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Comparative efficacy of immunisation with inactivated whole tachyzoites versus a tachyzoite-bradyzoite mixture against neosporosis in mice

Running title: Immunisation with inactivated whole tachyzoites and bradyzoites

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ABSTRACT

The worldwide economic impact of *Neospora caninum* infection has caused the development of effective vaccines to become one of the main goals in the field of neosporosis research. In this study, the protection conferred by antigens from inactivated whole tachyzoites (TZ) and a tachyzoite-bradyzoite mixture (TZ-BZ) of *N. caninum* (Nc-Spain7 isolate) incorporated into a water-in-oil emulsion (W/O) and aluminium hydroxide-ginseng extract (Al/G) was evaluated in mouse models of congenital and cerebral *N. caninum* infection. Immunisation with TZ-BZ induced congenital and cerebral neosporosis exacerbation that was mainly characterised by reduced neonatal median survival time and increased parasite presence in adult mouse brains. The immune response of mice immunised with TZ-BZ was characterised by an increase in IFN-γ expression prior to challenge and an increase in IL-4 expression accompanied with significantly higher levels of antibodies against two recombinant bradyzoite-specific proteins (rNcSAG4 and rNcBSR4) after challenge. Immunisation with TZ in W/O significantly reduced neonatal mortality, vertical transmission as well as parasite presence in adult mouse brains and induced a strong humoral immune response. The current study demonstrates the critical role of stage-specific antigens and adjuvants on the development of effective inactivated vaccines for the prevention of *N. caninum* infection.

Key words: *Neospora caninum* / inactivated vaccine / tachyzoite / bradyzoite / mice

INTRODUCTION

The tissue-cyst forming protozoan parasite *Neospora caninum* has emerged as an important cause of reproductive failure in cattle worldwide, leading to significant economic losses in beef and dairy cattle industries (Dubey et al. 2007). In cattle, *N. caninum* is able to persist in the brain of an immunocompetent host in a cyst form containing slowly dividing bradyzoites (BZ). The cysts remain quiescent while awaiting an appropriate immunological scenario wherein they switch into fast replicating tachyzoites (TZ). In pregnant animals, TZ can disseminate and cause potentially fatal disease, resulting in abortion, birth of a weak calf or birth of a clinically healthy but persistently infected calf (Buxton et al. 2002; Innes et al. 2002).

Epidemiological evidence confirming the protective immunity against vertical transmission and abortion in some *N. caninum*-infected cows makes immunoprophylaxis a feasible alternative to control the disease (Dubey et al. 2007; Innes et al. 2002; Reichel and Ellis 2006). In recent years, most vaccine trials against *N. caninum* have focused on molecules from TZ in the form of live, inactivated or recombinant antigens, principally to control the acute phase of infection (Liddell et al. 1999; Lunden et al. 2002; Reichel and Ellis 2009; Ribeiro et al. 2009). Using vaccines containing parasite antigens from different life-cycle stages could be a promising strategy to improve the protection conferred by vaccines against neosporosis. Recently, we evaluated the role of the recombinant bradyzoite-specific SAG4 (rNcSAG4) protein of *N. caninum* in the protection against chronic and congenital infection in mice (Aguado-Martínez et al. 2009a). This study revealed that immunisation with rNcSAG4 failed to protect against parasite infection. However, a slight yet significant delay in the death of pups in the rNcSAG4-vaccinated group was observed, motivating future studies to evaluate the effect of vaccines made from bradyzoite antigens. To this end, a

mixture of whole *Neospora* TZ and BZ containing native organelle and membrane antigens from both stages could be a good candidate for a vaccine development not only against the rapid dissemination of parasite during the acute infection phase but also against the establishment of persistent *N. caninum* infection.

We previously observed that immunisation with whole inactivated TZ conferred protection against acute and chronic neosporosis in non-pregnant BALB/c mice when combined with appropriate adjuvants and antigen doses (Rojo-Montejo et al. 2011). Specifically, a water-in-oil emulsion (W/O) adjuvant containing 5×10^5 inactivated whole TZ reduced parasite presence in the brain during the chronic stage of infection, whereas aluminium hydroxide co-administrated with ginseng extract (Al/G) containing 5×10^5 inactivated whole TZ significantly reduced acute parasitaemia. These results suggest that the W/O may control the establishment of infection in brain, whereas the Al/G may control the vertical transmission of the parasite to progeny by reducing maternal parasitaemia and therefore the number of parasites reaching the placenta and foetus.

The objective of the present study was to investigate the protective efficacy of previously tested vaccine preparations containing inactivated whole *N. caninum* TZ as well as a new formulation of a tachyzoite-bradyzoite mixture (TZ-BZ) combined with W/O or Al/G against congenital and cerebral neosporosis with both pregnant and non-pregnant BALB/c mouse models (López-Pérez et al. 2006; López-Pérez et al. 2008).

MATERIALS AND METHODS

Parasite Culture

Culture of N. caninum zoites for immunisation

The *N. caninum* Nc-Spain7 isolate, recently obtained from the brain of an asymptomatic but congenitally infected calf (Regidor-Cerrillo et al. 2008), was used for immunisation.

TZ were grown by continuous passage in MARC-145 cells following standard procedures (Pérez-Zaballos et al. 2005). TZ for vaccine formulations were harvested 3.5 days post-infection. For formulations containing TZ-BZ, *in vitro* stage conversion was induced by treatment of Nc-Spain7 tachyzoite- infected culture with 70 μM sodium nitroprusside (SNP) for up to 7 days (Risco-Castillo et al. 2004). Zoites were harvested from tissue culture on day 7 post-stress. Both TZ and TZ-BZ cultures were purified by washing three times in sterile phosphate-buffered saline (PBS, pH 7.4) and separated from host cell debris by passing the mixture through a 25-gauge needle following passage through disposable PD-10 desalting columns (GE Healthcare, Buckinghamshire, UK) (Hemphill et al. 1996). Cell-free Nc-Spain7 zoites were counted by Trypan blue exclusion followed by counting in a Neubauer chamber, adjusted to a final concentration of 5×10^7 zoites/ml and immediately inactivated as described in *"Vaccine formulations"* Section.

Conversion measurement assay

Seven days after stress treatment, the tachyzoite-to-bradyzoite conversion rate was measured by double immunofluorescence (Risco-Castillo et al. 2004). Identification of TZ and BZ was performed by labelling parasites with antibodies directed against the immunodominant *N. caninum* tachyzoite surface antigen NcSAG1 (Fuchs et al. 1998) and antiserum against *T. gondii* bradyzoite antigen 1 (BAG1), which exhibits cross-reactivity with *N. caninum* BZ (McAllister et al. 1996). Coverslips were labelled with a monoclonal mouse antibody directed against the tachyzoite surface antigen NcSAG1 (αSAG1) (1:2000) and a polyclonal rabbit antiserum against the intracytoplasmic bradyzoite antigen BAG1 (αBAG1) (1:100) (Risco-Castillo et al. 2004). Antibody binding was observed with an inverted fluorescence microscope (Model TE200 Nikon, 100× oil-immersion objective). The conversion rate of TZ to BZ was calculated by

random counting of 10 fields per coverslip and comparing the percentage of BAG1-positive zoites *vs.* total zoites. Double immunofluorescence revealed a TZ-to-BZ conversion rate around 30%, which was used for vaccine formulations containing TZ-BZ antigen.

Parasite culture for challenge

Tachyzoites of the Nc-Liverpool isolate (Barber et al. 1995) were maintained *in vitro* by continuous passage in MARC-145 cells using standard procedures (Pérez-Zaballos et al. 2005). For *N. caninum* challenge, TZ were prepared followed described procedures (López-Pérez et al. 2006; López-Pérez et al. 2008) at the required dose of 2×10^6 TZ in a final volume of 200 µl per mouse and used immediately to infect mice.

Vaccine formulations

Purified Nc-Spain7 TZ and TZ-BZ mixture were inactivated with 0.01 M (final concentration) of binary ethylenimine for a period of 96 h at 4ºC, followed by neutralisation with sodium thiosulphate (Andrianarivo et al. 2000). Inactivated whole Nc-Spain7 zoites were incorporated into the W/O or Al/G. The W/O was used at a concentration of 104 mg per dose. In the adjuvant Al/G, aluminium hydroxide was used at a concentration of 1.53 mg per dose combined with 0.2 mg of ginseng extract. Both adjuvant preparations were developed by HIPRA (Girona, Spain). The immunising doses were prepared in a final volume of 200 µl per mouse.

Immunisation assay and sample collection

All mouse handling procedures complied with EU legislation. Eight-week-old female BALB/c mice (Harlan Interfauna Ibérica, Barcelona) were randomly divided into 10 groups. Each mouse was injected subcutaneously twice at 3 week intervals with 5×10^5 inactivated whole *N. caninum* zoites (TZ or TZ-BZ) incorporated into the W/O, the Al/G or PBS. Other groups received the W/O, the Al/G or PBS alone (Table 1). For

logistical reasons, the experiment was carried out at two different times. First, vaccine formulations containing *N. caninum* TZ were inoculated. Next, mice were immunised with *N. caninum* TZ-BZ. Non-immunised/non-challenged (group 9) and non-immunised/challenged (group 10) mice were included at both time points (subgroups a and b) of the experiment to ensure the reproducibility of the technique. Three weeks after the booster immunisation, BALB/c mice were mated for 96 h following synchronisation of oestrus using the Whitten effect (Whitten, 1957). Day 0 of pregnancy was defined as the first day that females were housed with males. The challenge infection was achieved by subcutaneously inoculating animals with 2×10^6 Nc-Liverpool TZ at mid-gestation (between days 6 and 10 of gestation). Pregnant animals were housed individually and allowed to carry their pregnancy to term. Pups were evaluated daily from birth to day 30 postpartum (PP) for congenital neosporosis (López-Pérez et al. 2008). Dams and non-pregnant mice were evaluated for chronic infection until days 30 PP and 30 post-challenge, respectively (Collantes-Fernández et al. 2006; López-Pérez et al. 2008). Brains and lungs from neonates and brains from adult mice were removed aseptically and frozen at –80ºC until required for DNA extraction. Samples from some progeny could not be collected due to cannibalism by the dams. Immune responses were evaluated in 5 randomly selected mice from each group prior to the challenge (2 days after booster) and in dams at the chronic phase (day 30 PP). The number of pregnant mice analysed at this time point (day 30 PP) varied between 5 and 10 animals per group; the number of pregnant mice analysed was related to the number of dams that survived until the end of the experiment. To measure the humoral immune response, blood samples were collected by cardiac puncture, and the recovered sera were aliquoted and cryopreserved at -80 ºC until serological analysis. For measurements

of cytokine expression, spleen samples from each group were pooled (0.05 g of spleen tissue per mouse) and cryopreserved at -80 ºC until processed as described below.

Parameters evaluated for safety and efficacy

The safety of different formulations was determined by daily observation of mice for adverse reactions and by palpation for the presence of nodules at the inoculation site on day 10 after the booster vaccination. Vaccine efficacy studies were performed using both pregnant and non-pregnant BALB/c mouse models as previously described (Aguado-Martínez et al. 2009a; Rojo-Montejo et al. 2011). In the pregnant mouse model, data on litter size, hebdomadal mortality, temporal evolution and the rate of neonatal mortality and vertical transmission were collected. Litter size was defined as the number of pups delivered per dam. Hebdomadal mortality was defined as the number of full-term dead pups at the time of birth and those that died between birth and day 2 PP. Neonates were examined daily for morbidity and mortality. Neonatal mortality was defined as the number of dead pups from day 2 to day 30 PP. Temporal evolution of neonatal mortality was evaluated using Kaplan-Meier survival curves. Finally, vertical transmission of *N. caninum* was identified by the presence of parasites in the lungs or brains of pups that died within 48 h (hebdomadal mortality) and pups born alive. Vaccine efficacy against the chronic *N. caninum* infection phase was studied in dams and non-pregnant mice by checking clinical signs compatible with neosporosis, mortality and the presence of *N. caninum* DNA in the brain.

DNA extraction and nested PCR

Tachyzoites for PCR controls were prepared as previously described (López-Pérez et al. 2006). The Real Pure Extraction genomic DNA kit (Durviz, Valencia, Spain) was used to extract DNA from 10-20 mg of each host tissue and 10^7 *N. caninum* tachyzoites according to the manufacturer's instructions. Amounts of DNA were measured

spectrophotometrically, and samples used for DNA detection by nested PCR were diluted to a final concentration of 50 ng/µl. For the detection of parasite DNA, nested PCR on the internal transcribed spacer (ITS1) region of *N. caninum* was performed with four oligonucleotides as described by Buxton et al. (Buxton et al. 1998). Secondary amplification products were visualised by 1.8% agarose gel electrophoresis and ethidium bromide staining. To avoid false positives, DNA extraction, PCR sample preparation and electrophoresis were performed in separate rooms employing different sets of instruments, aerosol barrier tips and disposable gloves. Moreover, negative control samples were included in each set of DNA extractions and PCR reactions.

Analysis of humoral immune response

Serum levels of *N. caninum*-specific IgG1 and IgG2a were measured as identifiers of Th2 and Th1 immune responses, respectively, with an ELISA based on *N. caninum* soluble tachyzoite antigens (Collantes-Fernández et al. 2006; Long et al. 1998).

Specific IgG response developed against different life-cycle stages were measured by ELISA based on the stage-specific recombinant NcGRA7 and NcSAG4 proteins according to previously described methods (Aguado-Martínez et al. 2009a; Aguado-Martínez et al. 2009b). Similarly, a recombinant NcBSR4 protein-based ELISA was developed to measure serum levels of specific IgG against NcBSR4 protein. The dense granule protein NcGRA7 is an immunogenic antigen that is highly associated with active replication of the parasite (Aguado-Martínez et al. 2008; Jenkins et al. 1997). Therefore, we used recombinant NcGRA7 (ÁlvarezÁlvarez-García et al. 2007) as an antigen to evaluate the antibody response developed against TZ. The recently described *N. caninum* NcSAG4 (Fernández-García et al. 2006) and NcBSR4 (Risco-Castillo et al. 2007) proteins are stage-specific antigens expressed at early and late bradyzoite stages, respectively. We employed both of them to measure the humoral immune response

specifically developed against this slowly dividing parasite stage. The ELISA results were expressed as a relative index percent (RIPC) using the following formula: RIPC = (OD_{405} sample-OD_{405} negative control)/(OD_{405} positive control-OD_{405} negative control) × 100, where OD is the mean value of the optical density. For recombinant protein-based ELISA (GRA7, SAG4 and BSR4), 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 sera from non-immunised/non-infected mice.

Analysis of cytokine expression

Cytokine expression was evaluated in spleen by real-time RT-PCR as previously described (López-Pérez et al. 2010). Briefly, pooled spleens (0.05 g per mouse) were homogenised in the commercial TRI REAGENT (Sigma, St. Louis, MO, USA) by a Polytron PT1600E homogeniser (Kinematica, AG, Lucerne, Switzerland). Total RNA was extracted following manufacturer's instructions, and the integrity was checked by agarose gel electrophoresis. cDNA synthesis was performed with SuperScript II Reverse Transcriptase (Invitrogen, Paisley, United Kingdom) following the manufacturer's recommendations. The primer sequences used for the amplification of interferon γ (IFN-γ), interleukin 4 (IL-4) and β-actin cDNA have been previously published (Varona et al. 2005). Real-time PCR was performed using the ABI PRISM 7300 Sequence Detector Machine (PE Applied Biosystem, Foster City, California, USA) with the commercial kit Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Paisley, United Kingdom). Each sample was analysed in triplicate, and the cycle threshold (Ct) value was obtained with Sequence Detection System Software. All cycle threshold values were normalised to the expression of the housekeeping gene β-actin. For relative quantification of gene expression, the Comparative Threshold Cycle method was used. The relative n-fold change of each target cytokine expression,

normalised to the endogenous reference and relative to the control group, is given by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

Data analysis.

All immunised groups were compared to the non-immunised/challenged group to evaluate the protective efficacy of the vaccine. The influence of the stage-specific antigens and adjuvant type was evaluated by comparing the different antigens (TZ *vs.* TZ-BZ) within the same adjuvant group and the different adjuvants (W/O *vs.* Al/G) with the same type of antigen, respectively.

Differences in morbidity, mortality, parasite detection and vertical transmission rates were organised in a contingency table. A Chi-squared test or Fisher F-test was performed, and Bonferroni´s adjustment was applied to the *P* value. Post-natal mortality was analysed by the Kaplan-Meier survival method (Bland and Altman 1998) to estimate the percentage of surviving individuals at each time point (days PP). To compare the survival curves between groups, the log-rank statistical test was applied (Bland and Altman 2004). Litter size and serological data were compared using a one-way analysis of variance (one-way ANOVA) test, followed by Tukey's multiple comparison test. All statistical analyses were carried out using Statgraphics Plus v. 5.1 (StatPoint, Inc., Herndon, VA, USA) and GraphPad Prism 5 v. 5.01 (San Diego, CA, USA) software.

RESULTS

Vaccine safety

No systemic side effects were observed in any animal after immunisation. Upon palpation, nodules at the injection site were found in 64.7% to 100% of mice receiving W/O or Al/G, respectively.

Protective efficacy against congenital neosporosis

Different degrees of protection against congenital parasite infection afforded by the different vaccine formulations tested were observed (Tables 2 and 3 and Fig. 1). Dams vaccinated with TZ-BZ (groups 2, 5 and 8) and TZ (group 7) incorporated into PBS transmitted the infection more efficiently to their offspring in comparison with the non-immunised/challenged group (group 10) and displayed the most severe outcome of congenital neosporosis. A significant increase of hebdomadal mortality (groups 2 and 5 *vs.* group 10; $P<0.0001$) and a shorter median survival time of neonates (groups 2, 5, 7 and 8 *vs.* group 10; $P<0.05$) were observed in these groups. In contrast, the group vaccinated with TZ and W/O (group 1) had a lower mortality rate ($P<0.005$), longer median survival time ($P<0.005$) and reduced vertical transmission ($P<0.0001$) in comparison with the non-immunised/challenged group (group 10).

When the influence of the antigen type was evaluated, higher hebdomadal mortality rates were observed in all groups vaccinated with TZ-BZ and W/O, Al/G or PBS (group 2 *vs.* groups 1 and 3; group 5 *vs.* groups 4 and 6; group 8 *vs.* group 7; $P<0.01$). Moreover, pups born alive from dams immunised with TZ-BZ with the Al/G exhibited a shorter median survival time (group 5 *vs.* groups 4 and 6; $P<0.0001$). Conversely, among the groups vaccinated using the W/O adjuvant, pups from dams given TZ displayed a lower neonatal mortality rate, longer median survival time and lower vertical transmission than pups from groups immunised with TZ-BZ or PBS (group 1 *vs.* groups 2 and 3; $P<0.0001$).

When the adjuvants were compared, mice vaccinated with TZ-BZ and Al/G showed higher hebdomadal mortality than mice given the same antigen and PBS (group 5 *vs.* group 8; $P<0.001$). A protective adjuvant-dependent effect was observed in mice vaccinated with TZ and W/O; the pups of these mice that were born alive showed a

lower mortality rate, longer median survival time and lower vertical transmission than pups from groups immunised with Al/G or PBS (group 1 *vs.* groups 4 and 7; $P<0.005$). Taken together, these results show that immunisation with the TZ-BZ antigen exacerbated congenital neosporosis and allowed parasite transmission to offspring during pregnancy. In contrast, immunisation with TZ and W/O partially limited vertical transmission of parasite to progeny, preventing offspring infection.

Protective efficacy against cerebral neosporosis

Data on *N. caninum*-related clinical signs, mortality and parasite detection in brain during chronic infection phase are summarised in Table 4. Mice immunised with TZ-BZ (groups 2, 5 and 8) and TZ (group 7) with PBS exhibited more severe cerebral neosporosis than the non-immunised/challenged group (group 10), showing a higher frequency of clinical signs (groups 2, 5, 8 and 7 *vs.* group 10; $P<0.001$) and higher mortality rates (groups 5, 8 and 7 *vs.* group 10; $P<0.0001$). On the contrary, immunisation with W/O and TZ reduced the presence of *N. caninum* in the brain compared with mice from the non-immunised/challenged group (groups 1 *vs.* group 10; $P<0.01$).

The effect of the type of antigen was observed in the group inoculated with Al/G and TZ-BZ, in which clinical signs and mortality were significantly increased compared with those vaccinated with the same adjuvant and TZ or PBS (group 5 *vs.* groups 4 and 6; $P<0.01$).

When the influence of adjuvant type was evaluated, an increase in morbidity and mortality rates was observed in groups immunised with TZ or TZ-BZ plus PBS or Al/G when compared to groups given the W/O adjuvant (groups 4 and 7 *vs.* group 1; groups 5 and 8 *vs.* group 2; $P<0.01$). Similarly, an adjuvant-dependent increase in mortality rate

was observed in mice given TZ or TZ-BZ and PBS with respect to those given the Al/G adjuvant (group 7 *vs.* group 4; group 8 *vs.* group 5; $P<0.01$).

Together, these data show that the TZ-BZ antigen did not limit the establishment of chronic infection in brain and in fact exacerbated cerebral neosporosis. Conversely, immunisation with TZ with the W/O adjuvant reduced the parasite multiplication in brain during chronic infection.

Immune response prior to challenge

Production of IgG1 was observed in all groups that were immunised with *N. caninum* antigen combined with an adjuvant, but the highest levels were observed after immunisation with TZ or TZ-BZ plus W/O (group 1 *vs.* groups 7 and 9; group 2 *vs.* groups 5, 8 and 9; $P<0.0001$) (Fig. 2A). With respect to the IgG2a isotype, the group given TZ-BZ and W/O produced the highest antibody levels (group 2 *vs.* groups 5, 8 and 9; $P<0.01$).

The anti-rGRA7 IgG response was significantly higher in mice immunised with TZ-BZ and W/O when compared with groups immunised with the same adjuvant, antigen or with non-immunised group (group 2 *vs.* groups 1, 3, 5, 8 and 9; $P<0.0001$) (Fig. 3A). No *N. caninum*-specific rNcSAG4 and rNcBSR4 IgGs were detected prior to challenge in any immunised groups (Fig. 3B and 3C).

IFN-γ mRNA expression was induced in groups immunised with *N. caninum* antigen-containing preparations (TZ or TZ-BZ) and was highest in groups immunised with bradyzoite-specific antigens (groups 2, 5 and 8). No up-regulated IL-4 mRNA expression was detected in any immunised group (Fig. 4A).

Immune response after challenge

During the chronic infection phase, mice exhibited a strong anti-*N. caninum* response by producing IgG1 and IgG2a (Fig. 2B). Significant differences in IgG2a production

were detected only between some immunised groups when compared with the non-immunised/challenged group (groups 1, 5, 7 and 8 *vs.* group 10; $P<0.0001$). When the effect of adjuvant or antigen was evaluated, no influence on antibody response was observed.

Regarding the specific antibody response against *N. caninum* recombinant proteins, significantly higher levels of anti-rNcGRA7 IgGs were detected in groups immunised with TZ-BZ (groups 2, 5 and 8 *vs.* group 10; $P<0.0001$) (Fig. 3A). Interestingly, TZ-BZ immunised groups produced significantly higher levels of antibodies against the recombinant bradyzoite-specific proteins rNcSAG4 (groups 2, 5 and 8 *vs.* group 10; $P<0.0001$) and rNcBSR4 (groups 5 and 8 *vs.* group 10; $P<0.0001$) (Fig. 3B).

With regard to the cytokine mRNA expression levels in spleens from dams, groups vaccinated with TZ-BZ (groups 2, 5 and 8) showed the highest IL-4 and IFN-γ transcript levels, with a predominance of IL-4 expression (Fig. 4B).

DISCUSSION

The present study was conducted to evaluate the protective effect of inactivated whole *N. caninum* TZ and TZ-BZ compositions formulated with two different adjuvants against congenital and cerebral *N. caninum* infection. The ability of whole TZ antigens to reduce acute parasitaemia and parasite brain infection when combined with Al/G and W/O, respectively, during chronic neosporosis was previously demonstrated in a non-pregnant mouse model (Rojo-Montejo et al. 2011). To determine if these formulations conferred protection not only against acute and cerebral neosporosis but also against congenital infection, a well-established pregnant BABL/c mouse model was used (López-Pérez et al. 2006; López-Pérez et al. 2008; Regidor-Cerrillo et al. 2010). This model is a highly stringent tool for testing the efficacy of vaccine formulations against the transmission of the parasite to progeny (Aguado-Martínez et al. 2009a) because

there is a high transmission rate of *N. caninum* to the offspring after inoculation of dams at the second trimester of gestation. The results obtained here indicate that different degrees of protection are strongly dependent on the type of antigen and the co-administered adjuvant.

An important feature of cyst-forming protozoan parasites such as *N. caninum* is their ability to establish a chronic infection by converting from rapidly proliferative TZ into BZ that remain hidden from the host immune system contained within tissue cysts. Vaccine preparations composed of antigens from different life-cycle stages may be advantageous to confer protection against the rapid dissemination and invasion of host cells by *N. caninum* TZ and the establishment and maintenance of a chronic infection after conversion of TZ to BZ (Innes and Vermeulen 2006). To our knowledge, BZ-specific antigens have only been employed as recombinant vaccine candidates against *N. caninum* infection in one instance (Aguado-Martínez et al. 2009a). In this previous study, we evaluated the induced immune response, safety and efficacy of immunisation with rNcSAG4 in a pregnant mouse model. Although no significant protection was found with this vaccine formulation, the decrease in pup mortality indicates that adjusting some aspects of vaccine development, such as ensuring the correct balance of the Th1/Th2 immune response or introducing new vaccine candidates, could lead to more encouraging results. One drawback of using recombinant preparations is that antigens may not retain their native conformation; consequently, their recognition by immune system may be different, causing a loss of immunogenicity (Aguado-Martínez et al. 2009a; Cannas et al. 2003; Srinivasan et al. 2007). In addition, most BZ-specific antigens from the closely related parasite *T. gondii* appear to be poorly or not at all immunogenic during infection, and this condition may be one of the mechanisms by which BZ escape immune surveillance (Di Cristina et al. 2004; Kim and Boothroyd

2005). Due to the complex interaction of the parasite and host, a inactivated whole TZ-BZ mixture composed of a combination of antigens from different life-cycle stages may induce better protective immunity than when the antigens are administered singly, as occurs in our previous study. Recent studies have shown that whole TZ (live, frozen-inactivated or heat-inactivated) preparations achieve a stronger type 1 immune response compared to other *N. caninum* antigen preparations (lysates in form of total, soluble or insoluble antigen). This response was characterised by increasing number of IFN-γ-producing NK cells and eliciting IL-12 and TNF-α production by bone marrow-derived DCs and high levels of IFN-γ by spleen cells (Feng et al. 2010; Klevar et al. 2007; Strohbusch et al. 2009). Therefore, in the present study, we compared the effect of immunisation with two different inactivated whole antigen preparations (TZ and TZ-BZ). Zoites for vaccine formulations were obtained from the Nc-Spain7 isolate of *N. caninum* (Regidor-Cerrillo et al. 2008), which transforms from TZ into BZ after stress by the SNP agent in cell culture. Because the conversion to BZ is a process that can be triggered by immune-derived stress, this *in vitro* procedure mimics early events of the TZ-BZ switch, in which mixed parasitophorous vacuoles composed of both TZ and BZ are obtained on day 7 after stress induction. Recently, we identified differentially expressed proteins during the tachyzoite-bradyzoite stage conversion, which are likely involved in early events of bradyzoite development, using the same *in vitro* conversion procedure and DIGE technology (Marugán-Hernández et al. 2010). Therefore, our initial hypothesis was that vaccination of mice with a TZ-BZ mixture, containing bradyzoite proteins up-regulated early during the conversion of TZ to BZ, may induce an effective immune response against initial steps of bradyzoite cyst formation.

Surprisingly, TZ-BZ-vaccinated groups presented the shortest median survival time of pups and the highest morbidity and mortality rates in adult mice, indicating exacerbated

congenital and cerebral neosporosis. These findings were associated with higher antibody levels against the *N. caninum* stage-specific proteins rNcGRA7, rNcSAG4 and rNcBSR4 after challenge. As mentioned above, the immunogenic dense granule protein NcGRA7 is highly involved in active replication of the parasite and host cell invasion (Augustine et al. 1999; Cho et al. 2005). The high antibody production against rNcGRA7 points to widespread tachyzoite proliferation that may explain the fatal outcome of infection. On the other hand, the magnitude of antibody levels against both bradyzoite-specific proteins rNcSAG4 and rNcBSR4 may indicate an antigenic restimulation as a consequence of the conversion to BZ by some parasites after challenge, probably to escape detection by the host immune system and to establish a persistent infection. In this study, immunisation with TZ-BZ induced a polarised type 1 immune response that was associated with a dramatic dissemination of parasites throughout the organism, where they crossed biological barriers such as the placenta or the blood-brain barrier and actively invaded foetal tissues and brain from adult mice. These results are in contrast with previous conventions describing the protective role of the Th1 immune response against *N. caninum* by parasite clearance via IFN-γ production (Khan et al. 1997; Nishikawa et al. 2001a; Yamane et al. 2000). The immunological mechanisms by which TZ/BZ immunisation led to the exacerbation of the disease are not clear. The immune-derived stress could favour the rapid dissemination of TZ to immune-privileged tissues, such as the brain, to evade the immune response, potentially allowing uncontrolled TZ invasion and replication to occur. The detection of the highest levels of antibody against bradyzoite-specific proteins may indicate that, once in the brain, some parasites began the conversion process and that immunisation was not able to block the first steps of the conversion from TZ to BZ as we initially hypothesised. Because proper techniques to detect tissue

cysts in the brain were not used, further studies are required to determine whether vaccination with TZ-BZ antigens is able to impair both cyst formation and successful establishment of a chronic infection. Alternatively, inflammatory cytokine-mediated immunopathological changes may also contribute to disease severity. Dysregulation of the balance between Th1 and Th2 responses by the hyper-production of Th1-related cytokines such as IFN-γ was also associated with a failure in the protection against toxoplasmosis and neosporosis in different vaccine assays (Aguado-Martínez et al. 2009a; Kim and Boothroyd 2005; Ribeiro et al. 2009). Interestingly, a different pattern of cytokine expression was observed during chronic infection in the spleens of mice immunised with TZ-BZ, where IL-4 expression was up-regulated. For other intracellular protozoa such as *Leishmania*, it has been suggested that parasite load might affect the type of immune response developed. High parasite load would favour a Th2 response, which acts directly to down-regulate Th1 cells (Hondowicz and Scott 2002). The more rapid dissemination and uncontrolled parasite multiplication within host tissues in TZ-BZ-vaccinated groups may induce a progressive impairment of immune functions, dysregulating the delicate balance between infection control and host survival and finally resulting in an exacerbated neosporosis.

In accordance with previous results (Rojo-Montejo et al. 2011), vaccination with inactivated whole TZ and the W/O emulsion induced partial protection to congenital infection by significantly reducing neonatal mortality (from 84% to 51.6%) and vertical transmission (84% to 33.3%) and conferred complete protection in 2 out of 5 (40%) litters. A strong humoral immune response with predominant IgG1 production and a cellular response dominated by IFN-γ cytokine expression were produced prior to challenge with W/O and TZ. This result suggests a mixed Th1/Th2 responses that may be beneficial to limit dissemination and protect offspring against congenital infection of

N. caninum. Previously, a study in congenital neosporosis showed that protection against vertical transmission in mice immunised with different recombinant vaccinia viruses was related to the cellular immune response and high IgG1 antibody levels. These results suggest a role for a high level of IgG1 production in the clearance of *N. caninum* at early stage of infection and the role of a T cell response in later stage of infection (Nishikawa et al. 2001b).

Immunisation of mice with TZ and Al/G failed to confer protection against transplacental transmission and cerebral infection of *N. caninum,* as we have observed previously (Rojo-Montejo et al. 2011). A slight improvement in vaccine formulation was detected because no exacerbated neosporosis was observed in mice immunised with TZ in the vaccine containing Al/G. However, the immune response induced by immunisation with TZ and Al/G was clearly insufficient to generate significant protection against cerebral and congenital neosporosis.

Although the focus of the present study was the evaluation of safety and efficacy, antibody and cytokine responses were also measured. Interestingly, differences in cytokine expression levels depending on the type of antigen inoculated were observed between immunised groups, as indicated above. While the cytokine mRNA expression data indicate a strong trend, all cytokine measurements were performed using pooled spleen samples, and consequently, the data could not be statistically analysed. Thus, the data should be interpreted with caution.

In summary, the present study highlights the critical role of stage-specific antigens and different adjuvants in the development of effective inactivated vaccines for the prevention of *N. caninum* infection. Immunisation with TZ-BZ considerably affected the immune response generated against *N. caninum* infection, after which the most severe congenital and cerebral neosporosis were detected, probably due to an inadequate

balance of immune response. On the contrary, partial protection including reduced vertical transmission and neonatal mortality was observed in the group given inactivated whole *N. caninum* TZ combined with the W/O adjuvant. Although the present study showed discouraging results on the use of bradyzoite antigens as vaccine candidates, immunisation against different stages of *N. caninum* is still a promising approach in vaccine developments. Further studies are required to identify the bradyzoite-specific proteins that induced protection against chronic infection and reactivation of the parasite by blocking the TZ-to-BZ conversion and vice versa.

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Table 1. Summary of group characteristics.

Group	Adjuvant	Parasite antigen	Challenge dose (Nc-Liv)
1[a]	W/O	TZ	2×10^6
2[b]	W/O	TZ-BZ	2×10^6
3[a]	W/O	PBS	2×10^6
4[a]	Al/G	TZ	2×10^6
5[b]	Al/G	TZ-BZ	2×10^6
6[a]	Al/G	PBS	2×10^6
7[a]	PBS	TZ	2×10^6
8[b]	PBS	TZ-BZ	2×10^6
9a, 9b[c]	---	---	PBS
10a, 10b[c]	---	---	2×10^6

[a] Mice were immunised with *N. caninum* whole TZ incorporated into W/O, Al/G or PBS in groups of 22 mice.

[b] Mice were immunised with the *N. caninum* whole TZ-BZ mixture incorporated into W/O, Al/G or PBS. The number of mice per group was expanded to 32 mice to make the statistical analysis more consistent by increasing the number of pregnant mice per group.

[c] Non-immunised/non-challenged (group 9) and non-immunised/challenged (group 10) mice were included at both time points (subgroups a and b) of the experiment to ensure the reproducibility of the experimental design. The number of mice in subgroups a and b was 22 and 32, respectively.

Table 2. Litter size, hebdomadal mortality and neonatal mortality of pups in each group.

Group	Litter size[a]	Hebdomadal mortality		Median survival time (days) [a]	Neonatal mortality	
		Per pups[b]	Per litters[c]		Per pups[d]	Per litters[e]
1 (W/O-TZ)	6.8 ± 0.8	3/34 (8.8%)	2/5 (40%)	24.7 ± 1.3	16/31 (51.6%)	2/5 (40%)
2 (W/O-TZ-BZ)	5.1 ± 1.8	22/42 (52.4%)	6/8 (75%)	7.3 ± 1.4	18/20 (90%)	7/7 (100%)
3 (W/O-PBS)	4.6 ± 1.1	4/42 (9.5%)	4/9 (44.4%)	19.4 ± 1.0	35/38 (92.1%)	9/9 (100%)
4 (Al/G-TZ)	4.6 ± 1.4	1/37 (2.7%)	1/8 (12.5%)	18.7 ± 1.0	32/36 (88.8%)	8/8 (100%)
5 (Al/G-TZ-BZ)	5.3 ± 1.2	35/53 (66%)	10/10 (100%)	3.1 ± 0.6	18/18 (100%)	10/10 (100%)
6 (Al/G-PBS)	3.8 ± 1.4	5/34 (14.7%)	4/9 (44.4%)	23.4 ± 1.4	19/29 (65.5%)	9/9 (100%)
7 (PBS/TZ)	5.6 ± 1.6	9/79 (11.4%)	4/14 (28.5%)	15.7 ± 0.5	66/70 (94.2%)	14/14 (100%)
8 (PBS/TZ-BZ)	7.0 ± 1.7	13/42 (30.9%)	4/6 (66.6%)	6.2 ± 0.7	29/29 (100%)	6/6 (100%)
9a (non-immunised/non-challenged)	5.1 ± 1.5	0/37 (0%)	0/8 (0%)	30.0 ± 0	0/37 (0%)	0/8 (0%)
9b (non-immunised/non-challenged)	5.0 ± 1.6	2/45 (4.4%)	2/10 (20%)	30.0 ± 0	0/43 (0%)	0/10 (0%)
10a (non-immunised/challenged)	5.2 ± 1.7	8/58 (13.7%)	5/11 (45.5%)	23.2 ± 0.9	42/50 (84%)	11/11 (100%)
10b (non-immunised/challenged)	5.4 ± 1.7	14/65 (21.5%)	6/12 (50%)	20.0 ± 0.9	43/56 (76.8%)	12/12 (100%)

[a] Average ± SD

[b] No. of hebdomadal dead pups/no. of pups born in the group (percentage).

[c] No. of litters with at least one stillbirth/no. of litters in the group (percentage).

[d] No. of pups dead from day 2 to 30 PP/no. of pups born alive (percentage).

[e] No. of litters with at least one pup dead from day 2 to 30 PP/no. of litters in the group (percentage).

Table 3. Vertical transmission in hebdomadal dead pups and pups born alive as detected by nested PCR.

Groups	Hebdomadal dead pups [a]	Pups born alive [b]	Per litter [c]
1 (W/O-TZ)	3/3 (100%)	9/27 (33.3%)	3/5 (60%)
2 (W/O-TZ-BZ)	16/17 (94.1%)	13/14 (92.8%)	7/7 (100%)
3 (W/O-PBS)	2/3 (66.6%)	22/25 (88%)	9/9 (100%)
4 (Al/G-TZ)	ND[d]	31/32 (96.9%)	8/8 (100%)
5 (Al/G-TZ-BZ)	27/28 (96.4%)	9/10 (90%)	10/10 (100%)
6 (Al/G-PBS)	3/4 (70%)	25/27 (92.6%)	9/9 (100%)
7 (PBS/TZ)	6/7 (85.7%)	47/49 (95.9%)	14/14 (100%)
8 (PBS/TZ-BZ)	13/13 (100%)	17/18 (94.4%)	6/6 (100%)
9a (non-immunised/non-challenged)	0/0 (0%)	0/37 (0%)	0/8 (0%)
9b (non-immunised/non-challenged)	0/2 (0%)	0/43 (0%)	0/10 (0%)
10a (non-immunised/challenged)	3/5 (60%)	42/50 (84%)	11/11 (100%)
10b (non-immunised/challenged)	8/11 (72.7%)	43/56 (76.8%)	12/12 (100%)

[a] No. of infected stillborns/no. of stillborns analysed in the group (percentage).

[b] No. of positive pups born alive/no. of analysed pups born alive (percentage).

[c] No. of litters with at least one positive pup/no. of analysed litters (percentage).

[d] ND: not determined. No brain samples from hebdomadal dead born pups could be recovered and analysed as a result of cannibalism of the dams observed in all the experimental groups.

Table 4. Morbidity and mortality rates and detection of parasite DNA in adult mice at chronic infection phase.

Group	Morbidity [a]	Mortality [b]	Parasite presence [c]
1 (W/O-TZ)	3/16 (18.8%)	2/16 (12.5%)	6/16 (37.5%)
2 (W/O-TZ-BZ)	14/27 (51.9%)	9/27 (33.3%)	24/27 (88.9%)
3 (W/O-PBS)	6/17 (35.3%)	1/17 (5.9%)	10/17 (58.8%)
4 (Al/G-TZ)	11/17 (64.7%)	5/17 (29.4%)	15/17 (88.2%)
5 (Al/G-TZ-BZ)	26/27 (96.3%)	20/27 (74.1%)	26/27 (96.3%)
6 (Al/G-PBS)	3/17 (17.6%)	0/17 (0%)	11/17 (64.7%)
7 (PBS/TZ)	17/17 (100%)	17/17 (100%)	16/17 (94.1 %)
8 (PBS/TZ-BZ)	26/26 (100%)	26/26 (100%)	27/27 (100%)
9a (non-immunised/non-challenged)	0/17 (0%)	0/17 (0%)	0/17 (0%)
9b (non-immunised/non-challenged)	0/24 (0%)	0/24 (0%)	0/24 (0%)
10a (non-immunised/challenged)	1/16 (6.25%)	0/16 (0%)	13/16 (81.25%)
10b (non-immunised/challenged)	2/24 (8.3%)	2/24 (8.3%)	21/24 (87.5%)

[a] No. of mice with clinical signs compatible with neosporosis/no. of mice in the group (percentage).

[b] No. of sacrificed mice due to severity of clinical signs/no. of mice in the group (percentage).

[c] No. of nested PCR positive mice/no. of analysed mice in the group (percentage).

Figure captions

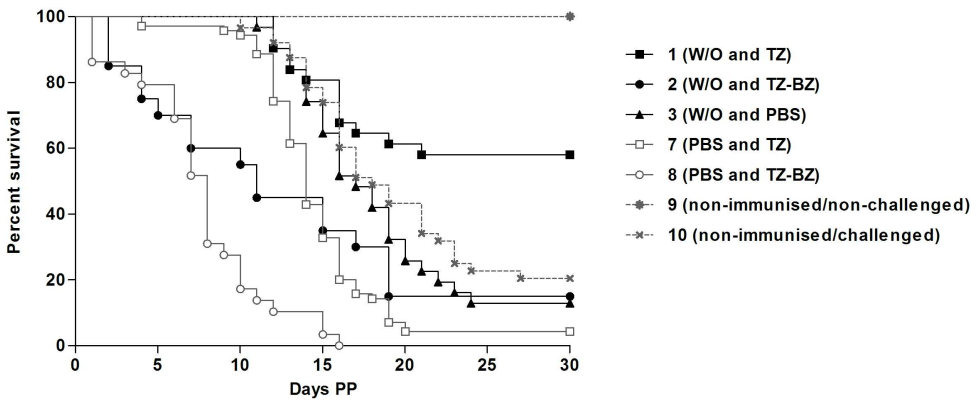
Fig. 1. Kaplan–Meier survival curves for neonates born from dams immunised with adjuvant W/O (panel A) or adjuvant Al/G (panel B). The curves represent the percent survival as the proportion of all individuals surviving over a period of 30 days PP. Vertical steps downward correspond to days PP when a death was observed. Symbols indicate censored observations.

Fig. 2. ELISA anti-*N. caninum* IgG1 and IgG2a both from BALB/c mice prior to challenge at day 2 after booster (panel A) and from dams at day 30 PP (panel B). Bars represent the mean relative index percent (RIPC), and error bars indicate the standard deviation for each group. A total number of 5 mice prior to challenge and all of the surviving dams at day 30 PP were included in the analysis. Descriptions of groups 1–10 are summarised in Table 1.

Fig. 3. ELISA results for anti-rNcGRA7 (panel A), anti-rNcSAG4 (panel B) and anti-rNcBSR4 (panel C) antibodies in both BALB/c mice prior to challenge at day 2 after booster and from dams after challenge at day 30 PP. Bars represent the mean relative index percent (RIPC), and error bars indicate the standard deviation for each group. Positive cut-offs were established in GRA7-based ELISA at ≥ 7.6 RIPC, in SAG4-based ELISA at ≥ 7.8 and in BSR4-based ELISA at ≥ 10 RIPC. Five mice prior to challenge and all of the surviving dams at day 30 PP were included in the analysis. Descriptions of groups 1–10 are summarised in Table 1.

Fig. 4. Cytokine expression in both BALB/c mouse spleen prior to challenge 2 days after booster (A) and after challenge in dams at day 30 PP (B). The results of real-time RT-PCR are given by $-2^{\Delta\Delta Ct}$. The $-2^{\Delta\Delta Ct}$ value for the control is 1. Bars represent the cytokine expression in the pool of each group. Five mice prior to challenge and all the

surviving dams at day 30 PP were included in the analysis. Descriptions of groups 1–10 are summarised in Table 1.



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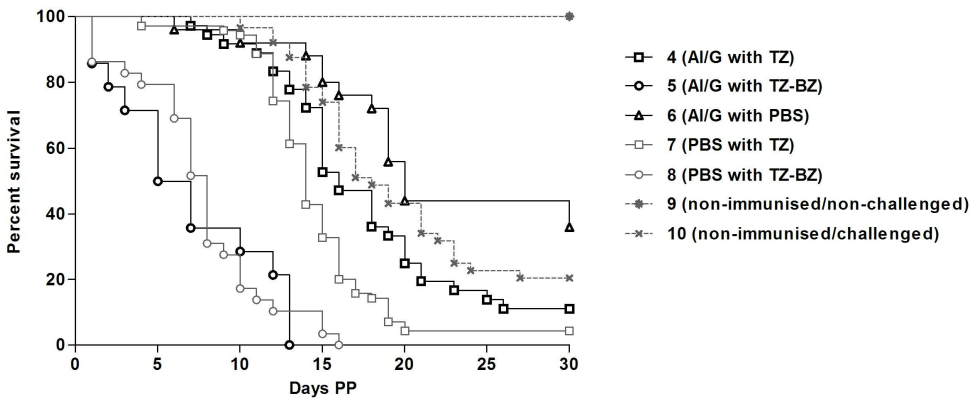

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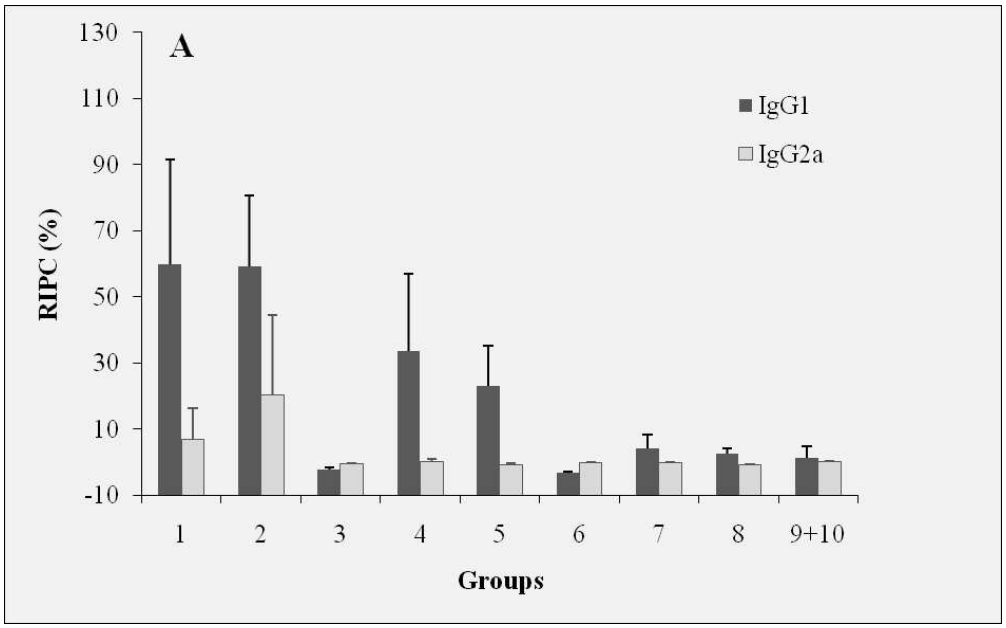

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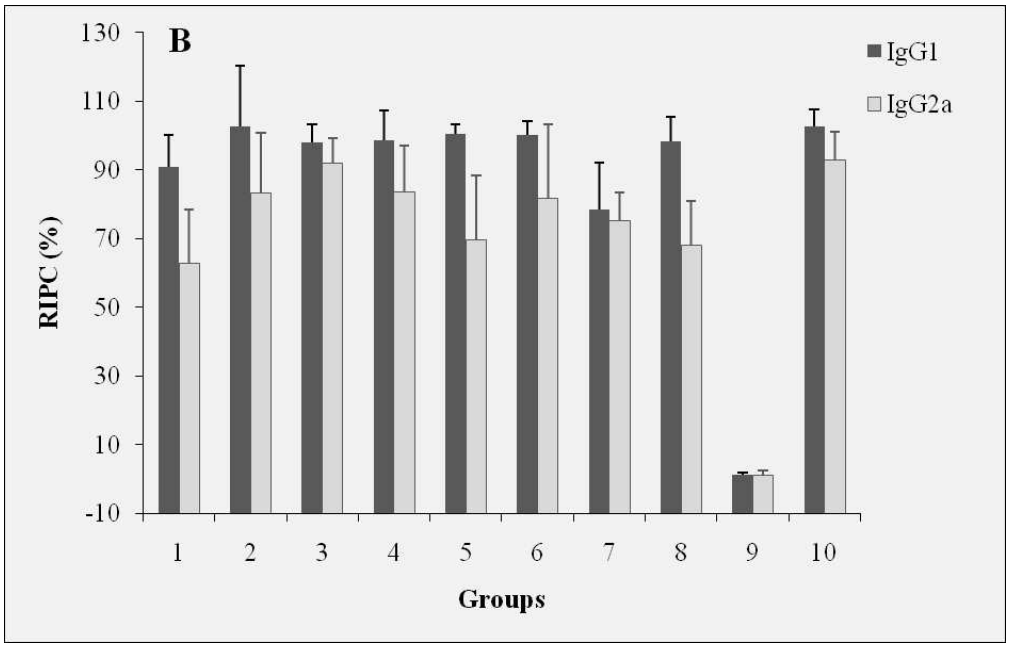

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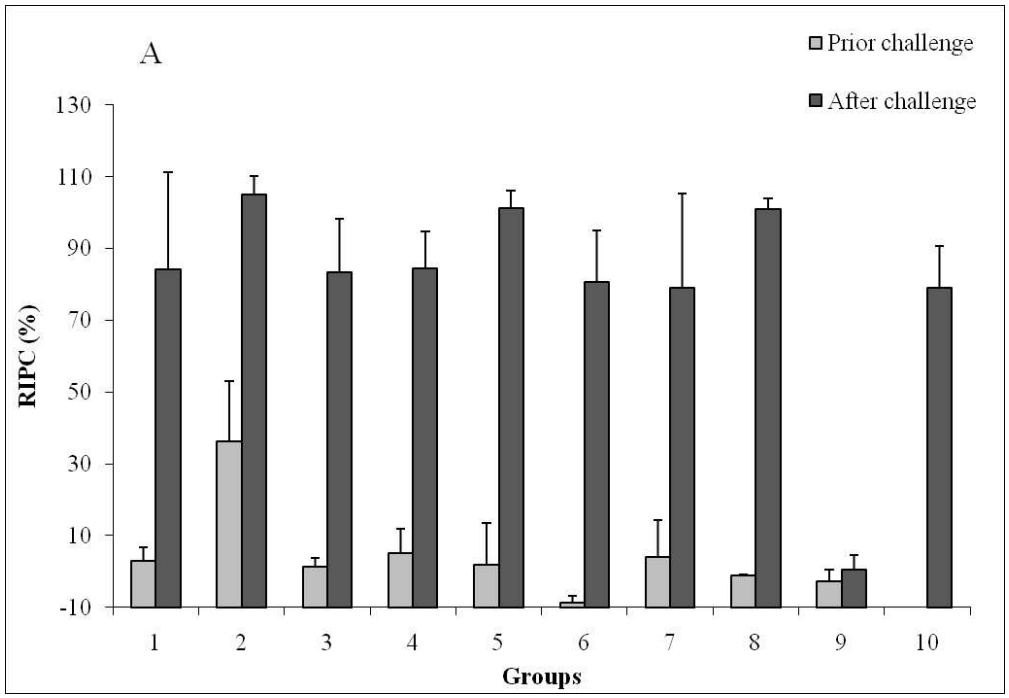

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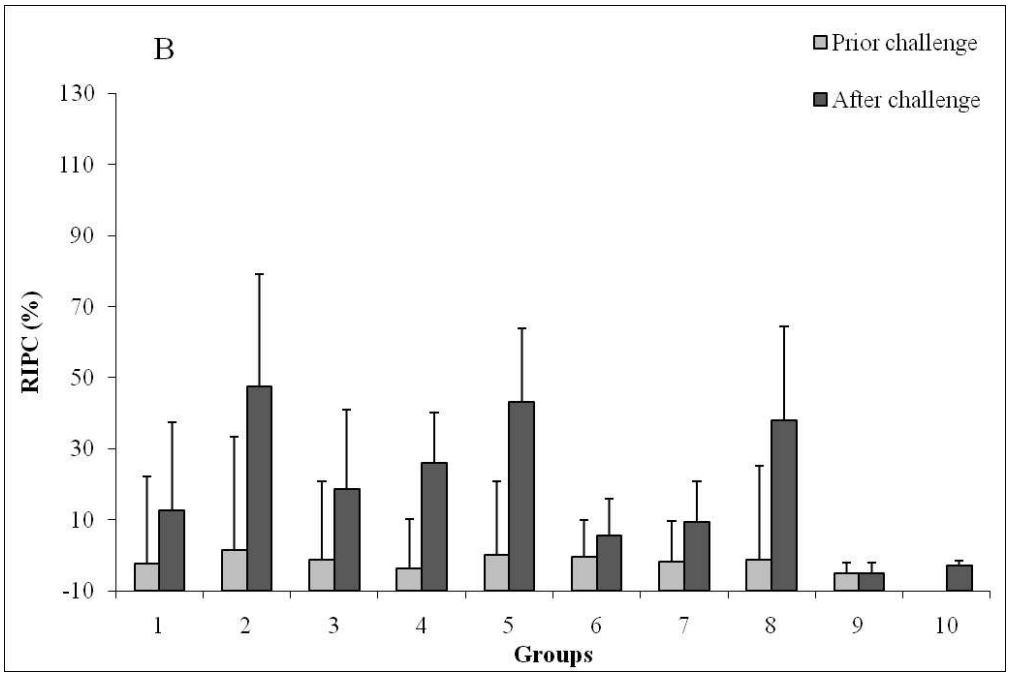

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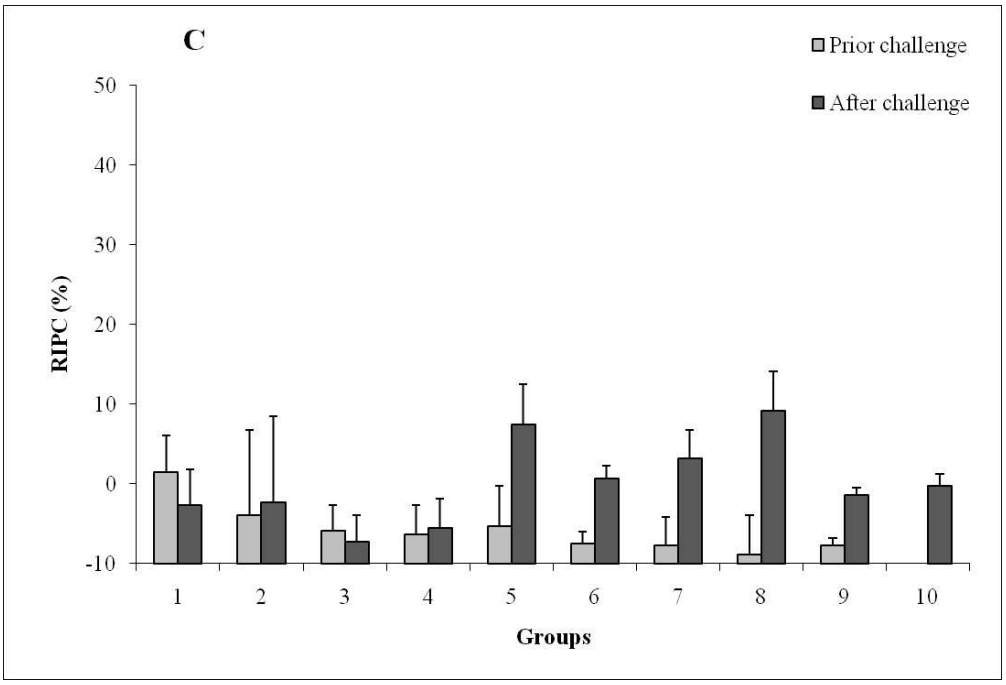

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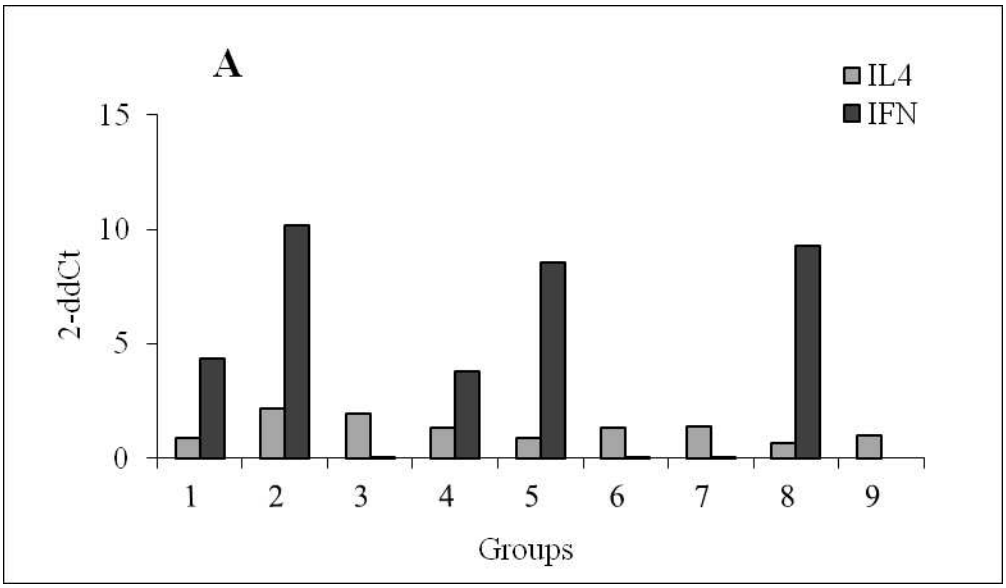

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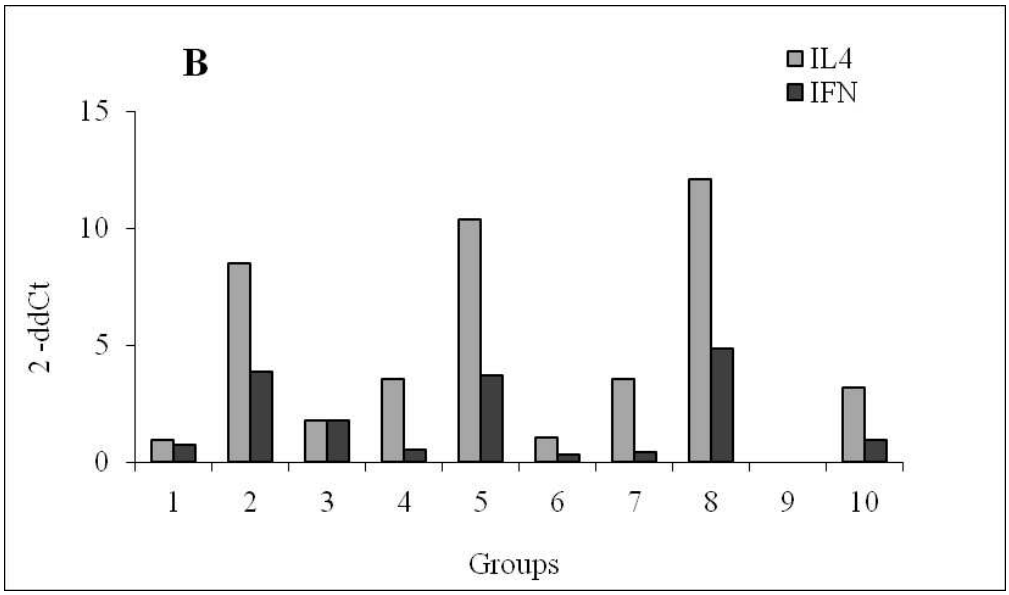

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