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

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ABSTRACT

In view of the wide variety in the composition of fixative solutions used for spiders, five different fixative formulae were tested for fixing male brown spider (*Loxosceles intermedia*) gonad tissues. The brown spider represents a public health problem in the city of Curitiba. The morphological study of its gonads may aid in the understanding of its reproductive strategies and possibly in the development of a growth 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. The fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde, in 100 mM phosphate buffer with 200 mM sucrose, or in 200 mM sodium cacodylate preserved well mitochondria, Golgi apparatus, and membranes in general. These formulas were nearly isosmotic (439 mOsm/kg H_2O e 455 mOsm/kg H_2O respectively) to the brown spider hemolymph (478 mOsm/kg H_2O). Concerning the fixative agents, the combination of glutaraldehyde and paraformaldehyde is advised to give optimal fixation of *Loxosceles intermedia* cells. For other species of spiders, hemolymph osmolality should be considered, but the fixative formulae cited above will also probably give good results.

AVALIAÇÃO DE SOLUÇÕES FIXADORAS PARA A ANÁLISE ULTRA-ESTRUTURAL DOS TECIDOS DA ARANHA MARROM *LOXOSCELES INTERMEDIA* (ARANEAE: SICARIIDAE)

RESUMO

Dada a variabilidade na composição de soluções fixadoras utilizadas em aranhas, cinco diferentes fixadores foram elaborados para a análise ultraestrutural 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 (478mOsm/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.

1. INTRODUCTION

The genus *Loxosceles* includes the most poisonous spiders in Brazil. The brown spider *L. intermedia* Mello-Leitão - 1934 (Araneae, Sicariidae) (Platnick - 2004) is the prevailing species in the urban environment of the city of Curitiba, state of Paraná. It is abnormally widespread in domestic habitats, resulting in increasing number of reports of accidents. Its poison has proteolytic action (Silveira et al. - 2002) and causes a local necrotic skin lesion, frequently with systemic effects that can result in death (Gonçalves de Andrade et al. - 2000), thus representing a local public health problem. The study of *L. intermedia* reproduction and its morphological details at electron microscopical level can provide basis for a better understanding of the biology and reproduction strategies of this spider. Such an understanding should aid in the establishment of a growth control program for the brown spider.

Considerable variation in the composition of buffer and fixative solutions is found in the literature on ultrastructural studies of spider tissues. It is well known that a detailed analysis of any tissue or organ at the ultrastructural level requires excellent preservation of the material, and in order to achieve this optimal tissue preservation, the fixatives, buffer composition and osmolality, and the type of added osmolytes should all be carefully considered.

This work reports the treatment of brown spider male gonads, the tissue of choice here, with five different fixative solutions, of variable osmolality and buffer composition.

2. MATERIAL AND METHODS

2.1 Animals

Adult brown spider (*Loxosceles intermedia*) males were donated by inhabitants of the city of Curitiba, Paraná, Brazil (25°25’40’’ S/ 49°16’23’’ W). The animals were

maintained in the laboratory inside small plastic vials, receiving *Tenebrium sp* larvae and water (in a saturated small cotton ball) twice a month. A total of 15 adult male *L. intermedia* specimens were utilized in the present study, with testis of 3 spiders being fixed with each of the 5 different fixative solutions tested. Spider's hemolymph was extracted from the cephalothorax at the point of insertion of the legs. Two hemolymph pools were obtained, each using 5 - 6 spiders.

2.2 Fixative solutions

The compositions of the 5 fixative formulae tested are displayed in Table 1. The pH of all the solutions was 7.4. Fixative solutions (FS) were prepared with glutaraldehyde alone (FS 4) or glutaraldehyde plus paraformaldehyde; glutaraldehyde being 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 as osmolytes were tested NaCl (FS 1) and sucrose (FS 2). Phosphate buffer was first tested as a nontoxic alternative to cacodylate. The 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 of 211 mOsm/kg H_2O.

2.3 Osmolality measurements

The osmolality of 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, and the means are shown in Table 1.

2.4 Dissection, fixation, and embedding method

Dissection of the testes was performed with the spider immersed in the respective fixative solution. The dissection took about 15 minutes for each spider, and fixation time (that includes the dissection time) was always 3 hours, at 4 ^{o}C, followed

by several washes in the respective buffer. After post-fixation with 1% osmium tetroxide, prepared with the same buffer of 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.

2.5 Sectioning, staining and analysis

Ultrathin sections (70 ηm) were obtained using an ULTRACUT ultramicrotome Leica, Germany, and were contrasted with 5% uranyl acetate for 30 minutes, followed by Reynold's lead citrate for 10 minutes. Sections were examined in a JEOL JEM 1200 EXII Transmission Electron Microscope and electron micrographs were obtained with the aid of GATAN-MULTISCAN 600W Software. The analyses were accomplished by comparative examination of the acquired electron micrographs, considering general tissue preservation, the aspect of structures such as mitochondria, Golgi apparatus, and preservation of cellular membranes.

"TABLE 1 CAN BE INSERTED HERE"

3. 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 the 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 the hemolymph osmolality.

Table 2 summarizes the results obtained with the five different fixative solutions (FS). Electron micrographs of the testes fixed with FS 1 showed the cells with a swollen aspect, despite the addition of 50 mM NaCl to phosphate buffer. Their

mitochondria had frequent swollen crista (Fig.1), and the images of the Golgi apparatus were poorly defined.

“TABLE 2 CAN BE INSERTED HERE”

The general aspect of the tissue treated with FS 2 had clear indication of swelling and poor preservation of the tissue, despite the addition of 200 mM sucrose. The cells, however, displayed intact and much more defined cytoplasmic membranes, and their mitochondria presented a less dense matrix**,** their membranes were better preserved, but swelling of cristae was still evident (Figs. 2 and 5). The use of 200 mM phosphate buffer in FS 3 resulted in a completely dried tissue, which fragmented when sectioned (Table 2).

The use of FS 4, which contained 2.5% glutaraldehyde (without paraformaldehyde) and 200 mM sodium cacodylate instead of phosphate resulted in a slightly better preservation of tissues when compared to all previous fixative solutions. Only occasional swelling was observed, and the general aspect of cells and organelles was well preserved (Figs. 3 and 6). However, with the use of this solution the cytoplasm had a flocculated appearance. The best result was obtained using FS 5, which contained 2.5% glutaraldehyde with the addition of 2% paraformaldehyde in 200 mM sodium cacodylate buffer (Figs. 4 and 7). The tissue appeared uniform, presenting rare swelling or tissue disruption; cell membranes were intact and mitochondria very well preserved, with a clear matrix and intact membranes, with only occasional swelling (Table 2). Thus, highest image quality of the structures such as mitochondria, Golgi apparatus, and membranes was obtained with the use of this last fixative solution.

PLATES WITH FIGURES 1-4 AND 5-7 CAN BE INSERTED HERE.

4. DISCUSSION

This work reports the search for an appropriate buffer and fixative solution to be used for ultrastructural study of *Loxosceles intermedia* tissues, using the male gonad as a model. In spider ultrastructure literature there is wide variation in the reported compositions of buffer and fixative solutions. 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, are reported for various species of spiders.

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

Regarding osmolality, buffer 1 (100 mM phosphate buffer plus 50 mM NaCl) was not adequate, probably because it was hyposmotic (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 osmolality is important, but also the composition of the buffer and fixative solution (Hayat - 1970). An ionic osmolyte was used in FS 1 (NaCl), and a non ionic osmolyte in FS 2 (sucrose), this one rendering the osmolality value (439 mOsm/kg H_2O) close to the hemolymph osmolality, and giving better results in comparison to FS 1 (Tables 1 and 2, Figs. 2 and 5). The association of phosphate buffer and sucrose was successfully used by Suzuki & Kondo - 1994 and Michalik et al. - 2004, such as was observed in the cited studies, this combination stabilized membranes in brown spider cells (Figs. 2 and 5). Better results with nonionic osmolytes, such as sucrose or dextran instead of NaCl have been cited before (Hayat -1970). Although the membranes were well defined, FS 2 was not efficient to preserve mithocondria, and a little swelling of the tissue was still observed.

Cacodylate as buffer (200 mM) in FS 4 and FS 5 (Table 1), without any added osmolyte, was nearly isosmotic to the brown spider hemolymph, yielding 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 dried tissues, impossible to be sectioned, in all 3 preparations attempted, thus allowing the conclusion that cacodylate is a better buffer than phosphate for spider testes and other animal tissues (Hayat – 1970). Phosphate at a concentration of 100 mM, in association with 200 mM sucrose, could be an option for brown spider tissues when avoiding the use of toxic agents such as cacodylate is an important issue for the user.

The use of glutaraldehyde with paraformaldehyde in FS 5 (Tables 1and 2; Figs. 4 and 7) yelded a better preservation of the testis tissue when compared to the use of glutaraldehyde alone (FS 4, Tables 1 and 2, Figs. 3 and 6). With glutaraldehyde alone, cytoplasmic organelles were visible, but the cytosol seemed coagulated. In FS 5, the cytosol had a more uniform aspect, and all membranes were perfectly preserved (Tables 1 and 2, Figs. 4 and 7). Glauert -1975 reports that the combination of glutaraldehyde and paraformaldehyde gives better preservation of tissues than either aldehyde alone, because paraformaldehyde penetrates into tissues much more rapidly, stabilizing the structures for the glutaraldehyde to achieve a subsequent and more permanent fixation. Our results with FS 4 and FS 5 on the brown spider tissue confirm Glauert`s observation.

The results gave clear evidence that, in order to achieve optimal tissue preservation, one should pay attention to the osmolality of the extracellular fluid of the animal of interest, the addition of the aldehydes, buffer quality (cacodylate or phosphate buffer), added osmolytes (sucrose or NaCl), and the total osmolality of the solution. The fixative formulas used in the present work with success for ultrastructural studies of brown spider testes could also be attempted for other species of spiders and others tissues, especially if their hemolymph is osmotically similar to that of *Loxosceles intermedia*'s.

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Table1. 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; SUC = Sucrose.

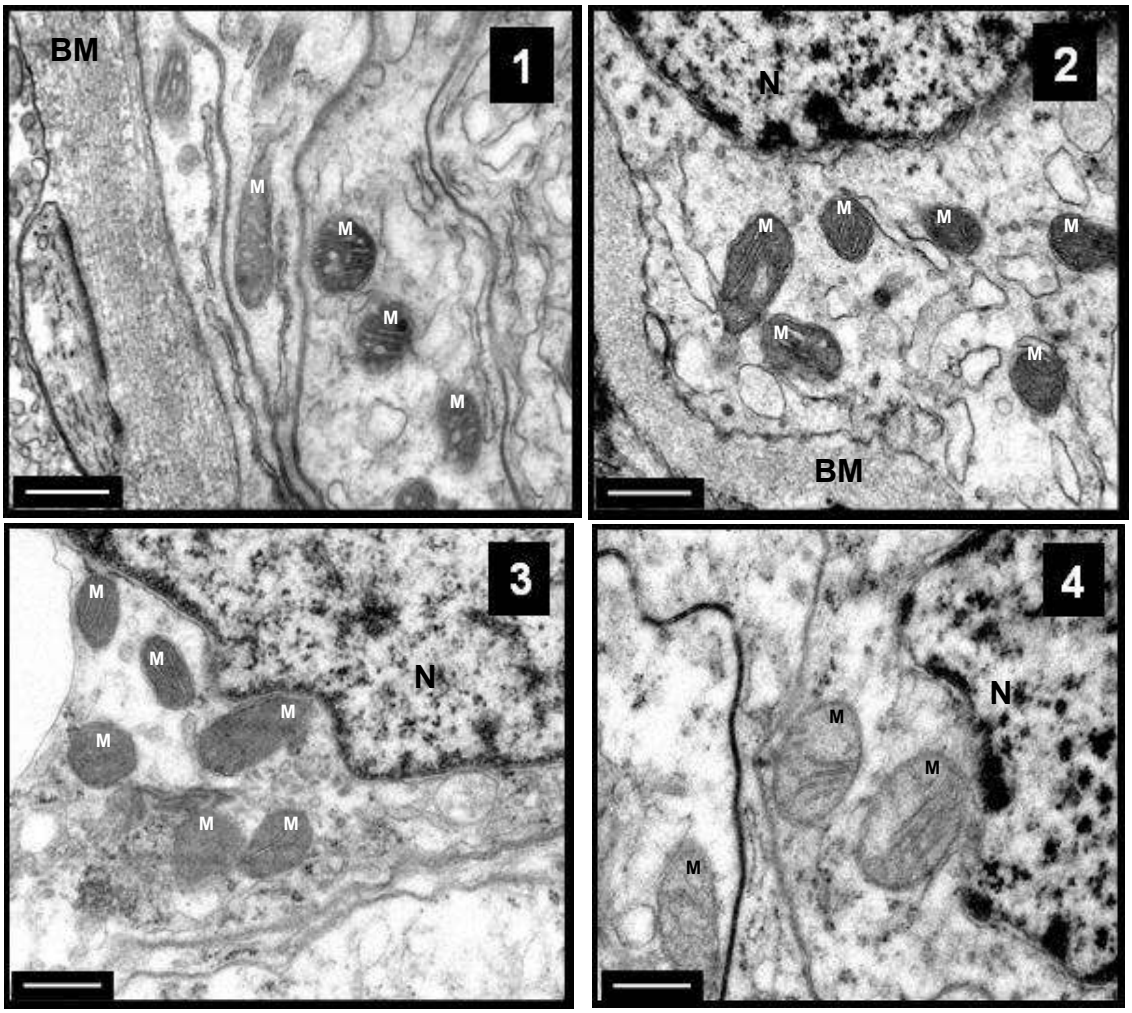
Table 2. General aspect of tissues of brown spider testes fixed with the five solutions tested.

FIXATIVE SOLUTION	GENERAL ASPECT OF THE TISSUE			
	Cell preservation	Plasma membranes	Mitochondria aspect	Golgi apparatus
1	-	+	-	-
2	-	++	+	++
3	*	*	*	*
4	+	++	±	++
5	++	++	++	++

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

FIGURES

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


BM
1
M
M
M
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N
2
M
M
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BM
3
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4
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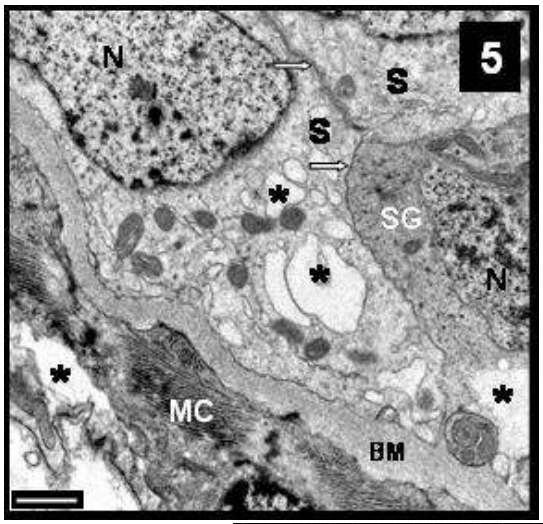
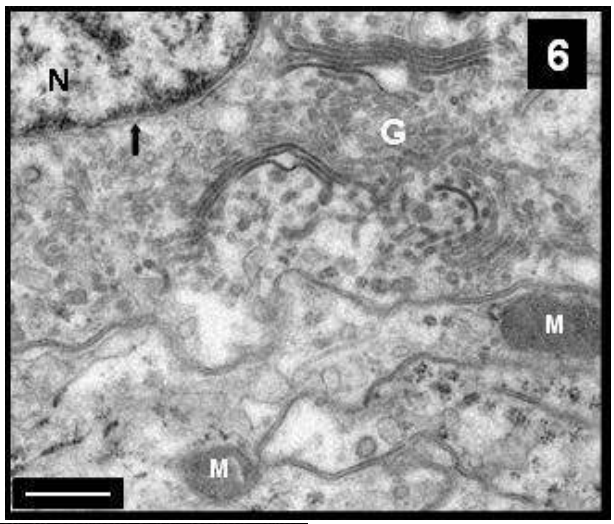
M

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


5
N
S
S
SG
N
MC
BM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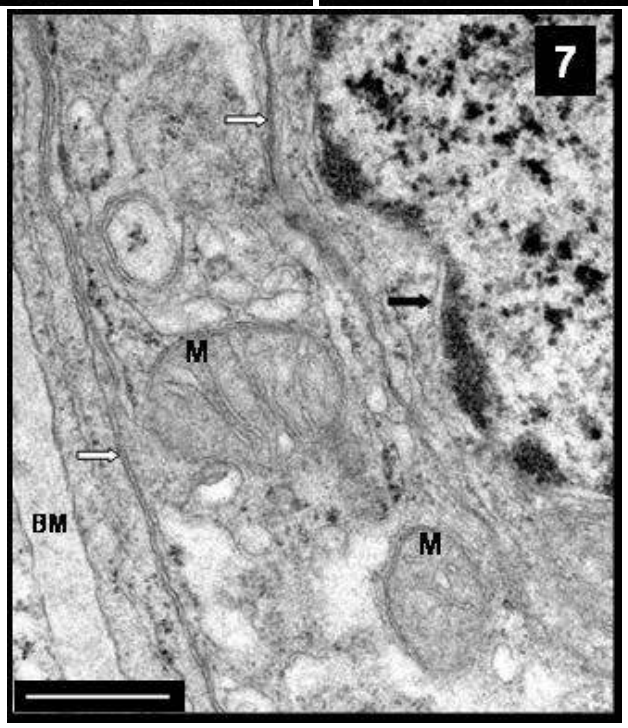
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