Zygote **7** (May), pp 143–149. © 1999 Cambridge University Press Printed in the United Kingdom

Proteolytic activity of rabbit perivitelline spermatozoa

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Date submitted: 7.7.98. Date accepted: 12.10.98

Summary

Acrosin, an acrosomal serine protease, has been associated with binding of spermatozoa and their penetration through the zona pellucida. This study was aimed at determining whether the remaining proacrosin/acrosin system on rabbit perivitelline spermatozoa still has proteolytic activity and whether this activity is involved in further penetration of unfertilised rabbit eggs. Eight hundred and sixty-five rabbit perivitelline spermatozoa were evaluated by the gelatin-substrate film technique for the detection of acrosin on individual spermatozoan. Fifteen per cent of the studied spermatozoa showed small digestion halos on the gelatin film. The proteolytic activity of rabbit perivitelline spermatozoa was inhibited in the presence of 1 mg/ml of soybean trypsin inhibitor (SBTI) or with 20 µg/ml of a mixture of the monoclonal anti-proacrosin/acrosin antibody. *In vitro* fertilisation occurred in 21.8% of rabbit oocytes co-incubated with perivitelline spermatozoa and was completely inhibited when oocytes were incubated with 600 µg/ml of a mixture of three anti-acrosin monoclonal antibodies (ACRO-A8C10, ACRO-C2B10 and ACRO-C5F10). Inseminations in the presence of anti-cholera monoclonal antibody (irrelevant to spermatozoa) resulted in 17.6% fertilisation. These results support the idea that the residual proacrosin/acrosin system in perivitelline spermatozoa might be involved in spermatozoal binding and/or second penetration through the zona pellucida.

Keywords: Acrosin, Fertilization, Perivitelline spermatozoa, Zona pellucida

Introduction

Acrosin has been localised on intact spermatozoa of the ram (Huneau *et al.*, 1984), human (Tesarik *et al.*, 1988; Barros *et al.*, 1990, 1992; Capote *et al.*, 1992), pig (Castellani-Ceresa *et al.*, 1983), rabbit (Valdivia *et al.*, 1994), hamster (Barros *et al.*,1990, 1993), guinea pig (Barros *et al.*, 1990, 1993) and bull (De Los Reyes & Pérez, 1991). The natural substrate for this enzyme is the zona pellucida, which behaves as a competitive inhibitor of BANA (*N*-α-Benzoyl-DL-Arginine-β-NaphthylAmide), a specific substrate for acrosin (Urch, 1986).

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It has been suggested that the proacrosin/acrosin system could contribute to the binding and/or enzymatic penetration of the spermatozoon through the zona pellucida after the acrosome reaction (O'Rand & Fisher, 1987; De Ioannes *et al.*, 1990). On the other hand, it has been shown that anti-acrosin monoclonal antibodies can inhibit *in vivo* (Dudkiewicz, 1983) and *in vitro* (De Ioannes *et al.*, 1990) fertilisation. Similarly, trypsin inhibitors such as soybean trypsin inhibitor (SBTI) can inhibit fertilisation (Stambaugh *et al.*, 1969; Zaneveld *et al.*, 1971; Saling, 1981; Elce *et al.*, 1986; De Ioannes *et al.*, 1990).

Immunocytochemical studies show that after the acrosome reaction the proacrosin/acrosin system is rapidly lost from the surface of the inner acrosomal membrane of hamster spermatozoa, while in rabbit, human and guinea pig this system remains associated for a certain period (Barros *et al.*, 1992; Valdivia *et al.*, 1994), but eventually is completely lost (Kopecny & Fléchon, 1981, 1987; Sakai & Yasuda, 1981; Barros *et al.*, 1992; Valdivia *et al.*, 1994). Different results were reported, however, by Sakai & Yasuda (1981), who

https://doi.org/10.1017/S0967199499000507 Published online by Cambridge University Press

found that in the guinea pig the acrosomal protease was lost immediately after the acrosome reaction.

Digital images of rabbit spermatozoa induced to react with solubilised zona pellucida and labelled with anti-acrosin monoclonal antibodies showed that, at the beginning of the acrosome reaction, the proacrosin/acrosin system appears localised at the apex of the spermatozoon head, then at the acrosomal region, and finally on the equatorial and post-acrosomal region (Richardson *et al.*, 1991).

By *in vitro* fertilisation it was shown that perivitelline rabbit spermatozoa were able to fertilise other freshly ovulated rabbit oocytes at rates of around 25% (Kuzan *et al.*, 1984). Later studies (Valdivia *et al.*, 1994) showed that in about 26% of rabbit spermatozoa recovered from the perivitelline space it was possible to immunolocalise the proacrosin/acrosin system. The label was found at the equatorial and post-acrosomal regions. Similar studies using the hamster showed that it was not possible to localise the proacrosin/acrosin system in any of the perivitelline spermatozoa (Yunes *et al.*, 1992).

The finding that rabbit perivitelline spermatozoa had residual proacrosin/acrosin provides a basis to hypothesise that the ability of perivitelline spermatozoa to fertilise freshly ovulated rabbit oocytes (Valdivia *et al.*, 1994) could be due, at least in part, to the presence of residual proacrosin/acrosin system. This hypothesis could find support if those perivitelline spermatozoa do indeed have proteolytic activity. Therefore, the purpose of this work was to study the proteolytic activity of perivitelline rabbit spermatozoa and their ability to fertilise freshly ovulated oocytes, and to discover whether these activities could be inhibited.

Materials and methods

Animals

Adult New Zealand rabbits were kept at a 14:10 h light:dark cycle. Females were superovulated as previously described (Brackett & Oliphant, 1975) by an intramuscular injection of 150 IU pregnant mares' serum gonadotrophin (PMSG), followed 96 h later by intravenous injection of 75 IU human chorionic gonadotrophin (hCG). Males of proven fertility were used as semen donors.

Recovery of spermatozoa

Rabbit semen samples were obtained by means of an artificial vagina (Bredderman *et al.*, 1964) and washing was performed as described elsewhere (Valdivia *et al.*, 1994). The ejaculate was washed twice in BWW (Biggers *et al.*, 1971) culture medium by centrifugation at 400 *g* for 5 min each time. Finally, new culture medium, supplemented with bovine serum albumin (15 mg/ml) was added to the pellet of spermatozoa and incubated at 37 °C under a 5% CO_2 atmosphere for 30 min to allow spermatozoa migration into the culture medium (Barros *et al.*, 1979).

Monoclonal antibodies to proacrosin/acrosin

We used the anti-human acrosin monoclonal antibodies ACRO-A8C10 and ACRO-C5F10 (Valdivia *et al.*, 1994) and ACRO-C2B10 (Sillerico *et al.*, 1996), all of IgG1 isotype. These antibodies cross-reacted with mouse and rabbit, as determined by indirect immunofluorescence on methanol-fixed spermatozoal smears. Production and characterization of monoclonal antibodies has been described previously. Briefly, the monoclonal antibodies were developed using Balb/cJ mice immunised with human acrosin, affinity purified on a SBTI column and eluted with a pH acid shift (Leyton *et al.*, 1986). The supernatants from the hybridoma cell cultures were tested for the presence of anti-acrosin monoclonal antibodies by ELISA using purified acrosin on the wells. Further assessment of specificity was carried out by immunofluorescence on smears of fixed and permeabilised spermatozoa from human and other mammals. It is important to note that non-permeabilised spermatozoa are negative with the anti-acrosin antibodies. Finally they were characterised by western blotting after SDS reducing and non-reducing conditions. All the monoclonal antibodies used in the present study show reactivity to rabbit acrosin by the above techniques (Valdivia *et al.*, 1994; Sillerico *et al.*, 1996).

Recovery of perivitelline spermatozoa

Fertilised eggs of superovulated females were used as donors of perivitelline spermatozoa. Immediately after intravenous hCG injection, females were anaesthetised with 1% acepromazine (Acedan, Hollyday; 2–3 drops/kg) and then injected intramuscularly with 11–20 mg/kg of ketamine (Ketostop, Drugpharma). The uterine horn was then exteriorised through a dorsolateral incision and 0.3 ml of spermatozoal suspension (3×10^6 spermatozoa/ml) injected in the lumen of the uterine horn. The females were killed 15 h after insemination. Ovaries, oviducts and the proximal uterine segments were removed and placed in a tissue culture dish and covered with culture medium. The oviducts were washed, and eggs with perivitelline spermatozoa were collected. The eggs were carefully pipetted and thoroughly washed to remove all spermatozoa bound to the outer surface of the zona pellucida. The success of this procedure was evaluated with an inverted phase contrast microscope with the ×40 objective. The eggs

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were then transferred, using a dissecting microscope, to either a gelatin film for experiments on proteolytic activity, or a culture dish for fertilisation experiments. The perivitelline spermatozoa were released by using a small-bore pipette to break the zona pellucida.

Proteolytic ability of perivitelline rabbit spermatozoa evaluated by the gelatin-substrate film technique

The proteolytic ability of ejaculated spermatozoa treated with 1 µg/ml of the calcium ionophore A23187 and perivitelline rabbit spermatozoa was evaluated by the gelatin-substrate film technique. In this technique, the gelatin films (Ficsor *et al.*, 1983) were placed in a moisturised box in a 4 °C refrigerator for 24 h. Ten minutes before use, the films were removed from the refrigerator and allowed to warm to 37 °C. Thoroughly washed fertilised rabbit eggs were placed onto the gelatin film and the zona pellucida was ripped off with a pair of watchmaker´s forceps to allow perivitelline spermatozoa to settle over the film. As controls we used freshly ejaculated spermatozoa selected by the swim-up technique, and incubated for 15 minutes with 1 µg/ml of the calcium ionophore A23187. Spermatozoa were washed again by centrifugation, spread over the films and incubated for 2 h at 37 °C. The films were stained with amido black, rinsed in distilled water and studied with the light microscope for evidence of a digestion halo. Halos were recognised by the absence of staining around the spermatozoon head. Some films were processed as described elsewhere (Sillerico *et al.*, 1996) to be studied with a scanning electron microscope.

Ejaculated (control) and perivitelline spermatozoal spreads were used to evaluate the presence and size of: (a) the digestion halo; (b) the digestion halo in the presence of 1 mg/ml of SBTI; and (c) the digestion halo in the presence of 20 µg/ml of a mixture (ACRO-A8C10, ACRO-C2B10 and ACRO-C5F10) of anti-human acrosin monoclonal antibodies.

Fertilising ability of perivitelline spermatozoa

Fertilised rabbit eggs, containing perivitelline spermatozoa, were obtained from the oviducts of artificially inseminated females and treated as described above to eliminate any spermatozoa bound to the outer surface of the zona pellucida. The zygotes containing perivitelline spermatozoa were placed in 200 µl drops of BWW (Biggers *et al.*, 1971) culture medium supplemented with 15 mg/ml of bovine serum albumin. The zona pellucida was broken using watchmaker's forceps to release the perivitelline spermatozoa. The zygote and debris of the zona pellucida were removed using a fine-bore pipette and the freshly ovulated eggs added to the sperm suspension. The gametes were incubated at 37 °C in an atmosphere of 5% CO_2 in air, for 24–26 h. At the end of the incubation period they were fixed with 1.2% glutaraldehyde and stained with the fluorochrome Hoechst 33258 to evaluate the rate of fertilisation, as judged by the presence of two pronuclei.

Forty rabbit zygotes were used as the source of perivitelline spermatozoa and were used in the following experimental groups:

Group a: Spermatozoa from 20 rabbit zygotes were used to inseminate 32 freshly ovulated rabbit eggs (control).

Group b: Spermatozoa from 10 rabbit eggs were used to inseminate 14 freshly ovulated rabbit eggs in the presence of 600 µg/ml of a mixture of anti-acrosin monoclonal antibodies (ACRO-A8C10, ACRO-C2B10 and ACRO-C5F10), and analysed as described above.

Group c: Spermatozoa from 10 rabbit zygotes were used to inseminate 17 freshly ovulated rabbit eggs in the presence of 600 µg/ml of the monoclonal antibody 5C12 specific to *Vibrio cholerae* 01 to assess the specificity of the inhibition. This is a negative control in the *in vitro* fertilisation assay, because the anti-*V. cholerae* 01 antibody belongs to the same IgG subclass as the anti-acrosin antibody and is directed against LPS (Castillo *et al.*, 1995). Thus it is irrelevant for the spermatozoa

Statistical analysis

The presence of digestion halos in perivitelline spermatozoa was evaluated with the chi-squared test (Table 1). The percentage of *in vitro* fertilisation using perivitelline spermatozoa was determined with the Mann–Whitney test (Table 2) (Sokal & Rohlf, 1981).

Results

Recovery of perivitelline spermatozoa

In each experiment, eggs containing perivitelline spermatozoa were selected and all the recovered spermatozoa were motile The recovered spermatozoa were used to evaluate (a) their proteolytic activity on gelatin films, and (b) their fertilising ability.

Proteolytic activity of perivitelline rabbit spermatozoa

Ejaculated spermatozoa

Eighty-three per cent (414/499) of freshly ejaculated rabbit spermatozoa treated with the calcium ionophore A23187 displayed digestion halos on gelatin films (Table 1). Those digestion halos were of three different mean diameters: 75.6 ± 3.5 µm, 42.5 ± 9.4 µm and 25.8 ± 0.9 µm. When ejaculated spermatozoa were incubated in the presence of 1 mg/ml of SBTI, no digestion halos were observed (0/425). When they were incubated with a mixture of anti-acrosin monoclonal antibodies the digestion halos were inhibited in 91% of cases.

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Table 1 Proteolytic activity of rabbit ejaculated and perivitelline spermatozoa

Treatments	No. of spermatozoa with digestion halos (%)			No. of spermatozoa with no digestion halo (%)	Total no. of spermatozoa
	Large 76 µm	Medium 43 µm	Small 11–26 µm		
Ejaculated + A23187	324 (65)	65 (13)	25 (5)	85 (17)[a]	499
Ejaculated A23187 + SBTI	–	–	–	425 (100)[b]	425
Ejaculated A23187 + oligoclonal antibody	4 (4)	1 (1)	5 (4)	96 (91)[b]	106
Perivitelline	–	–	130 (15)	735 (85)[c**]	865
Perivitelline + SBTI	–	–	–	115 (100)[d]	115
Perivitelline + oligoclonal antibody	–	–	–	105 (100)[d]	105

SBTI, soybean trypsin inhibitor.
*a significantly different from *b* ($p < 0.001$, chi-squared test).
**c significantly different from *d* ($p < 0.001$, chi-squared test).

Table 2 *In vitro* fertilisation of rabbit oocytes inseminated with perivitelline rabbit spermatozoa

Experimental design	No. of fertilised oocytes/no. of inseminated oocytes (%)
No antibody (control) ($n = 3$)	7/32 (21.8)[a]
Irrelevant antibody ($n = 2$)	3/17 (17.6)[a]
Anti-acrosin monoclonal antibody mixture ($n = 2$)	0/14[b]

Values with different superscripts are significantly different ($p < 0.001$, Mann–Whitney test).

Perivitelline spermatozoa

Fifteen per cent (130/865) of perivitelline rabbit spermatozoa (Table 1) obtained from 26 rabbit zygotes exhibited digestion halos (Fig. 1*A*), of a mean diameter of 11 ± 3 µm. No digestion halos were observed when 115 perivitelline spermatozoa recovered from three rabbit zygotes were incubated with 1 mg/ml of SBTI. There were also no digestion halos when 105 spermatozoa obtained from three rabbit zygotes were incubated with a mixture of anti-acrosin monoclonal antibodies (ACRO-A8C10, ACRO-C2B10 and ACRO-C5F10).

Similar observations made with a scanning electron microscope (Fig. 1*B*) showed the formation of spermatozoal digestion halos.

Fertilising ability of rabbit perivitelline spermatozoa

Seven (21.8%) of 32 rabbit oocytes inseminated with perivitelline spermatozoa were fertilised (Table 2). Fourteen oocytes incubated with perivitelline spermatozoa in the presence of the anti-human acrosin oligoclonal antibodies remained unfertilised, while three (17.6%) oocytes were fertilised when the gamete mixture was incubated in the presence of an irrelevant antibody (anti-*V. cholerae* 01) (Table 2).

Discussion

We confirmed the results of Kuzan *et al*. (1984), which showed that rabbit perivitelline spermatozoa retain the ability to fertilise other unfertilised rabbit oocytes.

In previous studies (Valdivia *et al.*, 1994) we showed the presence of the proacrosin/acrosin system on 26% of rabbit perivitelline spermatozoa by means of the silver-enhanced immunogold technique, using anti-human acrosin monoclonal antibodies. This percentage correlates well with the 21.8% fertilisation reported here using perivitelline rabbit spermatozoa. The presence of this system on the spermatozoa could support the idea that the retention of the fertilising ability of perivitelline rabbit spermatozoa is associated with the presence of the proacrosin/acrosin system. In this study we have shown that 15% of rabbit perivitelline spermatozoa retain their proteolytic activity, as judged by the formation of digestion halos on gelatin films. These halos observed with a scanning electron microscope (Fig. 1*B*) resemble the spermatozoal halos left on the zona pellucida surface by penetrating spermatozoa (figs. 10 and 11 of Barros *et al.*, 1984). In contrast, hamster spermatozoa, once they undergo the acrosome reaction, lose their ability to cross the zona pellucida (Barros *et al.*, 1992). It has also been shown that hamster spermatozoa recovered from the perivitelline space lack the proacrosin/acrosin system (Yunes *et al.*, 1992).

Olson & Winfrey (1994) reported that the acrosomal laminae of the apical segment of rabbit spermatozoa become associated with the crystalloid matrix. Once the reaction begins the laminae–matrix complex could provide a structural framework to immobilise matrix-associated proteins and prevent their rapid diffusion

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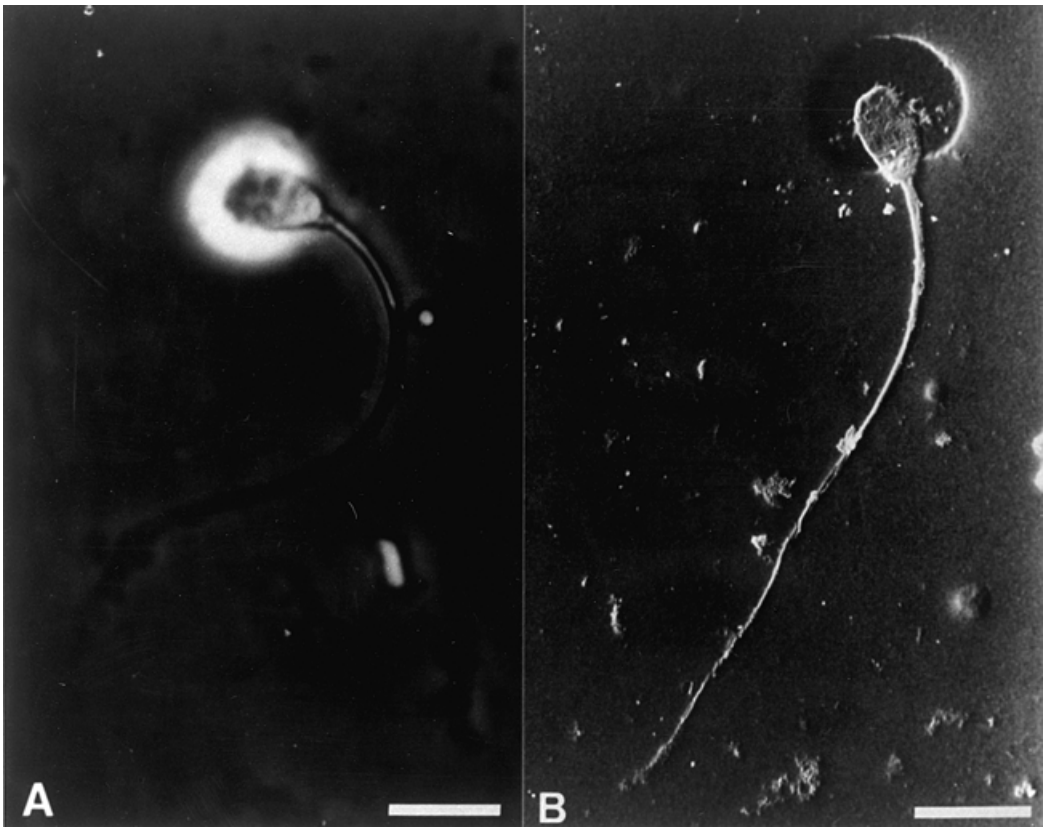


Figure 1 Proteolytic activity of perivitelline rabbit spermatozoa on gelatin films. Note the digestion halos as observed with light microscopy (*A*) and scanning electron microscopy (*B*). Scale bar represents 10 μm.

from the spermatozoal heads during acrosomal exocytosis (Olson & Winfrey, 1994). This evidence supports the notion that perivitelline rabbit spermatozoa retain the proacrosin/acrosin system (Valdivia *et al.*, 1994).

The proteolytic activity of perivitelline spermatozoa on gelatin films was inhibited by SBTI and by a mixture of three anti-human acrosin monoclonal antibodies. This supports the idea that this proteolytic activity may be related to the presence of residual proacrosin/acrosin system on perivitelline rabbit spermatozoa. The finding that in perivitelline spermatozoa the size of the digestion halo is half the size of the halo produced by ejaculated spermatozoa induced to acrosome react by means of the calcium ionophore A23187, would suggest that a minute amount of the proacrosin/acrosin system is still present in some perivitelline spermatozoa. Moreover, such small amounts would seem to be sufficient to allow spermatozoa to bind and penetrate the zona pellucida. The proportion of perivitelline spermatozoa that have proteolytic activity (15%) is related to the percentage (26%) of perivitelline spermatozoa in which the proacrosin/acrosin system can be demonstrated by the silver enhanced immunogold technique using anti-human acrosin monoclonal antibodies (Valdivia *et al.*, 1994).

Another interpretation of our results, based on the fact that there are three other serine proteases present in the mammalian spermatozoon acrosome (Akama *et al.*, 1994; Kohno *et al.*, 1998), is that the enzymatic activity on gelatin plates observed in our results and inhibited by trypsin inhibitors could be exerted on other serine proteases present in the acrosome and not necessarily only on acrosin. But the specificity of the anti-acrosin monoclonal antibodies is beyond doubt, as shown by previous results on western blots (Valdivia *et al.*, 1994; Sillerico *et al.*, 1996). These findings suggest that rabbit spermatozoa do not use all the serine proteases during penetration through the zona pellucida.

Acrosome-intact and acrosome-reacted rabbit spermatozoa can interact with the rabbit zona pellucida (O'Rand & Fisher, 1987). This further supports the hypothesis that perivitelline rabbit spermatozoa can interact with the zona pellucida of other non-fertilised rabbit oocytes. It has been shown that after the acrosome reaction in the rabbit there is a redistribution of acrosin from the acrosomal matrix to the equatorial segment and post-acrosomal regions (Richardson *et al.*, 1991).

In spite of the evidence indicating that acrosin is the enzyme responsible for the digestion of the zona pellucida, the other three serine proteases present in the mammalian spermatozoon acrosome (Akama *et al.*,

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1994; Kohno *et al.*, 1998) could also participate in spermatozoal penetration through the zona pellucida. One of these enzymes, acrosin-like, shares 80% homology with acrosin but can be extracted only in the presence of detergent. Recently, the role of acrosin has been challenged by experiments in which mice carrying a targeted mutation of the acrosin gene (Baba *et al.*, 1994) were shown to be fertile, but although they were able to fertilise homologous oocytes *in vitro*, it took them 30 min longer than the controls. Moreover, it has been shown that in *in vitro* fertilisation assays in which wild-type spermatozoa compete with those lacking a functional acrosin gene, only wild-type spermatozoa were able to fertilise the oocytes (Adham *et al.*, 1997). These authors concluded that spermatozoa lacking the acrosin gene have a selective disadvantage when they are in competition with those having the active gene. Taken together, these results may suggest that during normal fertilisation the acrosin gene, and maybe the acrosin-like gene (Akama *et al.*, 1994), are required for successful fertilisation. Probably for a second fertilisation, with most of the spermatozoal enzymatic machinery being exhausted, all the serine proteases would be required for zona pellucida penetration.

Acknowledgements

This work was supported by grant 1971234 from Fondecyt. One of the authors (M.V.) wishes to acknowledge a MUTIS Fellowship from the Spanish Agency for International Development. We would also like to acknowledge Dr Maria Inés Becker for her valuable assistance during the preparation of this manuscript.

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