Original Article

The Evaluation of *Androctonus crassicauda* Antivenom against the Effects of *Aegaeobuthus nigrocinctus* Scorpion Venom on Autophagy, Apoptosis and Necroptosis

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Abstract

Background: In this study aimed to show the role of autophagy acting as a seesaw between apoptosis and necroptosis in certain vital organs under the effects of the *Aegaeobuthus nigricinctus* venom and different dosages of the *Androctonus crassicauda* antivenom administration in mice.

Methods: In the venom group (VG), mice (n= 6) were inoculated with $2LD_{50}$ *A. nigrocinctus* venom. In the antivenom administered groups (AVG), the effects of the potency of the *A. crassicauda* antivenom were evaluated to have a neutralization effect against $20LD_{50}$ of the *A. nigrocinctus* venom. After histopathological examination, expressions of mammalian target of rapamycin (mTOR) as an autophagy activator, receptor-interacting serine/threonine-protein kinase 3 (RIPK3) as a necroptosis activator, and caspase-3, caspase-9 as the markers of apoptotic cell death signals were evaluated by the immunoperoxidase method in addition to DNA in-situ fragmentations by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method.

Results: Only in VG, caspases and TUNEL expressions were found to be higher after the envenomation process in contrast to the elevated RIPK3 expressions. mTOR expressions remained almost stable in the organs. In AG, mTOR expressions were further increased in the $30LD_{50}$ and $40LD_{50}$ groups.

Conclusion: There were an increased mTOR expression and stabilized caspases and TUNEL expression in these subgroups, the RIPK3 expressions were found to be low when compared with all of the antivenom administration groups. Increasing doses of the antivenom drifts more the cells to autophagy while cell fate in organs under envenomation getting rid of apoptosis and necroptosis pathways.

Keywords: Scorpion; *Aegaeobuthus nigrocinctus* venom; *Androctonus crassicauda* antivenom; Cell death mechanisms

Introduction

Venom secretions from scorpions are comprised of a complex mixture of salts, mucoproteins, histamine, serotonin, biogenic amines, low molecular weight peptides and high molecular weight proteins. The venom of each scorpion species has a different component profile. Low molecular weight peptides which are neurotoxins is the most important components in scorpion venoms, and it is also the component that is believed to be responsible for envenomation. These peptides stimulate the ion channels of cells such as sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and chloride (Cl^-) (1). Scorpion venoms can cause oxidative stress on cells and mitochondrial instability depending on overproduction of reactive oxygen species (ROS) (1–5). Against this situation, autophagy machinery is activated (2, 3). Mammalian target of rapamycin (mTOR), a serine/threonine kinase, is likely to be the chief of this orchestra. It has a pivotal role in cellular metabolism, such as cell growth and proliferation (6). However, mTOR creates an inhibition in the autophagy induction (7). Another reaction in response to cellular damage like mitochondrial distress is necroptosis to be known as a regulated type of necro-

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sis. It includes the spilling of the cellular contents and therefore, the triggering of the chemoattractant factors to independently develop, arising from the absence of caspase activations. The mechanism is continued mainly by the serine-threonine kinase receptor-interacting protein (RIP) although the absolute activation is unknown (8–12). However, in programmed cell death or apoptosis, cellular contents cannot always spill out in response to several damages. The cells sometimes prefer silent deaths, triggered by intrinsic and/or extrinsic pathways under the effect of chemoattractant or immune-mediated signals (12, 13). Amongst these, the cysteine-dependent aspartate driven proteases (caspases) are known to have a well-defined role in the apoptosis complex for a prolonged time. Caspase-9 as initiator of apoptosome and caspase-3 as effector have pivotal roles after the mitochondrial cytochrome c. Caspases, furthermore, lead to cleaving in cellular proteins to be like in receptor-interacting serine/ threonine-protein kinase 1–3 (RIPK1-RIPK3) complex in necrosome of dying cells (14, 15). Therefore, caspases can take a role in a number of non-apoptotic molecular interaction independently of apoptotic cell death (16).

Up to now, scorpionism has been one of the lasting global health problems in tropical and subtropical countries (17). Therefore, it is extremely important to know about the medical importance of scorpion venom (17, 18). In Turkey, the *Aegaeobuthus nigrocinctus* scorpion was reported to exist in the Adıyaman, Erzincan, Gaziantep, Hatay, Kahramanmaras, Kilis and Malatya provinces of the South-eastern Mediterranean and Eastern Anatolian regions. In these regions, therefore, this scorpion species may be responsible for most of the cases of scorpion stings (19, 20).

Nowadays, although there are no vaccines or other effective agents against animal venoms, hence the serotherapy stands as the only and unique treatment option available as reported before (21). The *Androctonus crassicauda* monovalent antidote has been used in the treatment of all cases of scorpion stings in Turkey (22).

As far as we know, currently there are a few studies about the *A. nigrocinctus* scorpion venom. Moreover, cell death pathways, unfortunately, has have not been observed on different organs, although there have been numerous reports on cellular damage-stemming from a few scorpion species. Therefore, the species at hand is a neglected scorpion species regarding studies all over the world and in Turkey (20). In this respect, there is a requirement to fill the gap over the cell death cascade. Both different cell death types have been comparatively evaluated in different organs and the supremacy of the antivenom over the neglected *A. nigrocinctus* scorpion has been shown by this study. So, the presented study is one of the first studies conducted on the *A. nigrocinctus* scorpion venom.

The aim of this study was to measure the neutralization efficiency of the *A. crassicauda* antivenom against the *A. nigrocinctus* venom; (a) the effect of autophagy on the seesaw role between apoptosis and necroptosis (b) and to measure the response in different cell death reactions in the various vital organs.

Materials and Methods

The usage of animals and their care

In this study which was approved by the local ethics committee (2017/04), 30 healthy CD-1 mice of 20±2g were used in total. Until the end of the experiment, mice (n= 30) were housed in a polycarbonate mouse cage (EU Type 2) and they were maintained at 22±2 °C on a 12h light / dark cycle with free access to food and water.

Venom and antivenom handling

In all the experimental procedures, the *A. nigrocinctus* venom was collected from The Nemrut Mountain National Park, which is in the Adıyaman Province in the Southeastern Anatolia Region of Turkey, and its LD_{50} is 0.38

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mg/kg on mice (20). The monovalent *A. crassicauda* antivenom produced in horses by the Ministry of Health was used by The General Directorate of Public Health. The potency capacity (ED_{50}) of 1mL of antivenom neutralizes $50LD_{50}$ of the venom.

Experimental procedure

The mice modelling system is performed simply according to two main caption: antivenom administered envenomation group (AVG), only venom administered group (V), and the control (C) group which was not administered any agent.

The Effect of the monovalent antivenom and the *A. nigrocinctus* venom in mice: Pathological examination

In antivenom group, mice were classified into three sub-groups with six mice in each group (n= 18). For each antivenom group, individually, 1ml of the antivenom was mixed with an equivalent volume of doses of $20LD_{50}$ (GI), $30LD_{50}$ (GII), and $40LD_{50}$ (GIII) of the *A. nigrocinctus* venom and incubated for 45 min at 37 °C. Then, mice in each AG were subcutaneously (s.c.) injected with 200µL of each of the mixtures. After injection, mice were monitored for abnormal reactions and signs of envenomation for 12h. The mice in the AG were euthanized with overdose of the mixtures of ketamine and xylazine at the end of the observation. The animals (n= 6) in the venom group (VG) as positive control were injected with 2 LD_{50} of *A. nigrocinctus* venom in 200 µL physiological saline solution (PSS), while the negative control group (CG) were injected with 200 µL of the PSS venom.

Macroscopical and Histopathological Examinations

Autopsy procedures were performed immediately on dead mice in VG and the animals (n= 6) in CG and AVG groups for the examination of macroscopic changes and histochemical analysis after the animals were euthanized. Briefly, the peritoneal cavities of the mice were opened, and tissue samples were collected from the livers, kidneys, lungs, hearts, and brains of the mice in each group and were immediately placed in 10% v/v formalin solution. After embedding in paraffin, the sections at 4µm-thicknesses were taken. They were placed in slides and were stained with hematoxylin and eosin (H and E) for microscopic examination.

Immunohistochemical analysis

Detection of mTOR, RIPK3, caspase-3 and caspase-9 expressions

The Strep Avidin-Biotin Complex Peroxidase (strep ABC-P) method was applied following the manual instructions described in the kit (Peroxidase Detection System, RE7110-K, Leica, Novocastra). The sections at 4µm-thickness were passed through xylol and alcohol series (5min for each), and then de-paraffinized and rehydrated. The sections were boiled in citrate buffer (pH 6.0) at 160 ºC for 15min to reveal the antigenic determinants (Bioptica, Italy). To eliminate endogenous peroxidase activity, the tissues were kept in 3% hydrogen-peroxide (H_2O_2)-methanol solution for 15min. Non-specific protein activity was prevented with the use of blocking serum (Novocastra, Leica). Incubation with primary antibodies (mTOR-1:250 dilution Gene Tex, GTX48628, RIPK3-1:200 Antibodiesonline.com, ABIN2792102, caspase-3 LS-B22845, LSBio, USA, 1:100 dilution, anti-caspase-9 ADI-AAP109, EnzoLife Sciences, USA, 1:150) was left overnight at +4 °C. Biotin-linked antibody and streptavidin-linked antibody were dripped onto tissue sections and incubated at 37 ºC for 15min. Thereafter, they were rinsed twice for 5min, using PBS at the end of each phase, except in the protein blocking phase. For the control sections, PBS was used instead of primary antibody as the negative control. Diaminobenzidine (DAB) was used as chromogen, while Gill's hematoxylin was used as ground staining. The slices were fixated using Entellan® which is a non-aqueous mounting medium.

Detection of DNA in-situ fragmentation

The terminal deoxynucleotidyl transferase

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mediated nick end labeling (TUNEL) staining assay method was applied according to the kit procedure (In situ Cell Detection Kit, Roche, USA, Cat no: 11684795910). For control sections, a labeling solution without terminal transferase was dripped onto the slices.

Evaluation of results

All histopathological and immunoexpression results were illustrated in an Olympus BX51 and photographed with an Olympus DF 25 camera attachment. Mean scores were performed semiquantitatively counting 10 high power field at 400 magnifications for each collected organs in all groups. Histopathological scoring was found to be as follows; negative (-): 0–10%, mild (+): 10–30%, moderate (++): 30–70%, strong (+++): 70–100%.

Statistical analysis

Immunoexpressions were evaluated using a Two-way ANOVA test to compare the variability in reactions among the groups. The post-hoc Bonferroni test was used in multivariate comparisons. The data was analyzed using the Graphpad (8.0 version) software. A value of $p< 0.05$ was accepted as statistically significant.

Results

Histopathological findings

The Venom group and Control groups

Regarding the results of the negative control group; the organs aforementioned were not affected by any degeneration or necrotic changes. There was also no inflammatory reaction at all. The only hyperemic changes were present in the liver and kidney vessels at some of the cases (Fig. 1). Findings of the positive control as VG in liver suggests that there was hyperemia in central and portal vessels. Acute cell swelling to vacuolar degeneration were ended in karyolysis and cytoplasmic shrinkage in hepatocytes. In the kidney: hyperemic capillaries and glomerulus were present. Acute cell swellings as well as vacuolar degeneration were evaded in particularly cortical tubules. In the spleen, follicular hyperplasia in lymphoid follicle as well as haemorrhage was observed. In the lungs, hyperemic capillary vessels and neutrophil extravasation were considered. In the heart, hyperemic capillaries, parenchyma degeneration as well as inflammatory cell infiltration were again found (Fig. 1).

Antivenom group (AVG)

In the liver, the degeneration associated with acute cell swelling and vacuolar degeneration was not found in hepatocytes at every field as being in VG. In the kidney, acute cell swelling and vacuolar degeneration in cortical tubules were present although there were not any findings in the medullary region of the kidneys. In the spleen, follicular hyperplasia in lymphoid follicles, intrafollicular hemorrhage as well as the presence of megakaryocytes was observed in some of the cases. In the heart, individual parenchyma degeneration with shrinkage pink cytoplasm in some cardiomyocytes were observed. The findings were not observed in all the cases (Fig. 1).

Immunohistochemical findings

mTOR expressions

Expressions were localized in the membrane and cytoplasm of cells**.** In VG, the expressions were scattered diffusively from the central to peri-central region of the lobules of the liver, on the periphery of lymphoid follicles of the spleen, on the cortical tubule epitheliums of the kidney and cardiomyocytes in the heart. Any statistical differences were not found between these groups ($p> 0.05$). In the control group, there were no expressions ($p< 0.05$ between this and other groups).

In all the antivenom subgroups (AVG) including from GI to GIII, the expressions were increased when compared to previous subgroups in envenomed mice organs. The expressions were localized in the periphery of lobules of the liver, in the periphery of the

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lymphoid follicles of the spleen, on the cortical tubule epitheliums of the kidney, and cardiomyocytes in the heart. Any statistical differences were not found between these groups ($p> 0.05$). However, there was a meaningful statistical difference in comparison between the envenomed and antivenom administration groups ($p< 0.05$). In the control group, no expressions were found ($p> 0.05$ between this and other groups).

Caspase-3 and caspase-9 expressions

These expressions were localized in the membrane and cytoplasms of the cells. In VG, both expressions were found at high degrees regarding the aforementioned cellular localization which stated in mTOR sections. However, when compared between caspase-3 and caspase-9 expressions, there was not any statistical difference between subgroups ($p> 0.05$). In comparison of two caspases, both had the same distribution with no statistical difference ($p< 0.05$). In the control group, there were no expressions ($p< 0.05$ between this and other groups). In GI to GIII, both expressions had similar characteristics in terms of localization, the expressions were found at lower degree of positivities ($p< 0.05$). A meaningful statistical difference was found between envenomed and antivenom administration groups ($p< 0.05$). In the Control group, there were no expressions ($p< 0.05$ between this and other groups).

TUNEL reactions

Expressions were the same with the previous ones. In VG, the expressions were at high levels. They were found at the same localization in all of the organs as mentioned in previous markers. In the control group, there were no expressions ($p< 0.05$ between this and other groups). The expressions were found at the same localization in all of the organs as mentioned in previous markers. However, the positivity degrees were lower in GII and GIII of AVG when compared to that of GI ($p< 0.05$). When these expressions were compared to the envenomed groups, such positivities were more elevated and the distribution of positivities were more evaded in tissues in antivenom groups ($p< 0.05$). In the control group, there were no expressions ($p< 0.05$ between this and other groups).

RIPK3 expressions

In VG, expressions were seen in the cytoplasms of cells. The expressions had the same localizations as being previous markers. The distribution of positivities were stronger and more prevalent in the tissues when compared to that of GI in AVG. Among groups, there was a statistical difference ($p< 0.05$). In the control group, there were no expressions ($p< 0.05$) between the previous two groups.

The expressions were decreased in all of the antivenom subgroups when compared to that of all envenomed groups ($p< 0.05$). The distribution of positivities were the same as the previous ones. Nevertheless, the number of positive cells decreased particularly in the GII and GIII of AVG. There was a statistical difference with the comparison of GI ($p< 0.05$). In the control group, there were no expressions ($p< 0.05$ between this and other groups). All the expressions according to vital organs were shown in Figure 2, 3 and Fig. 4.

Statistical evaluations of tabular data were presented in Table 1.

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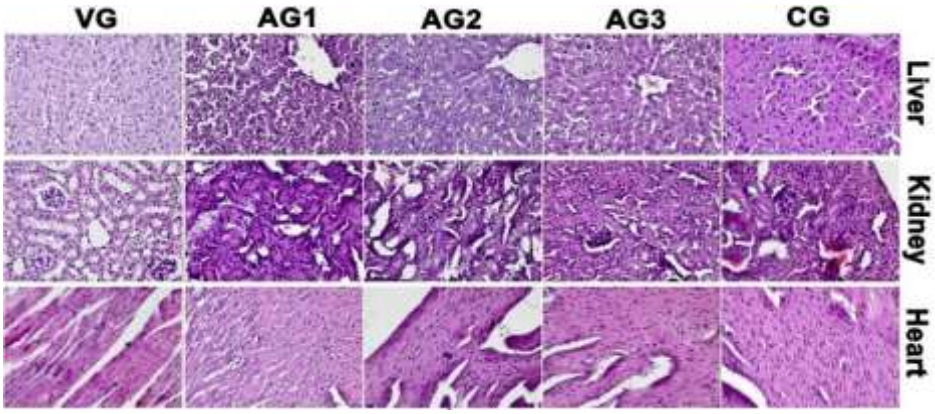

Fig. 1. Description of the degeneration in the liver, kidney, and heart in the envenomed groups (**VG**), and antivenom groups (AG1 to 3), no findings to report in the control group (CG), x400, Hematoxylin-Eosin (H and E) staining

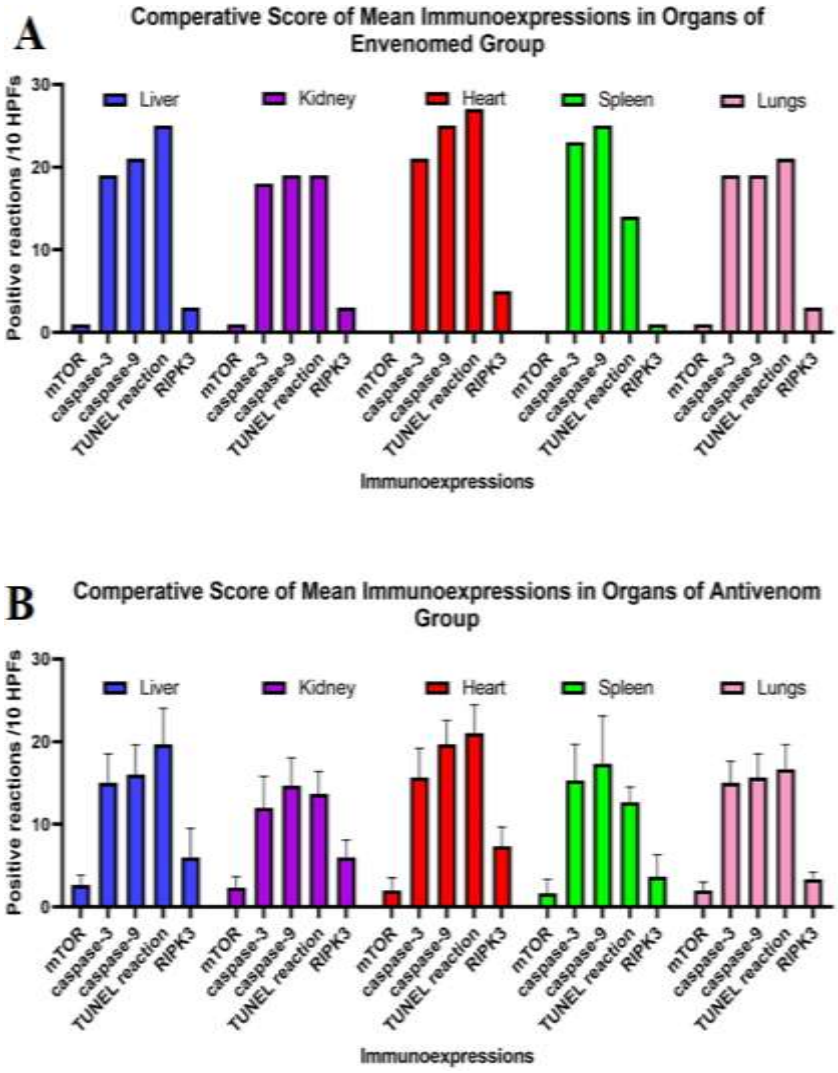

Fig. 2. Immunoexpressions of mTOR, caspases, DNA in situ fragmentation and RIPK3 in the experimental group (A) and the antivenom groups (B)

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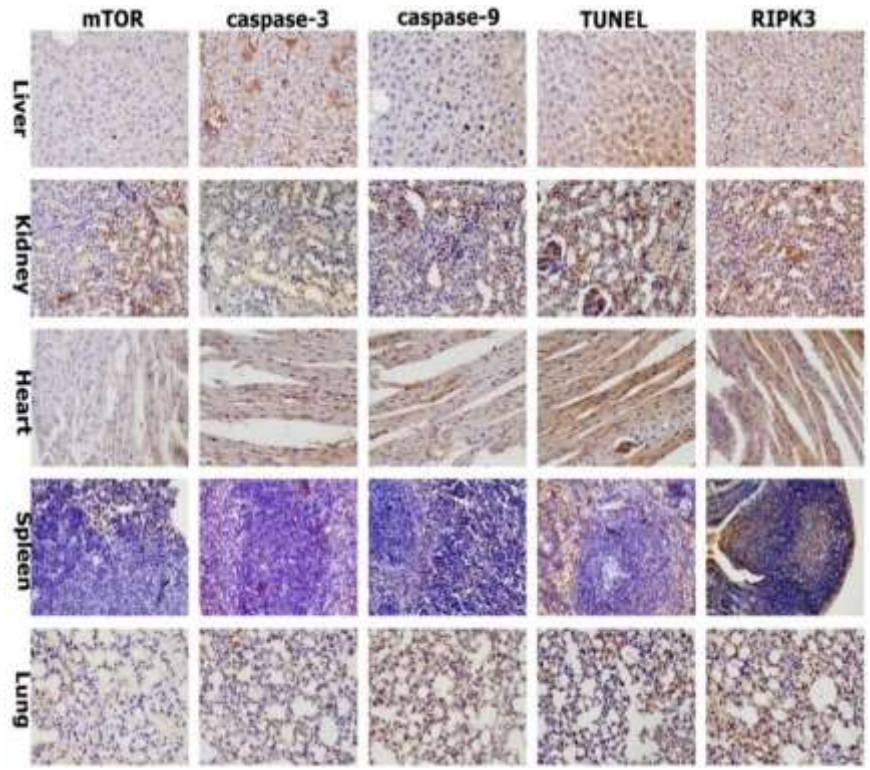

Fig. 3. Immunoexporessions in the organs of the envenomed animals, Diaminobenzidine (DAB) chromogen, ABC-P, x200

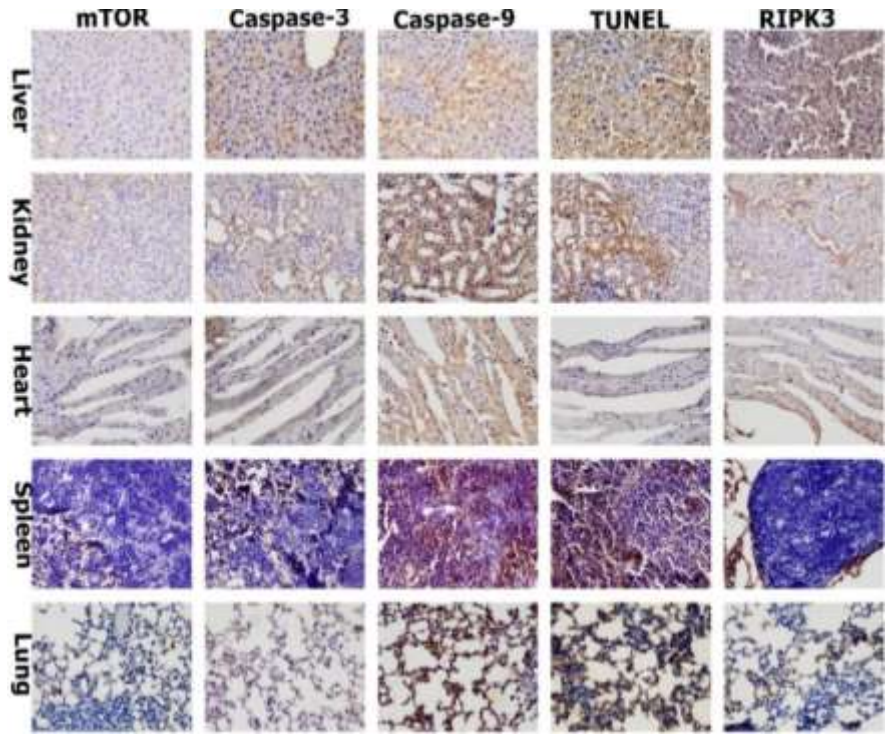

Fig. 4. Immunoexporessions in organs of the antivenom administered animals, Diaminobenzidine (DAB) chromogen, ABC-P, x200

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Discussion

Cells may be exposed to numerous dangerous factors during their lifetime. In this case, cells, due to continuous damage-may be drifted to death by using self-activation of specific molecular pathways (23). These possible molecular mechanisms and some triggering linkages are a controversial matter for a long time whether if this is a real self-suicide or only a biological result or reaction against lethal factors. Therefore, several terminologies have been produced meeting those mechanisms (24, 25). Cell death mechanisms can be diversified within different categories including apoptosis, necroptosis, autophagy, piroptosis etc. In particular, first three mechanism are related to formerly known necrosis. Serine-threonine kinase receptor-interacting protein (RIP) and kinase (RIPK), activate signaling pathways under cellular distress by excessive free radical accumulation. On the other hand mTOR which is taken as a role regarding the PI3K/Akt/mTOR signaling pathway, in terms of creating a downstream in autophagic activation. Caspases, cysteine-dependent aspartate driven proteases, are taken into role within necroptosis. Caspases, have a role of cleaving in cellular proteins in necrosome. Herein, the key role of caspase-9 is associated to be an activator for apoptosis and a deactivator for autophagy. Downregulation of caspases, reactivated mTOR change autophagic mechanism in order to provide autolysosomal activity. By negative feedback, increased autosomal vesicles can reverse autophagy. If free radical (i.e ROS activity) related-damages are excessive and being out of controling mechanism, RIP activation, namely necroptosis, is generated because mitochondrial distress is triggered. As seen herein, each mechanism is closely related to each other. These mechanisms can be easily developed under cellular distress to be like in scorpion envenomation (thanks to toxin contents) (8–12).

In the light of the current knowledge, autophagy is known to provide cellular homeostasis by inhibiting catabolic products and generating nutritional substance and some molecular precursors for cells; namely, a cell survival mechanism (25). However, more recently, the subject of autophagy machinery or self-eating has been given emphasis (24).

Against the cellular damage resourced from toxication, some damages happen in cells due to intoxication. Many toxins and metabolites, cause cytotoxicity by their effects on vital organs such as the heart, kidney, spleen, brain, and skin. In addition to the cellular alterations, hemorrhage and disseminated intravascular coagulation can develop as a result of cytotoxicities (5, 26–29). The toxic effects primarily start in the mitochondria by increasing oxidative stress and over time, it begins to affect all organs. As a result of cellular alterations, cells can be drifted by many mechanisms into death. Several factors can determine the fate of the cell. These mechanisms include autophagy, programmed cell death or apoptosis and necroptosis which results in necrosis (5, 29, 30).

To begin with programmed cell death, it is reported that the execution of cell death plays an orchestrate role between the mentioned processes. Apoptosis triggers cellular membrane receptor facilitating to emit death signals to cytosol. In some events, cells under oxidative stress can release cytochrome c from the mitochondria (4, 5, 31). In such situations, the apoptosis cascade begins to develop autonomously by the activation of Cysteinyl-aspartases or caspase family of proteases (32). Among the caspases family, caspase-9 is known to have an essential role for the mitochondrial signaling pathways. Apoptotic cascades continue by the activation of caspase-3 (33). Another idea on cell death is related to autophagy. It takes critical responsibilities on cell death as well as many roles including tissue development, differentiation and homoeostasis and taking under control of health and aging in a healthy organism. However, the role of housekeeping

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and checking the vital functions of cells whether they behave normally is among the evitable responsibilities (34, 35). In our study, we observed that the caspase-3 and caspase-9 expressions in VG, were high. In AVG, GI to GIII, both expressions had similarly and lower caspases expression of VG. There is statistical significance between VG and AVG. But there is no meaningful difference between both caspases' level.

The autophagy mechanism provides this by regulating some proteins to send signals. mTOR or rapamycin, phosphoinositide 3-kinase (PI3K), GTPases, calcium and elements of protein synthesis machinery are included among them (36). Some regulatory factors control mTOR activity in cells according to whether the cell posing a threat or not. The decreased PI3K activations generally show parallel situation and function mTOR signaling via Akt-mediated phosphorylation. Therefore, PI3K/Akt/mTOR pathway signaling cascade leads to a decreasing activity in the autophagy mechanism (37). On the contrary, the induction of autophagy may lead to cell survival even the cell under stress conditions by oxidative stress. In addition to, it has been stated that autophagy might prevent cells from undergoing apoptosis (38). Therefore, for the autophagy mechanism, it has a pro-survival effect to antagonize apoptosis. In this respect, the current study results are consistent with these data. On the other hand, programmed necrotic cell death or necroptosis is triggered by serine/ threonine kinases receptor-interacting protein 3 or RIPK3 activation, binding to RIPK1 after the regulation by caspases and ubiquitination. Thus, these enzymes facilitate to loss of the cellular carbohydrate deposits and to increase glutamine metabolism (39). In our study, we observed that mTOR in VG were expressed in the liver, spleen, kidney and heart although there was no statistical difference between remained groups.

On the other side, the present study deter mined that venom exposure triggers increased RIPK3 activity. However, we observed that of all the dosages of antivenom administration, mTOR expression continues to increase under toxin stress. Therefore, we concluded that mTOR and RIPK3 have adverse effects when each dose of antivenom was administered. In our study, the RIPK3 expressions of VG were much stronger and more prevalent in the tissues when compared to that of GI in AVG. RIPK3 expressions at both remained groups and control groups were similar and did not give meaningful statistical results because the expressions were decreased in particularly GII and GIII of AVG. We believe that the sole envenomation proven increase in RIPK3, i.e., necroptosis. So, this situation shows that the hypothesis makes the current study right on envenomation-necroptosis interaction. On the other hand, mTOR expressions, i.e., autophagy, were decreased in some organs of VG in spite of increasing in AVG. Decreasing mTOR show that autophagy can be increased in some organs. But other organs were not affected from triggering autophagy as being like in control group. So, these results prove–partly the hypothesis regarding the vice-versa effect between RIPK and mTOR activities. Cells' fate in the way of surviving and drifting to death can be triggered at the same way in some vital organs against venom-associated cellular distress.

Accordingly, we found that cellular death pathways were triggered by high apoptosis and necroptosis as well as low autophagic activity which resulted in cellular DNA breaks.

TUNEL reactions have proven such kind of DNA breaks. In VG, the TUNEL reactions were at high levels. However, these reactions in AVG were lower than that of the VG. When compared within the AVG, the reactions were found lower in GII and GIII than GI. These results showed that antivenom co-administration in the envenomed group inhibit cellular death mechanism. So, DNA breaks can be stopped thanks to diminished cellular death and possibly ROS-related cellular stress.

Another important point in our study is the antivenom or immunotherapeutic usage, how

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it can affect cellular damage or can reverse the adverse effect in such envenomation. It has been reported that the improvement of the immunotherapeutic treatment in such envenomation events require a better knowledge of the pharmacological actions of the scorpion venom and of the mechanism of its in vivo neutralization by the antivenom. Selection of the proper antivenom dose has a vital effect on immediate and durable intervention with regards to sting events by complete neutralization of the toxins. As such, the cellular and vascular or other damages can be better prevented by the effective diffusion of antivenom to all the organs (40).

In the current study, decreasing mTOR and RIPK3 expressions as well as relatively increasing caspases and DNA in-situ fragments in all the antivenom subgroups show us that necroptosis decreases, and cell death increases as a result of the autophagy mechanism controlling cell survival in the way of preventing apoptosis. By evaluating the results of the current study (a) there is a close relationship between autophagy-apoptosis and necroptosis. (b) In mice, the *A. nigricinctus* venom and the monovalent antivenom administration can be a useful model for coming to a better understanding of the potential harmful effects over cells in various vital organs such as the liver, kidneys, spleen, heart, and lungs. (c) The monovalent antivenom against high LD value of the venom may reverse the potential necroptotic effects on cells due to envenomation. (d) Caspase signals apart from apoptotic cell death can also aid in decreasing mTOR expressions in envenomed animals. Thus, combined expressions may trigger the activation of autophagy. (e) RIPK3 may solely change the fate of cells in the course of necroptosis.

Conclusion

Envenomation by scorpion toxin cause a cellular damage in several organs. Excessive free radicals disturb *de facto* cellular membran, cytoplasmic organels and nuclear structure. In this situation is known that cells are drifted to degeneration or necrosis. However, by this experimental study, we show there are definitive mechanisms which is related to each other. According to exposure degree of toxin and serum support, cell fate can be easily changed under free radical distress. These mechanisms can be turned appear as apoptosis, necroptosis and autophagy. We obtained sustainable information from this experimental that autophagic mechanism shows parallel to decreased of venom capacity and increased serum support. At the same time, we understood that this condition gets the irreversible necroptosis capacity decreased. By this, we have also seen that apoptotic mechanism is more effective in initial phase of envenomation. But we have concluded that apoptotic signals get less effective in presence of higher serum support. In this situation, cell makes a decision living or death after such mechanisms are run in cell at the same time. The obtained results can facilitate in order to understand the relations amongst different cell death mechanisms as well as reversal of the monovalent antivenom effectivity on organ damage. At the same time, we inferred from result of this study that serum support the more earlier time is early getting started and suitable dose is selected, the less organ damages are developed. However, the results should be confirmed by correlating with other markers taking place in apoptotic, necroptotic and autophagic cascades.

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Ethical considerations

The animal care and all of the experimental protocols were performed in accordance with the guidelines defined by the local ethical committee in Experimental Animal Research Comitee, Health Ministry (2017-E330341).

Conflict of interest statement

The authors declare no conflict of interest with any researchers.

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