Cancer micro-environment immune modulation by Egyptian cobra (*Naja haje*) crud venom

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Background
Cancer can control immune system suppression mechanisms by activating regulatory T cells; myeloid-derived suppressor cells (MDSCs) and increasing the expression of co-inhibitor proteins. Snake venoms showed anticancer activity by targeting specific molecular pathways.
Objective
Here, we investigate the immunomodulatory effects of Egyptian cobra (*Naja haje*) venom different doses compared with cisplatin in healthy and cancer murine models.
Materials and methods
Female Balb/c mice aged 2–3 months, are separated into three general groups (control groups, solid (subcutaneous) tumors, and soft (ehrlich ascites) tumors. Mice were inoculated with ehrlich ascites carcinoma cells about 2×10^6 and 1.5×10^6 cells subcutaneously and intraperitoneal for 28 and 10 days, respectively.
Results
MDSCs decreased nonsignificantly in control groups treated with cisplatin, 1/10, 1/30 LD50 also, in ascites tumor group treated with 1/30 LD50 (P=0.055). While it increased non-significantly in healthy control treated with 1/20 LD50, all treated solid tumor groups and in ascites tumor groups treated with cisplatin and 1/20 LD50, on the other hand, Regulatory T cells in control groups decreased significantly in groups treated with cisplatin and 1/30 LD50 on the other hand it increased nonsignificantly in groups treated with 1/20 and 1/10 LD50. In solid tumor groups, T regs increased with no statistical significance in all treated solid tumor groups also, in ascites tumor groups treated with 1/20 LD50 and cisplatin.
Conclusion
Low doses of (*Naja haje*) crud venom reduce MDSCs and T reg in the microenvironment of tumor while higher doses increase them, further investigation will be needed.

Keywords:
cancer treatment, egyptian cobra, myeloid-derived suppressor cells (MDSCs), regulatory T cells

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Introduction

Recent studies reported that antitumor chemotherapy produced its antitumor effects by not only direct cytotoxic effects against tumor cells but also the elimination or inactivation of cells with suppressive effects on tumor immunity such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) [1,2].

MDSCs, are a heterogeneous population of the myeloid lineage that can enhance cancer progression by stimulating cancer cell invasion, metastasis and tumor angiogenesis [3]. The main feature of MDSCs is the ability to switch off adaptive and innate immune responses [4]

In many solid tumors, MDSCs play a pivotal role in the tumor microenvironment, leading to the failure of immunotherapy. Studies demonstrated that inhibition of MDSCs recruitment or function effectively enhanced the efficacy of immune checkpoint blockade [5,6]. Also, recent studies showed that MDSCs have elevated concentrations in the peripheral blood and in the tumor microenvironment of cancer patients, and it is associated with poor prognosis [7–10].

Phenotypically, MDSCs have two subtypes in mice monocytic (mononuclear) (M-MDSC) and granulocytic (polymorphonuclear) (PMN-MDSC).

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They both express the granulocyte differentiation antigen Gr1 and CD11b surface markers. M-MDSC expresses Ly6C and lower levels of Gr1, while PMN-MDSC expresses Ly6G and higher levels of Gr1 [11].

Recent studies aiming to target or understand the mechanisms of MDSCs expansion and activation in the tumor microenvironment confirm the involvement of factors like granulocyte-macrophage colony-stimulating factor (GM-CSF) [12], granulocyte CSF [13], Prostaglandin (PG) E2 [14], IL-6 [15], IL-8 [4], IL-10 [16], transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α [17], They also facilitate neovascularization through their production of VEGF, and production of matrix metalloproteases [14,16,18].

MDSC immune suppress T cells by production of NO and ROS including peroxynitrite PNT [19,20], and depleting L-arginine via Arg1 which is needed for T cell proliferation [21], uptake of L-cysteine which is vital for activation and function [22] or express high levels of Indole Amine 2, 3 Dioxygenase (IDO) which reduce local tryptophan levels due to the activity [23].

Many recent studies have proposed that a high T reg percentage in peripheral blood and tumor infiltrate may be correlated with poor prognosis [24–28]. Activated T regs suppress the immune system by many mechanisms and molecules such as interactions between checkpoint molecules and their ligands involving PD-1, PD-L1, CTLA-4 (cytolytic T lymphocyte-associated antigen), GITR (glucocorticoid-induced TNFR family-related gene), Tim-3 (T-cell immunoglobulin and mucin-domain containing-3), and galectin-9. Furthermore, Tregs can contribute to a strong immunosuppressive tumor environment by releasing cytokines such as transforming growth factor (TGF) – beta, IL-10 and IL-35 [29,30].

Another cancer immune-suppression mechanism is to diminish the immune co-stimulator axis programmed cell death receptor (PD-1)/programed cell ligand (PDL-1).PDL-1 is expressed on antigen-presenting cells (APCs), non-hematopoietic cells, including cells in many tissues and tumor cells [31] while PD-1 (CD279) is expressed on activated T cells, NK cells, and B cells [32]. Naturally, PD-1/PDL-1 interaction protects from the auto-immune response [33] but tumor cells use this interaction to suppress T cells by inhibiting T cell receptors this effect is induced by (inhibiting Lck and ZAP-70 phosphorylation signaling pathway, PI3K-Akt pathway, Ras-MEK-ERK this leads to alterations in the expression of interferon γ (IFNγ), basic leucine zipper transcription factor (BATF), tumor necrosis factor α (TNFα), and interleukin-2 (IL-2) [31,34] and stimulating T cell apoptosis [31].

Vascular endothelial growth factor (VEGF) plays a role in cancer angiogenesis. It increases vessel permeability and stimulates endothelial cells prelibation [35]. Also, it plays a role in cancer immunosuppression it can recruit Treg cells and (MDSCs) [36].

Cisplatin, [Cis-diamine-dichloroplatinum (II)], is effective against various types of cancer. However, it develops drug resistance and undesirable side effects such as severe kidney problems, allergic reactions, and decrease immunity to infection. Cisplatin covalently binds to DNA bases, forming DNA adducts. It reacts with the nucleophilic N7-sites of purine bases, and a double reaction may covalently link purines, causing DNA damage, and subsequently inducing apoptosis in cancer cells [37,38].

Many compounds purified and characterized from snake venom act as anti-cancer agents they can target specific molecular pathways, cytotoxins from (*Naja haje*, *Naja oxiana*, and *Naja kaouthia*) can markedly accumulate in lysosomes causing leakage of lysosomes and plasma membrane injury [39,40].

We are hypothesizing that (*Naja haje*) venom possesses anti-tumor activity *in vitro* which can be replicated *in vivo* by inducing direct anti-tumor effects by killing cancer cells and indirect immunomodulatory effects by reducing suppressor cells (MDSCs & Treg) and modulating VEGF and PDL-1 expression.

Materials and methods

Mice: Female Balb/c mice aged 2–3 months were obtained from Helwan animal house of the biological products and vaccines (VACSERA), Cairo Egypt, and the animals were maintained in a 12 h light/12 h dark cycle under a suitable temperature (20±4°C), a commercial diet and tap water were provided. Any mouse with a palpable mass, weight loss, or change in body condition was excluded so that only healthy elderly mice were examined and all sample collection performed in the morning. All experiments were performed as described below at faculty of science Suez Canal University.

Cell line: Ehrlich ascites carcinoma cell line (EAC) was obtained from the National Institute of Cancer, Cairo,

Egypt. Fresh ehrlich ascites carcinoma (EAC) cells were grown in mice by serial intraperitoneal transplantation of 2×10^6 cells, 15-day-old EAC cells were used for testing. Mice were separated into three general groups Table 1.

The solid tumor model was produced by inoculating the ehrlich ascites carcinoma cell line for 28 days by injecting about 2×10^6 cells subcutaneously. The soft (ascites) tumor model is produced by inoculating ehrlich ascites carcinoma cell line for 10 days by injecting about 1.5×10^6 cells intraperitoneal.

Cobra venom was milked and lyophilized at the faculty of science Suez canal university, Ismailia. We determined cobra venom LD50 experimentally, doses 1/10 LD50, 1/20 LD50, and 1/30 LD50 in normal saline have been used, each mouse injected with 0.5 ml containing the desired dose of venom or 40 U of cisplatin twice, onset (28 days for solid tumor and 10 days for soft tumor) and after 5 days then sacrificed at day 10. Blood samples were collected from all groups, Peritoneum tissue collected from control groups, tumor tissue collected from solid tumor groups, and ascites tumor cells collected from soft tumor groups.

Immunophenotyping: Single-cell suspensions were prepared from mouse peritoneum and tumor specimen tissues were teased apart gently with scissors and filtered through 40 μm nylon strainers. Ehrlich ascites carcinoma cells from soft tumors that are ready for use. All the cells were kept at –20°C for 4 days before immunophenotyping. Only ehrlich ascites carcinoma cells from soft tumors processed directly for Annexin V (a protein marked with florescent stain) to evaluate necrosis.

Flow cytometry: A single-cell suspension was stained with allophycocyanin (APC)-labeled anti-mouse CD4 (BD Biosciences, USA), phycoerythrin (PE)-labeled antimouse CD25 (BD Biosciences, USA) for regulatory T cells and fluorescein isothiocyanate (FITC)-labeled antimouse LY6G (BD Biosciences, USA), APC-labeled antimouse CD11B (Bioscience, USA) for MDSCs. For cell cycle analysis, cells were stained with propidium iodide (PI) (Sigma, USA). Annexin V cells were stained by Annexin V (PE) (BD Biosciences, USA) the stained cells were measured using flow cytometry (Acuri C6, BD Biosciences, USA) and analyzed by (Acuri C6, BD Biosciences, USA) software.

Immunohistochemical procedures: Solid tumor sections were de-waxed and immersed in a solution of 0.05 M citrate buffer, pH 6.8 for antigen retrieval. These sections were then treated with 0.3% H_2O_2 and protein block. After that, they were incubated with polyclonal anti-VEGF antibodies (Thermofisher Scientific, Catalog # PA5-16754, dilution 25 μg/ml), and polyclonal PDL-1 antibody (Thermofisher Scientific, Catalog # PA5-20343, dilution 1/100). After rinsing with PBS, they were incubated with a goat antirabbit secondary antibody (cat. no. K4003, EnVision+ System Horseradish peroxidase labelled polymer; Dako) for 30 min at room temperature. Slides were visualized with DAB kit and eventually stained with Mayer's hematoxylin as a counterstain. The staining intensity was assessed and presented as a percentage of positive cells in about 8 high power fields [41,42].

Statistical analysis: Statistical analysis was performed on SPSS v 14 software. The data was presented as mean±SD. Statistical significance was analyzed by Mann–Whitney *U* test comparing the two groups. $P<0.05$ was considered a significant difference.

Results

Cell cycle study

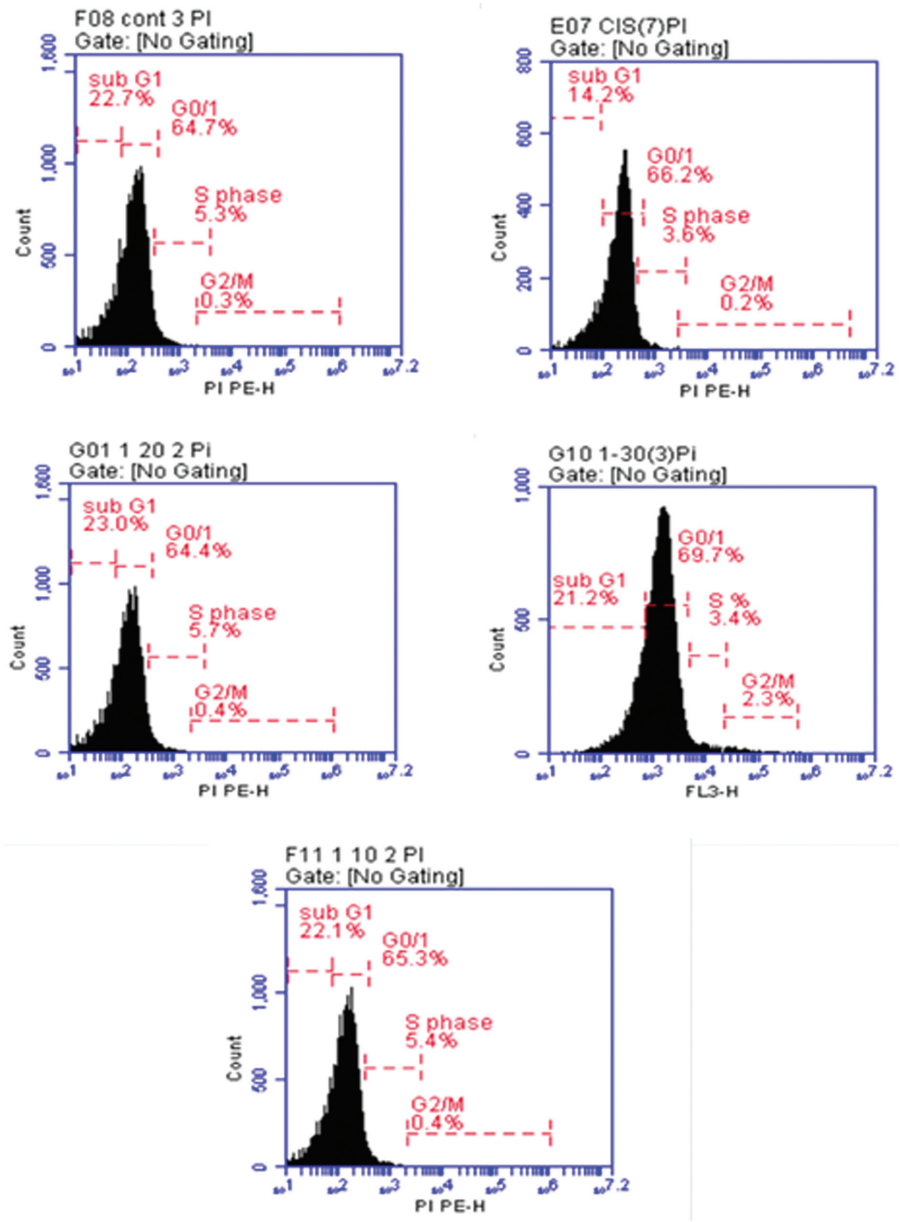
The cells in G0/G1 phase Fig. 1, decreased significantly in the treated control groups with

Table 1 Experimental design

Control groups	Solid tumor groups (subcutaneous tumor)	Soft tumor groups (Ehrlich ascites tumor)
Healthy control group treated with saline.	Solid tumor group treated with saline.	Ascites tumor group treated with saline
Cis control group treated with cisplatin.	Solid tumor + Cis group treated with cisplatin.	Ascites tumor (Asc + Cis) group treated with cisplatin
1/10 Control group treated with a crud venom dose 1/10 LD50	Solid tumor + 1/10 group treated with crud venom dose 1/10 LD50	
1/20 Control group treated with a crud venom dose 1/20 LD50	Solid tumor + 1/20 group treated with crud venom dose 1/20 LD50	Ascites tumor (Asc + 1/20) group treated with crud venom dose 1/20 LD50
1/30 Control group treated with a crud venom dose 1/30 LD50		Ascites tumor Asc + 1/30) group treated with crud venom dose 1/30 LD50

Mice were separated into three general groups (control groups, solid tumor (subcutaneous tumor), and soft tumor (ehrlich ascites tumor).

Figure 1



Cell cycle analysis of a sample from each control group. Healthy control animals injected with saline, Cis control treated with cisplatin, 1/10 Control treated with a crud venom dose 1/10 LD50, 1/20 Control treated with a crud venom dose 1/20 LD50 and 1/30 Control treated with a crud venom dose 1/30 LD50.

venom doses 1/20 but the decrement was not significant in treated control groups with venom doses 1/10, cisplatin, and venom dose 1/30 when compared with healthy control. Cells in the G2/M phase increased nonsignificantly in treated control groups with cisplatin, venom doses 1/10, 1/20 and significantly increased in the control group treated with 1/30 when compared with healthy control as shown in Table 2. There was no statistical significance in cell cycle results when compared with solid nontreated tumor

The cells in the sub G1 phase, Fig. 2, increased significantly in ascites tumor groups treated with venom doses 1/20, 1/30, and cisplatin when compared with nontreated ascites tumor Table 2. Cells in G0/G1 phase increased in ascites tumor treated groups when compared with the nontreated ascites tumor. The cells in the S phase decreased significantly in the ascites tumor groups treated with cisplatin, venom doses 1/20 and 1/30 when compared with the nontreated ascites tumor. The cells in the G2/M phase increased in the ascites tumor group treated

Table 2 The total cell count in the cell cycle phases

	Sub G1	G0/G1	S	G2M
Control negative	18.33±6.91	67.38±4.40	4.28±2.98	0.18±0.15
Cis control	19.85±3.86	65.45±0.51	5.30±1.15	0.25±0.13
1\10 control	19.38±3.80	62.03±4.36	5.93±0.99	0.30±0.08
1\20 control	21.67±1.67	54.33±15.82**	5.23±0.90	0.40±0.10
1\30 control	16.03±3.95	66.55±11.39	10.15±5.14	3.50±2.29**
G1 S. T+cis	23.38±1.44	49.66±17.44	12.58±4.11	1.58±0.48
G2 S. T +1/10	20.12±6.95	52.90±12.82	17.52±7.84	2.82±3.67
G3 S. T +1/20	30.96±13.29	43.34±13.90	15.34±9.09	2.28±2.24
G4 S. T	24.72±6.08	57.58±5.11	10.58±3.37	1.52±1.64
Ascites control	3.94±1.04	31.42±3.42	44.66±5.49	19.93±6.57
Asc + Cis	14.60±4.64**	38.26±19.32	23.96±10.77**	23.63±14.40
Asc + 1/20	19.75±8.65**	31.78±10.19	18.55±7.10**	21.50±14.06
Asc + 1/30	16.05±0.42**	70.98±0.24**	6.53±0.22**	2.13±0.10*

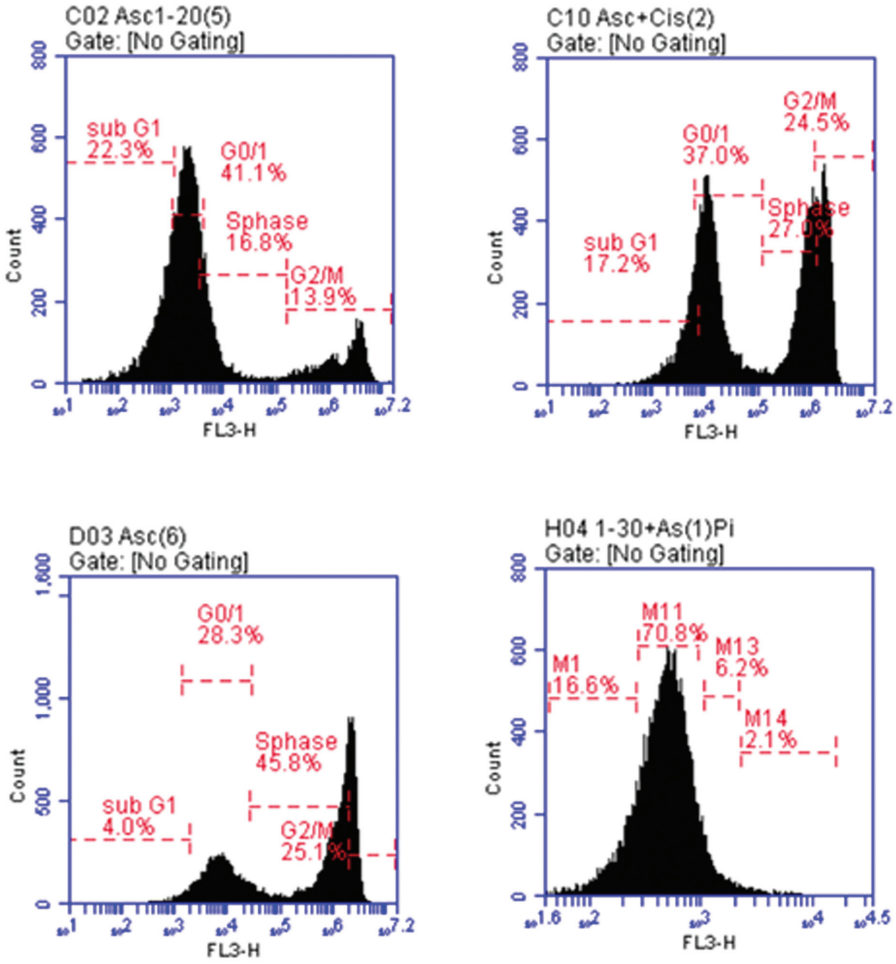
Data are expessed as mean±SD. G is Growth phase, S is DNA synthesis phase, and M is the mitotic phase. 1/10, 1/20, and 1/30 LD50 of the crude venom. Cis is cisplatin. *$P \leq 0.05$ when compared with healthy control group. **$P \leq 0.05$ when compared with nontreated ascites tumor.

with cisplatin and venom dose 1/20 while decreased significantly in the ascites tumor group treated with venom dose 1/30 when compared with nontreated ascites tumor.

Myeloid derived suppressor cells (MDSCs)

MDSCs were reduced in control groups treated with cisplatin, 1/10 venom dose, and 1/30 venom dose compared with the healthy control group, on the

Figure 2



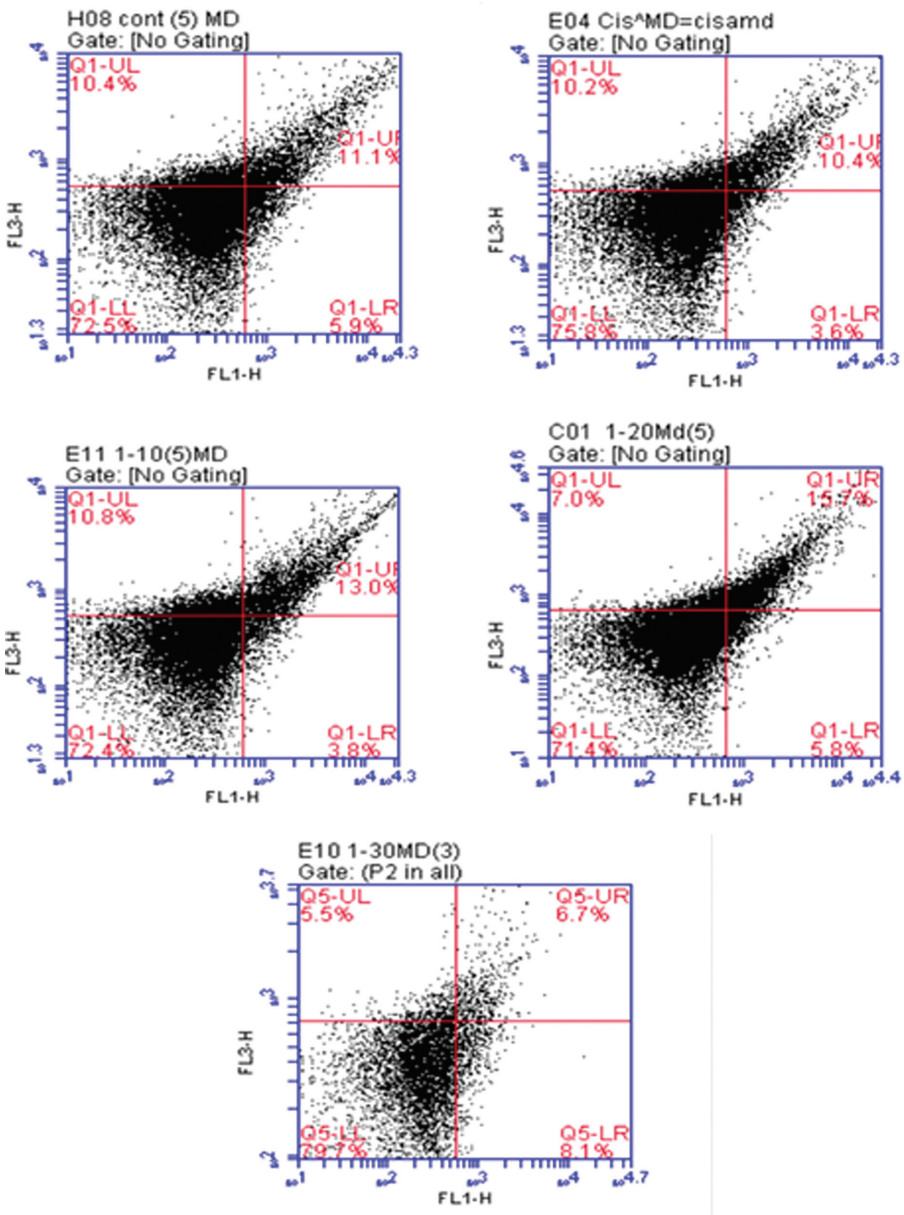
Cell cycle analysis of a sample from each soft tumor group. Ascites tumor animals injected with saline, cisplatin (Asc + Cis), 1/20 LD50 crud venom (Asc + 1/20) and 1/30 LD50 crud venom (Asc + 1/30).

Table 3 The mean percentage of MDSCs.

	$CD11b^+$	$IY6G^+$	$CD11b^+IY6G^+$
Control negative	28.5±10.5	26.7±9.5	20.2±7.9
Cis control	28.9±8.8	22.2±9.5	17.2±7.8
1\10 control	29.7±16.6	23.4±18.6	19.1±16.8
1\20 control	40.6±16.6	37.4±17.5	30.4±15.3
1\30 control	28.0±13.6	35.8±19.4	20.0±12.1
G1 S. T+cis	35.6±11.8	33.5±12.5	25.3±10.7
G2 S. T +1/10	33.1±13.2	30.4±17.5	22.4±13.3
G3 S. T +1/20	41.0±13.3	39.4±16.9	30.2±14.4
G4 S. T	29.1±8.7	25.5±9.6	19.3±6.6
Ascites control	35.0±13.3	47.9±16.9	31.0±13.8
Asc + Cis	37.7±12.2	56.9±21.9	34.2±12.6
Asc + 1/20	41.8±5.4	60.1±21.1	37.4±7.7
Asc + 1/30	21.0±6.9	26.7±10.1	13.0±5.9

Data are expessed as mean±SD. CD11b positive cells for CD11b only, IY6G positive cells for IY6G only, CD11b+IY6G+ positive MDSCs. 1/10, 1/20, and 1/30 LD50 of the crude venom. Cis is cisplatin. *$P \leq 0.05$ when compared with healthy control group. **$P \leq 0.05$ when compared with nontreated Ascites tumor.

Figure 3



The percentage of MDSCs from each control group. Healthy control animals injected with saline, cisplatin, 1/10 LD50 crud venom, 1/20 LD50 crud venom and 1/30 LD50 crud venom. CD11B on (X axis FL3-H) and LY6G on (Y axis FL1-H).

other hand, it increased in control group treated with 1/20 venom dose Table 3 Fig. 3. MDSCs increased in ascites tumor group treated with cisplatin and ascites tumor group treated with 1/20 venom dose compared with nontreated ascites control tumor group Fig. 4. While it decreased in ascites tumor group treated with 1/30 venom dose (P=0.055).

Venom dosing effect on CD11b, LY6G and MDSCs cells

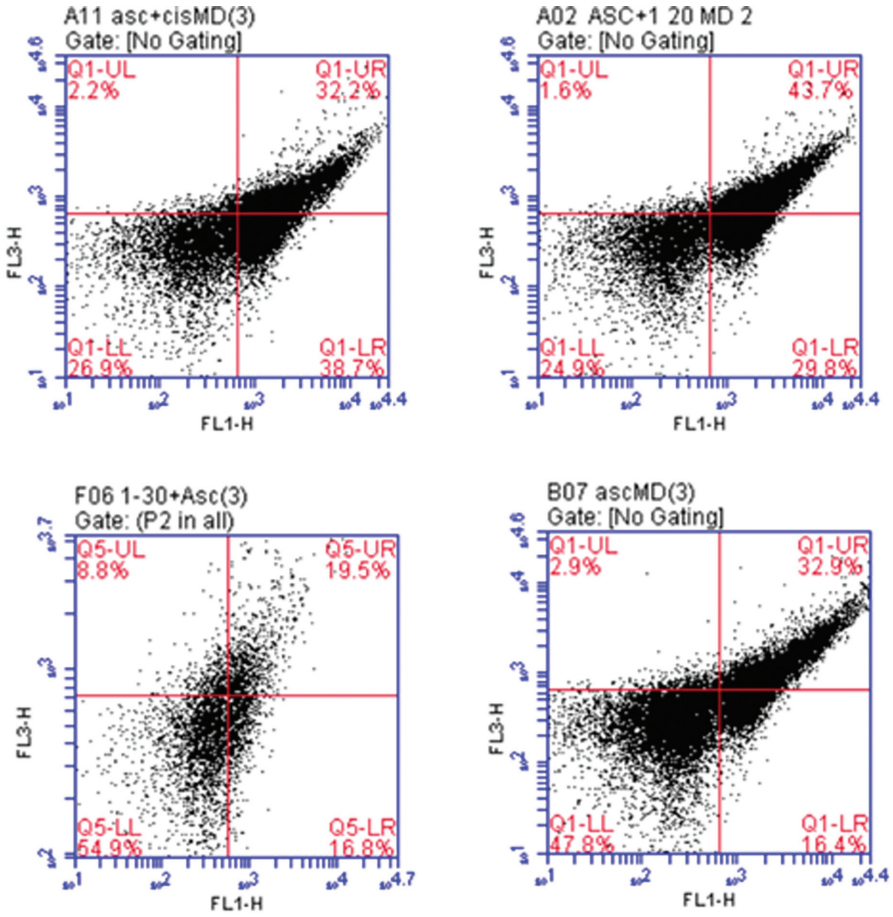
MDSCs increased in the control group treated with 1/20 venom dose compared with 1/10 and when compared with 1/30 on the other hand it decreased in the control group treated with1/10 compared with the control group treated with 1/30. MDSCs increased in solid tumor group treated with 1/20 venom dose compared with solid tumor group treated with 1/10 and 1/30 venom doses. MDSCs increased in solid tumor group treated with1/10 venom dose, cisplatin and with 1/20 venom dose compared with nontreated solid tumor group.

CD11b expression increased significantly in the ascites tumor group treated with 1/20 venom dose compared with ascites tumor treated with 1/30 venom dose (P=0.010). LY6G expression increased in the ascites tumor group treated with 1/20 venom dose compared with ascites tumor treated with 1/30 venom dose (P=0.055). MDSCs (CD11b+ LY6G+) increased significantly in the ascites tumor group treated with 1/20 venom dose compared with ascites tumor treated with 1/30 venom dose (P=0.011).

Venom dosing effect on CD4+, CD25+, CD4+CD25+ T_{reg} cells

Cisplatin reduced the expression of $CD4^+$, $CD25^+$ and $CD4^+CD25^+$ cells compared with control negative group with a statistical significant (P= 0.023, 0.012, 0.051, respectively). 1/30 venom dose also reduced the expression of $CD4^+$, $CD25^+$ and $CD4^+CD25^+$ cells with the lowest values compared with control negative with a statistical significant (P=0.032, 0.003, 0.007) respectively (Table 4) and Fig. 5. In solid tumor groups the percentage of $CD4^+$, $CD25^+$and $CD4^+Cd25^+$ cells increased among treated groups 1/10, 1/20, and cisplatin compared with controlled solid tumor. In ascites tumor groups Figs. 6, 1/30 venom dose

Figure 4



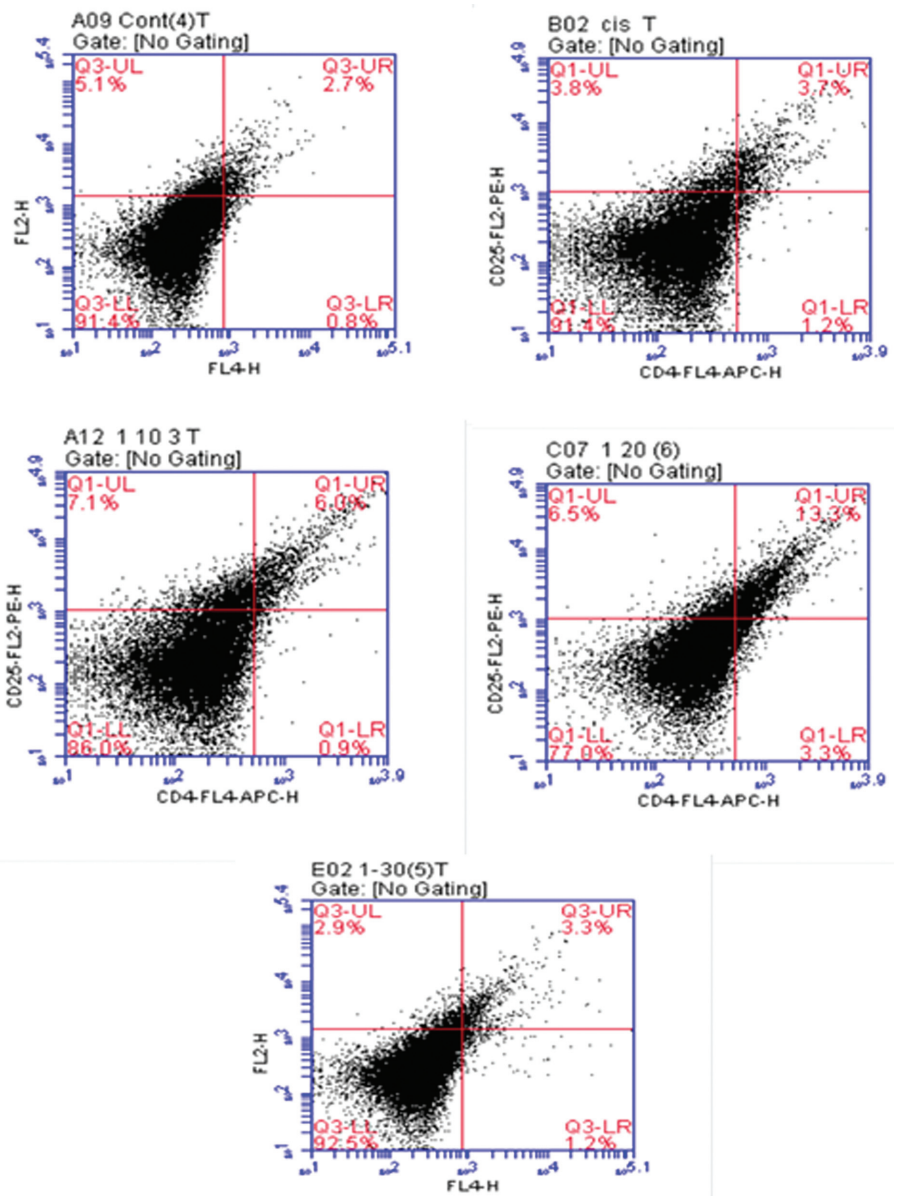
The mean percentage of MDSCs from each soft tumor group. Ascites tumor animals injected with saline, cisplatin (Asc + Cis), 1/20 LD50 crud venom (Asc + 1/20) and 1/30 LD50 crud venom (Asc + 1/30). CD11B on (X axis FL3-H) and LY6G on (Y axis FL1-H).

Table 4 The mean percentage of T_{reg}

	$CD4^+$ T cells	$CD25^+$ T cells	$CD4^+CD25^+$ T cells
Control negative	7.9±4.0	11.0±4.0	6.1±3.2
Cis control	3.0±1.4	4.8±2.9	2.3±1.3
1\10 control	11.5±6.9	14.8±5.2	8.9±5.1
1\20 control	9.4±6.5	11.1±6.5	7.0±5.0
1\30 control	2.7±2.0	3.2±2.1	1.5±1.2
G1 S. T+cis	10.2±9.5	11.6±6.4	5.9±5.6
G2 S. T +1/10	6.0±2.8	11.1±5.4	5.0±2.4
G3 S. T +1/20	6.7±4.1	13.1±8.5	5.6±3.9
G4 S. T	4.7±3.4	9.6±5.7	4.1±3.0
Ascites control	12.3±6.6	22.2±9.5	11.7±6.6
Asc + Cis	17.5±13.1	31.3±22.2	17.0±13.2
Asc + 1/20	21.6±11.5	39.2±18.3	21.1±11.5
Asc + 1/30	7.8±5.4	12.8±6.0	6.2±5.2

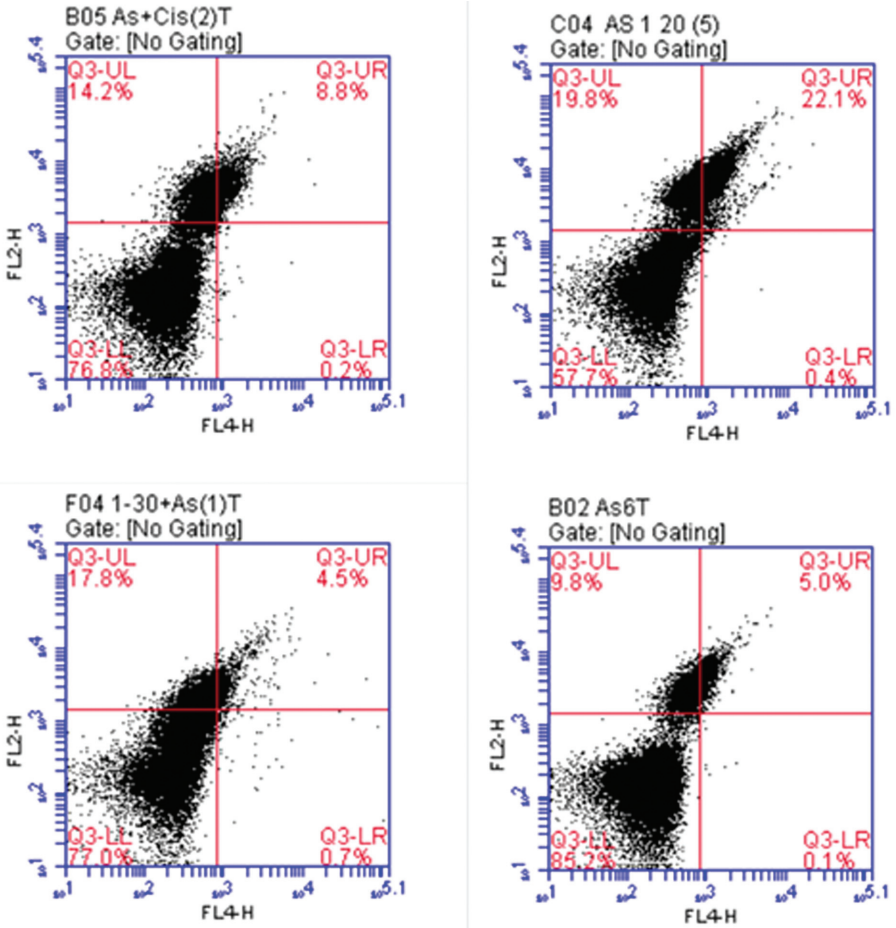
Data are expessed as mean±SD. $CD4^+$ positive T cells, $CD25^+$ positive cells, $CD4^+CD25^+$ positive T_{reg} cells. 1/10, 1/20, and 1/30 LD50 of the crude venom. Cis is cisplatin. *$P \leq 0.05$ when compared with healthy control group. **$P \leq 0.05$ when compared with nontreated Ascites tumor.

Figure 5



The mean percentage of T_{reg} from each control group. Healthy control animals injected with saline, cisplatin, 1/10 LD50 crud venom dose, 1/20 LD50 crud venom and 1/30 LD50 crud venom. CD25 on (X axis FL2-H) and CD4 on (Y axis FL4-H).

Figure 6



The mean percentage of T_{reg} cells from each soft tumor group. Ascites tumor animals injected with saline, cisplatin (Asc + Cis), 1/20 LD50 crud venom (Asc + 1/20) and 1/30 LD50 crud venom (Asc + 1/30). CD25 on (X axis FL2-H) and CD4 on (Y axis FL4-H).

reduced the expression of $CD4^+$, $CD25^+$, and $CD4^+CD25^+$ cells compared with control ascites non-treated On the other hand, 1/20 venom dose increased the expression of $CD4^+$, $CD25^+$, and $CD4^+CD25^+$ cells, also cisplatin increased the expression of $CD4^+$, $CD25^+$, and $CD4^+CD25^+$ cells.

The expression of $CD4^+$, $CD25^+$, $CD4^+CD25^+$ decreased as the venom dose decreased this decrement between 1/10 and 1/20 was not statistically significant (0.361, 0.36, 0.361) while it was statistically significant between 1/10 and 1/30 (0.006, 0.006, 0.006 while between 1/20 and 1/30 (0.055, 0.01, 0.016). $CD4^+CD25^+$ cells increased in the solid tumor group treated with 1/20 venom dose compared with the solid tumor group treated with 1/10 venom dose (P=0.728). $CD4^+$, $CD25^+$ and $CD4^+CD25^+$ expression increased in ascites tumor group treated with 1/20 venom dose compared with ascites tumor treated with 1/30 venom dose (P=0.053, 0.053 and 0.053 respectively).

Necrosis in ehrlish ascites cells

Necrotic Annexin V positive cells percentile increased in all treated ascites tumor groups cis (3.6), 1/20 (5.14), and 1/30 (3.05) compared with the nontreated Ascites tumor control group with no statistically significant.

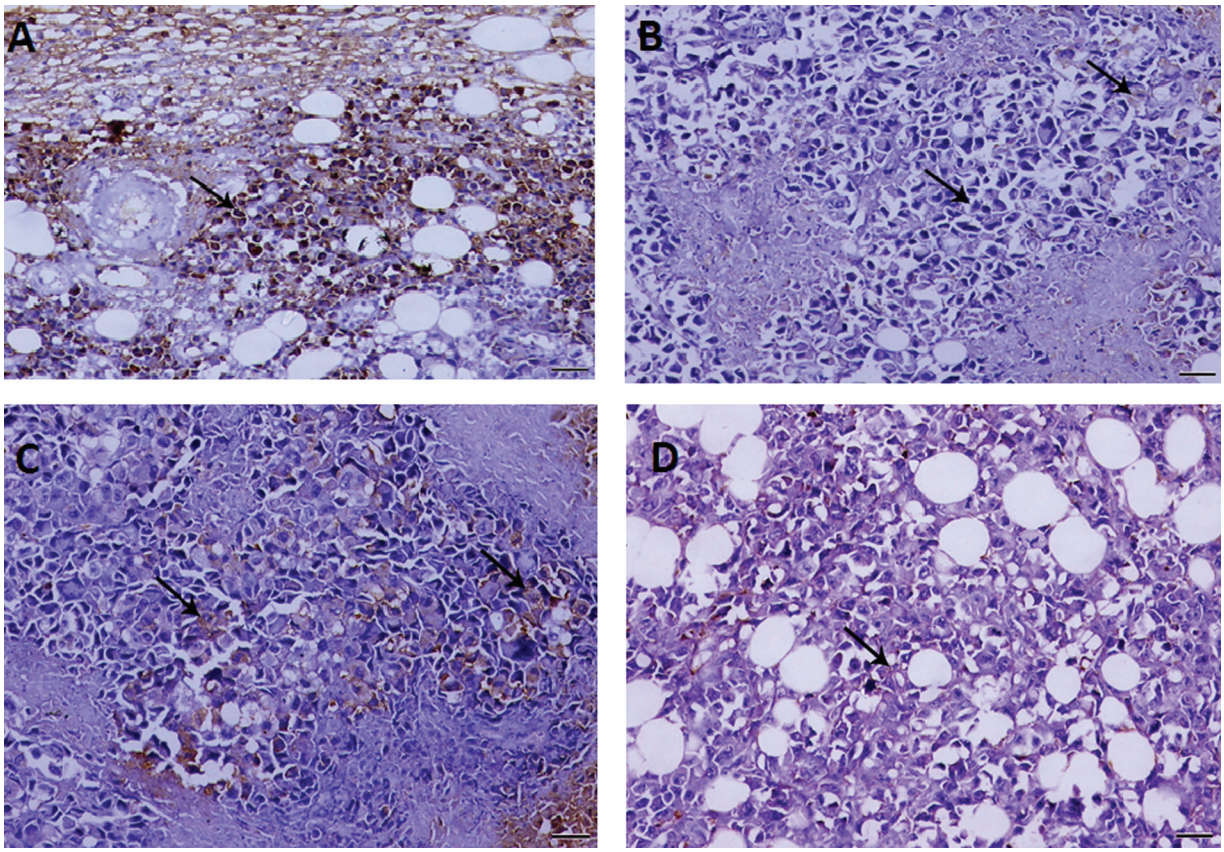
Venom dosing effect on PDL-1 and VEGF

Our results revealed that expression of both PDL-1 and VEGF decreased in solid tumor groups treated with 1/10 LD50 venom dose with the lowest percent (9.9%, P= 0.4 and 21.7%, P= 0.902), 1/20 LD50 venom dose (30.6%, P= 0.695 and 31.2%, P= 0.226) and cisplatin (28.6%, P= 0.528 and 33.6%, P= 0.580) compared with nontreated group. Also, there is a dose-dependent effect in reducing PDL-1 and VEGF in solid tumor-bearing mice treated with 1/10 and 1/20 LD50 (Figs. 7 and 8).

Discussion

Our study revealed that the cell cycle of peritoneum tissue infiltrates treated with cisplatin, nonsignificantly

Figure 7



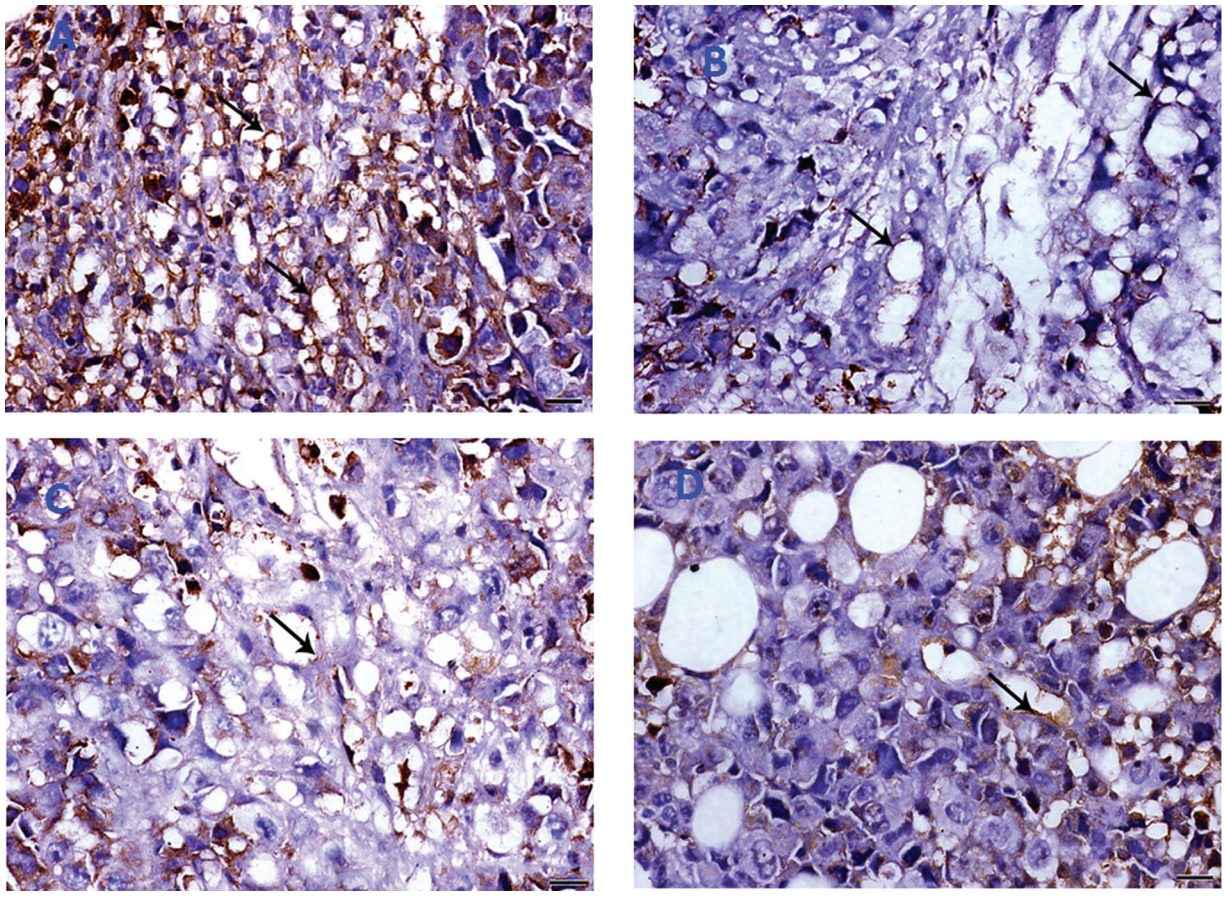
Immunohistochemistry of PDL-1 on different solid tumor groups. Subcutaneous tumor mass of control positive animal showing marked expression of PDL-1 within the neoplastic cells (arrow), PDL-1 IHC, X200, bar= 80 μm. (B) Subcutaneous tumor mass of animal treated with 1/10 LD50 Naja haje venom showing scanty immunoexpression of PDL-1 expression within the neoplastic cells (arrows), PDL-1 IHC, X200, bar= 80 μm. (C) Subcutaneous tumor mass of animal treated with 1/20 ld50 Naja haje venom showing marked decrease of PDL-1 expression within the neoplastic cells (arrows), PDL-1 IHC, X200, bar= 80 μm. (D) Subcutaneous tumor mass of animal treated with cisplatin (40 u) showing marked decrease of PDL-1 expression within the neoplastic cells (arrow), PDL-1 IHC, X200, bar= 80 μm.

increased sub G1, S, and G2M phases, it also reduced G0/G1phase this also occurred in both doses of venom 1/20 and 1/10 with significant decrees in G0/G1 for dose 1/20 this may be caused by the direct cytotoxicity of cisplatin and venom doses. The 5 days between each treatment dose may give the cells sufficient time to recover, this effect appear in increased S and G2M phases. 1/30 venom dose reduced sub G1and G0/G1 on the other hand, S and G2M phases increased.

Also, our results showed increased levels of cells in sub G1, G0/G1, S and G2M phases in tumor tissue compared with peritoneum tissue infiltrates in the healthy control groups this may be caused by the proliferative activity of cancer cells. The solid tumor group treated with cisplatin showed reduced cells in G0/G1 phase and increased cells in the S phase compared with the solid tumor nontreated group. 1/10 LD50 venom dose reduce cells in sub G1 and G0/G1 phases and increased S and G2M phases, also 1/20 LD50 venom dose reduced cells in G0/G1 and increased cells in Sub G1 it also increased the S phase as well as it increased the G2/M in Solid tumor. Among all treated solid tumor groups cisplatin had the lowest S Phase percentile the increment in the S phase may be caused by cell recovery and proliferation this effect may be reduced by decreasing the time between doses also using enhancers that keep cisplatin and venom inside cells for a longer time.

Ascites soft tumor showed vigorous growth and remarkable decrease in sub G1 phase and G0/G1 on the other hand, increased S phase compared with all groups and G2M compared with both normal control and solid tumor. In the ascites treated groups, cisplatin increased sub G1, G0/G1 phases this may be caused by the direct effect of cisplatin on cancer cells, it also decreased the synthetic S phase. The increased G2M in cisplatin may be caused by tumor resistance. Dose 1/20

Figure 8



Immunohistochemistry of VEGF on different solid tumor groups. (A) Subcutaneous tumor mass of control positive animal showing marked expression of VEGF within the blood capillaries (arrows), VEGF IHC, X200, bar= 40 μm(B) Subcutaneous tumor mass of animal treated with 1/10 of ld50 Naja haje venom showing marked decrease of VEGF expression within the neoplastic mass (arrows), VEGF IHC, X200, bar= 80 μm. (C) Subcutaneous tumor mass of animal treated with 1/20 of ld50 Naja haje venom showing decrease of VEGF expression within the neoplastic mass (arrow), VEGF IHC, X200, bar= 80 μm. (D) Subcutaneous tumor mass of animal treated with cisplatin (40 u) showing decrease of VEGF expression within the neoplastic mass (arrow), VEGF IHC, X200, bar= 80 μm.

significantly increased sub G1 and decreased S phase, this dose has a therapeutic effect although the G2M phase also increased, the increment was not significant compared with ascites nontreated group and from the 1/20 LD50 venom dose treated control group this concentration activates G2M. 1/30 venom dose showed a significant increase in sub G1, G0/G1 this may be caused by cell recovery by correcting cell cycle pathways and significantly reducing S and G2M phases to the lowest levels compared with other doses of venom and cisplatin.

Our results agreed with the previous studies that *Naja haje* crude venom arrest treated cancer cells at G0/G1 in 24 tissue culture study Sharkawi and colleagues [39]. Cisplatin arrested the cell cycle, providing adequate time for DNA repair mechanisms to remove the lesions. In cases of impaired repair or excessive damage, the cells undergo apoptosis Siddik [38], low doses of cisplatin arrest cell cycle in G0/G1 after 24, 48, 72, and 96 h of treatment in promyelocytic leukemia cells Velma and colleagues [43].

This is the first study on the dose-effect of (*Naja haje*) Egyptian cobra crud venom on G-MDSCs among the three doses we used on control groups, the dose 1/20 LD50 had the highest levels of G-MDSCs this effect might be caused by the accompanying immune regulation against venom. This results repeated in the solid tumor and soft tumor when treated with this dose having the highest positive necrotic cells. On the other hand, G-MDSCs levels decreased in the control group treated with dose 1/10 but increased insignificantly in solid tumor treated with dose 1/10. G-MDSCs levels significantly reduced in ascites tumor treated with 1/30 compared with nontreated ascites tumor and other ascites treated tumors, this may be caused by the venom affects the cancer cells properly

this explanation agrees with the cell cycle study of this dose which arrests Ehrlish ascetic cancer cells in G0/G1 phase with significant decrees in the synthetic phase and G2/M phase. Our results agree with recent study Cao and colleagues [44] that found G-MDSCs increased in both tumor tissue and peripheral blood of tumor-bearing mice compared with normal control.

Our data revealed that $CD4^+CD25^+$ regulatory T cells are significantly reduced in the control group treated with 1/30 LD50 venom dose and cisplatin respectively these results suggested that both 1/30 venom dose and cisplatin depleted T-reg cells directly. On the other hand, both venom doses 1/10 and 1/20 increase T-reg cells this may be explained as an immune effect for the venom dose also accompanied by high levels of $CD4^+$ T cells compared with cisplatin, 1/30 and control group. In the solid tumor microenvironment levels of T-reg cells are increased in all treated groups compared with nontreated we predicted these results for venom doses 1/10 & 1/20 but not for cisplatin these results may be regarded to tumor resistant; These results didn't agree with the previous studies that found significant reduction on Treg cells percentage in tumor infiltrate treated with cisplatin Huang [45]. Our results on the effect of cisplatin on $CD4^+$ T cells are agreed with the previous result that found $CD4^+$ T cells are increased non-significantly in tumor infiltrate of mice treated with cisplatin Wu and colleagues [46]. While at dose 1/30 of venom and at the ascetic soft tumor the levels of T-reg cells decreased compared with all treated and nontreated groups caused by both direct effects of this dose on suppressing the tumor cell cycle on G0/G1 and depleting T-reg cells.

Our findings referred to decrease PDL-1 expression on solid tumor microenvironment when treated with venom doses this may be caused by venom direct anti-tumor effect as shown in the cell cycle of solid tumor or as a result of a direct or indirect effect on PDL-1 expression further study on this point still needed, also the levels of PDL-1 was decreased on the solid tumor microenvironment treated with cisplatin. These findings disagree with previous studies which demonstrated that PDL-1 increased on cancer cells when treated with cisplatin Grabosch and colleagues [47] and Tran and colleagues [48]. This may be regarded to cisplatin resistance which may be noticed from the solid tumor cell cycle in our results. Also, another study Qin and colleagues [49] recorded that low doses of cisplatin unregulated PDL-1 but decreased with higher doses.

Our results showed that decreased VEGF expression in the solid tumor treated with cobra venom doses 1/10, 1/20 LD50, and cisplatin. These results agreed with the previous research on cisplatin by Duyndam and colleagues and Zhong and colleagues [50,51] and disagree with another study by Tsuchida and colleagues [52] on osteosarcoma (HOS), neuroblastoma (SK-N-BE2) and rhabdomyosarcoma (RH-4) cell lines). Solyanik and colleagues [53] suggested that the increased VEGFR on Lewis lung carcinoma (LLC) cell line, when treated with cisplatin compared with its cisplatin-resistant variant (LLC/R9), is an intracellular adaptation tumor reaction underlie their drug resistance.

Conclusion

Our data showed that cancer cells can resist cisplatin and cancer may be much more aggressive, Egyptian cobra (*Naja haje*) venom may also represent a kind of unique cancer-targeting treatment, as we described earlier doses 1/20 and 1/10 LD50 have an anticancer effect either by cytotoxicity on the cancer cell and affecting cell cycle positively although both concentrations didn't show significant reduction of MDSCs and T-reg cells in the tumor microenvironment, it showed a remarkable increase for both cells in healthy control mice. These results are very valuable for treating auto-immune diseases. Also both doses reduced VEGF and PDL-1 in cancer microenvironment. 1/30 LD50 dose showed a remarkable decrease in MDSCs and T-reg cells in the tumor microenvironment and resting the cell cycle in soft ascites tumors. Further investigation should be done to understand the mechanisms of action and purifying or mimicking active ingredients especially if we know that the venom dose is very.

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The authors declare that they have no relationships or conflicts to disclose.

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