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Bothrops moojeni Venom: A New Tool to Investigate Osteoclasts Differentiation

Fernanda D'Amélio [1,2,*]; Hugo Vigerelli de Barros [1,3], Álvaro Rossan de Brandão Prieto da Silva [1], Eduardo Osório Frare [1,3], Isabel de Fátima Correia Batista [3], Daniel Carvalho Pimenta [4] and Irina Kerkis [1,3]

[1] Laboratory of Genetics, Butantan Institute, São Paulo, Brazil; Laboratory of Biochemistry, Butantan Institute, São Paulo, SP, Brazil
[2] The Postgraduate Program in Toxinology, Butantan Institute, São Paulo, Brazil
[3] Centre of Excellence in New Target Discovery (CENTD), Butantan Institute, São Paulo, Brazil
[4] Laboratory of Biochemistry, Butantan Institute, São Paulo, Brazil; e-mail@e-mail.com
* Correspondence: fernanda.damelio@butantan.gov.br

Abstract: *Bothrops moojeni*, a Brazilian lanced-head viper, presents a rich, but not well explored, venom composition. This venom is a powerful tool for the discovery of new molecular targets in many different biological processes. Osteoclasts (OC) are extremely important for bone maintenance, calcium physiology, and balance of tissue regeneration being involved in such diseases as osteoporosis and rheumatoid arthritis. The goal of our study was to evaluate the effect of *Bothrops moojeni's* venom and its fractions of human peripheral blood mononuclear cells derived OCs in vitro differentiation.After the induction of OCs differentiation, on day 4 the venom was added at different concentrations (5, 0.5, and 0.05 μg/mL), and the reduction of tartrate-resistant acid phosphatase positive (TRAP+) osteoclasts, which was more prominent at the concentration of 5 μg/mL were observed. Phalloidin staining was used for morphological analyzes of F-actin rings integrity. Venom provoked F-actin ring disruption in treated versus control OCs. We obtain high molecular weight (HW) and low molecular weight (LW) venom fractions. Both fractions induced the reduction of TRAP+ OCs (HW fraction at a concentration of 5 μg/mL and LW fraction at 1 μg/mL, respectively).We performed a secretome analysis of OCs treated with venom and its fractions using mass spectrometry (LC-MS/IT-Tof). The data obtained demonstrate possible pathways and mechanisms involved in OCs reduction after the treatment. Example giving is catabolic mechanisms for HW and proteins correlated with genetic modifications for LW. New experiments are in progress, aiming to discover the molecules that possibly interfering in the osteoclasts differentiation.

Keywords: *Bothrops moojeni*; Osteoclasts; Cell Differentiation

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1. Introdution

Osteoclasts (OC) are extremely important for bone maintenance, calcium physiology, and balance of tissue regeneration being involved in such diseases as osteoporosis and rheumatoid arthritis. The goal of our study was to evaluate the effect of *Bothrops moojeni's* venom and its fractions of human peripheral blood mononuclear cells derived OCs in vitro differentiation.

2. Results and Discussion

2.1. Effect of B.moojeni Crude Venom on Cell Viability, TRAP+ Ocs Number, F-acting Rings Integrity

The effect of *B.moojeni* venom in OCs differentiation model was evaluated using phenotypic assays based on characteristics of mature OCs, such as number of TRAP+ cells, F-acting rings integrity and OCs multinuclearity.

To evaluate the toxic effect of the *B.moojeni* venom on OCs, we performed the mature OCs viability test on day 15 of differentiation. For this purpose, differentiation into OCs was induced using RANKL immediately after PBMCs plating. The venom was added at different concentrations (5, 0.5, and 0.05 μg/mL) on day 4 after plating, and it was maintained before the end of differentiation (day 15). CCK8 method was adopted to evaluate OCs primary culture viability based on hydrogenase activity measuring. For this, the absorbance value was reversed in the percentage of living cells. According to Figure 1A, no statistically significant difference in OCs viability was observed between mature OCs at all tested concentrations.

TRAP is a specific marker of mature OCs; therefore, we perform the TRAP staining at the end of PBMC differentiation protocol in the groups treated with crude venom at the same concentrations used in the viability assay. Besides, this staining was performed in two control groups, one with PBMC, induced for differentiation and, the other with PBMC in the basal medium. TRAP staining demonstrates, in the positive control, multinucleated and active OCs appearing in a purple color where it is possible to observe the stained nuclei. Cells not competent to metabolize become very dark in color (Figures B–E). Figure 1B demonstrates TRAP+ OCs control culture and TRAP+ OCs treated with crude venom at concentration 0.05 μg/mL (Figure 1C), 0.5 μg/mL (Figure 1D), and 5 μg/mL (Figure 1E).

Next, we counted the number of TRAP+ OCs in positive control e in OCs treated with venom. The results showed that OCs treatment with 5 μg/mL of venom significantly reduces their number compared with the positive control (Figure 1F).

OCs are bone-resorbing cells acting as fundamental mediators of bone conditions. Mature OCs polarize and reorganize their cytoskeleton to create an F-actin-rich ring upon adhesion to the bone. Staining of F-actin rings with phalloidin allowed us to observe the conservation and integrity of these structures. The OCs treated with venom show a difference in the integrity of the ring (Figure 1G,H). Figure 1-G demonstrates intact F-actin ring formation in positive control. After OCs treating with different venom concentrations, the rings' gradual disruption was observed, which depends on venom concentration. Representative Figure 1-H shows the OCs that demonstrate the intact ring on one side, while on the other side ring shows topic disruptions. This effect is stronger at a concentration of 0.5 μg/mL (Figure 1-I) and, in Figure 1-J destroyed F-actin ring is shown. The same procedures were performed with high and low mass, the decreasing number of osteoclasts it's observed.

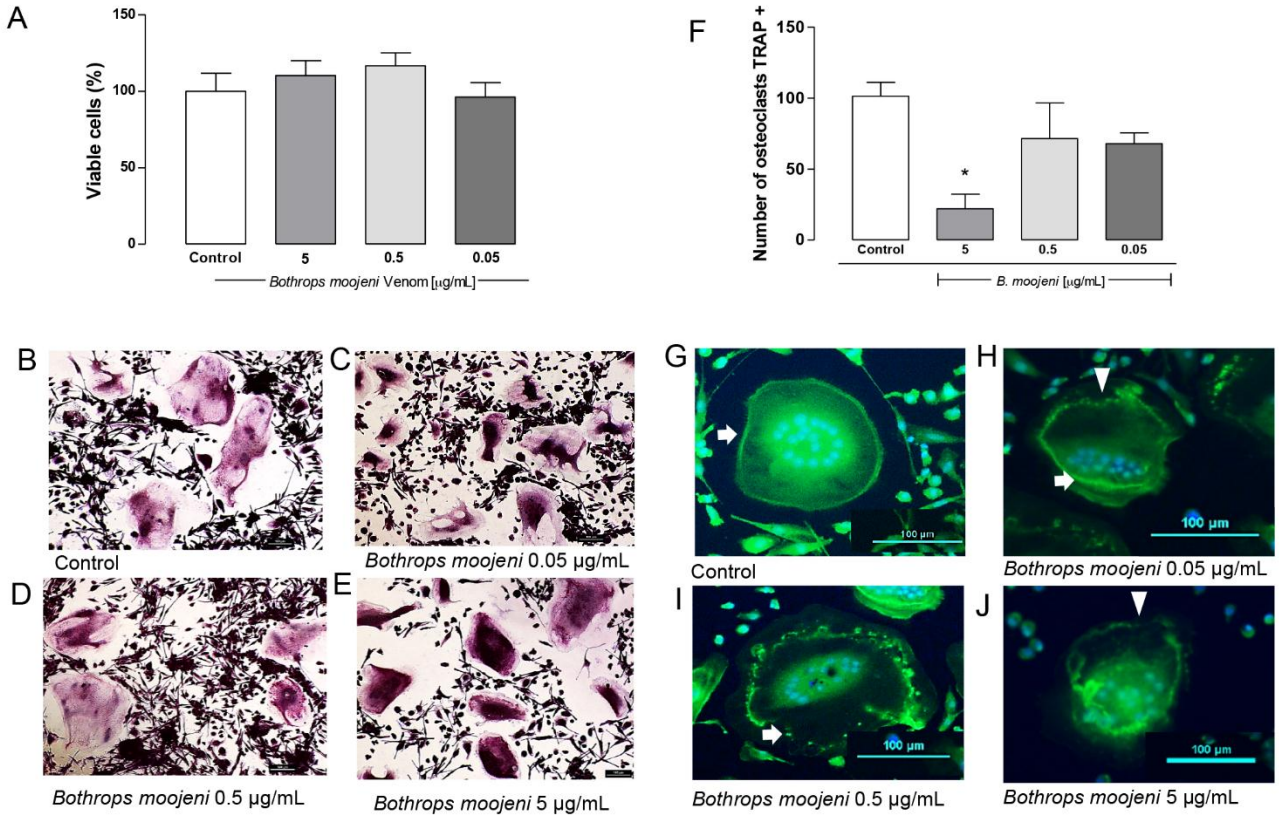

Figure 1. Osteoclast viability, TRAP - staining, TRAP+ OCs counting, and F-ring morphology after the treatment with B. moojeni venom. (**A**) CCK8 assay of mature OCs treated with crude venom viability. (**B**–**E**) OCs tartrate-resistant acid phosphatase (TRAP) staining. (**B**) TRAP+ OCs – positive control. (**C**–**E**) TRAP OCs staining after the treatment with B. moojeni venom at concentrations of 0.05, 0.5, and 5 µg/mL, respectively. Multinucleated TRAP+ purple cells can be observed. (**F**) Response rate curve for counting the number of TRAP + osteoclasts * p <0.05. (**G**–**J**) Staining the F-actin rings with phalloidin (green), nuclei stained with DAPI (blue). OCs treated with venom at concentrations of 0.05, 0.5, and 5 µg/mL, respectively. White arrows indicate intact F-rings. White arrowheads indicate F-rings' gradual disruption. (**H**–**J**). Scale bar: 100 µm.

2.3. Cell Culture Medium Soluble Protein Analysis of Mature Ocs (Day 15)

We identified 120 proteins for the positive control, 53 proteins for the negative control, 103 proteins for the group treated with *B. moojeni* crude venom, 22 proteins for the group with high weight, and 22 proteins for the low weight group. It is noteworthy that on a par with secreted proteins, the proteins originated from different extracellular vesicles, and proteins released by OCs during the fusion process can be found. *B.moojeni* venom has proteases and other components that are not yet described. Our data show that all three groups present a distinct pattern of identified proteins. We selected some of these proteins for further discussion.

Using Panther software analysis, several categories of enrichment data were detected. Pathway analysis indicates those proteins important to the OCs differentiation in the positive control group. Having Hedgehog signaling pathway (P00025), histamine H1 receptor-mediated signaling pathway (P04385), oxytocin receptor-mediated signaling pathway (P04391) highlighted. In the group treated with *B. moojeni* crude venom, interleukin signaling pathway (P00036), inotropic glutamate receptor pathway (P00037), metabotropic glutamate receptor group III pathway (P00039), PI3 kinase pathway (P00048), and pyrimidine metabolism (P02771) are evidenced. In the group treated with HMW fraction, the PI3 kinase (P00048) and Pyrimidine Metabolism (P02771) pathways are of importance, while the group treated with LMW shows a single pathway involved in Blood coagulation

(P00011). All pathways found in the treated groups are exclusive to each group, except blood coagulation, observed in the group treated with LMW fraction and negative control.

Regarding the pathways identified in the *B. moojeni* group treated with venom, we can emphasize that, in the Interleukin signaling pathway (P00036), cytokines are essential in playing a regulatory role in osteoclastogenesis, acting together as anti-osteoclastogenic and pro-osteoclastogenic modulators [1]. For the glutamate-related pathway, it was reported that glutamate-type receptors and glutamate have the function of ensuring bone impact. Inotropic glutamate receptors' activation demonstrates regulating the OCs phenotype in vitro and maintaining bone mass [2,3]. The PI3K-AKT pathway, which we also found in the group treated with the venom, is related to the increase in the number of osteoclasts and cytokines, with greater precursors' effectiveness [4]. We also identify the pyrimidine pathway; it is known that extracellular nucleotides can play a role in bone regulating, signaling, and in cartilage metabolism. These can be stimulating during differentiation [5].

In the group treated with HMW, we detected the pathways for pyrimidine and PI3. In the group treated with LMW, only a single pathway related to coagulation was revealed, which may be influenced by thrombin that stimulates bone resorption mediated by OCs.

3. Preliminary Conclusions

We demonstrated that *B. moojeni* crude venom, and LMW, and HMW fractions are responsible for reducing mature OCs number without interfering with cell viability. The venom compounds, like metalloproteases, phospholipases, L amine oxidase acid, and serine proteases, have biological activity on cellular membranes and pathways related to inflammation [6–9] and may cause multiple effects on mature OCs formation.

The venom and its components were shown to be responsible for causing some morphological and cytoskeletal changes. F-actins rings are classic OCs phenotypic characteristics [10]. Besides being involved with the cytoskeleton and the cell`s sustentation, also presents essential role functionally, including motility, conformation, and fixation for absorption [11]. We analyzed the format of rings and observed that F-actin ring formation in some treated cells was affected by crude venom and it's both fractions, when used in studied concentrations, suggesting commitment of bone resorption capacity of mature OCs.

The analysis of the OCs proteins in culture medium is an important parameter not yet investigated by the scientific community. The exposure to inflammatory components naturally can causes changes in the differentiation OCs. The present study indicates that treatment with crude venom, HMW, and LMW cause morphological, functional, and molecular changes in mature OCs.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Amarasekara, D.S.; Yun, H.; Kim, S.; Lee, N.; Kim, H.; Rho, J. Regulation of Osteoclast Differentiation by Cytokine Networks. *Immune Netw.* **2018**, *18*, e8, doi:10.4110/in.2018.18.e8.
2. Biosse-Duplan, M.; Baroukh, B.; Dy, M.; de Vernejoul, M.-C.; Saffar, J.-L. Histamine Promotes Osteoclastogenesis through the Differential Expression of Histamine Receptors on Osteoclasts and Osteoblasts. *Am. J. Pathol.* **2009**, *174*, 1426–1434, doi:10.2353/ajpath.2009.080871.
3. Brakspear, K.S. Glutamate signaling in bone. *Front. Endocrinol.* **2012**, doi:10.3389/fendo.2012.00097.
4. Espinosa, L.; Itzstein, C.; Cheynel, H.; Delmas, P.D.; Chenu, C. Active NMDA glutamate receptors are expressed by mammalian osteoclasts. *J. Physiol.* **1999**, *518*, 47–53, doi:10.1111/j.1469-7793.1999.0047r.x.
5. Adapala, N.S.; Root, S.; Lorenzo, J.; Aguila, H.; Sanjay, A. PI3K activation increases SDF-1 production and number of osteoclast precursors, and enhances SDF-1-mediated osteoclast precursor migration. *Bone Rep.* **2019**, *10*, 100203, doi:10.1016/j.bonr.2019.100203.

6. Pereira-Crott, L.S.; Casare-Ogasawara, T.M.; Ambrosio, L.; Chaim, L.F.P.; de Morais, F.R.; Cintra, A.C.O.; Canicoba, N.C.; Tucci, L.F.F.; Torqueti, M.R.; Sampaio, S.V.; et al. Bothrops moojeni venom and BmooLAAO-I downmodulate CXCL8/IL-8 and CCL2/MCP-1 production and oxidative burst response, and upregulate CD11b expression in human neutrophils. *Int. Immunopharmacol.* **2020**, *80*, 106154, doi:10.1016/j.intimp.2019.106154.
7. Assakura, M.T.; Reichl, A.P.; Asperti, M.C.A.; Mandelbaum, F.R. Isolation of the major proteolytic enzyme from the venom of the snake Bothrops moojeni (caissaca). *Toxicon* **1985**, *23*, 691–706, doi:10.1016/0041-0101(85)90374-5.
8. Tempone, A.G.; Andrade, H.F.; Spencer, P.J.; Lourenço, C.O.; Rogero, J.R.; Nascimento, N. Bothrops moojeni Venom Kills Leishmania spp. with Hydrogen Peroxide Generated by Its -Amino Acid Oxidase. *Biochem. Biophys. Res. Commun.* **2001**, *280*, 620–624, doi:10.1006/bbrc.2000.4175.
9. Stábeli, R.G.; Sant'Ana, C.D.; Ribeiro, P.H.; Costa, T.R.; Ticli, F.K.; Pires, M.G.; Nomizo, A.; Albuquerque, S.; Malta-Neto, N.R.; Marins, M.; et al. Cytotoxic l-amino acid oxidase from Bothrops moojeni: Biochemical and functional characterization. *Int. J. Biol. Macromol.* **2007**, *41*, 132–140, doi:10.1016/j.ijbiomac.2007.01.006.
10. Matsubara, T.; Kinbara, M.; Maeda, T.; Yoshizawa, M.; Kokabu, S.; Takano Yamamoto, T. Regulation of osteoclast differentiation and actin ring formation by the cytolinker protein plectin. *Biochem. Biophys. Res. Commun.* **2017**, *489*, 472–476, doi:10.1016/j.bbrc.2017.05.174.
11. Han, G.; Zuo, J.; Holliday, L. Specialized Roles for Actin in Osteoclasts: Unanswered Questions and Therapeutic Opportunities. *Biomolecules* **2019**, *9*, 17, doi:10.3390/biom9010017.