EVALUATION OF FIXATIVE SOLUTIONS FOR ULTRASTRUCTURAL ANALYSIS OF BROWN SPIDER *Loxosceles intermedia* (ARANEAE: SICARIIDAE) TISSUES

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(With 7 figures)

ABSTRACT

In view of the widely varying compositions of fixative solutions used for studying spiders, five different fixative formulas were tested for fixing male brown-spider (*Loxosceles intermedia*) gonad tissues. The brown spider represents a public health problem in Curitiba (Paraná State, Brazil). Morphological study of its gonads may aid in understanding the reproductive strategies of this species, and possibly in developing a reproduction control program. The fixatives tested contained glutaraldehyde alone or combined with paraformaldehyde, and the buffers cacodylate or phosphate, with or without the addition of sucrose or sodium chloride as osmolytes. Those containing 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM phosphate buffer with 200 mM sucrose, or in 200 mM sodium cacodylate, satisfactorily preserved mitochondria, the Golgi apparatus, and the membranes in general. These formulas were nearly isosmotic (439 mOsm/kg H_2O and 455 mOsm/kg H_2O respectively) to brown spider hemolymph (478 mOsm/kg H_2O). With respective to the fixative agents, a glutaraldehyde-paraformaldehyde combination resulted in optimal fixation of *Loxosceles intermedia* cells. For other species of spiders, hemolymph osmolality should be considered, but the fixative formulas cited above would also probably yield good results.

Keywords: brown spider tissues, fixative solutions, *Loxosceles intermedia*, testis, ultrastructure.

RESUMO

Avaliação de soluções fixadoras para a análise ultra-estrutural dos tecidos da aranha marrom *Loxosceles intermedia* (Araneae: Sicariidae)

Dada a variabilidade na composição de soluções fixadoras utilizadas em aranhas, cinco diferentes fixadores foram elaborados para a análise ultra-estrutural dos tecidos da aranha marrom *Loxosceles intermedia*. A aranha marrom representa um problema de saúde pública na cidade de Curitiba, e o estudo morfológico de suas gônadas pode auxiliar na compreensão de suas estratégias reprodutivas e, possivelmente, no desenvolvimento de um programa de controle da sua população. As fórmulas usadas continham glutaraldeído com ou sem paraformaldeído, tampão cacodilato ou fosfato, e NaCl ou sacarose como osmólitos. As soluções fixadoras compostas por 2.5% glutaraldeído e 2% paraformaldeído, em tampão fosfato com adição de sacarose ou em 200 mM cacodilato de sódio, preservaram bem estruturas como mitocôndrias, aparelho de Golgi e membranas em geral. Os tampões são praticamente isosmóticos (439 mOsm/kg H_2O e 455 mOsm/kg H_2O, respectivamente) à hemolinfa da aranha marrom (478 mOsm/kg H_2O). Ainda, com relação aos agentes fixadores, a combinação do glutaraldeído e paraformaldeído levou a uma melhor preservação das células. Para outras espécies de aranhas, a osmolalidade da hemolinfa deve ser medida e considerada, mas as fórmulas acima citadas podem ser testadas, com chance de sucesso.

Palavras-chave: tecidos da aranha marrom, soluções fixadoras, *Loxosceles intermedia*, testículo, ultra-estrutura.

INTRODUCTION

The genus *Loxosceles* includes the most poisonous spiders in Brazil. In the urban environment of the city of Curitiba, capital of Paraná State, the brown spider *L. intermedia* Mello-Leitão - 1934 (Araneae, Sicariidae) (Platnick - 2004) is the most common species, specially in domestic habitats where - according to recent reports - it poses an increasing danger. The poison of *L. intermedia* acts proteolytically (Silveira *et al*., 2002), causing a local necrotic skin lesion, frequently with systemic effects that can be lethal (Gonçalves de Andrade *et al*., 2000). The study of *L. intermedia* reproduction and its morphological features by electron microscopy can promote better understanding this spider's biology and reproductive strategies. Furthermore, it should be useful in developing a reproduction control program. Considerable variation in composition of buffer and fixative solutions for studying spider tissues is found in ultrastructural studies in the literature. It is well known that a detailed analysis of any tissue or organ's ultrastructure requires optimal preservation of the material. Therefore, fixatives, buffer compositions and osmolality, and type of osmolytes should all be carefully considered before being selected.

This work reports treatment of male brown-spider gonads: the tissue chosen, and the five different fixative solutions tested, all of which were of different osmolalities and buffer compositions.

MATERIAL AND METHODS

Material analyzed

Adult brown-spider (*Loxosceles intermedia*) males were donated by inhabitants Curitiba, Paraná State, Brazil (25° 25' 40'' S and 49° 16' 23'' W). The spiders were kept in small plastic vials in the laboratory, and twice a month received *Tenebrium* sp. larvae and water in a small, saturated cotton ball). A total of 15 adult male *L. intermedia* specimens were used, along with 3 spider testes fixed with each of the 5 different fixative solutions tested. Spider hemolymph was extracted from the cephalothorax at the point where the legs are inserted. Two hemolymph pools, each from 5-6 spiders, were obtained.

Fixative solutions

The compositions of the 5 fixative formulas tested appear in Table 1. In every case, the pH was 7.4. Fixative solutions (FS) were prepared with glutaraldehyde alone (FS 4) or glutaraldehyde plus paraformaldehyde. Glutaraldehyde was tested in two different concentrations (2% in FS 1, and 2.5% in FS 2 - 5). Phosphate (FS 1, 2, and 3) and sodium cacodylate (FS 4 and 5) were tested as buffers, and osmolytes tested were NaCl (FS 1) and sucrose (2). Phosphate buffer was first tested as an nontoxic alternative to cacodylate. Measured osmolality of the fixative solutions was 423-523 mOsm/kg H_2O above buffer osmolality, except for fixative 4, in which the difference was 211 mOsm/kg H_2O.

TABLE 1
Composition and osmolality of buffers and fixative solutions.

Fixative solutions	*Composition						Osmolality (mOsm/kg H_2O)	
	Gluta (%)	Paf (%)	Buffer (mM)					
			PB	CACO	NaCl	SUC	Buffer	Fixative solution
1	2	2	100	-	50	-	302	725
2	2.5	2	100	-	-	200	439	871
3	2.5	2	200	-	-	-	453	976
4	2.5	-	-	200	-	-	454	665
5	2.5	2	-	200	-	-	454	899

* Gluta = Glutaraldehyde; Paf = Paraformaldehyde; PB = Phosphate buffer; CACO = Sodium cacodylate buffer; and SUC = Sucrose.

Osmolality measurements

Osmolality of the two hemolymph pools and of all fixative solutions and buffers was measured using a vapor pressure osmometer (VAPRO 5520, Wescor, USA). Each sample was measured in triplicate.

Dissection, fixation, and embedding method

Testes dissection, which took about 15 min/spider was done with the spider immersed in the fixative solution chosen. Fixation, including dissection, required 3 h and was done at 4 °C, followed by several washings in the buffer being tested. Following post-fixation with 1% osmium tetroxide, prepared with the same buffer used in the primary fixative, samples were washed in distilled water, dehydrated in a graded series of ethanol and embedded in Spurr's resin, according to standard protocols.

Sectioning, staining and analysis

Ultrathin sections (70 ηm) were obtained using a Leica Ultracut ultramicrotome (UCT), and were contrasted with 5% uranyl acetate for 30 min, and for 10 min with Reynold's lead citrate. Sections were examined in a transmission electron microscope (TEM), jeol JEM-1200 EX II; electron micrographs were obtained with Gatan Digital Micrograph software for image acquisition. The analyses were done by comparing tissue preservation - particularly of the mitochondria, the Golgi apparatus, and the cellular membranes - based on the electron micrographs.

RESULTS

The mean osmolality of the two pools of brown spider hemolymph was 478 mOsm/kg H_2O. As described in Table 1, buffer solution 1 (100 mM phosphate buffer plus 50 mM NaCl) was hyposmotic to brown spider hemolymph. Buffer solution 2 (100 mM phosphate buffer plus 200 mM sucrose) was slightly hyposmotic, and the others (solution 3: 200 mM phosphate buffer, and solutions 4 and 5: 200 mM sodium cacodylate) were nearly isosmotic to hemolymph osmolality.

Table 2 summarizes the results obtained with the five fixative solutions (FS). In the electron micrographs of the testes fixed with FS 1, the cells appeared swollen, despite the addition of mM NaCl to the phosphate buffer. Mitochondria presently frequently swollen cristae (Fig. 1); the images of the Golgi apparatus were poorly defined.

The tissue treated with FS 2 presented clear indications of swelling and poor preservation, despite the addition of 200 mM sucrose. The cells, however, displayed intact, much more clearly defined cytoplasmic membranes, and their mitochondria presented a less dense matrix and were better preserved than the membranes themselves. But cristae swelling was still evident (Figs. 2 and 5).

As for FS 3, the use of 200 mM phosphate buffer resulted in completely dried out tissue, which fragmented when sectioned (Table 2).

With respect to FS 4, which contained 2.5% glutaraldehyde (without paraformaldehyde) and 200 mM sodium cacodylate instead of phosphate, the result was slightly better tissue preservation, as compared to the results of all previous fixative solutions. Only occasional swelling was observed, and cells and organelles were generally well preserved (Figs. 3 and 6). However, this solution made the cytoplasm appear flocculated. The best result was obtained using FS 5, which contained

TABLE 2
General appearance of tissues of brown spider testes fixed with the five solutions tested.

Fixative solution	General aspect of the tissue			
	Cell preservation	Plasma membranes	Mitochondria	Golgi apparatus
1	-	+	-	-
2	-	++	+	++
3	*	*	*	*
4	+	++	+	++
5	++	++	++	++

(-) non-preserved; (±) poorly preserved; (+) preserved; (++) well preserved; and (*) could not be analyzed due to very poor preservation.

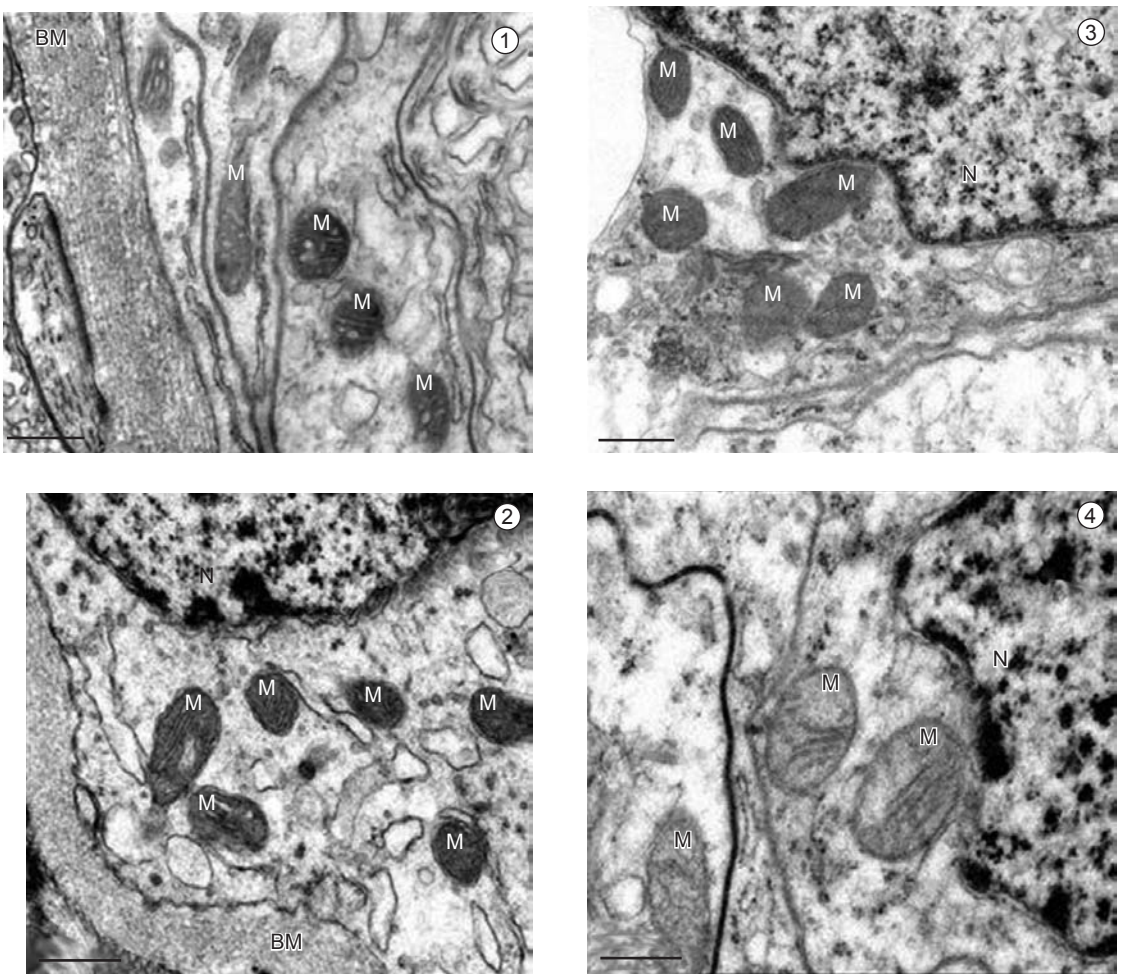

Fig. 1-4 — Transmission electron micrographs showing brown spider testes fixed with 1) FS 1; 2) FS 2; 3) FS 4; and 4) FS 5. Scale bar: 0.5 µm. BM: basal membrane, M: mitochondria, N: nucleus.

2.5% glutaraldehyde with the addition of 2% paraformaldehyde in 200 mM sodium cacodylate buffer (Figs. 4 and 7). The tissue appeared uniform, and presented little swelling or tissue disruption; cell membranes were intact, mitochondria and the Golgi apparatus very well preserved, with a clear matrix, intact membranes, and only occasional swelling (Table 2).

DISCUSSION

This study recounts a search for a suitable buffer and fixative solution for ultrastructural study of *Loxosceles intermedia* using male gonad tissue. In the literature on spider ultrastructure, a wide variety exists in buffer and fixative solution composition. Solutions buffered with either phosphate (Suzuki & Kondo, 1994; Suzuki, 1995; Michalik *et al*., 2004), or sodium cacodylate (Uhl, 2000) in different concentrations, with or without salt addition, have been used for various species of spiders.

In the present study, five different fixative formulae were prepared and tested, based on the formulas described in the above references, and modified in order to adapt them to brown spider hemolymph osmolality, which is 478 mOsm/kg H_2O and within the 400-600 mOsm/kg H_2O range for spiders cited by Foelix (1996).

With respect to osmolality, buffer 1 (100 mM phosphate buffer plus 50 mM NaCl) was not adequate, probably because it was hyposmotic

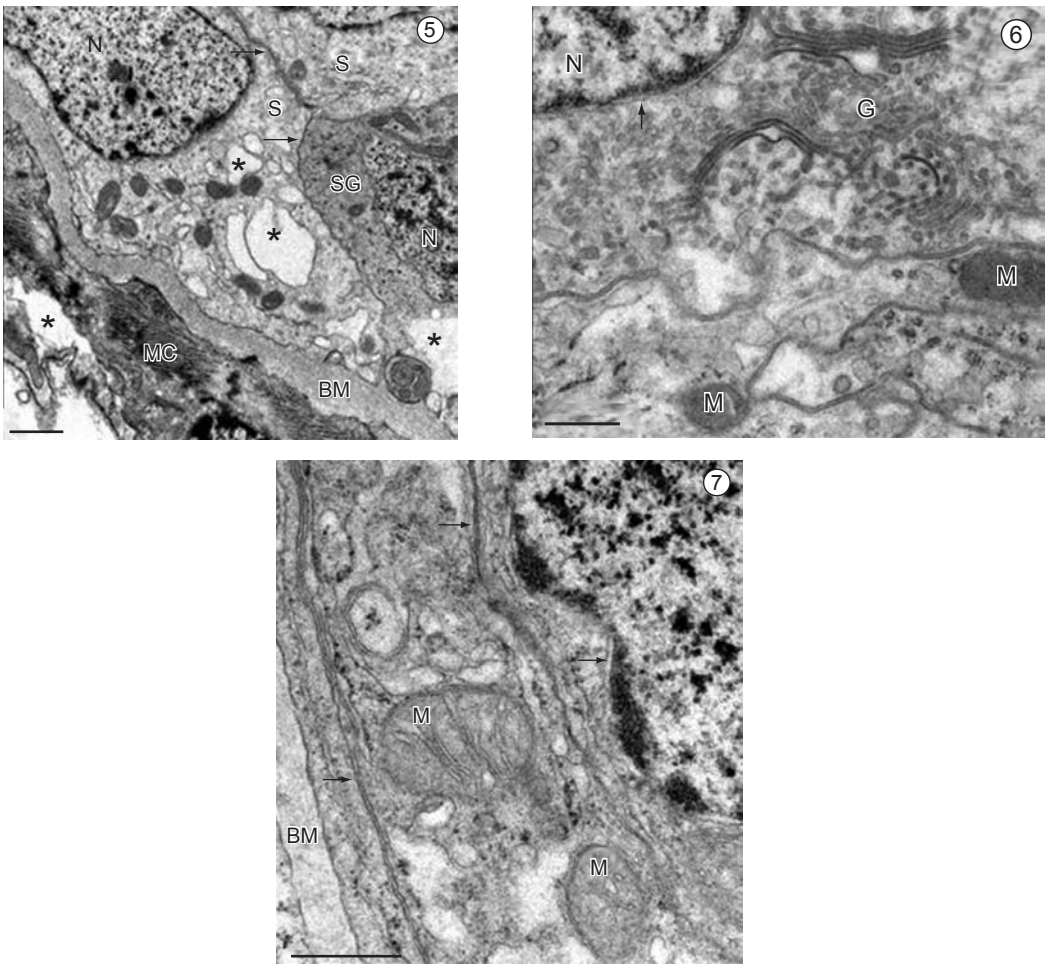

Fig. 5-7 — Transmission electron micrographs showing details of cells treated with FS 2, 4, and 5. 5) FS 2, showing poor cell preservation, with signs of swelling. Scale bar: 1.0 µm; 6) FS 4, scale bar: 0.5 µm; 7) FS 5, scale bar: 1.0 µm. (*) swelling points, BM: basal membrane; G: Golgi apparatus; M: mitochondria. MC: myoepithelial cell; N: nucleus; S: somatic cell; SG: spermatogonia. Black arrow: nuclear membrane; white arrow: cell membrane.

(302 mOsm/kg H_2O) to the brown spider hemolymph (Tables 1 and 2, Fig. 1). The other buffer solutions were nearly isosmotic to the hemolymph. However, not only is osmolality significant, but also the buffer and fixative solution composition (Hayat, 1970). An ionic osmolyte was used in FS 1 (NaCl), and a non-ionic osmolyte in FS 2 (sucrose), which rendered the osmolality value (439 mOsm/kg H_2O) close to that of the hemolymph, and yielded better results in comparison to those of FS 1 (Tables 1 and 2, Figs. 2 and 5). The successful association of phosphate buffer and sucrose (Suzuki & Kondo, 1994; Michalik *et al*., 2004) is confirmed in the present work, in which this combination stabilized membranes of brown spider cells (Figs. 2 and 5). Better results with nonionic osmolytes, such as sucrose or dextran, than NaCl have already been reported (Hayat, 1970).

As for FS 2, although it produced well-defined membranes, it was unsatisfactory in preserving mithocondria, in which some tissue swelling was observed.

Cacodylate - used as the buffer (200 mM) in FS 4 and FS 5 (Table 1), with no osmolyte added - was nearly isosmotic to the brown spider hemolymph, and produced good results (Table 2; Figs. 3, 4, 6, and 7). At this same concentration (200 mM), phosphate buffer in FS 3 (Table 1) resulted in unsectionable tissue in these 3 preparations. Thus, cacodylate stands out as a better buffer than

phosphate for spider testes (Hayat, 1970). Phosphate at a concentration of 100 mM, in association with 200 mM sucrose, should be considered in studying brown spider tissues when use of toxic agents such as cacodylate is ruled out.

Use of glutaraldehyde with paraformaldehyde in FS 5 (Tables 1 and 2; Figs. 4 and 7) resulted in better preservation of the testes tissue, when compared to the results of glutaraldehyde alone (FS 4: Tables 1 and 2; Figs. 3 and 6), with which cytoplasmic organelles were visible, but the cytosol seemed coagulated. With FS 5, the cytosol appeared more uniform, and all membranes were perfectly preserved (Tables 1 and 2; Figs. 4 and 7). Glauert (1975) reported that the glutaraldehyde and paraformaldehyde combination results in better tissue preservation than either alone, because paraformaldehyde penetrates tissues much more rapidly and stabilizes the structures, while glutaraldehyde produces more permanent fixation. Our results with FS 4 and FS 5 on brown spider tissue confirm Glauert`s observation.

The present results clearly evidenced that for optimal tissue preservation, attention should be paid to the extracellular fluid osmolality of animals being studied, and to the aldehydes added, buffer (cacodylate or phosphate), osmolytes added (sucrose or NaCl), as well as to the total osmolality of the solution. The fixative formulas successfully used in the present work for ultrastructural studies of brown spider testes could also be tried for the tissues of other spider species, specially if their hemolymph is osmotically similar to that of *Loxosceles intermedia*.

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