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Original Article

Development of a monoclonal antibody against a synthetic peptide of Buthotus saulcyi scorpion venom: a novel diagnostic and neutralizing tool

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Abstract: Background: Hybridoma technology is an essential method used to produce monoclonal antibodies, providing equal specificity and scalability for biomedical applications. This methodology utilizes a hybridoma formation process through fusing short-lived, antibody-producing B lymphocytes with immortalized myeloma cells, thus providing hybridoma clones that produce monoclonal antibodies that are highly specific. Monoclonal antibodies produced through hybridoma technology have a consistent and reproducibility benefits over polyclonal forms of antibodies, making monoclonal antibodies an essential product in diagnostics and therapeutics. In this study, we attempted to produce monoclonal antibodies in order to target a synthetic peptide from venom of Buthotus Saulcyi, a medically important scorpion in the family Buthidae, native to Iran, known for having potent toxicity that is most dangerous in children and the elderly. Methods: Balb/c mice were immunized with the synthetic peptide P4 before fusion to Sp2/0-Ag14 myeloma cells using polyethylene glycol at a 5:1 ratio. Hybridoma cells were cultured in HAT selective media with a single clone isolated using limiting dilution. Result: Cell production was confirmed with an enzyme-linked immunosorbent assay (ELISA) and determined specificity to recognize *B. Saulcyi* venom. Neutralization was determined using MTT and SRB cell lines HepG2 and determined the monoclonal antibody treatment for *B. Saulcyi* venom had efficacy. Conclusion: These findings highlight the potential of this mAb as a diagnostic tool for rapid detection of *B. Saulcyi* venom in clinical settings, paving the way for improved management of scorpion envenomation.

Keywords: Hybridoma technology, monoclonal antibody, *Buthotus saulcyi*, scorpion venom, diagnostics

Introduction

Scorpion stinging continues to be a significant public health issue in Iran's and the world's subtropical and tropical regions. In Iran alone, there are at least seven species considered to be of medical importance and include, most prominently, the Buthotus saulcyi scorpions commonly found in western Iranian provinces playing a significant role in cases involving envenoming [1, 2]. Scorpion stings may cause severe clinical symptoms, including pain, swelling, high blood pressure, and abnormal heart rhythms, particularly in risk groups such as children and the elderly [3]. Globally, scorpionism accounts significantly for morbidity, with significant annual cases reported in endemic areas [4].

The primary treatment for scorpion envenomation is serotherapy, which utilizes polyclonal antivenoms derived from immunized equines. Yet, the effectiveness of these antivenoms is compromised by the small molecular size of scorpion toxins that facilitates quick systemic spread and impedes neutralization by larger-sized immunoglobulin G (IgG) molecules of around 100 kDa. Additionally, heterologous polyclonal antivenom therapy poses potential risks of adverse effects, such as acute anaphylactic shock and delayed serum sickness [5]. These constraints demonstrate the critical necessity for new approaches, e.g., the creation of monoclonal antibodies (mAbs) with higher specificity and reproducibility than polyclonal antibodies [6].

Hybridoma technology, a traditional technique for the production of mAbs, is comprised of fusing B cells that secrete antibodies with immortal myeloma cells to yield hybridomas

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capable of secreting highly specific mAbs [7, 8]. These mAbs have also been promising in therapeutic and diagnostic applications for scorpion envenomation, as seen in previous work against other scorpion toxins [5, 9]. In the case of *B. saulcyi*, a synthetic peptide, P4, from its venom was identified as a candidate target for mAb generation due to its bioactive properties, such as reported anti-cancer activity [10]. P4, a 61-amino-acid peptide with a molecular weight of 3.94 kDa, shares 82% homology with PBP2, making it a suitable candidate for immunological studies [11].

This study aims to develop a monoclonal antibody against the synthetic P4 peptide from *Buthotus saulcyi* scorpion venom, with the goal of creating a diagnostic tool for rapid venom detection in clinical settings. By addressing the limitations of current serotherapy, this mAb could contribute to improved management of scorpion envenomation, particularly in regions where *B. saulcyi* stings are prevalent.

Materials and method

Preparation of synthetic antigen

The synthetic peptide P4, derived from Buthotus saulcyi venom, was used as the antigen. P4 consists of 61 amino acids, has a molecular weight of 3.94 kDa, and shares 82% homology with PBP2 [11]. It was synthesized by Shinegene Molecular Biotech, Inc. (China) with > 95% purity, reconstituted in sterile PBS (pH 7.4), and stored at -20°C.

Myeloma cell culture

One week before the fusion operation, myeloma cells were cultured in DMEM (High Glucose, L-Glutamine) containing 10% fetal bovine serum, FBS (Fetal bovine serum, Sigma-Aldrich, USA) and 1% antimycotic antibiotic 100X (Sigma-Aldrich, USA). They were propagated inside a 37°C CO_2 incubator and were ready to merge with spleen cells.

Preparing feeder layer

Before the fusion process, a feeder layer must be prepared for the growth of hybridoma cells. A healthy Balb/c mouse was used for this purpose. After anesthetizing the mouse with 50 µl of ketamine, the mouse was disinfected with 75% ethanol, and the skin on its ventral side was cut under sterile conditions, and by injecting serum-free culture medium into its peritoneum and then removing it, the mouse lymphocytes and macrophages were removed. 96-well plates containing 100 µl of feeder layer in each well were kept for 24-48 hours in a CO_2 incubator to cultivate hybridoma cells.

Cell fusion and hybridoma cell production

The preparation of spleen cells in sterile conditions was done for cellular fusion after a week of injection into the mouse tail's vein with the highest specific antibody. The mouse's spleen tissue was taken out of the body after anesthesia and crushed under the hood of the lamina using a surgical razor and the end of the piston, then isolated with a syringe of 22 G. Myeloma cells were subjected to centrifugation at 2000-2500 rpm for a duration of 10 minutes and the supernatant was drained. The supernatant drop was mixed together with sperm cells and myeloma cells at a ratio of 1:5. Then, with PEG 4000 (Polyethylene glycol), 40% cell fusion was done. Cell suspension in culture medium containing 20% serum and HAT (1X) (Sigma-Aldrich, USA) supplement was placed in 96-well plates containing feeder layer and kept in a CO_2 incubator. After 10 days of fusion, to prevent the toxicity of aminopterin for hybridoma cells, the HT (1X) culture medium was replaced with HAT (1X) selective culture medium.

Hybridoma screening

We utilized an indirect ELISA to assess antibody titers in the serum of immunized mice and to screen the culture supernatant of hybridoma cells for specific antibody production. To begin, 96-well flat-bottom ELISA plates were coated with 100 µL per well of the synthetic P4 peptide (20 µg/mL), dissolved in 0.05 M carbonate-bicarbonate buffer (pH 9.6), and incubated overnight at 4°C to ensure proper antigen binding. We used Row H as a negative antigen control, adding only the coating buffer to it. Next, we washed the plates three times with 200 µL of PBS-T (PBS with 0.05% Tween 20) using an ELISA washer (BioTek ELx50, USA). To prevent non-specific binding, we added 200 µL of blocking buffer (5% skim milk in PBS) to each well and let the plates sit at 37°C for an hour.

To measure serum antibody levels, we washed the plates again. Then, we added 100 µL of mouse serum, diluted in PBS-T starting at 1:100 and further diluted in a series (1:200, 1:400, 1:800, 1:1600, and 1:3200), to the wells in duplicate. Row G was our antibody-negative control, getting only 100 µL of PBS-T. After letting the plates incubate at 37°C for an hour, we washed them three more times with PBS-T. We then added 100 µL of HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA), diluted 1:10,000 in PBS-T, to each well and incubated the plates again at 37°C for another hour. After a final wash, we added 100 µL of OPD substrate (Sigma-Aldrich, USA) and left the plates in the dark at room temperature for 10 minutes. To stop the reaction, we added 100 µL of 2.5 M H_2SO_4, and then measured the optical density (OD) at 492 nm using an ELISA reader (Epoch BioTek, USA).

To screen hybridoma cells, we tested the culture supernatant two weeks post-fusion using the same ELISA protocol. Specifically, 100 µL of supernatant was added in duplicate to P4-coated wells, and the plates were processed as described above. We selected the wells with the highest OD values at 492 nm, and the corresponding hybridoma cells were subcloned via the limiting dilution method to isolate monoclonal antibody-producing clones.

Evaluation of monoclonal antibody neutralization

The capacity of monoclonal antibodies (mAbs) to counteract the toxic effects of Buthotus saulcyi scorpion venom was investigated through MTT and sulforhodamine B (SRB) cytotoxicity assays, conducted on HepG2 cells.

MTT assay

In this study, we explored the ability of monoclonal antibodies (mAbs) to counteract the toxicity of Buthotus saulcyi scorpion venom through an MTT-based cytotoxicity assay. We diluted the venom to concentrations of 10, 50, and 100 µg/mL in DMEM containing 2% fetal bovine serum (FBS). Two experimental conditions were established: one set of wells was treated with both venom and mAbs, while a control set received only venom. These mixtures were carefully pipetted into 96-well plates, and then 2×10^4 HepG2 cells per well, in their logarithmic growth phase, were added. The plates were placed in an incubator at 37°C with 5% CO_2 for 22 hours. Following this, we added 5 µL of tetrazolium salt solution to each well and allowed an additional 2 hours of incubation. After removing the supernatant, 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the resulting formazan crystals. The plates were gently shaken for 6 hours in the dark at room temperature, and the optical density was recorded at 570 nm using a microplate reader.

SRB assay

To investigate how well monoclonal antibodies (mAbs) could counteract the venom of Buthotus saulcyi scorpions, we carried out a sulforhodamine B (SRB) assay. We started by plating HepG2 cells at a concentration of 2×10^4 cells/mL, dispensing 100 µL into each well of a 96-well plate. These cells were then left to settle and adhere for 24 hours at 37°C in an environment with 5% CO_2. We diluted the venom to three different strengths - 10, 50, and 100 µg/mL - and tested it on two sets of cells: one group received the mAbs alongside the venom, while the other served as a control without antibodies. After letting them incubate for another 24 hours, we carefully discarded the medium and added 20 µL of chilled 5% trichloroacetic acid (TCA) to each well with gentle handling. The plates were then maintained at 4°C for 10 minutes, followed by 4-5 washes with deionized water and air-drying. For staining, 20-35 µL of 0.04% (w/v) SRB solution was applied to the cells and left for 1 hour at room temperature. Unbound dye was removed through four washes with 1% (v/v) acetic acid, supplemented by two additional water washes. To release the protein-bound dye, 30 µL of 10 mM Tris-base (pH 10.5) was added per well, and the plates were agitated gently for 10 minutes. Optical density was subsequently measured at 520 nm using a microplate reader.

Results

Immunization

The results of the indirect ELISA conducted on serum samples from experimental and control mice following the third injection revealed that mice immunized with the synthetic P4 peptide produced specific antibodies, in contrast to the control group, which showed no such response.

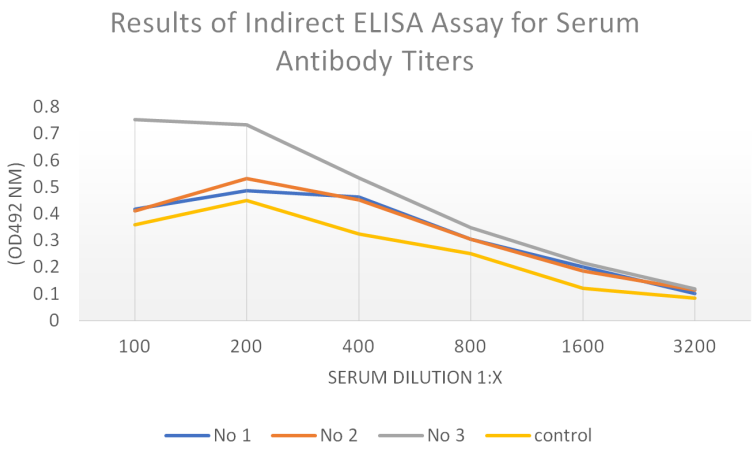

Figure 1. Results of indirect ELISA assay for serum antibody titers. This graph shows the optical density (OD) at 492 nm for serial dilutions of serum from three immunized BALB/c mice (No. 1, No. 2, and No. 3) and a control group, following the third and fourth immunizations with the synthetic P4 peptide from Buthotus saulcyi scorpion venom. Serum was collected after the third injection, and mouse No. 3, with the highest antibody titer, was selected for spleen cell isolation.

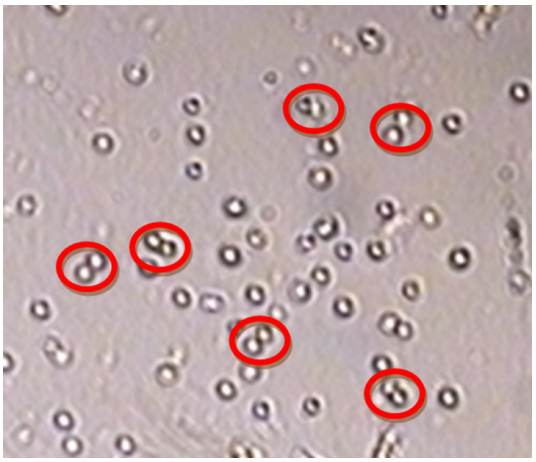

Figure 2. Merged cells after the addition of PEG (100×).

After the fourth injection, blood was collected from the mice via the retro-orbital method, and the resulting serum was subjected to an indirect ELISA assay. Based on these findings, mouse number 3, which exhibited the highest antibody titer, was selected for spleen cell isolation (**Figure 1**).

Fusion

Following the fusion of Sp2/0-Ag14 myeloma cells with spleen cells from mouse number 3, the wells containing the fused cells were imaged using a Zeiss inverted microscope (see **Figure 2**). Subsequently, the cells adapted to the HAT medium were photographed at various time points (**Figure 3**).

As shown in **Figure 3**, the cell number decreased initially following fusion, attributable to the selective pressure of the HAT medium, with only hybridoma cells persisting and multiplying after one week. Ten days after fusion, the culture medium was changed from HAT (1X) to HT (1X) selective medium to support continued hybridoma development.

Hybridoma screening

Following 17 days of fusion between myeloma cells and mouse spleen cells, an indirect enzyme-linked immunosorbent assay (ELISA) was employed to detect antibody-producing wells specific to the P4 peptide. The ELISA results identified four wells containing hybridoma cells that exhibited the highest specificity for producing polyclonal antibodies. These cells were subsequently transferred to a 24-well plate for further proliferation. The results for the four positive wells are summarized in **Table 1**.

Limiting dilution

To isolate single-cell hybridomas, the limiting dilution technique was employed. Following the proliferation of hybridoma cells in four wells of a 24-well plate, an enzyme-linked immunosorbent assay (ELISA) was conducted to identify cells with the highest specificity for the P4 peptide. The selected cells were then diluted to a concentration of one cell per 100 microliters and seeded into a 96-well plate. The culture medium used for this process consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 40% OPI (oxaloacetate, pyruvate, and insulin), 20% fetal bovine serum (FBS), and 1% antibiotic-antimycotic solution.

Characterization of monoclonal antibody

MTT assay: The MTT assay was conducted to evaluate the cytotoxic effects of *Buthus saulcyi*

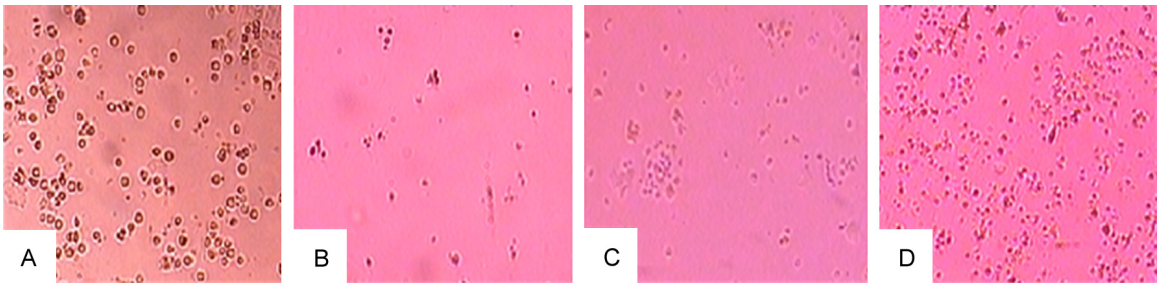

Figure 3. In this image, spleen and myeloma cells were photographed after being merged. The cells were observed at four different time points: (A) 2 days after fusion, (B) 5 days after fusion, (C) 8 days after fusion, and (D) 11 days after fusion (40×).

Table 1. Results for the four wells showing positive readings at 492 nm wavelength

Clone name	Plt3G9	Plt1H10	Plt3A7	Plt3H6
ELISA OD	1.075	1.020	0.604	0.549

For naming clones: 'P' represents the plate number, and the number in front of 'P' indicates the plate number. The letters following 'P' represent the specific well address on the plate.

scorpion venom on HepG2 cells across varying concentrations. The results demonstrated that treatment with increasing doses of scorpion venom, in the absence of specific monoclonal antibodies (mAbs), led to a dose-dependent increase in cell mortality. Conversely, the presence of specific mAbs during treatment with equivalent venom concentrations conferred significant resistance, resulting in 100% cell viability at all tested doses. These findings are illustrated in **Figure 4**, which presents the mean viability data derived from three independent experiments. Statistical analysis using one-way ANOVA confirmed significant differences in cell viability between venom-treated groups with and without mAbs ($P < 0.05$), highlighting the protective effect of the antibodies across the dose range.

SRB assay: The sulforhodamine B (SRB) assay was additionally employed to assess the neutralization efficacy of specific monoclonal antibodies (mAbs) against *Buthus saulcyi* scorpion venom. The results, presented in **Figure 5**, corroborate the findings of the MTT assay, demonstrating a dose-dependent reduction in cell viability with increasing venom concentrations in the absence of mAbs. In contrast, the presence of mAbs maintained high cell viability, with values approaching 100% across all tested doses. These data, derived from the mean of three independent experiments, underscore the protective role of mAbs against venom-induced cytotoxicity. Statistical analysis using one-way ANOVA revealed significant differences in viability between venom-treated groups with and without mAbs ($P < 0.05$).

Discussion

Scorpion stings are a major health concern in many parts of the world, including Iran, where they pose a significant public health challenge [4]. Among the various scorpion species found in the region, seven are particularly dangerous to humans [2]. One of these, Buthus saulcyi, accounts for about 6% of reported stings and is widespread across six southwestern provinces of Iran, where it thrives in large numbers [12, 13].

For years, the go-to treatment for severe scorpion stings has been intravenous antivenom. While this can help, the antivenom often contains high levels of unrelated proteins (1-2.5%), which can reduce its effectiveness and sometimes cause side effects [5]. This has driven researchers to explore better options, like developing targeted antibodies that can specifically neutralize scorpion venom and lessen the harmful effects of a sting.

The effort to create these antibodies kicked off in 1988 when El Ayeb and Rochat made a groundbreaking discovery. They developed the first monoclonal antibody (mAb) targeting the AaH2 toxin from Androctonus australis venom. They produced two antibodies that could bind to the toxin, but only one could neutralize its effects [14].

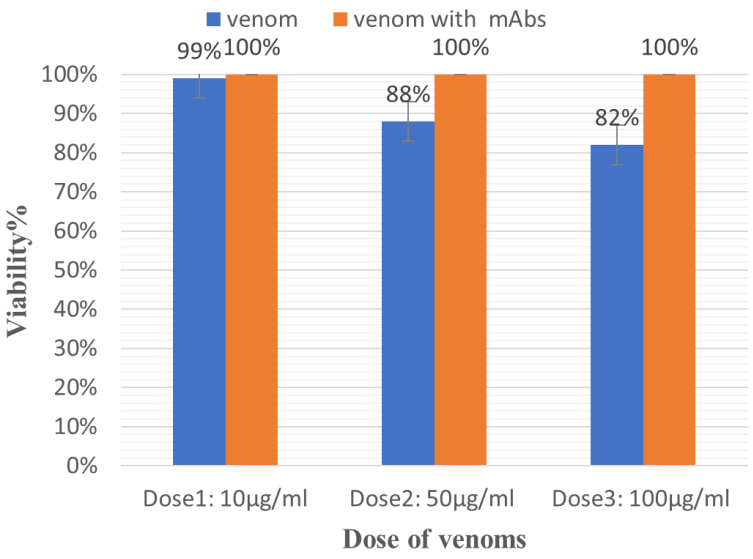

Figure 4. Cell Viability of HepG2 cells treated with Buthotus saulcyi scorpion venom in the presence and absence of specific monoclonal antibodies (mAbs). Cell viability (%) was assessed using the MTT assay following exposure to venom at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL, with and without mAbs. Data represent the mean ± standard deviation from three independent experiments. Blue bars indicate venom-only treatment, while orange bars represent venom treatment in the presence of mAbs. Statistical analysis by one-way ANOVA indicated significant differences ($P < 0.05$) between groups, underscoring the protective role of mAbs.

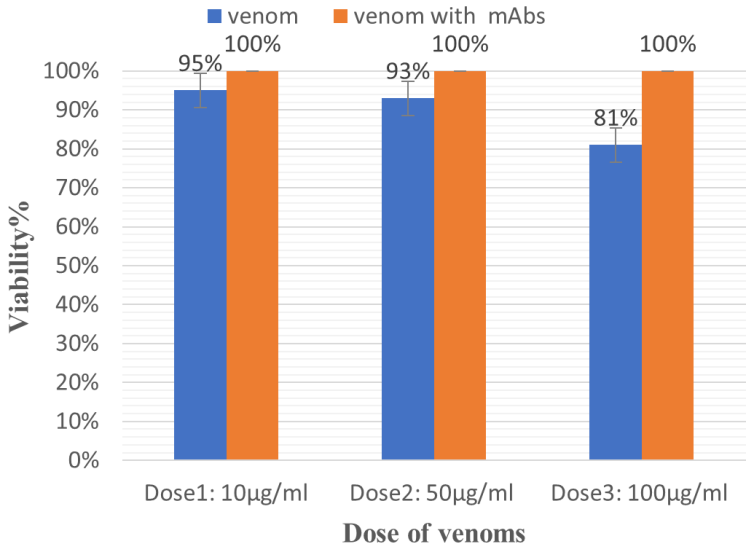

Figure 5. Cell viability of HepG2 cells treated with buthus saulcyi scorpion venom in the presence and absence of specific monoclonal antibodies (mAbs) assessed by SRB assay. Cell viability (%) was determined using the SRB assay following exposure to venom at concentrations of 10 µg/mL, 50 µg/mL, and 100 µg/mL, with and without mAbs. Data are presented as mean ± standard deviation from three independent experiments. Blue bars represent venom-only treatment, while orange bars indicate venom treatment in the presence of mAbs. One-way ANOVA confirmed significant differences ($P < 0.05$) between groups, supporting the neutralizing effect of mAbs.

Scorpion venom is a complex and dangerous mix of toxins, but researchers like Figen Caliskan are making strides in tackling its deadly effects. Caliskan's team developed a monoclonal antibody, 5B9, which zeroes in on the Acra3 peptide found in the venom of Androctonus crassicauda. Even though Acra3 is only a small part of the venom, it plays a big role in its toxicity. Their 5B9 antibody successfully recognized and dialed back the neurotoxic punch of Acra3 [15].

Meanwhile, Christiane Devaux and her group took a different approach, using hybridoma technology to create antibodies against α-neurotoxin I (Aah I), a particularly nasty toxin from Androctonus australis hector venom. To help the immune system spot Aah I, they paired it with a modified toxin, (Abu) 8 Aah II, and bovine serum albumin (BSA). The antibodies they produced not only detected Aah I but also neutralized its effects. Devaux's team didn't stop there - they also worked on AahII, another toxic component of the same venom. By using a non-toxic synthetic version, (Abu) 8-AahII, they crafted antibodies that could safely counteract the real thing [9]. In a similar vein, Guillermo Fernández-Taboada's team developed an antibody called mAb B7, which partially neutralized the Cl13 toxin using hybridoma methods [16].

Inspired by these efforts, our research took a parallel path. We immunized Balb/c mice with a synthetic peptide, P4, first described by Zargan and colleagues [10]. By fusing spleen cells from these mice

with Sp2/0-Ag14 myeloma cells and culturing them in HAT-selective medium, we established a stable hybridoma cell line that churns out monoclonal antibodies. To verify their effectiveness, we ran ELISA tests to confirm the antibodies could detect Buthus saulcyi venom. We also used MTT and SRB assays on HepG2 cells to measure how well they neutralized the venom's toxic effects. The results were promising - our antibodies performed just as well as those in previous studies, effectively counteracting the venom in these cell-based tests.

Our antibody could be a game-changer for scorpion sting treatment. It not only neutralizes Buthotus saulcyi venom but also shows potential for use in diagnostic tools, quickly identifying the venom in a patient's blood or serum [5]. This could pave the way for faster diagnoses, more targeted treatments, and better outcomes for people stung by scorpions.

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Disclosure of conflict of interest

None.

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