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SKIN AMPHIBIANS PEPTIDES: MORE THAN SHORT PROTEINS

SUMMARY

In Amphibians, the skin does not play the physiological role of mere integument, but it has a plethora of functions related to respiration, osmoregulation and thermoregulation, thus allowing individuals to survive and thrive in the terrestrial environment. For this purpose, Amphibians have developed some defence strategies that include the production and secretion of mucus with protective activities for skin, often accompanied by behavioural strategies. Amphibians' skin secretions contain several molecules, including peptides able to exert beneficial effects since they are involved in the defence against environmental and pathogenic insults. The study of amphibian peptides can contribute to the understanding of why some species resist to various environmental insults and can also help to limit the decline of Amphibians by developing appropriate strategies particularly against diseases such as viral and fungal infections.

Key words: Keratinized tegument, wound healing, skin defences, eco-physiology, animal physiology, *Batracochytrium dendrobatidis*

RIASSUNTO

Peptidi cutanei di anfibio: molto di più che corte proteine. Negli Anfibi, la pelle non svolge il ruolo fisiologico di mero tegumento, ma presenta una moltitudine di funzioni legate alla respirazione, all'osmoregolazione e alla termoregolazione, consentendo così agli individui di sopravvivere e prosperare nell'ambiente terrestre. A tale scopo gli Anfibi hanno sviluppato alcune strategie di difesa che prevedono la produzione e secrezione di muco con attività protettive per la cute, che è spesso accompagnata da strategie comportamentali. Le secrezioni cutanee degli Anfibi contengono diverse molecole, tra cui i peptidi in grado di esercitare effetti benefici poiché sono coinvolti nella difesa contro gli insulti ambientali e di patogeni. Lo studio dei peptidi anfibi può contribuire alla comprensione del motivo per cui alcune specie resistono a vari insulti ambientali e può anche aiutare

a limitare il declino degli anfibi sviluppando strategie appropriate in particolare contro malattie come infezioni virali e fungine.

Parole chiave: Tegumento cheratinizzato, cicatrizzazione, difese della pelle, eco-fisiologia, fisiologia animale, *Batracochytrium dendrobatidis.*

INTRODUCTION

Amphibian skin is not a mere protective tegument: it has a multitude of functions related to respiration, osmoregulation, and thermoregulation, thus allowing the individuals to survive and thrive in the terrestrial environment (HASLAM *et al.*, 2014). The central role of this organ in Amphibians points out that any kind of insult can exert adverse effects on individual fitness. Skin antimicrobial peptides secreted by granular glands contribute to the survival of the amphibians suffering several insults such as bacteria and/or fungi infections, skin ruptures, UV irradiation etc. In this scenario, the production of different peptides in different species can ensure the survival of some species rather than others when environmental conditions become more adverse.

One of the most widely diffused death causes in amphibians is chytridiomycosis, a skin disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (Bd).

The life cycle of Bd includes invasion of amphibian skin with motile zoospores and forms a spherical thallus, which matures and produces new zoospores by dividing asexually, renewing the cycle of infection when zoospores are released to the skin surface (BERGER *et al.,* 2005; DASZAK *et al.,* 2010), thus causing the onset of chytridiomycosis, loss of the integument functionality, skin ulcers, and eventually animal death (VOYLES *et al.,* 2009).

Wound healing (WH) is an evolutionarily conserved process leading to tissue restoration that occurs in Amphibians without scar formation. Skin antimicrobial peptides secreted by granular glands contribute to speed up WH process thus increasing individual fitness.

A better understanding of why some species seem to resist several environmental insults can help to limit the ongoing amphibian decline through the development of appropriate strategies, particularly against pathologies such as viral and fungal infections.

MATERIALS AND METHODS

Two frog species *Gastrotheca nebulanastes* (GN) and *Gastrotheca excubitor* (GE), were collected in montane scrub, cloud forest and high elevation

grassland habitats near Manu National Park in southeastern Peru (permits to prof. Alessandro Catenazzi: Protocol approval: IACUC-19-042-CR02, Topaz reference: 200867, approval date: 04/06/21, expiration date: 05/13/22; Protocol approval: IACUC-19-042-CR01, Topaz reference: 200867, approval date: 04/24/2020, expiration date: 05/13/2021; Protocol approval: IACUC-19-042, Topaz reference: 200867, approval date: 05/13/2019, expiration date: 05/13/2020). Peptides secretion was stimulated by injection of norepinephrine into the dorsal lymph sacks. Peptides were then purified by chromatographic techniques. The human endothelial cell line HECV was treated with Peptide concentrations ranging from 0.005 to 50 g/mL. Cell viability was verified by MTT test. Wound Healing properties were analyzed by scratch wound assay. Briefly, the cells were seeded on 12 multiwell plates and cultured until confluence. Cell monolayers were scraped with a yellow tip making one line to create a "scratch" (POZZOLINI *et al.,* 2016; VERGANI *et al.,* 2018). After washing, scratch images were recorded under the microscope at time 0 (T0). Then the medium was replaced with fresh medium containing different concentrations of peptide mixtures. After 24 h, scratch images were acquired again (T24). Distance between edges was measured and compared with the value obtained before treatment (Fig. 1).

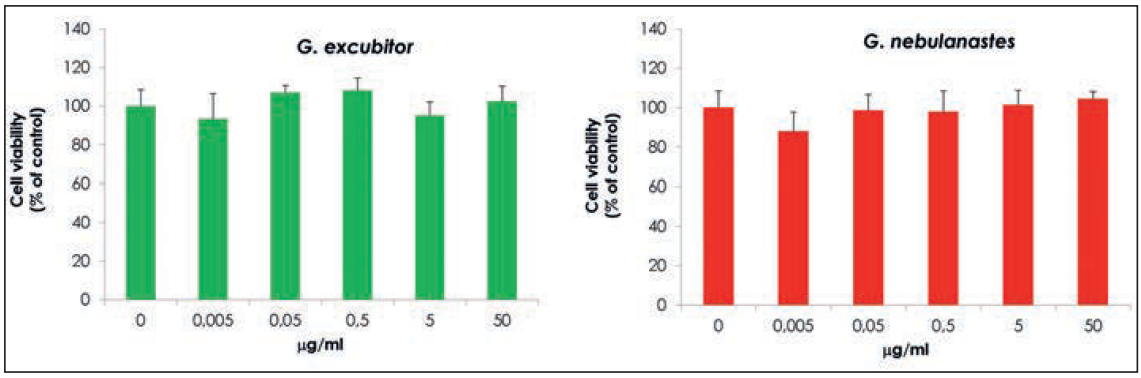

Fig. 1 — Assay performed in this work.

RESULTS

Peptide secretions derived from several individuals of *Gastrotheca nebulanastes* and *Gastrotheca excubitor* were tested at a concentration ranging 0.005-50 µg/ml with tenfold dilutions. Results showed no statistical differences in cell viability for any of the three peptide mixtures at any concentrations tested (Fig. 2). These results allowed us to evaluate other biological activities of peptide mixtures without any interference given by putative peptides cytotoxicity.

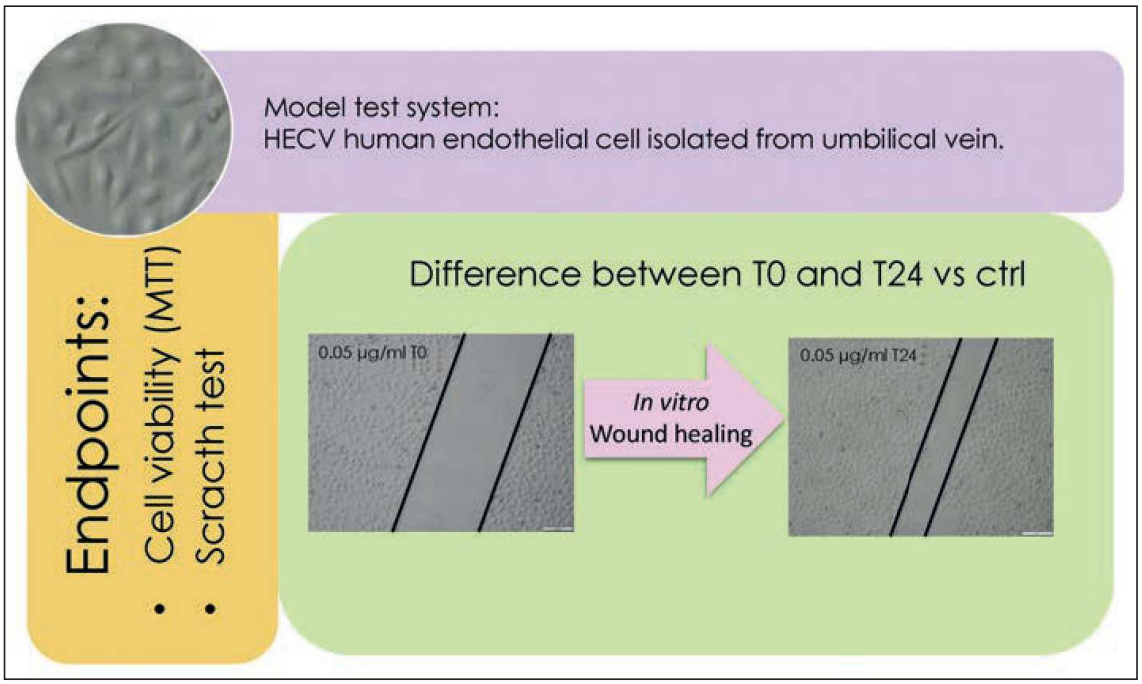

Fig. 2 — Cell viability of peptide secretions derived from several individuals of *Gastrotheca nebulanastes* and *Gastrotheca excubitor*.

Wound healing assay performed on human endothelial cells (HECV) is a two-dimensional simulation of a tissue rupture achieved by scratching cell monolayer with a yellow tip. Then the peptide mixture isolated from *Gastrotheca nebulanastes* or *Gastrotheca excubitor* were administered to the scratched monolayer for 24 h. The difference between T0 and T24 allowed us to establish if peptide mixture was able to increase velocity of heal. Unexpectedly, none of the two peptide mixtures at none of the concentrations tested differed from the controls.

CONCLUSIONS

The capability to speed up wound healing process can be viewed as a possible mechanism of defence against *Batrachochytrium dendrobatidis* (LOGCORE *et al.,* 1999). To this aim, we tested WH capability of peptide mixtures isolated from *Gastrotheca excubitor* and *G. nebulanastes* of displaying different susceptibility. The working hypothesis was to assess the presence of some correlation among WH capability and Bd susceptibility. However, we failed to demonstrate healing properties for peptide mixtures from *G. excubitor* and *G. nebulanastes* in an *in vitro* cellular model of WH.

All these observations confirm that defence mechanisms of amphibian skin

are complex and species-specific, involving different pathways that still need to be elucidated.

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