Host pregnancy influences the establishment of *Trichinella zimbabwensis* in Balb C mice

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Abstract In order to determine the effect of host pregnancy in the establishment of *Trichinella zimbabwensis*, 120 female Balb C mice were divided into 4 groups of 30 mice each. Group 1 animals were orally infected with 50 *T. zimbabwensis* larvae per gram (LPG) of body weight on day 0; group 2 were mated on day 0 and not infected; group 3 were mated at day 0 and infected with 50 LPG at day 7 post-mating and Group 4 were control animals which were neither mated nor infected. Six animals from each group were sacrificed and the number of adult parasites in the intestines as well as larvae in the muscles were determined at day 0, 7, 14, 21 and 28 post-infection for group 1; 0, 7, 14, 21 and 28 post-mating for group 2 and days 7, 14, 21, 28 and 35 post-mating for group 3. In addition, levels of progesterone and cortisol were measured in all groups at the same intervals. Our results showed that pregnancy reduced the number of larvae establishing in muscles with progesterone levels significantly higher in pregnant than in non-pregnant Balb C mice ($P < 0.05$). There were no significant differences in cortisol levels between pregnant and non-pregnant mice. High progesterone level in pregnant mice was assumed to have parasitocidal effect on the new-born larvae (NBL). Further research is needed to

determine the direct effect of progesterone on *Trichinella* NBL and how this can be exploited in designing remedies for preventing *Trichinella* infection in susceptible domestic animals and humans.

Keywords Balb C mice · Cortisol · Host-pregnancy · New born larvae · Progesterone · *Trichinella zimbabwensis*

Introduction

Various factors have been reported to affect the successful establishment of *Trichinella* infection and transmission, depending on the type of species and the respective host involved (Pozio 2000). These factors could be environmental, immunological, and physiological which includes the gender of the respective host and hormones present within the host (Wakelin et al. 1994; Pozio 2000; Escobedo et al. 2005). Female sex hormones have been reported to interact with the immune system (Grear et al. 2009; Hepworth et al. 2010), with the sex hormones reported to play a role in parasitic infections through the modulation of host immune-endocrine network (IEM) and/or direct regulation of parasite reproduction and differentiation (Cabrera-Munoz et al. 2010).

During pregnancy, there is production of progesterone (P_4), initially from the corpus luteum (Loeb and Quimby 1989; Graham and Clarke 1997) and subsequently from the placenta. Also produced in high levels during pregnancy is the corticotrophin-releasing hormone (CRH), which triggers the release of glucocorticoid stress hormones such as cortisol (Mastorakos and Ilias 2003).

P_4 has been reported to play a role on parasitic and viral diseases through the modulation of host immune-endocrine network (IEN) and direct regulation of parasite

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reproduction and differentiation (Cabrera-Muñoz et al. 2010). The elevated levels of P_4 during pregnancy has an impact on the immune system and incidentally on the resistance or susceptibility to parasite invasions (Lutton and Callard 2006), depending on both the parasite and host infected (Cabrera-Munoz et al. 2010).

The high P_4 levels associated with pregnancy have been shown to increase susceptibility to infection by *Toxoplasma gondii* through the down-regulation of IL12 by infected macrophages and upregulation of progesterone-induced blocking factor (PIBF) which inhibit CD8 and NK cell activity (Prigione et al. 2006; Jones et al. 2008). The same increased susceptibility in increased P_4 levels was observed by Fleming and Conrad (1989) in ewes infected with the nematode *Haemonchus contortus*. In contrast, a protective effect of P_4 in other parasitic infections in different host species has also been reported (Cabrera-Munoz et al. 2010). Administration of the P_4 analogue molecule, medroxyprogesterone acetate to female golden hamsters (*Mesocricetus aureatus*) infected with *Schistosoma haematobium* resulted in a decreased number of recovered worms and egg load (Soliman and Ibrahim 2005). P_4 has also been shown to restrict *Trypanosoma cruzi* (*T. cruzi*) establishment and development in ovariectomized female wild rodents (*Calomys callosus*) (do Prado et al. 1998). In *Trichinella spiralis* (*T. spiralis*), P_4 has been shown to restrict migration, reproduction and differentiation of the parasite and this is thought to be mediated through repression of the development and maturation of the newly born larvae (NBL) as well as activation of immune effector cells killing NBL in an antibody-independent manner (Nunez et al. 2005, 2008; Hernandez-Bello et al. 2011). The combined stimulation of Th2 responses by both pregnancy and parasite infection have been reported to result in increased NBL mortality (Nunez et al. 2002; Cabrera-Munoz et al. 2010; Hernandez-Bello et al. 2011).

In *T. spiralis* infected rats, high exogenous doses of cortisone have been shown to be immune-suppressant. Cortisone and hydrocortisone have been shown to suppress cellular responses to *T. spiralis* within the intestinal wall thus allowing for an increased parasite establishment and persistence resulting in more larvae migrating to the musculature (Stewart et al. 1982). Most documented studies are on the effect of exogenous and endogenous cortisol in pregnant and non-pregnant mice and rats infected with encapsulated *T. spiralis* (Stewart et al. 1982)).

No studies have been conducted to determine the effects of host-pregnancy on the establishment and larvae migration of the non-encapsulated *T. zimbabwensis*. The present investigation was therefore undertaken to fill in this void.

Materials and methods

One-hundred-and-twenty Balb C mice were randomly divided into four equal groups. In group 1; animals were infected at day 0 and not mated, Group 2; animals were mated at day 0 and not infected, Group 3; animals were mated at day 0 and infected at day 7 post-mating and Group 4; were control animals which were neither mated or infected.

The *T. zimbabwensis* strain used in our study was originally isolated from a crocodile (*Crocodylus niloticus*) naturally infected and has been maintained at BRU in rats. For preparation of infective material for the study animals, carcasses of infected rats were digested using a modified protocol of the HCl-pepsin digestion method described by Pozio et al. (2002). Muscle tissue from each rat was weighed and for every 100 g of tissue sample, 16 ml of 25% HCl, 20 g of 0.7 U/mg Pepsin (from porcine gastric mucosa), 2L H_2O was used to prepare the digestive fluid. Muscle tissue was digested for 35 min at 37 °C in a 2L beaker on a magnetic stirrer. After sedimentation for 40 min in a separating funnel, the larvae were collected by flushing the bottom 40 ml from the funnel into a 50 ml cylinder. After cleaning the digestion fluid with an additional 10 min sedimentation step, the top 30 ml supernatant in the cylinder was removed and the resulting suspension containing larvae was transferred to a marked petri-dish. The number of larvae was determined using a dissecting microscope 20 × objective. The number of larvae per gram (LPG) of muscle tissue per rat was calculated. The experimental animals were infected by a once-off gastric gavage of 50 LPG for Balb C mice.

Animals in group 1 were infected on day 0 of trial. Mating in group 2 and 3 females was achieved by pairing the female with male Balb C mice on day 0 of trial in a 2:1 ratio and enclosed in the respective cages for 24 h to allow for mating. Confirmation of conception was done by checking for a vaginal plug twice a day following mating (morning and afternoon) (Cui et al. 2006) and day of observation of a vaginal plug was taken as 1st day of pregnancy. On day 7 of pregnancy, animals from group 3 were infected with the same dose of larvae as group 1.

On days 0, 7, 14, 21, 28 post-infection for group 1; days 0, 7, 14, 21 and 28 post-mating for group 2 and on days 7, 14, 21, 28 and 35 post-mating for group 3, six animals were sacrificed from each group using a terminal dose of halothane. Recovery of adult worms from the intestines was done using a modification of the protocol described by Mukaratirwa et al. (2003) and muscle larvae were recovered as described by Pozio et al. (2002).

On days of sacrifice, blood was collected from mice by cardiac puncture into 1 ml blood tubes containing clotting

activator gel (dvac gel and clot activator tubes). The blood was subject to centrifugation using a Thermo scientific Heraeus Labofuge 200 microprocessor controlled table top centrifuge at $132\times g$ for 5–10 min. Following centrifugation, serum was collected into Eppendorf micro-centrifuge tubes and stored at -56 to -60 °C for later analysis.

Baseline levels of cortisol and progesterone at day 0 of trial were extrapolated from values obtained from group 2 prior to mating. Immediately after birth, off-springs were humanely sacrificed and screened for muscle larvae using the aforementioned method by Pozio et al. (2002).

Progesterone levels for group 4 were extrapolated from normal standard normal average progesterone levels of mice during estrous cycle (Loeb and Quimby 1989), assuming that animals were at estrus on day 0 of trial. Cortisol levels for group 4 were estimated from normal average cortisol levels of mice in relation to the time of day blood samples were collected from the other groups when taking into account the diurnal concentrations of cortisol. Normal serum cortisol levels in mice ranges from 3 to 12 µg/100 ml (Dracott and Smith 1979) which is equivalent to 3 000–12 000 pg/ml during the diurnal pattern of cortisol. The levels of blood cortisol peak in the early morning (approximately 8 am) and during the day reaching its lowest levels at about midnight—4 am.

Measuring serum cortisol and progesterone levels

Serum progesterone concentrations were measured using Demeditec Progesterone rat/mouse ELISA kit (No. DEV9988) from Biocombiotech. The assay procedure was conducted according to the manufacturer's instructions. A progesterone standard series (0, 0.4, 1.5, 6.5, 25 and 100) ng/mL was provided with the kit.

Standards were used to plot standard curve of absorbance versus concentration with the progesterone concentrations of samples determined through interpolation. The concentrations of progesterone in samples were determined using immunoassay software that measured optical densities and concentrations in samples. To convert to SI units (nmol/L), the following formula was used; Progesterone conc. (ng/mL) $\times 3 =$ nmol/L.

Serum cortisol concentrations were measured using the Enzo Life Sciences Cortisol EIA kit (No. ADI-900-071) purchased from Biocombiotech, which measures the amount of cortisol in serum, plasma, saliva, urine and faeces samples from any species. For quantitative measure of cortisol, the kit uses a monoclonal antibody to cortisol that binds in a competitive manner to cortisol in a sample or an alkaline phosphatase molecule which has cortisol covalently attached to it. The assay procedure was conducted according to manufactures instructions. A 100,000 pg/mL standard solution of cortisol was provided

with the kit and diluted with assay buffer to a standard series of (10,000, 5 000, 2 500, 1 250, 625, 313, 156) pg/mL to be used in the assay procedure.

The measured optical densities were used to calculate concentrations of cortisol. Standards were used to plot standard curve of percentage of bound cortisol versus concentrations of standards with the cortisol concentrations of samples determined through interpolation. The following equation was used to calculate the concentrations of cortisol:

1. The average net Optical Density (OD) bound for each standard and sample was calculated by subtracting the average NSB OD from the average OD bound; Average Net OD = Average Bound OD—Average NSB OD.
2. The binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), was calculated using the following formula; Percent Bound = Net OD $\times 100$ Net Bo OD.
3. Using Logit-Log paper, Percent Bound versus Concentration of Cortisol for the standards curve was plotted. Approximating a straight line through the points, the concentration of cortisol in the unknowns was determined by interpolation.

The assay has a sensitivity of 56.72 pg/mL, which allows for detection of low cortisol that range from 156 to 10,000 pg/mL from biological matrices.

Ethical considerations

Ethical clearance for the study was obtained from the UKZN Ethics Committee (069/12/Animal).

Data analysis

The parameters measured were the number of adult *T. zimbabwensis* in the small intestines, reproductive status of adult female parasite recovered from the intestines and the reproductive capacity index (RCI). The RCI was calculated as the number of muscle larvae recovered divided by the number of larvae inoculated (Krivokapich et al. 2012). The reproductive status of each female parasite from the intestines was evaluated by observing in a female parasite the presence or non-presence of larvae in the uterus. A female parasite was considered as “gravid” when there was presence of larvae in the uterus and as “non-gravid” when there was no presence of larvae in the uterus.

All data were expressed as mean $\pm$ standard error of mean (SEM) using Microsoft Office Excel 2007. Two-way analysis of variance (ANOVA) was used to compare mean values of adult worms and mean values of muscle larvae

Table 1 Mean ($\pm$ SEM) of *Trichinella zimbabwensis* adult worms and muscle larvae recovered from pregnant and non-pregnant Balb C mice at different days post-infection and the reproductive capacity index (RCI)

Days post-infection	Mean $\pm$ SEM adult worms recovered		Mean $\pm$ SEM larvae recovered		RCI	
	Pregnant	Non-pregnant	Pregnant	Non-pregnant	Pregnant	Non-pregnant
7	11.5 $\pm$ 2.72	26.5 $\pm$ 4.43	0	0	0	0
14	1.8 $\pm$ 1.28	2.0 $\pm$ 0.93	0	0	0	0
21	0	0	0	859 $\pm$ 305.09	0	0.66
28	0	0	0	3063 $\pm$ 335.60	0	2.62

SEM standard error of means

between male and female rats over time of exposure to infection. One-way ANOVA was used to compare mean number of female, gravid, non-gravid and male adult worms recovered within and between groups of animals (male and female rats). One-way analysis of variance (ANOVA) was used to compare mean number of female, gravid, non-gravid and male adult worms recovered between and within each group of animals (pregnant and non-pregnant). A non-parametric Spearman correlation analysis was used to examine the relationship between parasite establishment and the levels of progesterone and the relationship between parasite establishment and of cortisol levels post-infection. The statistical package SPSS 21 was used and $P < 0.05$ was considered significant.

Results

The establishment and development of *T. zimbabwensis* in pregnant and non-pregnant Balb C mice is shown in Table 1. There was no statistically significant difference in the numbers of adult worms recovered from both pregnant and non-pregnant mice ($P > 0.05$). Also there was no significant difference in the number of gravid and non-gravid worms recovered in both pregnant and non-pregnant mice ($P > 0.05$). On day 7, in both pregnant and non-pregnant mice, there were more adult females than males recovered, with the majority of the recovered females being gravid. Whilst gravid adult worms established in both pregnant and non-pregnant animals, there was no further parasite development in pregnant mice as evidenced by absence of muscle larvae in this group. Also none of the pups from the pregnant and infected mice were observed to have any infection at birth (no congenital infection).

On days 7 and 14, pregnant mice had higher progesterone than the non-pregnant ($P < 0.05$) (Fig. 1). There was also observed a weak non-linear correlation between

Fig. 1 Serum progesterone levels (nmol/L) in pregnant and non-pregnant Balb C mice infected with *Trichinella zimbabwensis* **Fig. 2** Serum cortisol level (pg/mL) in pregnant and non-pregnant Balb C mice infected with *Trichinella zimbabwensis*

infection establishment and progesterone levels of days 21 and 28 PI ($r = -0.21$; $P > 0.05$).

There was no significant difference in cortisol levels between pregnant and the non-pregnant ($P > 0.05$) (Fig. 2). A non-significant positive correlation was observed between infection establishment and cortisol levels on days 21 and 28 PI ($r = 0.139$; $P > 0.05$).

Discussion

Clinical and experimental evidence suggests that sex hormones regulate the immune system (Grossman 1985; Klein 2004; Escobedo et al. 2011). Changes in the level of sex steroids in a host can influence immune system response and hence outcome of infection (Lutton and Callard 2006). In our study, there was no significant difference in the numbers of established *T. zimbabwensis* adults in both the pregnant and non-pregnant groups. However, the infection only progressed in the non-pregnant group where the gravid females produced NBL which successfully established in the muscles of the host whilst there were no muscle larvae observed in the pregnant group. The failure of progression of infection in pregnant hosts can be explained by the reported parasitocidal effect of progesterone against NBL of *T. spiralis* (Nunez et al. 2005, 2008; Cabrera-Munoz et al. 2010; Escobedo et al. 2011).

Progesterone has been reported to act synergistically with Th2 immunity in an antibody-dependent cell cytotoxicity, targeting induction of effector cells responsible for mortality of *T. spiralis* NBL, thus resulting in reduced or no parasite establishment in the musculature (Nunez et al. 2005). In our study, there was an inversely proportional correlation observed between progesterone levels and presence of parasites within the musculature. Progesterone has also been reported to have parasitocidal effects against *Schistosoma haematobium* when injected into infected female golden hamsters (*Mesocricetus aureatus*) (Soliman and Ibrahim 2005). However, in *Taenia solium*, presence of P₄ has been shown to induce invagination of the scolex thus promoting parasite development into adult within the host (Escobedo et al. 2010).

Contrary to the congenital transmission observed by Matenga et al. (2006) when they infected rats (*Rattus norvegicus*) with *T. zimbabwensis* and assertions by Nuñez et al. (2002) that congenital infection is possible besides the helminthotoxicity and cytotoxic effects of progesterone on migrating larvae, in our study none of the pups from infected dams were infected. This difference could possibly be explained by the low infective dose used in our study in comparison to the one used by Matenga et al. (2006) (50 LPG vs 2,000 LPG). Indeed even with such a high infective dose, Matenga et al. (2006) only observed 1–3 larvae per positive offspring, which could indicate the difficulty of congenital infection in *T. zimbabwensis* infections. Also differences in stage of pregnancy at which infection was introduced and levels of progesterone in the hosts could have resulted in the observed differences (Nunez et al. 2002).

Whilst in our study cortisol did not have any effect on parasite establishment, it has been reported to have an effect on host-parasite interactions (Bushell and Hobson

1978; Haond et al. 2003). Mild cortisol stress response has been reported to enhance resistance of rainbow trout (*Oncorhynchus mykiss*) to infection by the crustacean ectoparasite *Argulus japonicus* (Haond et al. 2003). However, when added to the McCoy culture media, cortisol was shown to significantly increase the size and number of detectable *Chlamydia trachomatis* inclusion bodies (Bushell and Hobson 1978).

Conclusion

Our study showed that pregnancy had an effect on the development of *T. zimbabwensis* in mice, with the effect being noted in the failure of establishment of larvae in the musculature. This effect of pregnancy is likely associated with the higher progesterone levels, with progesterone affecting the larvae migration and establishment in muscles. Further studies are required to establish the effective helminthotoxic dose of progesterone and the stage of pregnancy when this is most likely to occur in the host in order to further explore the feasibility of this hormone as therapeutic compound.

Author contributions SM conceived the idea. LH & SC did the experimental work. BM did the analysis.

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