective killing of tachyzoites [16]. Champs et al. reported that increasing the *in vitro* pH up to pH 8 decreased the rate of

multiplication and the invasive power of tachyzoites [8]. Moreover, by frequently changing the media in cell cultures, McHugh et al. [21] and Wiess et al. [22] noticed that decreased tachyzoite multiplication is a prerequisite for cyst formation.

The developed forms in the present study failed to infect mice. This observation has been confirmed by many other reports [13,14,23]. Frenkle explained the long survival time of the inoculated mice in his study by the loss of virulence after *in vitro* cultivation [24]. Ferguson and Hutchison offered two possible explanations for the failure to observe cysts in infected mice: either there were too few of them (low parasite inoculum), or they were too small and irregular to be detected because they were insufficiently developed [9]. In the present study, the loss of infectivity was probably due to the development of attenuated organisms after the change in the pH of the medium. The parameters of attenuation were shown by the change in the shape and size, low cell activity and low proliferation rate.

We showed that it is possible to transform tachyzoites to a slowly proliferating stage simply by changing the pH of the culture medium. This will facilitate the study of the differences between the stages of conversion, which remain a major barrier to the understanding of the disease, especially in immunodeficient patients. Moreover, it would allow better control of toxoplasmic reactivation. Finally, attenuation of the organisms by this simple method, combined with assessment by morphometry, DNA and cell cycle phase measurements, and measurement of their infectivity may help in the development of vaccines against *Toxoplasma* and other parasites.

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