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Enhancing adjuvant efficacy with dispersed Ascorbyl Palmitate (ASC16)

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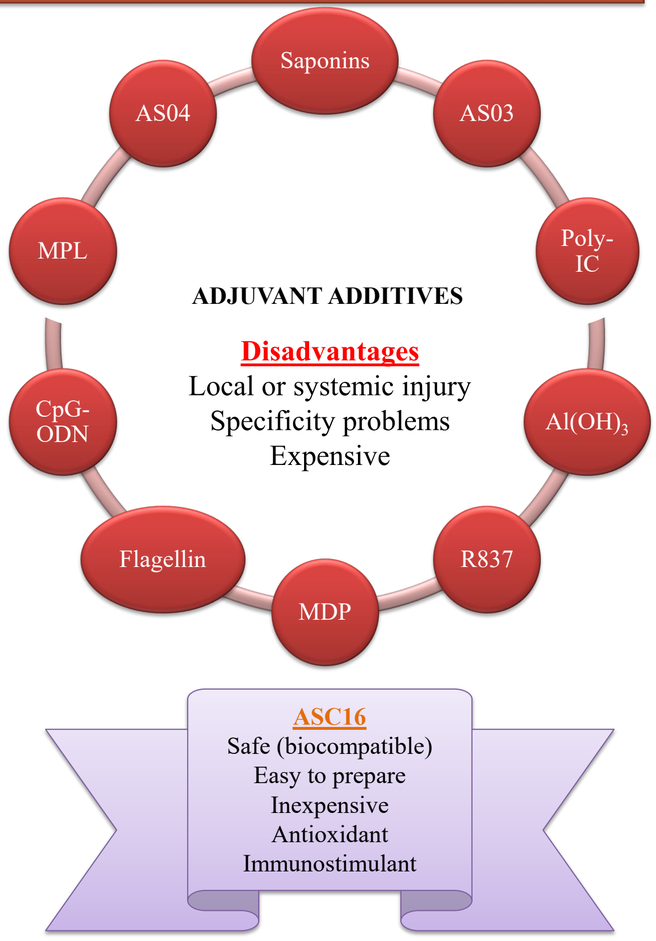
INTRODUCTION & AIM

Numerous molecules are currently used as adjuvant additives (Apostólico et al., 2016), but various factors determine the choice of an adjuvant to achieve optimal antibody production while ensuring the safety of the patient (for vaccines) or the producing animal (for antivenoms). In this respect, an ideal adjuvant should be safe, biocompatible, capable of directing the immune response, and able to enhance antigen presentation.

In this context, ascorbyl palmitate (ASC16) is a low-molecular-weight, fat-soluble derivative of vitamin C (GRAS-listed and included in the FDA Inactive Ingredients Database) that can form viscoelastic hydrogels (high concentration). It´s easy to prepare, inexpensive, and possesses antioxidant and immunostimulant properties (Palma et al., 2007; Vallecillo et al., 2015). However, ASC16, in its dispersed state (low concentration), exhibits an inhibitory effect on certain snake venom toxins, making it an attractive option for antivenom production (Maslovski et al., 2024). For these reasons, it was proposed to evaluate the effect of dispersed ASC16 as an adjuvant additive in the production of experimental antivenom against the venom of *Crotalus durissus terrificus*.



METHOD

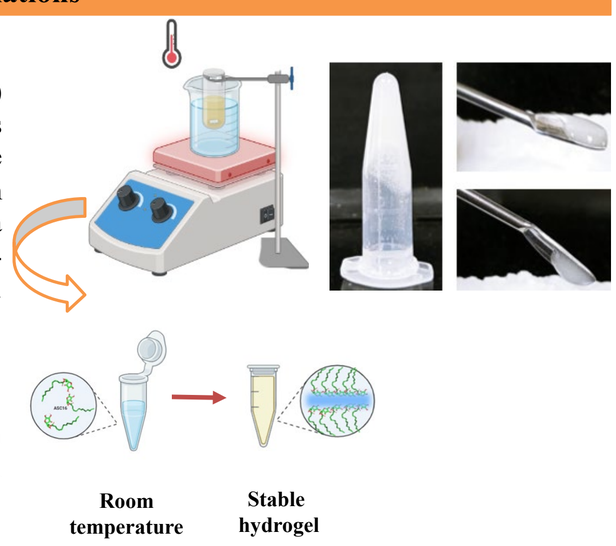
Formulations

Pa40:

- ASC16 (12,5 mg) and PEG400 solution (0.750 mL) were mixed in glasses tubes. This dispersion was heated to 63 °C in a thermostatic bath to complete solubilization (≤ 2 minutes). Then, this dispersion was cooled to 40 °C. After thermostabilization, a PBS solution (0.250 mL) with venom proteins (6-10 µg) was incorporated and mixed for 10 seconds. Finally, left to reach room temperature.

Pa40Mo:

- It was formulated in the same way as formulation Pa40, but the venom was pre-incubated with 120µM ASC16 (additive) for 30 minutes at 37 °C.



Immunisation

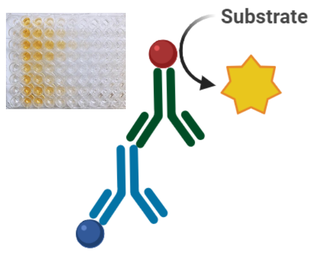
BALB/c mice (n = 5 per group) were subcutaneously immunised using Pa40 and Pa40Mo.

Each mouse was immunised with an entire dose (250 µL) equally distributed at five sites (50 µL/site). Blood samples were collected from the tail and sera aliquots were stored at − 70 °C.

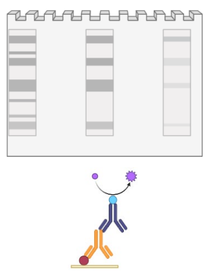
Immunisation protocol

Days	Proteins dose (µg/mice)	Bleeding
0	6	-
15	8	X
30	10	X
45	-	X

Evaluation of immune response

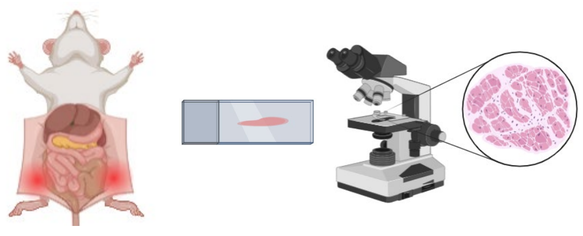


ELISA



Immunoblotting

Evaluation of local injury



For light microscopic examination, thick sections (4 µm) were prepared and stained with hematoxylin and eosin (H&E).

Statistical analysis

We used t-tests for multiple independent groups, each consisting of 5 independent samples, to determine if there were significant differences when comparing the results using GraphPad PRISM software. The level of significance was established at $p \leq 0.05$

RESULTS & DISCUSSION

Evaluation of immune response

The results indicate that immunisation with Pa40 stimulated a low immune response (Fig. 1A); in contrast, the Pa40Mo formulation showed a superior response, resulting in high antibody titers, with significant differences ($p<0.05$) compared to the formulation without the additive.

Immunoblotting studies to identify the specific antigens recognised by the different experimentally obtained antivenoms are shown in Figure 1B. The results indicate that the Pa40 formulation exhibits lower reactivity with the main venom proteins, such as phospholipase A2 (PLA2), crotoxin (CTX), thrombin-like enzyme (TLE), and phosphodiesterase (PDE). However, at the same serum dilution, the Pa40Mo formulation showed a higher signal.

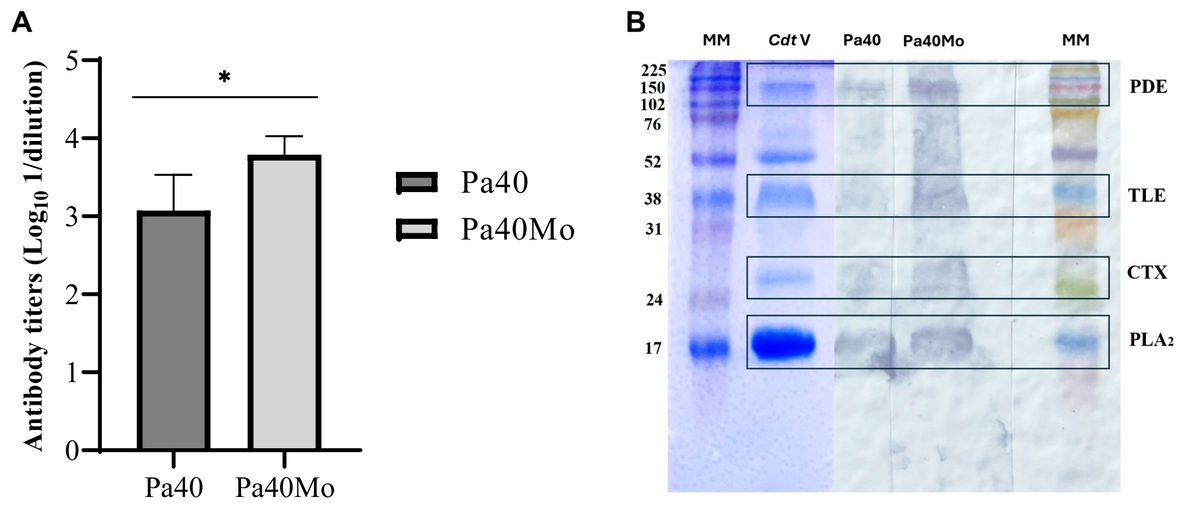


Figure 1. **A-** Antibodies titers against *C. d. terrificus* venom was measured by ELISA at 45 days. * $p<0.05$ indicates significant differences. **B-** The reactivity of antisera produced in mice against the main components of *C. d. terrificus* venom. **MM:** molecular markers expressed in kDa. **C.d.t V**: *C. d. terrificus* venom.

Evaluation of local injury

The Pa40 formulation generated local inflammation, with no evidence of abscess formation at the injection site. On the other hand, Pa40Mo also caused inflammation, and increased vascularisation was detected. Histological analyses indicate that both formulations are in a final stage of tissue recovery at the muscle level. The arrows indicate the region of regenerating tissue (presence of myoblasts and myotubules).

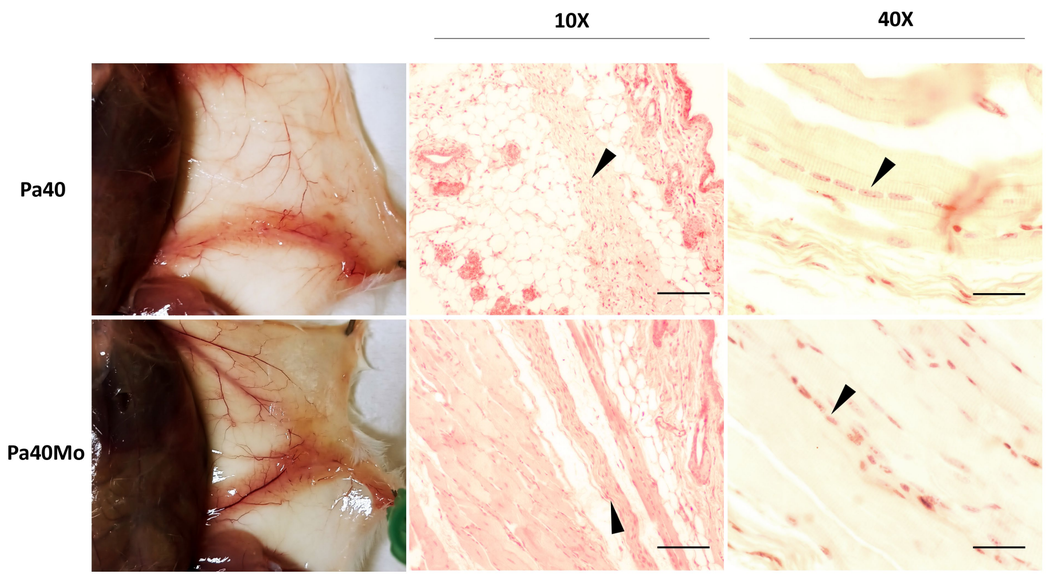


Figure 2. Macroscopic and microscopic analyses of the injection site. Panels show skin sections obtained at day 15 after last injection. **Scale bars:** low power magnification views = 100 µm; high power magnification views = 25 µm.

CONCLUSION

This study shows that dispersed (low concentration) ASC16, as an adjuvant additive, induces a higher immune response (higher titer and recognition of specific antibodies). Additionally, signs of acute local damage (presence of regenerative tissue with fibroblasts) were detected in both formulations. In this sense, our work highlights the potential of ASC16 hydrogel as a matrix and dispersed ASC16 as an additive, as a practical, and economical alternative for the production of specific antibodies. However, future studies are needed to determine the signalling mechanism of dispersed ASC16

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