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ORIGINAL PAPER

Toltrazuril treatment of congenitally acquired *Neospora caninum* infection in newborn mice

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Abstract C57BL/6 mice were infected with *Neospora caninum* tachyzoites during pregnancy, yielding a transplacental infection of developing fetuses. Subsequently, congenitally infected newborn mice were treated either once or three times with toltrazuril (or placebo) at a concentration of 31.25 mg compound per kg body weight. Both toltrazuril and placebo treatment had no negative effect on newborns, as noninfected treated pups developed normally without differences in mortality and morbidity to matching non-treated control animals. Already one application of toltrazuril was significantly ($p<0.01$) able to delay the outbreak of neosporosis in newborn mice, when compared to placebo-treated infected controls. We found significantly higher proportion of surviving newborns in one-time-toltrazuril-treated and three-time-toltrazuril-treated groups (34% and 54%, respectively) when compared to one-time-placebo-treated and three-time-placebo-treated groups (14% and 30%, respectively). There was no significant difference ($p=0.2$) in the proportion of surviving pups between one-time-toltrazuril and three-time-toltrazuril treatment. However, the number of diseased and *Neospora*-positive pups (46% and 47%, respectively) was markedly reduced after three-time-toltrazuril treatment compared to all other groups. Three-time-treatment also resulted in the highest antibody (IgG, IgG2a) response. Pharmacokinetic analyses using individual serum samples revealed that, although toltrazuril was absorbed and metabolized to toltrazuril sulfone by newborn mice, medicated animals exhibited an unexpected rapid turnover (half-life time) of the compound. Toltrazuril and the metabolite were also found in brain tissues, indicating that passage of the blood–brain barrier occurred. In conclusion, we could show that three times treatment with toltrazuril had a high impact on the course of infection in congenitally *N. caninum*-infected newborn mice.

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Introduction

The main problems associated with *Neospora caninum* and the corresponding disease, neosporosis, are (1) abortion in cattle (Dubey 2003), causing serious veterinary health issues and economic losses within livestock production (Hemphill and Gottstein 2000; Dubey et al. 2007), and (2) neuromuscular disease in dogs (Dubey 2003).

The need for the development of effective prophylactic or metaphylactic measures against bovine neosporosis has been widely addressed and discussed (Liddell et al. 1999; Gottstein et al. 2001; Innes et al. 2002; Kritzner et al. 2002; Häsler et al. 2006a, b). Although there are successful experimental vaccination approaches to prevent cerebral neosporosis (Cannas et al. 2003a, b; Debache et al. 2008) or vertical transmission (Nishikawa et al. 2001) in mice, there are currently no vaccines available to protect cattle from abortion (Andrianarivo et al. 2000; Innes and Vermeulen 2006).

Chemotherapy is being discussed as an interesting alternative to the vaccination strategy (Ortega et al. 2007). So far, a wide range of compounds have been tested in cell

culture-based assays and some pharmacologically active compounds, including lasalocid, monensin, piritrexim, pyrimethamine, and trimethoprim, were found to exhibit parasiticidal activity against *N. caninum* (Lindsay and Dubey 1989; Lindsay et al. 1994). More recently, it was reported that nitazoxanide (NTZ) and a series of NTZ derivatives efficiently inhibited *N. caninum* proliferation in vitro (Esposito et al. 2005; Esposito et al. 2007), and new generation diaminidines were shown to exhibit in vitro parasiticidal activity against *N. caninum* at the submicromolar level (Leepin et al. 2008). In vivo studies showed that oral drug treatment of *Neospora*-infected mice with sulfadiazine and amprolium was rather ineffective (Lindsay and Dubey 1990).

Toltrazuril, a symmetric triazinone derivative, was shown to exhibit anticoccidial activity against cyst-forming and noncyst-forming coccidian parasites (Haberkorn 1996). Toltrazuril is a weak acid compound (pK_a=6.8) with high lipid solubility. Pharmacokinetic studies in horses revealed that toltrazuril and its metabolites penetrated the blood–brain barrier (Furr and Kennedy 2000). The effect(s) of toltrazuril on the fine structure and multiplication of *N. caninum* were studied in cell culture demonstrating lethal damage in *N. caninum* tachyzoites (Darius et al. 2004a, b). The effect of toltrazuril on parasite survival after long-term treatments in cell culture, assessed by quantitative reverse transcription polymerase chain reaction, revealed a parasiticidal activity starting after a continuous 14-day treatment (Strohbusch et al. 2008). In the murine model of experimental *N. caninum* infection, toltrazuril treatment prevented severe clinical signs and formation of cerebral lesions (Gottstein et al. 2001; Darius et al. 2004a, b). However, an efficient metaphylaxis required at least a T cell-mediated immunological support in mice (Ammann et al. 2004). It was also reported that toltrazuril treatment of an acute *N. caninum* infection—induced during pregnancy in mice—resulted in a significant reduction of fetal losses (Gottstein et al. 2005). Furthermore, initially explorative approaches indicated a basic effectiveness of toltrazuril and its major metabolite, ponazuril, against experimental *N. caninum* infection in calves (Kritzner et al. 2002; Härdi et al. 2006).

In this study, we used the mouse model to determine whether treatment of neonates with toltrazuril has any effect, positive or negative, on the outcome of infection and disease.

Materials and methods

Tissue culture media, biochemicals, and drugs

If not otherwise stated, all tissue culture media were purchased from Gibco-BRL (Basel, Switzerland) and biochemical reagents were from Sigma (St. Louis, MO, USA). Toltrazuril and the corresponding placebo formulated for oral application were provided by Bayer HealthCare AG (Germany), stock solutions were diluted with water and used immediately.

Tissue culture and parasite purification

Cultures of Vero cells were maintained in RPMI 1640 medium (Gibco-BRL, Basel, Switzerland) supplemented with 5% fetal calf serum (FCS), 4 mM L-glutamine, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B at 37°C with 5% CO_2. Cultures were trypsinized at least once a week. *N. caninum* (Nc-1 isolate) tachyzoites were maintained in Vero cell monolayer cultures, during which time FCS was replaced with immunoglobulin G-free horse serum. Tachyzoites were harvested when they were still intracellular by trypsinization of infected Vero cells followed by repeated passage through a 25-gauge needle. Host cell debris were removed from parasites by separation on Sephadex-G25 columns as previously described (Hemphill et al. 1996). The tachyzoites were counted using a Neubauer chamber and parasite numbers were adjusted by adding RPMI medium as appropriate for experimental infection.

Mice, infection, treatment, and euthanasia

Twenty-eight pregnant wild-type C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Infection with *N. caninum* or mock infection with medium (control) was carried out at gestation day (GD) 13. Mice were maintained under conventional day/night cycle housing conditions as required by the animal welfare legislation of the Swiss Veterinary Office. Special food mix for breeding mice and water were provided ad libitum. For *N. caninum* infection, 18 mice were randomly selected and intraperitoneally (i.p.) inoculated with 6×10^5 live Nc-1 tachyzoites suspended in 200 μL RPMI medium. Matching control mice (n=3) received 200 μL medium without parasites. All mice were individually housed and checked daily for clinical signs, abortion, or preterm birth. Day of delivery was designated as day 0. Newborn mice were counted and daily checked for survival and health status. Infected dams and corresponding litters were randomly selected and divided into four treatment groups (groups C–F). Neonates from infected dams were treated either once (group C) or three times (group D) with toltrazuril or once (group E) or three times (group F) with placebo, respectively. Newborns from uninfected control mice were either treated three times with toltrazuril (group A) or placebo (group B).

In a parallel set of control experiments designed to temporally monitor plasma levels of toltrazuril (pharmaco-

kinetic study), seven pregnant C57BL/6 mice were used (group G).

For any treated animal, the first dose (toltrazuril at a concentration of 31.25 mg/kg body weight [bw]) was administered at days 3 and 4 of age, respectively. The treatment was done orally using a pipette, taking advantage of the sucking effect of newborns. Treatments were repeated identically at days 14 and 29 with 62.5 mg/kg bw, if applicable. For groups A–F, cages were daily checked and dead pups, if found, were removed as soon as possible. Pups and dams with clinical signs were euthanized immediately by CO_2 inhalation. All other dams were separated from pups and euthanized at day 28 (33 days post infection [dpi]), remaining pups were euthanized between days 64 and 100 of age. From all animals, pieces of brain, lung, heart, and liver were collected and stored at −20°C prior to DNA extraction. If possible, blood was taken for enzyme-linked immunosorbent assay (ELISA). For group G, three to five newborn mice were randomly selected before the first, second, and third treatment and at days 2, 5, 12, 17, 27, 32, and 45 posttreatment (counted after the first treatment onwards). Pups were killed and serum and brain tissues samples were frozen at −20°C prior to evaluation of toltrazuril and toltrazuril sulfone (major metabolite) concentration.

Vertical transmission

The proportion of vertical transmission from infected dams to fetuses was calculated retrospectively. All pups that died before initiation of treatment and pups from infected and placebo-treated groups (E, F) were tested for *Neospora* infection by conventional polymerase chain reaction (PCR) (see below). The number of PCR-positive pups was divided by the overall number of pups from these groups.

DNA extraction

DNA purification was performed using the DNeasy blood and tissue kit (Qiagen, Basel, Switzerland) according to the standard protocol suitable for animal tissues. Frozen organs were equilibrated to room temperature and then lysed overnight at 56°C in ATL buffer containing Proteinase K (12 mLAU/mL). DNA was eluted in 100 μL AE buffer, boiled at 95°C for 3 min, and frozen at −80°C prior to PCR.

Conventional PCR

Detection of parasite-specific DNA by PCR was done as previously described (Müller et al. 1996) with *N. caninum*-specific primers Np21plus and Np6plus.

Serology

Mouse serum samples were analyzed for the presence of antibodies against Nc-1 antigen by ELISA using the same somatic antigen as previously described (Eperon et al. 1999). Alkaline phosphate-conjugated goat antimouse IgG (Promega, Duebendorf, Switzerland), anti-IgG1, or anti-IgG2a (both from Southern Biotechnology Associates, Birmingham, AL, USA) were used as second antibody (conjugate). Absorbance was measured at 405 nm (A_{405}, reference filter 630 nm) using a Dynatech MR 7000 ELISA reader and the corresponding Dynatech Biocalc software (Dynatech, Embrach, Switzerland). The threshold value arbitrarily discriminating between "positive" and "negative" (cut-off) was defined by adding three standard deviations to the mean A_{405} value of the serum samples from noninfected and nontreated control mice.

Toltrazuril and toltrazuril sulfone concentration

Toltrazuril and toltrazuril sulfone concentrations were outsourced and thus determined at the laboratory for Animal Health (Bayer CropScience AG, BCS-D-ROCS, Monheim am Rhein, Germany). Serum samples were deproteinized with acetonitrile. Brain samples were extracted by homogenization with acetonitrile and water. After centrifugation, the quantitative determination was performed directly from the raw extract by high-performance liquid chromatography with detection by tandem mass spectrometry. The lower limit of detection was 0.025 mg/L for serum and 0.050 mg/L for brain tissue.

Statistical analyses

Comparisons of the IgG1 and IgG2a response in infected dams, number of cannibalized pups in noninfected and infected groups, as well as toltrazuril and toltrazuril sulfone concentrations in serum and brain samples were done using the Student's *t* test. Specific antibody responses (IgG, IgG1, and IgG2a) of the pups and the outbreak of the disease were analyzed using one-way analysis of variance (ANOVA). A nonparametric Kruskal–Wallis test was used wherever the distributional assumptions required for an ANOVA were felt to be unrealistic. When statistical differences were found, a nonparametric multiple comparison test was used to examine all possible pairwise comparisons. Survival curves were calculated using the Kaplan–Meier survival statistic and differences between groups were determined using the log-rank survival tests. Pups that died or became ill during the experiment were designated as failed, while healthy pups were designated as censored. Differences between the numbers of diseased or PCR-positive pups were analyzed by the chi-square test. A value of $p<0.05$

was considered statistically significant. All statistical analyses were carried out using the NCSS software version 2001 (NCSS, Kaysville, UT, USA).

Results

Infection of the dams and outcome of pregnancy

After infection at GD 13, none of control (n=3) and infected (n=18) dams showed abortion. All dams gave birth between GD 17 and 19. There was no difference (p=0.2) in the number of pups per litter between uninfected and infected animals (Table 1). Three out of 18 infected dams became sick at 19, 21, and 26 dpi, respectively. These three mice and also dams from which all pups died before the end of the lactation period (n=4; 6, 11, 13, 21 dpi) were killed. The remaining infected (n=11) and control (n=3) dams were killed at the end of the weaning period (33 dpi corresponding to 28 days after delivery). All dams yielded positive (conventional) PCR findings for *Neospora* DNA in the brain (data not shown). Furthermore, all 18 infected dams were seropositive at necropsy. Investigation for immunoglobulin subclasses by ELISA revealed a significantly higher level of IgG2a than of IgG1 (p=0.006) in infected dams (Fig. 1), indicating a rather Th1-oriented immune response after infection at GD 13. Uninfected control dams were all PCR-negative and *N. caninum* ELISA seronegative.

Treatment and subsequent proportion of surviving newborn mice

All treated pups were checked for their health status on a daily basis. Pups cannibalized by the dam were excluded from further investigations. There was no significant difference in the number of cannibalized pups from uninfected or infected dams (p=0.15). At day 3 of age, pups from uninfected dams were separated into two groups and treated either three times with toltrazuril (A, n=13) or three times with placebo (B, n=6). Chemotherapy itself with three temporally sequential applications of toltrazuril had no negative effect on the health status and survival of the newborn pups, as the uninfected control group (A) developed normally and showed no spontaneous morbidity and mortality after the last treatment until the end of the experiment.

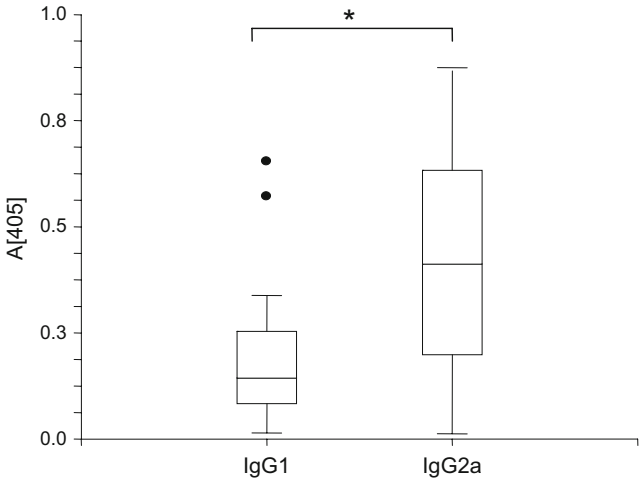

Fig. 1 Anti-*N. caninum* IgG isotype levels of infected dams as determined by ELISA, sera were sampled at 33 dpi. There was a significantly higher IgG2a level compared to IgG1 level in these dams. *p<0.05; significance hold true

Also at day 3 of age, pups from infected dams were separated in four different groups and treated one or three times with toltrazuril or placebo. When comparing the proportion of surviving pups of different infected treatment groups (C–F), significantly higher proportions were found for toltrazuril-treated groups (Fig. 2). The proportion of surviving three-time-toltrazuril-treated pups (D) was significantly higher than the proportion of surviving three-time-placebo-treated ones (F) (54% vs 30%; p=0.0072). A significant difference in the proportion of surviving newborns was also found between one-time-toltrazuril-treated (C) and one-time-placebo-treated (E) pups (34% vs 14%; p<0.01). Similar proportions of surviving pups were observed in the three-time-placebo-treated (F) and one-time-toltrazuril-treated groups (C). There was no significant difference between the toltrazuril treatment groups C and D (p=0.2), but three-time-toltrazuril treatment resulted in a 1.6 times higher proportion of surviving pups compared to one-time-toltrazuril treatment (Fig 2).

Table 1 Number of delivering dams and delivered newborns

	Uninfected control group	Infected group
No. of delivering dams	3/3	21/21
Average pups per litter	8.6 (7, 10)	7.2 (3, 10)
Total no. of newborns[a]	26	130
Died before treatment[b]	0	8
Cannibalized during experiment[c]	7	20

[a] Total number of live newborns at day 0 (day of birth)
[b] Number of pups that died before treatment, which started at day 3 of age. Pups were signed as untreated pups
[c] Number of pups that were cannibalized by mothers before or after treatment. These pups were logically excluded from the study because examinations were not possible

Outcome of the disease

The outcome of disease in very young diseased animals was characterized by spontaneous death. Older pups

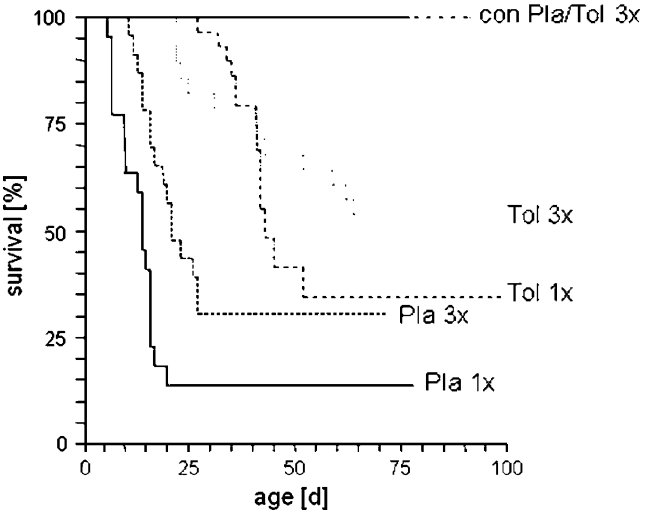

Fig. 2 Proportion of surviving pups. Proportions were calculated with Kaplan–Meier survival statistics. Untreated and cannibalized pups were excluded. Pups that died or became ill during the experiment were designated as failed, while healthy pups were designated as censored. Statistical differences were calculated with log-rank survival tests. Significant differences (*$p<0.05$) were found between the proportion of surviving of one-time-toltrazuril-treated and one-time-placebo-treated pups, as well as three-time-toltrazuril- and three-time-placebo-treated pups. No significant differences were observed between one-time-toltrazuril-treated and three-time-toltrazuril-treated pups, and also none between three-time-placebo-treated and one-time-toltrazuril-treated pups

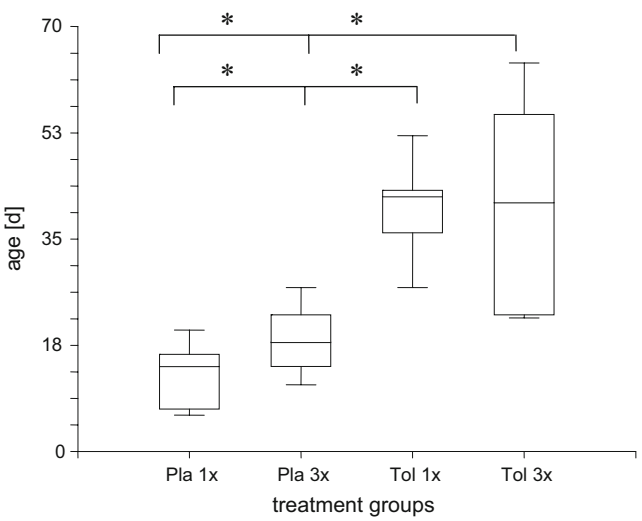

Fig. 3 Outbreak of disease depended on the age of pups. Outbreak of disease was defined when a pup died or became ill and was tested positive by *Neospora* PCR. There was a significant difference in the age when pups became ill between toltrazuril-treated and placebo-treated pups. *$p<0.05$; significance hold true

showed typical signs of murine neosporosis (apathy, bent back, rough hair coat) and were euthanized at the time the symptoms appeared.

Infected and placebo-treated pups (group E, $n=19$; group F, $n=16$) died or became sick within the first 27 days after birth. Most of infected and toltrazuril-treated pups (C, $n=19$; D, $n=13$), on the other hand, started to die or to show clinical signs from day 27 onwards (Fig. 3). This delay in the outbreak of neosporosis was significant ($p<0.01$) between toltrazuril-treated and placebo-treated groups but not between the two toltrazuril-treated groups with different application schemes.

Detection of *N. caninum* in congenitally infected and treated mice

Detection of parasite DNA was performed by PCR using DNA extracted from different organs of pups. The transmission, calculated retrospectively, revealed a proportion of vertical transmission of 74%. The proportion of PCR-positive pups in placebo-treated groups (E, F) ranged between 86% and 65%, whereas the proportion of PCR-positive pups in toltrazuril-treated groups (C, D) was between 66% and 47% (Table 2). There was a significantly different number of PCR-positive pups in the placebo-treated groups compared to the toltrazuril-treated groups ($p=0.03$). There was no significant difference, however, between one-time-toltrazuril-treated and three-time-toltrazuril-treated pups ($p=0.14$). We observed that those pups that became ill or died, independently of the treatment scheme, all exhibited cerebral infection, as determined by PCR.

Serological examination of congenitally infected mice

For serological studies, healthy pups killed at the end of the experiment and pups prematurely killed because of illness could be investigated. Pups that became ill during the experiment showed significantly ($p<0.01$) higher IgG levels than pups that survived until the end of the experiment, independently of the treatment schedule (Fig. 4a). As healthy pups exhibited negative or only very low antibody levels, they were not included anymore for further data analyses. Comparison of the IgG levels of only diseased pups, taking into account the different treatment groups, revealed a significantly higher antibody response for three-time-toltrazuril-treated pups (D vs C, $p=0.02$; D

Table 2 Proportion of PCR-positive pups in different treatment groups

	1xPla (%)	3xPla (%)	1xTol (%)	3xTol (%)
PCR+	86	65	64	47

PCR was performed with organs from pups that died or became ill during the experiment as well as from pups that were still alive at the end of the experiment. The age of the pups ranged between 0 and 100 days

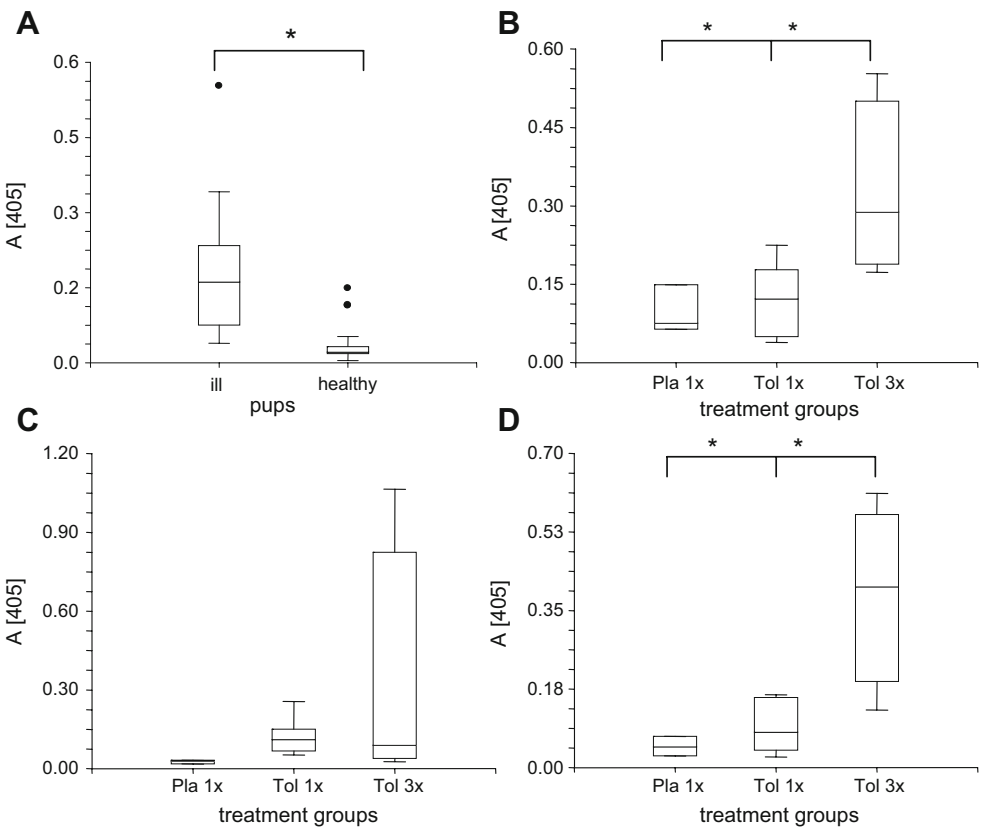

Fig. 4 Antibody response of pups from infected dams. **a** Comparison of IgG levels between pups that became ill during the experiment and pups that were healthy at the end of the experiment. There was a significant difference between the two groups. **b** Comparison of IgG levels between diseased pups of different treatment groups. There was a significant difference between three-time-toltrazuril-treated pups and all other groups. **c** Comparison of the IgG1 level between diseased pups of different treatment groups. There was no difference between the two treatment groups. **d** Comparison of the IgG2a level between diseased pups of different treatment groups. There was a significant difference between three-time-toltrazuril-treated pups and all other groups. *$p<0.05$; significance hold true

vs E, $p=0.03$) (Fig. 4b). We also determined the main IgG subclasses of diseased pups involved in the anti-*N. caninum* response. With regard to IgG1, there was no significant difference in antibody levels between the different treatment groups ($p=0.08$) (Fig. 4c). With regard to IgG2a, there was a significantly higher respective antibody level in group D when compared to group C ($p=0.007$) and group E ($p=0.03$) (Fig. 4d). Finally, there were no significant differences between IgG1 and IgG2a levels when comparing the results of different treatment groups. Consequently, this indicated that pups appeared to have mounted a mixed Th1/Th2 immune response following an in utero infection, independently of the treatment schedule subsequent to delivery.

Pharmacokinetic analyses

Temporal pharmacokinetic parameters of toltrazuril resorption and respective plasma and tissue levels were assessed using uninfected newborn mice (Fig. 5), which had been treated as outlined in the infection experiments. In serum samples and brain tissues, maximal toltrazuril concentrations were found 2 to 3 days after the respective treatments (Fig. 5a). Highest toltrazuril levels were observed 2 days after the second toltrazuril application in both serum samples (10.1 mg/L) and brain (9.2 mg/kg) tissues. After reaching maximal values 2 to 3 days after drug application, toltrazuril levels decreased until the next treatment. There was no difference in toltrazuril concentration measured between serum samples or brain tissues. Toltrazuril concentrations in serum samples and brain tissues were below detection limit from day 8 after the third treatment onwards. Newborn mice were able to metabolize toltrazuril, as the major metabolite toltrazuril sulfone was found in serum samples and brain tissues (Fig. 5b). Toltrazuril sulfone accumulated after two applications of toltrazuril and reached maximal concentration in serum samples (18.5 mg/L) and brain tissues (9.0 mg/kg) 2 days after the second treatment. Thereafter, the concentration decreased in serum samples and brain tissues until the next treatment. After the third toltrazuril application, maximal metabolite concentrations were found 3 days later in serum samples and brain tissues. Toltrazuril sulfone levels decreased thereafter below detection limit at day 21 after the third treatment. Over the whole time period, significantly higher toltrazuril sulfone concentrations were observed in serum samples when compared to the concentrations found in brain tissues.

Discussion

To date, the main mode of control of neosporosis is based upon culling persistently infected, seropositive reproducing

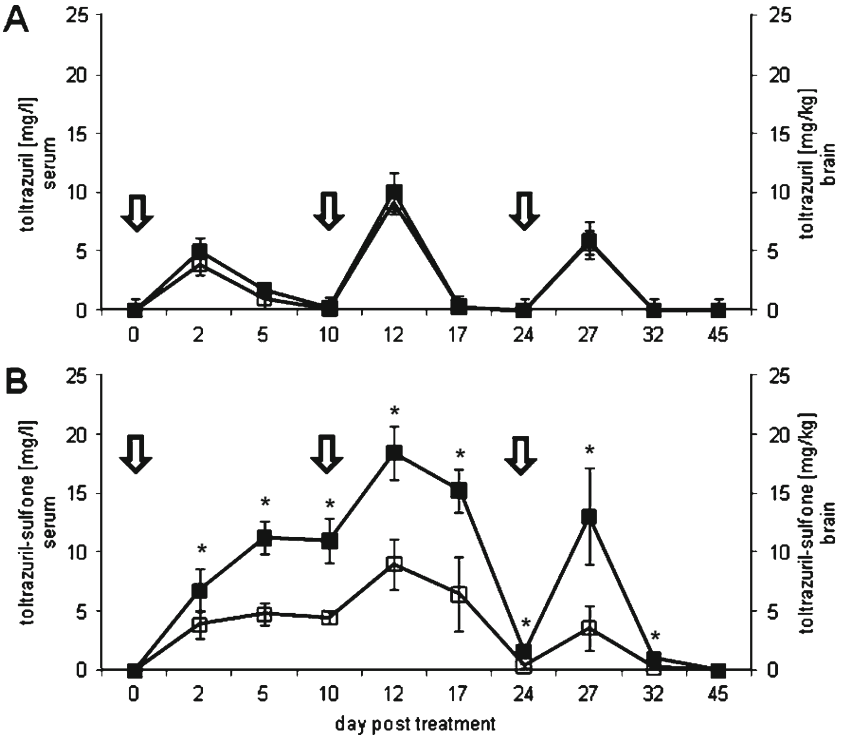

Fig. 5 Pharmacokinetic of toltrazuril. Concentration of toltrazuril (**a**) and its major metabolite toltrazuril sulfone (**b**) were evaluated over time after three oral applications of toltrazuril. Measurements were performed using serum samples (*closed squares*) and brain tissues (*open squares*) of uninfected and treated newborn mice. *Arrows* indicate day of treatment. *$p<0.05$; significance hold true

animals, as there is no effective treatment for cattle available so far (Andrianarivo et al. 2000; Innes and Vermeulen 2006). Toltrazuril is one of those few compounds that have demonstrated antiparasitic activity against *N. caninum* infections not only in cell culture-based assays, but also in vivo as shown in experimentally infected mice and even cattle (Darius et al. 2004a, b; Gottstein et al. 2001; Gottstein et al. 2005; Kritzner et al. 2002; Härdi et al. 2006).

The present study was designed as a further step in assessing the efficacy of toltrazuril by addressing the effect of toltrazuril in congenitally *N. caninum*-infected newborn mice. These experiments were intended to provide baseline data upon use of a small animal model, prior to carrying out similar experiments in the bovine system in the future. Recently, cell culture assays had indicated that toltrazuril required 14 days of in vitro treatment to yield parasiticidal activity; short-time exposures did not kill all parasites (Strohbusch et al. 2008). Extrapolating these findings to our mouse model, we should anticipate an appropriate and constant high toltrazuril concentration in vivo for at least 14 days; thus, repeated applications of toltrazuril seemed to be necessary. Congenitally infected newborn mice were treated with toltrazuril right after birth, either once or by three treatments. Control analyses showed that three consecutive control applications of toltrazuril alone (without concurrent infection) had no negative effect on the development and health status of newborns, thus justifying a multiple administration of the drug. With regard to the outcome of infection, both one and three toltrazuril applications significantly delayed the outbreak of congenitally acquired neosporosis in newborn mice, as pups treated once or three times with toltrazuril diseased at a significantly later time point when compared to placebo-treated pups. A marked delay of disease after toltrazuril treatment had already been observed in *N. caninum*-infected athymic nu^-/nu^- mice (Ammann et al. 2004), and these experiments had indicated that toltrazuril had a parasitostatic rather than a parasiticidal effect, requiring T cell-mediated support to control neosporosis. In the present study, the overall proportion of surviving animals in the three-time-toltrazuril-treated group was 1.6 times higher than the proportion of one-time-treated pups. Consequently, one application of toltrazuril appeared not as effective as repeated treatments. Similar findings were observed when comparing the number of diseased pups or the number of *N. caninum* PCR-positive pups. Both parameters were markedly reduced in three-time-toltrazuril-treated group compared to one-time-toltrazuril-treated and the placebo-treated pups. Finally, we conclude from these findings that a repeated toltrazuril application in newborn mice is mandatory to maintain an appropriate plasma and tissue level.

This became best apparent when the three-time-placebo-treated group yielded a proportion of surviving animals similar to that of one-time-toltrazuril-treated mice. We can only speculate why the three-time-placebo-treated group performed better than the one-time-placebo-treated group: intergroup variability may provide one explanation; another one would be that the repeated exposure of mice to the placebo may have supported nonspecific defense mechanisms in favor of the mouse and in disadvantage of the proliferating parasite.

It was shown earlier that newborn mice were as effective as adults in developing primary antigen-specific T cell populations within the first week of life (Adkins and Du 1998) and are thus fully immune competent at this age (Morein et al. 2002), although pathogen-specific immunity needs to be primarily developed upon first contact with respective organisms. Applied to our experiments, this allows the conclusion that congenitally infected pups appeared to have mounted a T cell immune response supporting toltrazuril treatment effectively. Antibodies also play an important role in the control of *N. caninum* infection, as B cell-deficient (μMT) mice were highly susceptible to infection (Eperon et al. 1999). Our data revealed that three-time-toltrazuril treatment yielded the highest antibody responses in treated pups. This is in line with the findings of Härdi et al. (2006), who observed the development of a strong humoral immune response after toltrazuril chemotherapy of congenitally (naturally) infected calves (Härdi et al. 2006). Similar results have also been documented in studies with *Eimeria*-infected chicken where an increase of parasite-specific antibody response after toltrazuril treatment was observed (Greif 2000).

Preliminary explorative experiments in cattle revealed a half-life time of toltrazuril sulfone (ponazuril) in serum samples of about 10 days (Kritzner et al. 2002). Toltrazuril sulfone was detectable up to 47 days after one application and up to 82 days after a 6-day treatment with 20 mg ponazuril per kg body weight. Pharmacokinetic evaluation of toltrazuril performed in the present study (unexpectedly) revealed that toltrazuril is much faster metabolized in newborn mice than in calves. The reason for this remains unclear. After three toltrazuril applications within 24 days, toltrazuril and toltrazuril sulfone concentrations already dropped below the limit of quantitation of 8 and 21 days, respectively, after the last medication. Furthermore, there was no accumulation of toltrazuril between the three treatments, indicating that time intervals between the application days may have been too long to establish a constant high toltrazuril concentration over a 14-day time period. Nevertheless, evaluation of drug concentration in brain tissues demonstrated for the first time that toltrazuril and toltrazuril sulfone passed the blood–brain barrier in mice. Similar findings were observed after treatment of horses with toltrazuril (Furr and Kennedy 2000).

The results presented in this study indicated that treatment with three applications of toltrazuril had the best positive effect to control the course of infection in congenitally *N. caninum*-infected newborn mice. On the other hand, we had to accept that both treatment schedules did not yield a complete end-point elimination of the parasite load. One explanation for this may have been the fact that the toltrazuril levels did not remain constantly high, as initially anticipated, but transiently dropped to low levels within intervals of a few days. Consequently, future studies will require a careful adaptation of the treatment schedule, based on the pharmacokinetic results, to obtain constant toltrazuril levels over a long time period. This might then increase the proportion of surviving congenitally infected newborn mice. Nevertheless, the present findings will be useful to address the question of efficacy in the experimental bovine model. Provided we can reach similar or even improved levels of efficacy, then the final aim will be to maturate an appropriate treatment schedule such as to allow controlling the problem at a national level of bovine production, anticipating a positive cost–benefit ratio as claimed by Häsler et al. (2006a, b; 2008).

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