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Whole venom of *Loxosceles similis* activates caspases-3, -6, -7, and -9 in human primary skin fibroblasts

Arthur Estanislau Dantas^a, Carolina Campolina Rebello Horta^{b,c}, Thais M. M. Martins^{a,d}, Anderson Oliveira do Carmo^b, Bárbara Bruna Ribeiro de Oliveira Mendes^b, Alfredo M. Goes^a, Evanguedes Kalapothakis^b, and Dawidson A. Gomes^{a,*}

^aDepartamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil

^bDepartamento de Biologia Geral, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil

^cPrograma de Pós-Graduação em Fisiologia e Farmacologia, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil

^dPrograma de Pós-graduação em Biologia Celular, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, 31270-901, Minas Gerais, Brazil

Abstract

Spiders of the *Loxosceles* genus represent a risk to human health due to the systemic and necrotic effects of their bites. The main symptoms of these bites vary from dermonecrosis, observed in the majority of cases, to occasional systemic hemolysis and coagulopathy. Although the systemic effects are well characterized, the mechanisms of cell death triggered by the venom of these spiders are poorly characterized. In this study, we investigated the cell death mechanisms induced by the whole venom of the spider *Loxosceles similis* in human skin fibroblasts. Our results show that the venom initiates an apoptotic process and a caspase cascade involving the initiator caspase-9 and the effector caspases-3, -6, and -7.

Keywords

Caspases; *Loxosceles similis*; venom; apoptosis; necrosis; cell death

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*To whom correspondence should be addressed: Dawidson A. Gomes. dawidson@icb.ufmg.br, Instituto de Ciências Biológicas, bloco Q4, sala 238. Universidade Federal de Minas Gerais. Av. Antônio Carlos, 6627. Belo Horizonte – MG, 31270-901, Brazil.

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Disclosure of potential conflicts of interest

The authors disclose any commercial associations that might create a conflict of interest in connection with the submitted manuscript.

Ethical Statement

This manuscript has not been published or presented elsewhere in part or in its entirety and is not under consideration by another journal. Informed consent was obtained for the human samples used in the study, and the study design was approved by the appropriate ethics review boards. All the authors have approved the manuscript and agree with its submission to your esteemed journal.

1. Introduction

The genus *Loxosceles* (Araneae, Sicariidae) comprises 105 species of spiders (Platnick, 2013), and many of the species of this genus have attracted the attention of researchers due to the human health risk arising from the systemic and necrotic effects of their bites. In Brazil, where approximately 10,000 such bites are reported annually, there are eleven species of *Loxosceles* spiders, although only a few, such as *L. laeta* (Nicolet, 1849), *L. gaucho* (Gertsch, 1967), and *L. intermedia* (Mello-Leitão, 1934), represent a great risk for problematic bites. Recently, *L. similis* has been found inside residences in Belo Horizonte in the state of Minas Gerais, demonstrating the adaptability of this spider to the urban environment (Machado et al., 2005).

The condition caused by the bites of *Loxosceles* spiders is referred to as loxoscelism, which is characterized by many symptoms but primarily by inflammation and dermonecrosis at the site of the bite. However, in some cases, systemic hemolysis and coagulopathy, leading to acute renal failure, are also present (da Silva et al., 2004; Guimarães et al., 2013; Ministério da Saúde, 2011). *Loxosceles* sp. venom is composed of a mixture of protein-based toxins, including ribonucleotide phospho-hydrolases (Futrell, 1992), serine proteases (Veiga et al., 2000), hyaluronidases (Barbaro et al., 2005; da Silva et al., 2007a), metalloproteases (Feitosa et al., 1998; da Silva et al., 2007b), and phospholipase-D (Chaim et al., 2006; Cunha et al., 2003; da Silva et al., 2007c; Tambourgi et al., 2002). Although the systemic effects of the venom of *Loxosceles* sp. are well described, little is known about the cell death mechanisms triggered after their bites. Thus, the aim of this study was to investigate effects of whole venom from the spider *Loxosceles similis* on the induction of necrosis and apoptosis.

There are studies linking *Loxosceles* venom and its components to apoptosis; however, the caspase effectors were not evaluated in these studies (Horta et al. 2013; Paixão-Cavalcante et al. 2007). There are two main pathways of apoptosis: caspase-dependent apoptosis and caspase-independent apoptosis (Galluzzi et al., 2012). Thus far, 14 human caspases have been characterized, although not all of them are related to apoptosis. For example, caspases-1, -4, and -5 are implicated in inducing pyroptosis, a form of death associated with the massive activation of inflammatory cells (Labbé and Saleh, 2008). Caspases related to apoptosis are classified into two groups, the “initiators”, consisting of caspases-8, -9, and -10, and the “effectors”, comprised of caspases-3, -6, and -7. The initiators are further classified as extrinsic (caspases-8 and -10) or intrinsic (caspase-9) based on the pathway that they trigger (Taylor et al., 2008). When activated, caspases selectively cleave vital cellular substrates, such as components of the cytoskeleton, nuclear envelope, cell-cell, and cell-matrix adhesion complexes. They also cause fragmentation of DNA by selectively activating DNases. All of these alterations characterize the cellular apoptotic morphology (Taylor et al., 2008; Ulukaya et al., 2011). The present work shows that the venom of *L. similis* triggers an apoptotic process that involves the activation of caspases-9, -6, -3, and -7 in human skin fibroblasts.

2. Materials and Methods

2.1 Spiders and venom

L. similis spiders were collected in a touristic cave, Gruta da Lapinha (geographic coordinates 43°57'W, 19°33'S), located in Parque Estadual Sumidouro, Lagoa Santa (Minas Gerais, Brazil), under license from the “Instituto Estadual de Florestas de Minas Gerais” (IEF/MG). The specimens were identified using the method described by Gertsch (1967). The venom glands were removed as described by Da Silveira et al. (2002), macerated, centrifuged, and the cleared supernatant was stored at –80°C until use. Protein quantification was performed in the venom using the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) (Sigma-Aldrich, CA, USA) as the protein standard. The absorbance was measured at 600 nm using an ELx 800 Universal Microplate Reader (Biotek Instruments, VT, USA) according to Chatzaki et al. (2012).

2.2 Skin fibroblast isolation

In this study, we used two different lines of primary skin fibroblasts, one isolated from human skin and other isolated from rabbit skin. Briefly, the rabbit skin tissue used for primary fibroblast isolation was a 1 cm² piece of skin obtained from a rabbit's back. All experimental protocols were performed in accordance with the guidelines for the humane use of laboratory animals established at our institution. This study was approved by the Ethics Committee on Animal Experimentation at the Federal University of Minas Gerais (CEUA/UFMG) (Protocol 305/2012).

The human skin tissues used for primary fibroblast isolation were obtained from patients subjected to blepharoplasty surgery. This procedure was approved by the Ethics Committee on Research at the Federal University of Minas Gerais (no CAAE: 02887512.6.0000.5149).

The fibroblast isolation protocol was the same for both tissue sources and was adapted from Seluanov et al. (2010). Briefly, the 1 cm² tissue sample was cut into pieces of approximately 1 mm², transferred to a 50 mL tube, and digested with 0.10% collagenase (Life Technologies, CA, USA) for 3 h. The same volume of DMEM (Sigma-Aldrich) plus 10% bovine fetal serum (Life Technologies) was then added to inactivate enzyme activity, and the mixture was centrifuged for 10 min at 350 × g. The cells were subsequently resuspended in DMEM + 10% serum, transferred to a T-25 cm² tissue culture flask (Sarstedt, Germany) and maintained in an incubator with a humid atmosphere at 37°C under 5% CO₂ (Thermo Scientific, MA, USA). The cells were used after reaching the third passage.

2.3 Analysis of cell viability and venom dosage: MTT assay

To assess the viability of cells treated with venom, we employed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Life Technologies), as previously described (Mosmann 1983). The cells used in this assay were Skhep-1 (human hepatic adenocarcinoma), Vero (a *Cercopithecus aetiops* kidney cell line), and MEF cells (BALB/3T3 clone A31) (ATCC, VA, USA) and human and rabbit primary skin fibroblasts.

Briefly, the cells were plated in 24-well culture plates at a density of 1×10^5 cells/cm² and incubated in a humidified 5% CO₂ incubator at 37°C for 24 h. The cells were subsequently incubated with 60 µg/mL of the spider's venom for 24 h, after which the medium was changed, and MTT (5 mg/ml) was added to the monolayer culture in each well.

After 2 h, the formazan salts were dissolved with 10% SDS-HCl overnight, and the optical density was measured at 595 nm using a microplate reader (Elx800, BioTek). Staurosporine from *Streptomyces* sp. (200 nM) (Sigma-Aldrich) was used as a positive control for cell death, and un-treated cells were used as the negative control. The values for the samples were expressed as a percentage of the negative control. All data were analyzed with GraphPad software.

This assay was also employed to assess the EC₅₀ value of the venom in human skin fibroblasts. The cells were plated and treated with serial dilutions (100, 33, 11, 3.66, and 1.22 µg/mL) of the venom for 24 h, after which the MTT protocol was performed. All data were analyzed using GraphPad software.

2.4 Cell death assay via fluorescence microscopy: apoptosis versus necrosis

Human skin fibroblasts were detached from the cell culture flask using a 0.05% trypsin-EDTA solution (Life Technologies), and trypsin was inactivated after 5 min by the addition of an equal volume of DMEM plus 10% serum. The cells were centrifuged at 350 × g and plated in 24-well culture plates at a density of 25×10^3 cells/well, after which they were maintained in a humid incubator with 5% CO₂ at 37°C for 24 h.

L. similis venom was added at the EC₅₀ of 40 µg/mL calculated based on the MTT assay, followed by incubation for 24 h. The cells were also incubated with staurosporine for 4 or 24 h as a positive control for cell death.

The cells were stained with YO-PRO-1 (Life Technologies) and propidium iodide (Life Technologies) for 30 min to quantify the percentages of cells undergoing necrosis and apoptosis, respectively. Images were captured using a Nikon TI-Eclipse Fluorescence microscope coupled with a Nikon CFI S Plan Fluor ELWD 20x (N.A. 0.45) objective (Nikon, JAP), a Lambda DG-4 illumination source (Sutter Instrument, CA, USA), and an EMCCD LucaEM R camera (Andor Technology, CT, USA) at the "Centro de Aquisição e Processamento de Imagem do Instituto de Ciências Biológicas - UFMG (CAPI)". The emission (EM) and excitation (EX) filters used to collect YO-PRO-1 fluorescence were EM 480/30 and EX 535/40, while EM 540/25 and EX 605/55 filters were used to collect the fluorescence from propidium iodide. Filters and components were controlled by NIS-AR imaging software (Nikon). Unstained cells were employed as a negative control, and cells stained only with one fluorochrome were employed to properly compensate for the propidium iodide channel.

2.5 Annexin V assay via flow cytometry

Human skin fibroblasts were plated in 24-well culture plates at a density of 50×10^3 cells/cm². The cells were maintained in a humidified incubator with 5% CO₂ at 37°C for 24 h.

Venom was added at the calculated EC₅₀. Again, the cells were also incubated with a solution of 200 nM staurosporine as a positive control for apoptosis and late necrosis for 4 or 24 h, respectively.

The cells were then detached from the cell culture surface, centrifuged, and stained using the Annexin V-FITC Apoptosis Kit (Sigma-Aldrich) for 10 min. The cells were subsequently analyzed with a Guava easyCyte 6-2L Flow Cytometer (Millipore, MA, USA). Annexin V-FITC fluorescence was collected using a 525/30 emission filter, and propidium iodide fluorescence was collected using a 583/26 emission filter. The fluorescence signals were properly compensated for prior to data collection using cells stained only with one of the probes. All data were analyzed using FlowJo Software (Tree Star, San Carlos, CA, USA).

2.6 Caspase inhibition assay via flow cytometry

This procedure was similar to the method described in section 2.5, but at the same time that the venom was added, the wells were also incubated with the following caspases inhibitors (20 μ M): Cell-Permeable Caspase-6 Inhibitor (Sigma-Aldrich) and Cell-Permeable Caspase-9 Inhibitor (Sigma-Aldrich).

Data acquisition and analysis were also performed as described in section 2.5.

2.7 Detection of caspases-3 and -7 activity

Fibroblasts were detached and plated according to section 2.4. Venom was added at the EC₅₀ and incubated with the cells for 24 or 48 h. A solution of 200 nM staurosporine was also added to the cells, followed by incubation for 4 h, as a positive control for cell death.

The cells were stained with the Vybrant FAM Caspase-3 and -7 Assay Kit (Life Technologies) for 30 min, and images were captured using a Nikon Eclipse TI fluorescence microscope as described in section 2.4. The EM and EX filters employed to collect Vybrant FAM Caspase-3 and -7 fluorescence were EM 480/30 and EX 535/40, respectively.

2.8 Statistical analysis

All of the presented data represent at least three independent experiments and are expressed as the mean $\pm$ standard error of the mean (SEM). The statistical analyses were performed with GraphPad software. The experiments with more than two data groups were compared using one-way ANOVA and the Bonferroni posttest.

3. Results

3.1 Treatment with *L. similis* venom decreases MTT metabolism only in primary isolated fibroblasts

The MTT assay showed a decrease in MTT metabolism in both the human and rabbit skin fibroblasts treated with 60 μ g/mL of *L. similis* venom for 24 h; compared to the control, the human fibroblasts (HFs) exhibited a decrease of $60 \pm 0.8\%$, and the rabbit fibroblasts exhibited a decrease of $45 \pm 1.6\%$ (Fig. 1). The other cell lines were not affected by the

venom treatment. We decided to perform all the ensuing experiments using the HFs as a model to test the effects of the *L. similis* venom.

An MTT assay was also conducted to calculate the EC_{50} of the activity of the venom (Fig. 2). The calculated EC_{50} for the venom in HFs was 40 $\mu\text{g/mL}$.

3.2 Human skin fibroblasts treated with *Loxosceles similis* venom for 24 h show an apoptotic response

After 24 h of treatment with the venom, $87 \pm 2.1\%$ of the HFs were labeled with YO-PRO-1 iodide (Fig. 3b). The YO-PRO-1 iodide dye is only able to penetrate the cell plasma membrane after apoptosis has begun, whereas propidium iodide can enter cells only when the membrane is compromised, pointing to a necrotic process. Our results indicate that the cells treated with venom entered an apoptotic process. Treatment with staurosporine for 24 h was used as a control for both necrotic and apoptotic cells. As there are no macrophages *in vitro* to phagocytose cells, the HFs entered into a secondary necrotic process and were therefore labeled with both dyes.

To confirm these results, we performed an Annexin V and propidium iodide assay using flow cytometry (Fig. 3C). Of the fibroblasts treated with the venom, $43.6 \pm 3.5\%$ were labeled with Annexin V; $12.8 \pm 3.3\%$ were double labeled; $0.41 \pm 0.02\%$ were labeled with propidium iodide; and $43.2 \pm 2.3\%$ were not labeled. Of the cells that were not treated with the venom, $0.82 \pm 0.09\%$ were labeled with Annexin V; $1 \pm 0.2\%$ were double labeled; $0.72 \pm 0.4\%$ were labeled with propidium iodide; and $97.4 \pm 0.6\%$ were not labeled. These results show that *L. similis* venom triggered an apoptotic process in HFs.

3.3 Inhibition of caspases-6 and -9 decreases the percentage of cells labeled with Annexin V

We used specific caspase inhibitors to determine whether inhibition of the main caspases involved in the intrinsic pathway would affect the apoptosis response caused by the venom. We observed that inhibition of caspase-9 decreased Annexin V staining from $67 \pm 3\%$ in the venom-treated group to $25 \pm 2.4\%$ in the caspase-9 inhibitor-treated group (Fig. 4A). Inhibition of the effector caspase-6 also decreased the percentage of cells labeled with Annexin V, from $67 \pm 3\%$ to $36 \pm 0.7\%$. Both groups exhibited differences compared to the group treated with the venom alone, as shown in the graphs in Fig. 4B. This was an expected result because caspase-9 is further upstream in the pathway. In this experiment, the cells were incubated with the venom for 48 h because we could not verify strong inhibition of the apoptotic response after 24 h. Considering that the caspase inhibitors were diluted in dimethyl sulfoxide (DMSO) and that the treatment group did not present a difference from the control group, all of the groups were compared to the group treated with the vehicle, DMSO. The cells treated with the caspase inhibitors that were positive for Annexin V or negative for both markers exhibited a difference from the group treated with venom for 48 h. The groups treated with the caspase inhibitors and the group treated with venom alone for 48 h did not show differences in double labeled and propidium iodide-labeled cells.

3.4 Caspases-3 and -7 are activated in cells treated with *L. similis* venom

To determine whether other effector caspases in the pathway were activated, we labeled the cells using the Vybrant FAM Caspase-3 and -7 Assay Kit and treated them with venom for 24 or 48 h. As we only wanted to verify caspase activation, we used staurosporine for 4 h as a control for apoptosis in this assay. The graph in Fig. 5A shows the intensity of the fluorescence in all groups, with the venom-treated groups exhibiting a higher fluorescence intensity than the control group, by 4.4 times in the 48 h group and by 4.8 times in the 24 h group. Caspases-3 and -7 were activated in both groups treated with the venom, and no difference between their activation levels was detected; therefore, we believe that the activation of these caspases was at its maximum after 24 h of incubation (Fig. 5B).

4. Discussion

To the best of our knowledge, this work is the first to explore the involvement of caspases in the death of cells treated with whole *Loxosceles* venom. We show that whole *L. similis* venom triggers an apoptotic signaling cascade that includes activation of the initiator caspase-9 and the effector caspases-3, -6, and -7 in human primary skin fibroblasts.

To our surprise, deleterious effects of *Loxosceles* venom were not observed in all of the cell types tested: the human and primate immortalized cell lines Skhep-1 and Vero, respectively, and primary mouse MEF cells were not affected by incubation with *L. similis* venom (Fig. 1A). There are several studies showing that whole venom from other spiders of the *Loxosceles* genus have cytotoxic effects on different primary cell types. For instance, *Loxosceles* venoms have toxic effects in human erythrocytes (Chaves-Moreira et al. 2009), human keratinocytes (Paixão-Cavalcante et al. 2006), rabbit endothelial cells (Nowatzki et al. 2012), and human fibroblasts (Horta et al., 2013). Our results suggest that it is mainly primary cell lines that are sensitive to the toxic effects of *Loxosceles similis* venom.

Venom interactions may occur at the cell membrane, as shown by Nowatzki et al. (2010), who tested whole *L. intermedia* venom for membrane interactions. Components of *L. intermedia* venom were found to interact with the cell membrane before being endocytosed by endothelial cells. Although venom components were found inside the lysosomes, no harm to the lysosome structure was detected, indicating that the cytotoxicity of the venom was not dependent on the disruption of this organelle (Nowatzki et al. 2010). Horta et al. (2013) showed that *L. similis* venom induces the release of cytokines and chemokines in human fibroblasts (HFF-1) through a mechanism that is dependent on lysophosphatidic acid (LPA) receptors; LPA is formed through the action of the toxin phospholipase-D on phospholipids in the cell membrane. Nevertheless, LPA receptors do not participate in the apoptosis induced by *L. similis* venom in human fibroblasts or endothelial cells (Horta et al., 2013). In the present work, we aimed to investigate the specific mechanism of cell death induced by *L. similis* venom. Our results indicate that, in human fibroblasts, *L. similis* venom activates several caspases that participate in the intrinsic apoptosis pathway (Fig. 4, Fig. 5). Because this cascade can be activated by interactions outside the cell, we speculate that the venom of *L. similis* may interact with receptors on the cell surface. Many studies have shown the binding of *Loxosceles* venom toxins upon the membrane of diverse cell types, showing direct induction of cytotoxicity and supporting the hypothesis of “planted

antigens” to different components of cell structures (Chaim et al., 2006; Chaves-Moreira et al., 2009; Dias-Lopes et al., 2010; Wille et al., 2013). Further research should be done to investigate which venom compounds can activate the caspases cascade investigated.

Our EC₅₀ results are in agreement with previously reported values for the venom of *Loxosceles* spiders from *in vitro* studies (Nowatzki et al., 2010; Nowatzki et al., 2012). However, we found an important difference in the percentage of cells labeled with the apoptosis markers YO-PRO-1 iodide and Annexin V (Fig. 3). We suggest that this difference can be explained by two factors, the first of which is that Annexin V is a more specific marker for apoptosis than YO-PRO-1 iodide, being indicated by the Nomenclature Committee on Cell Death as a detection method for apoptosis (Kroemer et al., 2009). The other possible reason for the difference between the two methods is the numbers of cells plated at the beginning of the assays. For the fluorescence microscopy assay, we plated 25×10^3 cells/cm²; for the cytometry assay, we plated 50×10^3 cells/cm², which was the same number used to establish the EC₅₀ for the venom. A smaller number of plated cells would be expected to suffer a stronger cytotoxic effect, justifying the higher percentage of stained cells observed in the microscopy assay. This inference also allows us to conclude that the effect of the venom is not only concentration dependent, but is also quantitatively dependent considering the number of cellular components available for interaction.

In vivo, *Loxosceles* venom causes cell death, but importantly, it also causes significant inflammation. We therefore believe that the inflammation triggered *in vivo* by the venom may be explained in part by the model we proposed as a control for the double-labeled cells in Fig. 3. We suggest that the apoptosis induced is so massive that macrophages may not be able to phagocytose all of the cells undergoing this process. These cells then enter into a secondary necrosis state in which their membranes lose integrity, showing leakage of their intracellular contents, stimulating an inflammatory reaction triggered by DAMP (damage-associated molecular pattern) receptor binding (Kerr et al., 1972; Kerr et al., 1994; Green and Reed, 1998).

We also found evidence of some endoplasmic reticulum stress-induced apoptosis, as inhibition of caspase-4 induced a decrease in the percentage of cells labeled with Annexin V, propidium iodide, or both (data not shown). However, this hypothesis is difficult to be examined because caspase-4 is poorly characterized and is associated with both pyroptosis and apoptosis induced via endoplasmic reticulum stress (Hitomi et al. 2004; Sollberger et al. 2012).

Considering that the whole venom of *Loxosceles* spiders is a mixture of several components, it is expected to activate many cell death pathways. In erythrocytes, phospholipase-D from the brown spider induces the metabolism of membrane phospholipids, such as sphingomyelin and lysophosphatidylcholine, generating bioactive products that stimulate calcium influx into red blood cells, mediated by L-type channels (Chaves-Moreira et al. 2011). In human keratinocytes, Paixão-Cavalcante et al. (2006) have shown that *Loxosceles* phospholipase-D induces apoptosis, associated with an increase in the expression of metalloproteinases-2 and -9. Tetracycline, a metalloproteinase inhibitor, reduces the expression of metalloproteinase-2 and prevents the induction of metalloproteinase-9 in

rabbit skin samples. In another study, topical tetracycline treatment of rabbits inoculated with the whole venom of *L. intermedia* or phospholipase-D resulted in a reduction of the progression of necrotic lesions (Paixão-Cavalcante et al. 2007). Additionally, it has been demonstrated that extracellular matrix molecules are targets for brown spider venom toxins such as proteases and hyaluronidases, leading to a disruption on cell-to-cell and cell-to-matrix contacts, loss of tissue integrity and spreading of the venom toxins (Veiga et al., 2000; Veiga et al., 2001; Ferrer et al., 2013). Thus, Nowatzki et al. (2012) showed that treatment with the venom of *L. intermedia* spider induces an anoikis process due to loss of cell anchorage in rabbit endothelial thoracic aorta cells. Therefore, there are likely several mechanisms through which the venom and its components can initiate cell death processes, and further studies using isolated venom components will be necessary to verify which cell death pathway is activated by each component. In addition, it will be important to evaluate whether the effects observed for whole venom are cell or species specific, as indicated by our preliminary results, and which mechanisms are involved in this specificity.

In summary, the present study provides data that advance our understanding of *L. similis* venom. We present results that indicate that human skin fibroblasts treated with the whole venom of the *L. similis* spider enter into a caspase-dependent cell death process involving activation of the initiator caspase-9 and effector caspases-3, -6, and -7. Inhibition of these caspases prevents or at least delays the onset of the apoptotic process.

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Abbreviations

BSA	bovine serum albumin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
EM	emission
EM	excitation
HF's	human fibroblasts
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Highlights

1. Only primary cell lines were sensitive to the toxic effects of venom in this study.
2. Our results show that the venom initiates an apoptotic process.
3. Caspases-3, -6, -7 and 9 were activated by the venom.

Figure 1. Treatment with *L. similis* venom decreases skin fibroblast viability

Cell viability was assessed by measuring MTT metabolization. The bar graph shows the percentage of metabolized MTT after 48 h of incubation with the venom in Skhep-1, Vero, mouse embryonic fibroblasts (MEFs), human fibroblasts, and rabbit fibroblasts. The control consists of the average for all untreated cells. All groups were compared to the control. (N=9, the experiment was performed on three independent days, *p<0.05).

Figure 2. *L. similis* venom affects fibroblasts in a dose-dependent manner

Human skin fibroblasts were treated with increasing concentrations of venom; the EC₅₀ was calculated to be 40 µg/mL. MTT assays were performed after 48 h of venom incubation (N=9, the experiment was performed on three independent days).

Figure 3. *L. similis* venom treatment induces apoptosis in fibroblasts

A Representative images of fibroblasts treated with staurosporine for 4 h (n=91) or 24 h (n=99), with venom for 48 h (n=124), or left untreated (n=145). Live cells were labeled with YO-PRO-1 iodide (green) to evaluate apoptotic cells, and propidium iodide (red) was used as a marker of necrotic cells. Bright-field images are presented on the left. The merged images show double-labeled cells. The data were collected from three different experiments. **B** Quantification of cells labeled with YO-PRO-1 iodide and propidium iodide. All groups were compared to the control (*p<0.001). **C** Representative cytometry plots for fibroblasts

treated with staurosporine for 24 h, with venom for 48 h, or left untreated (n=9, the experiment was performed on three independent days). The cells were labeled with Annexin V-FITC (green) and propidium iodide (red). A total of 5,000 events were collected per sample. **D** The bar graphs show the percentages of cells labeled with either Annexin V-FITC or propidium iodide (in this group, the Y axis was altered for better data visualization), or double labeled with Annexin V-FITC plus propidium iodide, or left unlabeled. All groups were compared to the control. (**p<0.01, ***p<0.001).

A **B**

Figure 4. Inhibition of caspases-6 and -9 decreases the percentage of apoptotic cells

A Representative cytometry plots of fibroblasts treated with staurosporine for 24 h, venom for 48 h, a caspase-6 inhibitor plus venom for 48 h, a caspase-9 inhibitor plus venom for 48 h, or left untreated (N=12, the experiment was performed on four different days). The cells were labeled with Annexin V-FITC (green) and propidium iodide (red). A total of 5,000 events were collected per sample. **B** The bar graphs show the percentages of cells labeled with either Annexin V-FITC or propidium iodide (in this group, the Y axis was altered for better data visualization), or double labeled with Annexin V-FITC plus propidium iodide, or left unlabeled. All groups were compared to the vehicle. (* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$).

Figure 5. Treatment with *L. similis* venom triggers caspases-3 and -7

A Representative images of fibroblasts treated with staurosporine for 4 h (n=133), with venom for 24 h (n=152) or 48 h (n=152), or left untreated (n=131). The cells were labeled with Vybrant FAM Caspase-3 and -7 (green). Bright-field images are shown in the panels on the right. **B** The bar graph shows the quantification of the fluorescence values of the labeled cells. All groups were compared to the control (***p<0.001).