Toxicity study of *Gloydius brevicaudus* venom on Hela and Hep G2 cells

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Abstract: The purpose of this study was to explore the cytotoxicity of *Gloydius brevicauda* venom on tumor cells. Hela and Hep G2 cells were used as the research subjects. The MTT assay was employed to investigate the proliferative activity of snake venom on tumor cells. The cell scratch assay was conducted to study the migration ability of tumor cells treated with snake venom. The Hoechst 33258 fluorescent staining method was utilized to examine the damage caused by snake venom on tumor cells. The results demonstrated that the half maximal inhibitory concentration (IC50) of the venom on Hela and Hep G2 cells at 48 hours was 8.176 µg/ml and 12.276 µg/ml, respectively. Furthermore, at a concentration of 4 µg/ml, the venom was able to inhibit the migration of tumor cells. Cell nuclear fluorescence staining revealed that the venom at concentrations of 8 µg/ml and 12 µg/ml induced DNA condensation in Hela and Hep G2 cells, respectively. In conclusion, the venom of *Gloydius brevicauda* was found to inhibit cell proliferation and migration, as well as induce cell apoptosis.

1. Introduction

Gloydius brevicauda is a species of Gloydius, Pit viper, Viperaceae. The body length did not exceed 70 cm. The amount of venom excreted ranged from 6 to 30 mg per dose (depending on the size of the snake). It is found mainly in China, Korea and North Korea[1,2]. The venom of *Gloydius brevicauda* contains four major components: neurotoxin, cytotoxin, thrombin inhibitor, and plasmin. Currently, anticoagulant components derived from the venom are primarily utilized in clinical practice, particularly for embolic diseases[3]. In recent years, researchers have isolated anti-tumor components from various snake venoms. However, there is limited research available on the cytotoxicity of *Gloydius brevicauda* venom towards tumor cells. Thus, this study aimed to expand the understanding of the physiological activity of *Gloydius brevicauda* venom[4].

Hela cells, collected in 1951 from a cervical cancer tissue sample from an African-American woman named Henrietta Lacks, were the first cell line ever to be successfully cultured in unlimited passaging, and are now widely used in biomedical research; Hep G2 cells, a cell line established in the 1970s and derived from the liver cancer tissue of a 15-year-old boy, has become one of the widely used models in liver cancer research[4].

2. Materials and Methods

2.1. Materials

2.1.1. Snake venom and tumour cells

Gloydius brevicauda venom was purchased from the Huangshan Snake Park in Huangshan City, Anhui Province, China. The venom was collected between July and August 2019 and stored at 4 °C for backup after low-temperature drying. The Hela and Hep G2 cell lines were purchased from Wuhan Pronosai Life Sciences Co., China.

2.1.2. Main instruments and consumables

Autoclave (ZEALWAY, USA), Ultra Clean Workbench (Zhejiang Baishun Experimental Equipment Co., Ltd.), Desktop centrifuge (Shanghai Disuo Instrument Co., Ltd.), Carbon dioxide incubator (SANYO Company, Japan), Inverted fluorescence microscope (OLYMPUS, Japan), High-speed freezing centrifuge (Hunan Xiangli Scientific Instrument Co., Ltd.), Enzyme-linked immunosorbent assay (Bio-Rad, USA).

T25 cell culture bottles and cell culture plates were purchased from Corning Inc. in the United States, and

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cell counting plates were purchased from Shanghai Xinrui Biotechnology Co., Ltd, China. MEM medium was purchased from Wuhan Pronosai Life Sciences Co., China. Fetal Bovine Serum (FBS) was obtained from Hangzhou Four Seasons Green Company, China. The BCA protein concentration detection kit, MTT cell proliferation kit, P/S, SDS-PAGE reagent kit, Kamas Brilliant Blue Staining Kit, and Hoechst 33342 fluorescent dyes are all from Biyuntian Biotechnology Co., Ltd, China.

2.2. Methods

2.2.1. SDS-PAGE electrophoresis

The electrophoresis procedure involved using a 12% concentration separation gel and a 5% concentration stacking gel. The snake venom supernatant was subjected to electrophoresis, which is a technique used to separate and analyze molecules based on their charge and size.

2.2.2. Tumor cell culture

The tumor cell culture medium consisted of 89% MEM (including NEAA), 10% FBS, and 1% P/S. The cells were cultured in a humidified incubator at 37 ℃, 5% CO_2 concentration, and with saturated humidity. Both Hela and Hep G2 cells exhibited adherent growth. Throughout the subsequent experiments, the culture medium formulation and the physicochemical parameters within the incubator remained constant.

2.2.3. The effect of snake venom on cell proliferation and vitality

Weigh 0.1 g of lyophilised Gloydius brevicauda snake venom dissolved in 1 ml of complete medium and then centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was then extracted for BCA protein concentration measurement.

Hela and Hep G2 cells in the exponential growth stage were seeded at a density of $1X10^5$ cells/well in a 96-well plate and incubated at 37 ℃ with 5% CO_2 for 24 hours. Both a control group and a snake venom group were established. The snake venom group had concentrations of 128 μg/ml, 64 μg/ml, 32 μg/ml, 16 μg/ml, 8 μg/ml, 4 μg/ml, 2 μg/ml, 1 μg/ml and 0.5 μg/ml, with 6 replicate wells and 3 replicates. The inhibition rate was calculated by measuring the absorbance at MTT after 48 hours[5].

2.2.4. Effect of snake venom on Cell migration

Inoculate Hela and Hep G2 cell suspensions at the logarithmic growth phase into separate wells of a 6-well culture plate and allow them to grow for 72 hours. Afterward, create a scratch in the cell monolayer. Both a control group and a venom group are established, with the venom group treated with a concentration of 4 μg/ml of snake venom. The cells are then cultured for an additional 48 hours, with each experiment repeated three times. The area of the scratch is measured using Image J software[6]. The cell migration rate is calculated using the following formula: (Area at 0-hour scratch - Area at 48-hour scratch) / Area at 0-hour scratch X 100%.

2.2.5. The damage effect of snake venom on cellular DNA

Hela and Hep G2 cells at the logarithmic growth stage were seeded in T25 flasks and incubated at 37 °C with 5% CO_2 for 24 hours. Afterward, the growth medium was removed, and the venom was added to the fresh medium. Hela cells were cultured with a concentration of 8μg/ml of venom, while Hep G2 cells were cultured with a concentration of 12 μg/ml of venom. The cells were incubated at 37 °C with 5% CO_2 for 24 hours. Following the incubation period, the cell culture was terminated. The cells were washed with PBS and then treated with Hoechst 33258 fluorescent staining solution at 37 °C with 5% CO_2 for 20-30 minutes. After the staining solution was discarded, the cells were washed 2-3 times with PBS in preparation for fluorescence microscopy.

2.3. Statistical analysis

SPASS 14.0 software was used to calculate the half inhibitory concentration (IC50) using t-test, $P \leq 0.05$ difference is significant, $P > 0.05$ difference is not significant.

3. Results

3.1. Snake venom protein purity

Weighing 0.1 g of snake venom lyophilised powder dissolved and centrifuged gave a protein content of 88.45% by the BCA method.

3.2. SDS-PAGE electrophoresis of snake venom proteins

After conducting SDS-PAGE electrophoresis, *Gloydius brevicauda* venom exhibited six distinct electrophoresis bands, with the most prominent bands observed at molecular weights of 25 kDa and 15 kDa. These results indicate the molecular weights of the main components present in the venom of *Gloydius brevicauda* (Figure 1).

3.3. *Gloydius brevicaudus* Venom Anti-Tumor Activity

The effect of *Gloydius brevicauda* venom on Hela cells revealed that at a concentration of 4 μg/ml, the cells exhibited aggregation and a decrease in adhesion ability (Figure 2). At 16 μg/ml, the cells started to detach and float, and at 32 μg/ml, the presence of flocculent substances was observed, suggesting possible cell death (IR = 98%) (Table 1). The half-maximal inhibitory concentration (IC50) was determined to be 8.176 μg/ml

(Figure 3). Similarly, the effect of *Gloydius brevicauda* venom on Hep G2 cells demonstrated that at a concentration of 8 µg/ml, the cells displayed aggregation and reduced adhesion ability (Figure 2). At 16 µg/ml, the cells started to detach and float, and at 64 µg/ml, the presence of flocculent substances indicated potential cell death (IR = 93.60%) (Table 1). The half-maximal inhibitory concentration (IC50) was found to be 12.276 µg/ml (Figure 4) [7].

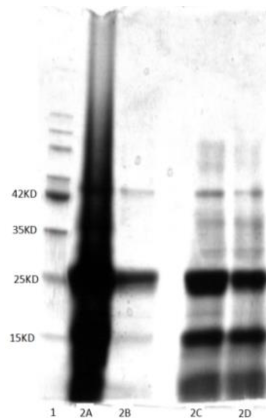


Figure 1 SDS-PAGE electrophoresis of snake venom. Note: 1: Mark; 2A: sample 15 µl; 2B: sample 3 µl; 2C: sample 10 µl; 2D: sample 6 µl.

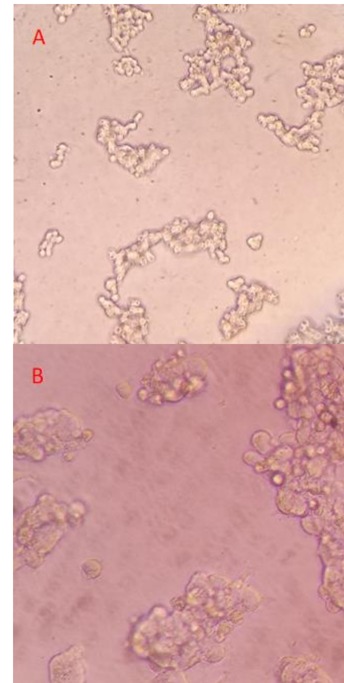
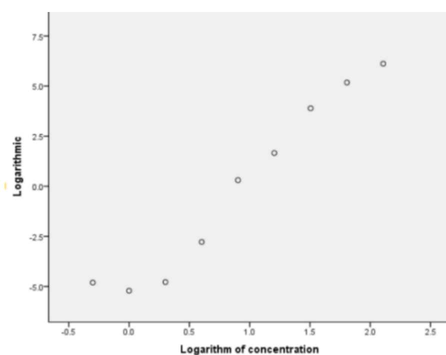


Figure 2 Morphology of Gloydius brevicaudus snake venom after its action on Hela and Hep G2 cells. Note: A. Hela cell morphology (4 µg/ml); B. Hep G2 cell morphology (8 µg/ml) (10 X 10).

Table 1 Inhibition of Hela and Hep G2 by Gloydius brevicaudus venom (IR%)

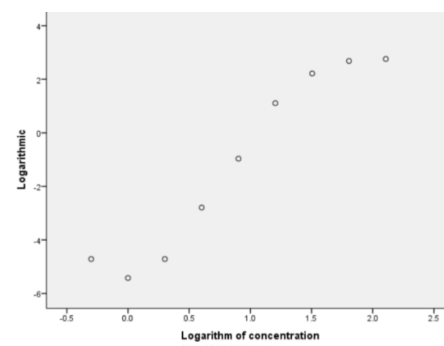
	0.5 µg/ml	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml
Hela	0.81±0.02	0.54±0.03	0.83±0.05	5.85±0.47	57.56±3.32	84±4.32	98±0.82	99.44±0.25	99.78±0.09
Hep G2	0.89±0.03	0.44±0.09	0.89±0.04	5.78±0.66	27.56±1.73	75.16±2.91	90.17±0.80	93.60±1.12	94.03±1.11



Confidence limit

	Probability	95% confidence limit of the concentration			95% confidence limit of log(concentration)[b]		
		Estimates	Lower limit	Upper limit	Estimates	Lower limit	Upper limit
LOGIT[a]	.010	1.598	.650	2.531	.204	-.187	.403
	.020	2.051	.945	3.070	.312	-.025	.487
	.030	2.378	1.177	3.444	.376	.071	.537
	.040	2.644	1.378	3.743	.422	.139	.573
	.050	2.872	1.557	3.998	.458	.192	.602
	.060	3.076	1.722	4.223	.488	.236	.626
	.070	3.262	1.877	4.427	.513	.273	.646
	.080	3.433	2.022	4.614	.536	.306	.664
	.090	3.594	2.161	4.790	.556	.335	.680
	.100	3.746	2.295	4.956	.574	.361	.695
	.150	4.415	2.903	5.688	.645	.463	.755
	.200	4.996	3.452	6.335	.699	.538	.802
	.250	5.534	3.969	6.948	.743	.599	.842
	.300	6.051	4.469	7.556	.782	.650	.878
	.350	6.562	4.961	8.181	.817	.696	.913
	.400	7.079	5.454	8.841	.850	.737	.946
	.450	7.614	5.953	9.554	.882	.775	.980
	.500	8.176	6.465	10.342	.913	.811	1.015
	.550	8.781	6.998	11.232	.944	.845	1.050
	.600	9.443	7.562	12.260	.975	.879	1.088
	.650	10.188	8.172	13.477	1.008	.912	1.130
	.700	11.048	8.847	14.964	1.043	.947	1.175
	.750	12.080	9.622	16.850	1.082	.983	1.227
	.800	13.380	10.552	19.374	1.126	1.023	1.287
	.850	15.143	11.752	23.037	1.180	1.070	1.362
	.900	17.848	13.489	29.143	1.252	1.130	1.465

Figure 3 Logarithmic response after conversion and Confidence limit of Hela



Confidence limit

	Probability	95% confidence limit of the concentration			95% confidence limit of log(concentration)[b]		
		Estimates	Lower limit	Upper limit	Estimates	Lower limit	Upper limit
LOGIT[a]	.010	1.139	.266	2.297	.056	-.576	.361
	.020	1.639	.469	3.027	.215	-.329	.481
	.030	2.032	.655	3.569	.308	-.184	.553
	.040	2.371	.831	4.021	.375	-.081	.604
	.050	2.675	1.001	4.419	.427	.000	.645
	.060	2.956	1.166	4.780	.471	.067	.679
	.070	3.220	1.328	5.114	.508	.123	.709
	.080	3.469	1.488	5.428	.540	.173	.735
	.090	3.708	1.646	5.727	.569	.216	.758
	.100	3.938	1.802	6.014	.595	.256	.779
	.150	5.003	2.574	7.336	.699	.411	.865
	.200	5.992	3.345	8.572	.778	.524	.933
	.250	6.953	4.131	9.802	.842	.616	.991
	.300	7.919	4.941	11.080	.899	.694	1.045
	.350	8.911	5.783	12.450	.950	.762	1.095
	.400	9.953	6.664	13.960	.998	.824	1.145
	.450	11.065	7.593	15.664	1.044	.880	1.195
	.500	12.276	8.582	17.631	1.089	.934	1.246
	.550	13.619	9.647	19.955	1.134	.984	1.300
	.600	15.141	10.810	22.768	1.180	1.034	1.357
	.650	16.911	12.106	26.270	1.228	1.083	1.419
	.700	19.031	13.587	30.780	1.279	1.133	1.488
	.750	21.673	15.342	36.853	1.336	1.186	1.566
	.800	25.152	17.527	45.555	1.401	1.244	1.659
	.850	30.119	20.462	59.266	1.479	1.311	1.773
	.900	38.265	24.937	84.717	1.583	1.397	1.928

Figure 4 Logarithmic response after conversion and Confidence limit of Hep G2.

3.4. Effect of snake venom on Cell migration

After 48 hours, the control group of Hela cells exhibited a migration rate of 45.61%, whereas the experimental group showed a significantly reduced migration rate of 1.48% (Table 2). Similarly, in the Hep G2 cell line, the control group had a migration rate of 40.81%, while the experimental group exhibited a migration rate of 6.96% (Table 2). These results indicate a significant difference in migration rates between the experimental and control groups for both cell lines (Figure 5, 6 and 7).

Figure 5 Hela cell migration experiment. Note: A. Control group scratch start; B. Control group incubated for 48 hours after scratching; C. Experimental group scratch start; D. Experimental group incubated for 48 hours after scratching (10 X 10).

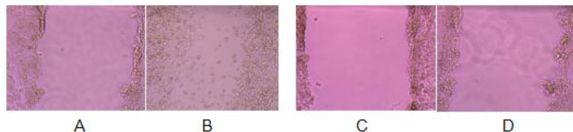


Figure 6 Hep G2 cell migration experiment. Note: A. Control group scratch start; B. Control group incubated for 48 hours after scratching; C. Experimental group scratch start; D. Experimental group incubated for 48 hours after scratching (10 X 10).

Table 2 Cell migration rate (%)

	Hela		Hep G2	
	Contorl	Venom	Contorl	Venom
migration rate (%)	50.14	1.31	43.18	6.78
	42.28	1.35	40.69	7.84
	44.40	1.79	38.55	6.16
Mean±SD	45.61±4.06	1.48±0.003	40.81±2.31	6.96±0.008

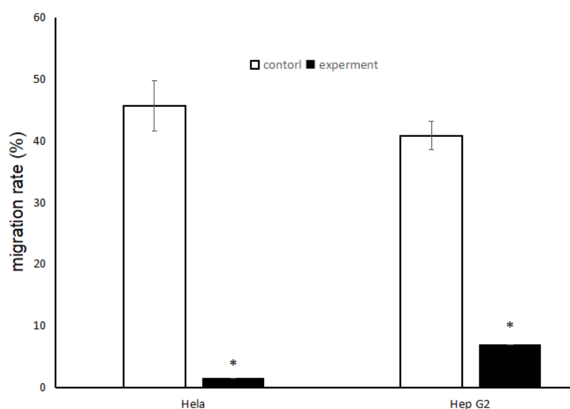


Figure 7 Cell migration histogram. Note: Hela: $P = 0.003 < 0.05$; Hep G2: $P = 0.001 < 0.05$.

3.5 The damage effect of snake venom on cellular DNA

Hoechst 33258 staining was used to stain Hela and Hep G2 cells after 24 h of the venom exposure, and both tumour cells showed nuclear chromatin condensation (Figure 8).

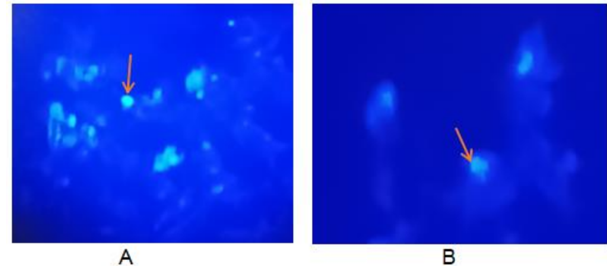


Figure 8 Hoechst 33342 fluorescent dye staining of nuclei. Note: A. Hela; B. Hep G2; Both cell lines showed dense and intense staining (10 X 40).

4. Discuss

The main components of snake venom are proteins, accounting for 88.45% of the dry matter of snake venom, of which the main molecular weights of the proteins in the venom are 25 kDa and 15 kDa (SDS-PAGE electrophoresis). The toxicity of snake venom proteins to Hela and Hep G2 cells was shown in two ways: firstly, inhibition of cell migration at low concentrations (4 μg/ml); secondly, inhibition of cell proliferation or induction of cellular DNA condensation at high concentrations. The effect of the inhibition of proliferation is that the higher the concentration the greater the inhibition[8]. Tumour cells no longer proliferate means that the tumour no longer grows, which is of great significance in the treatment of tumours. Inhibition of tumour cell migration is the key to curbing the metastasis of tumour cells in vivo, which in turn can prolong the survival time of patients after tumour surgery. DNA condensation is the initial stage of DNA fragmentation and is a sign of DNA damage. DNA damage is a necessary condition for apoptosis, which suggests that the Gloydius brevicauda venom may have a potential mechanism for inducing apoptosis[9,10].

5. Conclusion

Based on the above experimental analysis, the venom of *Gloydius brevicaudus* can not only inhibit the proliferation and migration of tumor cells, but also induce cell apoptosis.

Acknowledgments

This work was supported by the Innovation and Entrepreneurship Program for College Students of Anhui Province Education Department, China (No. S202210959202 and S202210959205) and the Natural Science Program of Anhui Sanlian University, China (No. PTZD2023008).

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