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EFFECT OF REFRIGERATION AND FREEZING ON SURVIVAL OF *Toxoplasma gondii* TISSUE CYSTS

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Ingestion of Toxoplasma gondii tissue cysts is a major route of infection with this parasite in nature. The viability of brain cysts of the Me49 strain of T. gondii after storage at the usual refrigerator and freezer temperatures of 4°C and -20°C, respectively, was studied by bioassay in mice. The source of brain cysts were mice infected with 10 cysts 8 weeks previously. Brains were homogenized in saline and cysts counted; aliquots assessed to contain 2000 cysts each were stored either at 4°C for 5, 6, 7, 8, 10 and 12 weeks or at -20°C for 24, 48 and 72 hours. At these time points, each aliquot was inoculated into 4 fresh mice (500 cysts per mouse), 2 each by the subcutaneous (s.c.) and peroral (p.o.) routes. All inoculated mice were examined for the presence of either tachyzoites in peritoneal exudates of animals dying within the first 3 weeks after inoculation, or brain cysts in animals that died later than 3 weeks after inoculation or in 6-week survivors. These findings were confirmed by serology. The results showed that infection was induced with cysts stored at 4°C for 5, 6, and 7 weeks, while storage for 8 weeks and longer resulted in complete loss of viability. Cysts stored at -20°C were able to induce infection after 24 and 48h of storage, but not after 72h. Since not all cysts are destroyed simultaneously, as shown by the decreasing percentage of animals infected with inocula stored at either temperature for increasing periods of time, and viability is not completely lost after 48h of storage at -20°C even in a small volume of homogenized tissue, these results suggest that the time of freezing meat for human consumption should be prolonged over 48h as a measure of prevention of T. gondii infection.

Key words: Toxoplasma gondii, tissue cysts, viability, refrigeration, freezing, bioassay, mice.

INTRODUCTION

Toxoplasma gondii is a ubiquitous parasitic protozoan capable of infecting all mammalian cell types, and toxoplasmosis is one of the most widely distributed anthropozoonoses. In man, the fetus and the immunosuppressed individual represent two categories of patients particularly prone to serious clinical disease due to *T. gondii*. While fetal infection may occur after maternal primary infection during pregnancy, life-threatening disease due to *T. gondii* in the immunosuppressed is generally thought to be due to reactivation of a previously latent infection.

The major route of infection in nature is by ingestion of oocysts shed in cat faeces, which quickly sporulate under environmental conditions and thus become highly infective, or of tissue cysts containing the asexual bradyzoite stage of the organism. The asexual developmental cycle of the parasite is perpetuated by carnivores preying or scavenging on rodents and other smaller mammals and birds. Ingestion of either form by an unimmunized host is followed by proliferation and dissemination of tachyzoites, eventually limited by the developing immune response which triggers conversion of the parasite into the slowly growing bradyzoite stage within tissue cysts, localized mostly within the brain and skeletal musculature. Tissue cysts are relatively inert, causing little pathology, but remain viable probably for the life of the host. However, it has long been known that they are temperature-sensitive and that good thermal processing of meat (internal temperature of 67°C) results in their destruction (Dubey 1996). While tissue cysts were shown to survive at 4°C in saline up to 68 days (Jacobs et al. 1960) and 56 days (Dubey 1997), less information is available on the survival of cysts at lower temperatures. Freezing at -8° to -12°C for 3 days, and at -9° and -20°C for 24 h and even less, has been shown to be lethal for tissue cysts in pork (Dubey et al. 1988, Kotula et al. 1991, Dubey 1974), as was freezing at -15 °C for 24 h for cysts in mouse brain tissue. It was thus accepted that short-term freezing for 18-24 h at -20°C gives ample time for cyst destruction (Remington and Desmonts 1990) and could represent one measure to prevent *T. gondii* infection. However, we show here that tissue cysts may remain viable beyond this period, suggesting a longer freezing time of meat for human consumption.

MATERIALS AND METHODS

Mice. Female Swiss Webster mice (Medical Military Academy Animal Research Facility, Belgrade), 5-6 weeks old, weighing 18-22 g at the beginning of experiments were used. Mice were housed 6 per cage, and offered drinking water and regular mouse feed *ad libitum*.

Parasites. Brain cysts of the Me49 strain of *T. gondii* (kindly provided by Dr. J. P. Dubey, Beltsville, MD), regularly maintained by passage through Swiss Webster mice twice a year, were used. For experimental infections, mice infected with Me49 cysts at least 8 weeks previously were killed, their brains removed and homogenized in a tissue homogenizer with 1ml saline each. For cyst enumeration, 25µl of the brain suspensions were placed on slides and counted under the microscope. The number of cysts per brain was calculated by multiplying the number counted in 2 drops by 2 different investigators by 20, giving a threshold sensitivity of 20 cysts per brain.

Experimental procedure. After counting, aliquots of the brain homogenates assessed to contain approximately 2000 each were placed either in a refrigerator at +4°C for 5, 6, 7, 8, 10 and 12 weeks, or kept frozen in a standard laboratory freezer (temperature controlled at -20°C) for 24, 48 and 72 h. Bioassay was carried out in fresh 6 week-old female Swiss Webster mice. At each time point, groups of 4 mice were inoculated with brain suspensions containing 2000 cysts (500 cysts / mouse), 2 each by intraoesophageal gavage and subcutaneously (s.c.). The experiment was performed twice, the repeat differing from the first in that inocula of 2000 cysts / mouse were used after 5 and 7 weeks of storage at 4°C, and inocula of 100 cysts / mouse after 24 h of storage at -20°C. Mice were observed daily and deaths recorded. Any animal that died was immediately examined for the presence of *T. gondii* as follows: peritoneal exudates were examined for tachyzoites in animals dying within the first 3 weeks after inoculation; if more than 3 weeks had elapsed between inoculation and death, the brains were searched for cysts, a procedure also performed for all survivors killed a minimum of 6 weeks after inoculation. Brain cysts were sought in unstained brain squash preparations, made by firmly pressing a small piece (approx. 2mm) of the front cerebral region between a microscopic slide and cover slip. If the presence of cysts was demonstrated in at least 1 of 2 such preparations, brain tissue was homogenized and cysts counted. Prior to killing, survivors were bled from the retroorbital sinus and blood collected for serology, performed at a later date to confirm negative results of examination for brain cysts.

Serology. IgG antibody was detected by the high sensitivity direct agglutination test (HS-DA) as described by Desmonts and Remington (1980), using formalin-fixed RH tachyzoites as antigen, kindly obtained from Dr. Ph. Thulliez, Paris, France. Sera were serially 2-fold diluted starting from 1:20; the first dilution considered positive was 1:40 (2 IU / ml according to both the WHO reference serum and a laboratory standard serum).

RESULTS AND DISCUSSION

Viability of brain cysts of the Me49 strain of *T. gondii* stored at 4°C, as determined by the ability to induce infection in fresh mice, is shown in Table 1. Brain cysts remained viable for at least 7 weeks, while no cysts were viable after storage for 8 weeks or longer, as determined by both negative brain squashes and negative serology. The percentage of infected animals inoculated with cysts refrigerated for 5, 6 and 7 weeks decreased with the increasing length of storage. Mice appeared more susceptible to the s.c. than to the p.o. route of infection, especially with increase in the length of storage, but these differences were not significant ($p<0.05$, X^2 test). Interestingly, the size of the initial inoculum appeared not to enhance the capacity to induce infection since in a repeat experiment in which larger inocula (2000 cysts / mouse) were given after 5 and 7 weeks of storage, the bioassay was positive in fewer animals. Thus, cyst destruction at 4°C seems to be more time- than inoculum size-related. The time-relatedness, however, does not appear absolute, since the decreasing percentage of animals infected with inocula stored for an increasing period indicates that not all cysts are destroyed simultaneously, leaving a critical number of viable bradyzoites.

Table 1. Viability of Me49 brain cysts stored at +4°C

weeks of storage	No infected/ /trial	No infected/ /route		%
		s.c.	p.o.	
5	4/4 3/4*	4/4	3/4	87.5
6	0/4 4/4	2/4	2/4	50
7	2/4 1/4*	3/4	0/4	37.5
8	0/4 0/4	-	-	0
10	0/4	-	-	0
12	0/4	-	-	0

* trial 2: 2000 cysts inoculated per mouse

On the other hand, cysts kept frozen at -20^{0}C for 24, 48 and 72 h remained at least partly viable for as long as 48 h, but not for 72 h (Table 2).

Table 2.Viability of brain cysts of the Me49 strain of *T. gondii* stored at -20°C

hours of storage	No cysts/ /mouse	No infected/ /trial	No infected/ /route		Total infected	%
			s.c.	p.o.		
24	500 100	4/4 1/4	2/4	3/4	5/8	62.5
48	500 500	1/4 1/4	0/4	2/4	2/8	25
72	500 500	0/4 0/4	0/4	0/4	0/8	0

While inoculation of 500 cysts per mouse after 24 h of freezing induced 100% infection, after 48 h of freezing infection was induced in 25% mice in 2 separate trials. No cysts remained viable after 72 h of freezing, as shown by both negative brain preparations and negative serology. To determine the effect of the inoculum size, in an experiment in which only 100 cysts frozen for 24 h were inoculated per mouse, infection was induced in 1 animal of the 4 inoculated (25%). Interestingly, this mouse was inoculated via the p.o. route, as were both infected animals inoculated with cysts frozen for 48 h. Similarly to refrigeration, freezing did not render all cysts non-viable simultaneously either, but its effect appeared to be inoculum size-related. These data show that infection may be induced by inoculation of cysts that have been frozen for 48 h, even if the brains containing cysts were homogenized before freezing, thus disturbing the original tissue structure, and were frozen in small volumes of 1 ml, which enabled quick freezing-through. In addition, the cysts which retained viability after 24 and 48 h of storage at -20°C were obtained from the same pool of animals infected with Me49 cysts as those which were stored at 4°C; these retained viability for 7 but not 8 weeks. This lower survival after storage at 4°C than the 68 days reported by Jacobs et al. (1960) and the 56 days reported by Dubey (1997) is probably due to different experimental conditions. In addition to differences in the numbers of cysts inoculated into mice, we homogenized brains before storage, while Dubey stored them intact. In conclusion, since most domestic freezers do not maintain temperatures below -16°C to -18°C, and since meat for human consumption generally has preserved original structure in addition to a far larger volume which is slower to freeze-through, the data presented have the epidemiological implication that, as a measure to prevent *T. gondii* infection, a freezing time above 48 h may be required to render meat safe for human consumption.

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PREŽIVLJAVANJE TKIVNIH CISTA *Toxoplasma gondii NA NISKIM TEMPERATURAMA*

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SADRŽAJ

U radu su prikazani rezultati ispitivanja vremena preživljavanja tkivnih cista *Toxoplasma gondii* čuvanih na temperaturama običnog frižidera (4°C) ili zamrzivača (-20°C). Kao izvor cista korišćeni su miševi inficirani sa 10 cista soja Me49; posle 8 nedelja, životinje su žrtvovane, mozgovi homogenizovani i ciste prebrojane. Alikvoti od po 2000 cista su odlagani u frižider na 5, 6, 7, 8, 10 ili 12 nedelja, ili u zamrzivač na 24, 48 i 72 sata. Posle isteka odgovarajućeg vremena, svaki alikvot je inokulisan u po 4 miša, i to u 2 subkutano a u 2 *per os*. Inokulisani miševi su ispitani na infekciju *T. gondii,* tako što je kod životinja koje su ugibale u toku prve 3 nedelje po inokulaciji pregledan peritonealni eksudat na tahizoite, a kod životinja koje su ugibale kasnije kao i kod preživelih koje su žrtvovane posle 6 nedelja pregledan mozak na tkivne ciste. Direktni nalaz je potvrđen i serološki, dokazivanjem specifičnih IgG antitela pomoću direktnog aglutinacionog testa. Rezultati su pokazali da je moguće inficirati miševe cistama koje su čuvane 5, 6 i 7 nedelja na 4°C, pri čemu se procenat inficiranih miševa smanjivao sa svakom nedeljom stajanja, a da su ciste koje su stajale 8 i više nedelja u potpunosti izgubile vijabilnost. S druge strane, bilo je moguće izazvati infekciju miševa cistama koje su bile zamrznute na -20°C tokom 24 pa i 48, ali ne i 72 h. Međutim, podatak da i posle 48 h zamrzavanja u malom volumenu homogenizovanog tkiva jedan broj cista zadržava vijabilnost, sugeriše potrebu da se u cilju prevencije toksoplazmatske infekcije meso za ljudsku ishranu zamrzava duže od uobičajeno u literaturi navođenih 24-48 h.