Genetics and Molecular Biology, 29, 4, 755-761 (2006)

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Research Article

Expression of *mdr* isoforms in mice during estrous cycle and under hormone stimulation

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Abstract

The multidrug resistance (MDR) phenotype is associated with the expression of P-glycoprotein (Pgp), coded by the multigenic *mdr* family. Mice present the isoforms mdr1 and mdr3, which are responsible for multidrug resistance, and mdr2, that is involved in the transport of phospholipids. mdr1 expression has more recently been associated also with the secretion of steroid hormones. This work presents an RT-PCR analysis of the expression of *mdr* isoforms, in several organs of mice during different phases of the estrous cycle. Additionally, females were ovariectomized, submitted to different hormone treatments, and their uterus was analyzed for the expression of mdr isoforms. The results show that in the adrenal gland and ovaries mdr1 is the main isoform during proestrus, and that progesterone or a combination of progesterone and estrogen induce the expression of all *mdr* isoforms in the uterus of ovariectomized females. We suggest that the functions of mdr1 and mdr3 are overlapping, that mdr3 may be the more efficient isoform in the detoxification function, and that mdr1 may be more closely related to the secretion of steroid hormones.

Key words: adrenal gland, multidrug resistance, ovaries, steroids, uterus.

Received: September 15, 2005; Accepted: April 4, 2006.

Introduction

The multidrug resistance (MDR) phenotype is associated with the expression of P-glycoprotein (Pgp), coded by the multigenic *mdr* family. Pgp acts as an energy-dependent efflux pump, preventing accumulation of cytotoxic drugs within the cell. The *mdr* gene family presents two members in humans (*MDR1/ABCB1* and *MDR3/ABCB4*) and three in rodents (*mdr1*, *mdr2*, and *mdr3*). MDR1, mdr1 and mdr3 are responsible for the MDR phenotype, whereas MDR3 and mdr2 seem to be involved only with the transport of phospholipids (Gottesman and Pastan, 1993; Shapiro and Ling, 1998).

The tissue distribution of Pgp has been investigated in humans and rodents through different approaches. In humans, Pauly *et al*. (1992) detected low levels of Pgp mRNA in the brain, bone marrow, esophagus, ovary and stomach, intermediate levels in the colon, liver and lung, and high levels in the adrenal gland, kidney and pancreas. In mice, *mdr1* mRNA was observed mainly in the pregnant uterus, adrenal gland, kidney and heart; high levels of mdr2 were expressed in the liver, spleen and muscles, and mdr3 was found in the intestine, brain, kidney, liver and spleen (Croop *et al*., 1989). It has been demonstrated that the placental *mdr3* Pgp is present in fetus-derived epithelial cells, and can greatly limit the passage of various toxic or beneficial Pgp substrate drugs into the fetus (Lankas *et al*., 1998; Smit *et al*., 1999). Ushigome *et al*. (2000) observed that Pgp (*MDR1*) is expressed on the brush-border membrane (maternal side) of human placental trophoblasts.

Borst *et al*. (1993) reviewed the possible physiological roles of Pgp isoforms as a function of their tissue distribution. However, the review was based mainly on the results published by Croop *et al*. (1989), who did not observe the expression of mdr3 in the adrenal gland, ovaries and uterus, or of mdr1 in the ovaries. The presence of Pgp in virtually all kinds of tumors, however, suggests that low levels of the protein are normally present in all tissues but could not be detected with the techniques available at that time. Alternatively, it was proposed that the MDR pheno-

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type could have been acquired as a consequence of chemotherapy (Herweijer *et al*., 1990; Goldstein *et al*., 1990; Schneider *et al*., 1989). It is possible that few pre-existing cells expressing Pgp are selected by chemotherapy (Noonan *et al*., 1990).

The role of Pgp expression in the secretion of steroid hormones has been studied in mice. Arceci *et al*. (1988) reported the predominant location of Pgp on the luminal surface of secretory epithelial cells of the endometrium. Yang *et al*. (1989) observed that progesterone interacts with Pgp in the endometrium of the pregnant uterus, and that the effect of steroids on the accumulation of other drugs is related to their hydrophobicity. The expression of *mdr* genes in the secretory epithelium of the endometrium was shown to be increased by the combination of estrogen and progesterone, although the level of expression of mdr mRNA did not seem to increase during the normal estrous cycle (Arceci *et al*., 1990). Pierkarz *et al*. (1993) reported that progesterone, whose level is increased during pregnancy, regulates the activity of the *mdr1* promoter, which has an element responsive to that hormone on its first untranslated exon.

The importance of mdr1 on the secretion of steroid hormones by the adrenal gland was also pointed out by Altuvia *et al*. (1993). According to Rao *et al*. (1994), the product of *MDR1* in humans is observed at high levels in tissues synthesizing steroid hormones. The authors detected ATPasic activity of Pgp with different steroids and concluded that progesterone was the most effective inducing agent for that activity, although â-estradiol could also be effective, but in higher concentrations.

The relationship of progesterone with Pgp remains unclear. Hamilton *et al*. (2001) showed that steroids vary in their ability to modulate Pgp. According to Lewin *et al*. (2002), although Pgp transports many steroids, it does not transport progesterone, unless it is treated with compounds that modify Pgp phosphorylation. On the other hand, Uhr *et al*. (2002) suggested that the endogenous steroid hormones corticosterone, cortisol, aldosterone and, to a lesser extent, progesterone are physiological substrates for Pgp. In *mdr1/3* (-/-) mice, the uptake of the four hormones into the brain is significantly increased compared to wild-type mice. In addition, the four endogenous steroid hormones were found to significantly accumulate in the testes of *mdr1/3* (-/-) mice. These organs need to be protected against the entry of a wide range of potentially toxic xenobiotics and drugs.

Kuo *et al*. (1995) observed that the rate of *mdr1* transcription in ovariectomized mice treated with estradiol and progesterone was only slightly higher than that of non-treated mice. However, the experimental conditions did not allow the distinction between the highly similar sequences of *mdr1* and *mdr3*. Morales *et al*. (2000) demonstrated that in renal tubular cells the mdr1 isoform activity can be modulated by aldosterone.

In a previous work (Schiengold *et al*., 2001), we investigated the expression of the mdr isoforms during murine ontogeny. The three isoforms were observed in all eight organs analyzed (spleen, brain, liver, adrenal gland, intestine, kidney, testes and ovaries), although with different frequencies. In adult mice, mdr3 was found to be the most frequently expressed isoform in the ovary and adrenal gland. The similarity observed among females was mainly due to absence of mdr1 expression. Interestingly, in all 20 females analyzed for the eight different organs (with the sole exception of the adrenal gland of a 45-day old animal), *mdr1* expression was always observed in coexpression with *mdr3*.

In this work, we investigated the expression of mdr isoforms during the different phases of the estrous cycle in normal mice, as well as in the uterus of ovariectomized animals submitted to different hormonal treatments.

Material and Methods

Mice

We used female BALB/c mice aged 3 to 6 months, raised in our animal house under standard conditions.

Identification of the estrous cycle phases

The females were analyzed twice daily, during one week, for the determination of the different phases of the estrous cycle (proestrus, estrus and diestrus). Vaginal secretion was collected by scraping the vaginal opening or by aspirating the suspension fluid with a Pasteur pipette. Individual slides from each sample were prepared and stained with Harris hematoxylin. Identification of the estrous cycle phases depends on the cell types present in the samples (Knobil and Neil, 1994), and was considered positive whenever both collection methods gave identical results.

Five females in each of the cycle phases were studied. Immediately after identification of the phase, the mice were sacrificed and organs were collected, under sterile conditions, for RNA extraction and analysis of mdr expression. The organs studied included the brain, spleen, liver, adrenal gland, intestinal tract, kidney and ovary.

Hormonal treatment

In this experiment, 21 animals were used. Twelve days prior to treatment, 30 females were ovariectomized bilaterally under ether anesthesia and allowed to recover, but only 18 were apt to experiment. Hormones were purchased from Sigma Chemical Company (St Louis, MO), prepared with ethanol and saline and injected intraperitoneally. The priming dose of 17 β-estradiol was 100 ng/100 μL, on 2 consecutive days. The subsequent daily hormone injections were given on the third day after the last priming dose. The ovariectomized animals were separated into groups of five and treated with 17 β-estradiol (20 ng/100 μL), 17 β-estradiol plus progesterone (500 μg/100 μL) or progesterone

alone, respectively, during 5 days. The control group was constituted of six females (three of them ovariectomized) that received saline injections. The mice were sacrificed 8 h after the last injection, the uteruses were collected, and total RNA was extracted for analysis of mdr expression.

RNA extraction

Total RNA was extracted with TRIZOL (Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions. From each organ, 0.5 g was used as source for RNA extraction. The organs were minced, TRIZOL was added, the suspension was homogenized, and chloroform was added. The mixture was centrifuged, and isopropanol was added to the collected aqueous phase, for RNA precipitation. Precipitated RNA was washed with 75% ethanol, suspended in diethylpyrocarbonate (DEPC, Sigma, St Louis, MO)-treated water and stored at -20 °C until used. RNA quality and integrity were tested by electrophoresis in 1.4% agarose gel containing ethidium bromide, by verifying the presence of ribosomal RNA.

cDNA synthesis

cDNA was synthesized from total RNA using the Bulk First Strand cDNA Reaction Mix kit (Amersham-Pharmacia Biotech, Buckinghamshire, UK). Briefly, 5 μL Bulk First Strand cDNA Reaction Mix, 1 μL DTT, 1 μL pd(N)6 at 0.2 μg/μL, and 8 μL total RNA were added together in a microcentrifuge tube. After 90 min of incubation at 37 °C and 5 min of incubation at 90 °C, the material was stored on ice until used.

PCR

Primers specific for the three *mdr* genes in mice (*mdr1*, *mdr2* and *mdr3*) were synthesized according to Vollrath *et al*. (1994); as a control for cDNA integrity, we used primers specific for 28S ribosome RNA (Muller *et al*., 1995). The primer sequences were 5'TGCTTATGGATCC CAGAGTGAC3' and 5'TTGGTGAGGATCTCTCCGG CT3' for *mdr1*; 5'CTCGTTAACATGCAGACAGCAG3' and 5'GACCAGGGAGAACATGTTACAC3' for *mdr2*; 5'AGCTATCACGGACAACATCTCC3' and 5'TGTCC GCTCTTCACCTTCAGAT3' for *mdr3*; and 5'GAAAGA TGGTGAACTATGCC3' and 5'TTACCAAAAGTGGCC CACTA3' for *rRNA*, as previously described by us in Schiengold *et al*. (2001).

The PCR reactions for each *mdr* gene were performed in individual tubes. When no amplification was detected for a given *mdr* gene, the PCR's were repeated using both primers, for 28S ribosome RNA and for the specific *mdr* gene, in the same tube, in order to rule out possible false-negatives. This multiplex system was tested, and in these cases a positive sample for the expression of both genes was also amplified as a control. Each PCR included all isoforms from all organs from different animals in different cycle phases or under different hormone treatments.

All PCR reactions consisted of: an initial denaturation cycle at 94 °C for 2 min, followed by 40 cycles at 95 °C for 30 s, 61 °C for 45 s, and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Each reaction contained 10 mM of each dNTP, 3 mM $MgCl_2$, 1 unit Taq polymerase (CENBIOT, Porto Alegre, Brazil), and 0.2 mM of each specific primer. The final volume was 25 μL, of which 10 μL were applied onto a 2% agarose gel for electrophoresis with 0.5 x TBE containing ethidium bromide.

Data analysis

Data were grouped according to positive or negative amplification (presence or absence, in the gel, of the specific band that indicates gene expression) in each organ of each individual studied. The results were analyzed for the different phases of the estrous cycle, according to two similarity coefficients (Sneath and Sokal, 1973) which can be expressed as fractions or as percentages. The Simple Matching Coefficient (S_{SM}) is based on both positive and negative concordances. The Jaccard Similarity Coefficient (S_J), on the other hand, does not consider the negative concordances, measuring similarity based only on the expression of the gene under analysis. The result (presence or absence of expression) for each individual organ was compared with those obtained for the same organ in each of the other four animals in the same phase of the estrous cycle. Thus, for each individual organ, an average index of association with the same organ of the other matched animals was calculated. These indexes were then used for the calculation of the average S_{SM} and S_J for each organ in each phase. The Kolmogorov-Smirnov one-sample test (Sokal and Rohlf, 1995) and the chi-square test using the Yates correction for continuity (Sneath and Sokal, 1973) were employed for testing the significance levels observed. Differences were considered significant when $p < 0.05$.

Results

Expression of mdr isoforms during the estrous cycle

The estrous cycle phase influenced the expression of *mdr* genes only in the adrenal gland and in the ovary. In the other organs, the patterns of expression were uniform throughout all phases (not shown). As detailed elsewhere (Schiengold *et al*., 2001), all isoforms are seen in all organs, with considerable individual variation.

Table 1 shows the results obtained for the presence or absence of mdr isoforms in the adrenal gland and ovary, for each estrous phase analyzed. The average similarity coefficients (S_{SM} e S_J) for the five females in the different estrous cycle phases are shown in Table 2.

In the adrenal gland it can be observed that, whereas *mdr2* expression is rare irrespective of the phase, all females in proestrus expressed *mdr1* ($S_{SM} = S_J = 1$), sometimes without the concomitant expression of *mdr3*. The Kolmogorov-Smirnov one-sample test showed that, al-

Table 1 - Presence (+) or absence (-) of *mdr* isoforms in the adrenal gland and ovary of females in different phases of the estrous cycle.

Mouse	Isoform	Proestrus		Estrus		Diestrus	
#		Adrenal	Ovary	Adrenal	Ovary	Adrenal	Ovary
	mdr1	+	+	-	-	+	-
1	*mdr2*	-	-	-	+	-	-
	mdr3	-	+	-	+	+	+
	mdr1	+	+	+	-	-	+
2	*mdr2*	-	-	+	-	-	+
	mdr3	-	+	+	+	-	+
	mdr1	+	+	-	-	+	-
3	*mdr2*	-	+	-	-	+	-
	mdr3	-	-	-	-	+	+
	mdr1	+	+	-	-	+	-
4	*mdr2*	+	+	-	-	-	+
	mdr3	+	+	+	+	+	+
	mdr1	+	+	+	+	-	-
5	*mdr2*	-	+	-	+	-	-
	mdr3	+	+	+	+	-	+

Table 2 - Average Jaccard Similarity Coefficient for the *mdr* isoforms in the adrenal gland and ovary in proestrus, estrus and diestrus.

Isoform	Coeff.	Proestrus		Estrus		Diestrus	
		Adrenal	Ovary	Adrenal	Ovary	Adrenal	Ovary
mdr1	S_{SM}	1.00	1.00	0.40	0.60	0.40	0.60
	S_J	1.00	1.00	0.20	0.00	0.30	0.00
mdr2	S_{SM}	0.60	0.40	0.60	0.40	0.60	0.40
	S_J	0.00	0.30	0.00	0.10	0.00	0.10
mdr3	S_{SM}	0.40	0.60	0.40	0.60	0.40	1.00
	S_J	0.20	0.60	0.30	0.60	0.30	1.00
Σ	S_{SM}	0.67	0.67	0.47	0.53	0.47	0.67
	S_J	0.40	0.63	0.17	0.23	0.20	0.37

though the detection of mdr2 and mdr3 was not variable (considering presence x absence) among the cycle phases ($p > 0.2$, $\alpha = 0.05$), mdr1 was significantly more detectable in proestrus ($p < 0.01$, $\alpha = 0.05$).

In the ovaries, mdr3 was the most frequently observed isoform, and an analysis of the different phases showed that the similarity coefficient due to expression of *mdr1* was 1.00 for females in proestrus, and 0.00 in estrus and diestrus (Kolmogorov-Smirnov one-sample test, $p < 0.01$, $\alpha = 0.05$). *Mdr2* expression was rarely seen, and expression of *mdr1* was observed in the absence of *mdr3* expression.

Expression of mdr isoforms in the uterus of females stimulated with steroid hormones

The expression of *mdr* genes was analyzed in the uterus of the six females in the control group, three of which were ovariectomized. The only isoform observed was mdr3, in two of the ovariectomized females (not shown).

Results for the mice treated with hormones are presented in Table 3. No *mdr* expression was detected in any of the five ovariectomized animals treated with estradiol. All mdr isoforms were frequently observed in ovariectomized females treated with progesterone. Estrogen plus progesterone also appears to induce the expression of the different mdr isoforms in the uterus of ovariectomized females. In the last case, *mdr1* expression was observed independently of *mdr3*, contrasting with the progesterone treatment, where *mdr1* expression, when detected, was concomitant with *mdr3* expression. Both progesterone and estrogen plus progesterone treatments induced *mdr* expression as compared to the control group ($\chi^2_y = 5.689$, $p = 0.017$, $\alpha = 0.05$)

Discussion

In rodents, the estrous cycle averages 4 to 5 days. The first phase is known as proestrus (proliferation) and is cyto-

Table 3 - Presence (+) or absence (-) of *mdr* isoforms in the uterus of 15 ovariectomized females submitted to different hormone treatments.

	Treatment														
	Estradiol					Progesterone					Progesterone + estradiol				
Animal #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
mdr1	-	-	-	-	-	-	+	-	+	-	+	-	-	+	+
mdr2	-	-	-	-	-	+	+	+	+	-	-	+	+	+	-
mdr3	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-

logically characterized by the predominance of nucleated epithelial cells, which are round, bear an easily visible nucleus and may appear in clusters or individually. Peaks of estradiol and progesterone secretion occur in this phase, which lasts about 12 h. The following phase is the estrus (sexual receptivity), which lasts 26 h and is cytologically characterized by large numbers of cornified squamous irregularly shaped epithelial cells, occurring in clusters. The next phase is the diestrus (sometimes divided into diestrus I and diestrus II), of relative sexual rest and in which the ovarian secretions prepare the reproductive tract for receiving the fertilized egg. If fertilization does not occur, the animal returns to proestrus. The diestrus lasts from two and a half to three days and is cytologically characterized by the predominance of small leukocytes interspersed by a few nucleated epithelial or cornified squamous epithelial cells (Knobil and Neil, 1994). Our results concerning the differential expression of the *mdr* genes along the various phases of the estrous cycle in ovaries and adrenal gland suggest an involvement of the P-glycoprotein in the secretion of steroid hormones. Special attention is given to *mdr1*, which presents a progesterone-responsive element on its first untranslated exon (Pierkarz *et al*., 1993).

High expression levels of the *mdr1* gene in the adrenal gland have been reported in mice (Croop *et al*., 1989), although Bradley *et al*. (1990) did not detect *mdr1* expression in the adrenal gland of Chinese hamster females. As there are no reports in the literature about *mdr* expression during the estrous phases of animals, our results may explain these contrasting results, showing that in females the adrenal gland does not display detectable expression of the *mdr* genes during most of the estrous cycle (estrus and diestrus), whereas in proestrus, possibly related to steroid synthesis, the expression of *mdr1* appears to be increased. This hypothesis is supported by the report of Altuvia *et al*. (1993), who observed that an increase in the steroid biosynthesis, induced by ACTH, resulted in an increase in the level of expression of *mdr1* in a murine adrenal gland cell line. In line with this finding, Sérée *et al*. (1998) observed that the inhibitory effect of dexamethasone on adrenocorticotropin hormone (ACTH) production can explain the decreased *mdr1* expression. The rare expression of *mdr1* during estrus and diestrus would be mostly related to its role as an adjuvant to mdr3 in detoxification rather than in hormone secretion.

mdr3 was the most frequently observed isoform in the ovaries, irrespective of the estrous cycle. For *mdr1*, however, expression was much higher in proestrus ($S_J = 1.00$), indicating its phase-related regulation. In the proestrus ovaries, it was also possible to observe the expression of *mdr1* in the absence of *mdr3* expression. Although this data concerns only one animal, this was never observed in ovaries before, as discussed in our previous study, where we stated that *mdr1* and *mdr3* expression were always concomitant (Schiengold *et al*., 2001). Noteworthy, considering the homologous genes to mouse *mdr1* and *mdr3* in humans, is that the activation of the *MDR3* gene seems to be independent of the activation of the closely linked *MDR1* gene (van der Bliek *et al*., 1988; Raymond *et al*., 1990; Chin *et al*., 1992).

The uterus is poor in *mdr* expression, and mdr3 is the main isoform present. Estradiol-treated females did not express *mdr* genes (Table 3), a result similar to that reported by Arceci *et al*. (1990), who employed *in situ* hybridization and the same experimental conditions used in the present study. As opposed to other results in that same report, however, in this study *mdr* expression was observed in all females treated with progesterone, with the expression of *mdr3* in all animals.

A similar situation was observed for the ovariectomized females treated with progesterone and estradiol. Moreover, in the present study, in one uterus, *mdr1* expression was observed in the absence of *mdr3* expression. Croop *et al*. (1989), using Northern blot, detected only mdr1 in the pregnant uterus and, although Arceci *et al*. (1990) believed that mdr1 was the isoform detected in their experiments, they also stated that the probes employed were not able to discriminate between the different *mdr* genes. Bello-Reuss *et al*. (2000) determined the role of MDR1 in the secretion of aldosterone by a human adrenal cell line. It is noteworthy that, whereas in humans MDR1 (equivalent to the murine mdr3 isoform) functions in detoxification and in the transport of steroids, mice present two isoforms to which different function have been ascribed. mdr3 is referred to as the most effective isoform in detoxification, and mdr1 as the isoform preferably associated with the transport of steroid hormones (Yang *et al.*, 1989; Gottesman and Pastan, 1993). Interestingly, Taylor *et al.* (1999) found no significant differences between the mdr3 and mdr1 isoforms in the nature of drug-binding sites and suggested that the presence of multiple isoforms of Pgp al-

lows subtle quantitative and qualitative regulations of their respective cellular activity.

Mice which are homozygous for a disruption of *mdr1* or *mdr3* are apparently healthy (Borst *et al*., 1993). In 1997, Schinkel *et al*. obtained mice which, although homozygously deficient for the *mdr1* and *mdr3* genes combined, were healthy and fertile. These results suggest that no strict functions of *mdr1* or *mdr3* are essential to survival, or that the mdr2 isoform can compensate for the absence of the other isoforms (Smith *et al*., 2000, demonstrated that the protein encoded by *MDR3*, although not concerned with the MDR phenotype, can transport drugs). Also, other proteins associated to the MDR phenotype can compensate for the absence of Pgp.

Our results suggest that the functions of mdr1 and mdr3 in mice are not restricted. mdr3 is probably more efficient in the detoxification function. The detection of *mdr1* expression independently of *mdr3* under hormonal stimulation and during proestrus is very surprising (according to Smit *et al*., 1999, the *mdr1* and *mdr3* genes are linked, and hence behave essentially as one locus) and indicates that its function is closely related to the secretion of steroid hormones. It is also interesting to observe that in mice the three *mdr* genes are located in tandem on chromosome 5 (*mdr3*, *mdr1*, *mdr2*), which suggests that transcription of more than one isoform due to an initial transcription of *mdr3* may be a common event. According to Lee and Ling (2003), while little is known about the molecular mechanism governing the changes in Pgp expression at the tissue level, accumulated evidence suggests that post-transcriptional control at the RNA stability level plays a key role.

In conclusion, we investigated the expression of the mdr isoforms during the phases of the estrous cycle in different organs of normal mice. We observed that only in the adrenal gland and the ovary the estrous cycle influenced the expression of *mdr* genes. In these organs we observed that *mdr2* expression is rare, irrespective of the phase. All females in proestrus expressed mdr1 in the adrenal gland. In the ovaries, mdr3 was the most frequently observed isoform. mdr1 expression in the absence of mdr3 was observed in the ovaries and in the adrenal gland in proestrus. These results could be related to peaks of secretion of estradiol and progesterone that are seen in proestrus. In the uterus, the only isoform observed was mdr3. Estradiol does not seem to induce *mdr* expression. Progesterone and estrogen plus progesterone induced the expression of all mdr isoforms in ovariectomized females, and this last treatment may also have induced *mdr1* expression alone in one animal. Our results suggest that the mdr1 and mdr3 functions are overlapping. While mdr3 may be the more efficient isoform in the detoxification function, the detection of *mdr1* expression independently of *mdr3* under hormonal stimulation indicates that its function is closely related to the secretion of steroid hormones.

Acknowledgments

We are grateful to Dr. Carmen C. R. Saavedra and Dr. Karen L. Haag for valuable comments during the preparation of this work and for critically reviewing the manuscript. The present work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Associate Editor: Klaus Hartfelder