The Cytotoxic Effect of Slow Loris (Nycticebus) Venom on Human Epidermal carcinoma cells

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Abstract

Within the Kingdom Mammalia, venom evolution is rare, occurring in only six orders. Arguably the most cryptic, and academically neglected venom occurs within primates among slow lorises (*Nycticebus* spp.). Venoms comprise novel biological compounds with a potential plethora of proteins and peptides available for utilisation in bio-medical research. We collected samples of slow loris saliva from eight captive-bred pygmy slow lorises (*N. pygmaeus*) at Paignton Zoo and Shaldon Wildlife Trust UK, given voluntarily as slow lorises chewed on Salimetrics children's swabs. From January to March 2017, we employed MTT assays, and microscopy assessments to determine cell survival on human epidermal carcinoma cells (A431 line) after the application of concentrations of slow loris salivary venom. Cell survival from both male and female derived saliva was half that of untreated cells. Cytotoxic action is demonstrated in concentrations as low as 0.01% venom. Results demonstrate a cytotoxic effect with ensuing physiological damage on human cancer cells, demonstrating the cytotoxic action of slow loris saliva only, without the admixture of brachial gland exudate. We show that even captive-bred slow loris saliva harbours potentially dangerous substances, with functional applications towards slow loris husbandry. Knowledge of slow loris salivary venom increases understanding of the novel salivary composition and supports discussions of slow loris conservation by proposing a functional narrative to oppose the illegal pet trade, by contradicting their 'cute and cuddly' appeal. Evidence of salivary venom shows that cytotoxic effects can result even in the absence of a bite puncturing the skin, and further demonstrates their inappropriateness as pets.

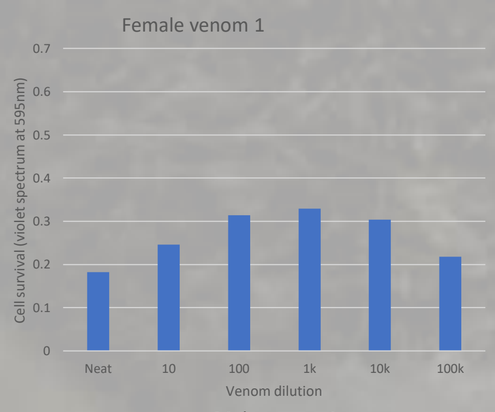
Introduction

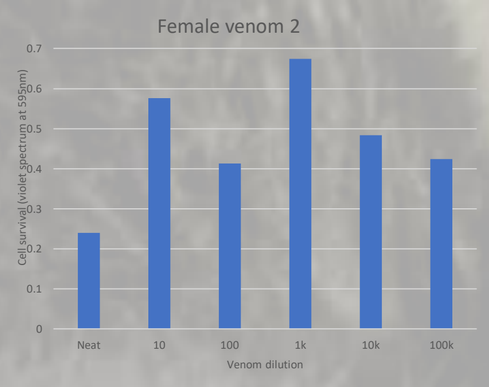
- **Slow lorises (*Nycticebus spp*.) are small bodied nocturnal primates found in southwest Asia[1]**
- **Wild populations are being decimated by the illegal pet trade[2], fuelled by social media presence[3].**
- **Slow lorises are the only known venomous primate[4]. Nycticebus venom has been demonstrated as comprising of two entities: on brachial gland exudate, with the ad-mix of saliva [5].**
- **The venom has been demonstrated as being fatal to mice[5], containing a variation of the cat allergen protein Fel-D1 [6] repelling certain predators[7] and acting as a anti-parasite agent[8].**
- **Wild and captive slow loris populations have been documented suffering prolonged (e.g. festering, necrosis and infection), and often fatal injuries following intraspecific bite wounds [9, 10].**
- **Documented accounts of human loris bite wounds detail near-fatal anaphylaxis [11, 12].**

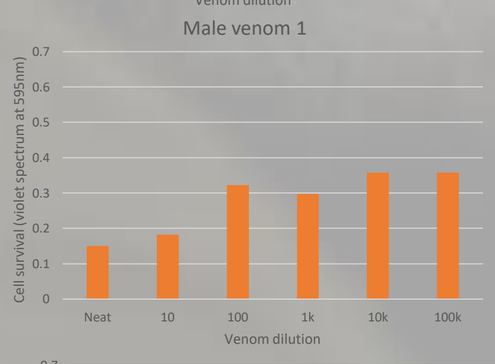
Methods

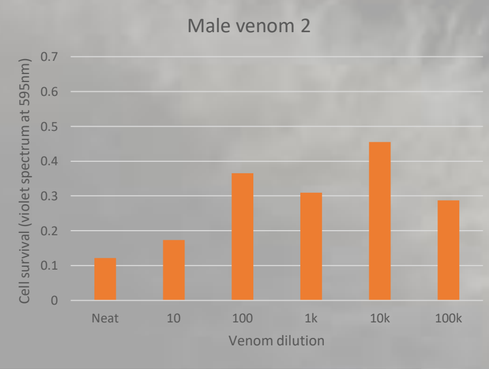
- ***N. pygmaeus* saliva samples were voluntarily collected from Paignton zoo, and Shaldon wildlife trust.**
- **I performed a MTT assay to evaluate the mitochondrial activity following the method of Mosmann [13] and Hussain et al [14].**
- **Cells were trypsinised to prevent adherence, 40,000 cells per well were, seeded in a 96-well plate. Twenty-four hours after seeding, I exposed the cells to freshly prepared dilutions of slow loris venom**
- **Cells were exposed to neat venom, and dilutions of 1/10, 1/100, 1/1000, 1/10000 and 1/100000 two hours, under incubation at 95% co^2 38.5 degrees Celsius**
- **venom media was discarded and MTT dye (5-diphenyltetrazolium bromide] was then added (100ul per well).**
- **Ensuing foramen crystals were solubilised in 50ul SDS and quantified by measuring absorbance at 595nm in a microplate photometers, (Synergy H1 multi-mode reader), using Gen5 software.**
- **Unpaired T-tests were performed using SPSS statistics 22 software.**
- **Five repetitions were performed from two male, and two female samples, and due to the sample collection technique a honey control was performed.**
- **Additional microscopic analysis was performed to visually assess cell destruction.**

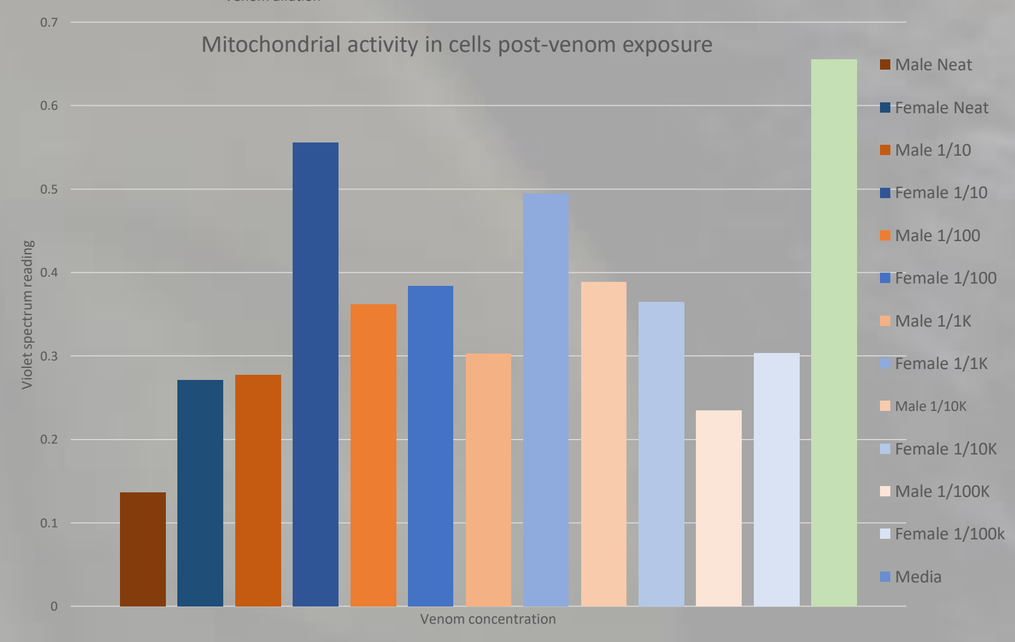
Figure 2. Detailing the mitochondrial activity (cell survival) post-MTT assay. Individual female and male samples are presented, followed by average male/female vales alongside the media control. Note all Y axis are set to a maximum of 0.7 to enable comparisons.











Results

- **The MTT dye Cleaves to mitochondria present in live cells, creating purple precipitate: a direct proxy for cell survival [13] – precipitates (and therefore cells) were showed a negative correlation with applied venom concentration (Figure 1).**
- **Results detail cytotoxicity in concentrations as low as 0.001% venom; this difference is statistically significant (T-test P = 0.05).**
- **Cytotoxicity in media-venom exposed cells displayed a survival rate up to half that of control measures, and significantly greater than the honey control.**
- **Male and female venom show cytotoxicity; male venom is more cytotoxic overall (figure 2).**
- **Microscopy details a high level of cell destruction without the forced cell permeation employed in MTT assays, at concentrations as low as 0.0001% venom (Figure 3)**

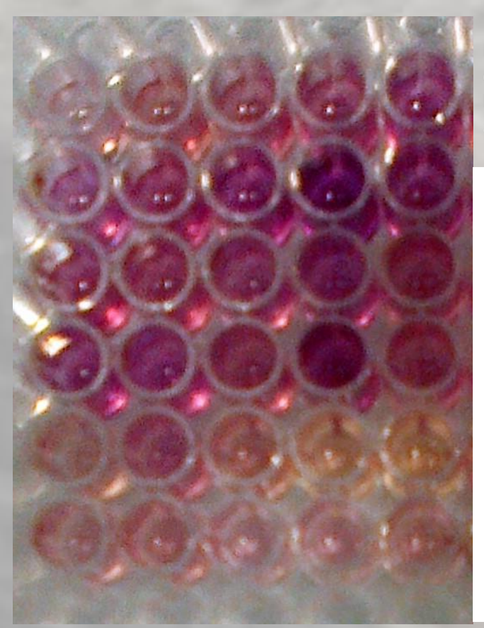
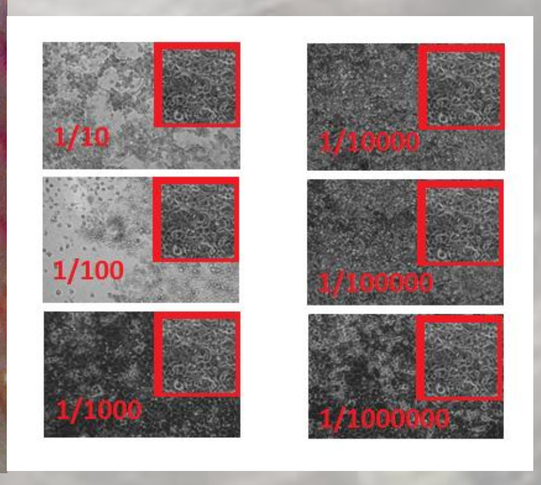


Figure 1. (left) detailing the purple precipitate, a proxy for cell survival in male venom sample 1.
Figure 3. (below) detailing cell degradation after the application of varying concentrations of venom (in red)



Concluding discussion

- **The MTT assay demonstrates slow loris saliva conforms to Fry et al 's (2009) widely accepted definition of a venom; without the ad-mix of brachial gland exudate.**
- **Venom possession contradicts slow lorises desired 'cute and cuddly' appeal, a driving force of illegal pet ownership. Increased knowledge of the chemical effects of slow loris venom therefore, helps to negate their appeal.**
- **Increased knowledge of slow loris venom aids in propagating a discourse highlighting their unsuitability as captive-companion animals.**
- **Additionally is has been demonstrated any new knowledge on a species aids in public engagement which increases support for related conservation initiatives.**
- **Knowledge of slow loris salivary venom can aid in captive management husbandry practices; as it demonstrates even small wounds have the potential to cause epidermal cell destruction and ensuing physiological damage.**

References cited:

1. Nekaris, K.A.I., 2014. *Extreme primates: Ecology and evolution of Asian lorises*. Evolutionary Anthropology: Issues, News, and Reviews, 23(5), pp.177-187.
2. Nijman, V., 2010. *An overview of international wildlife trade from Southeast Asia*. Biodiversity and conservation, 19(4), pp.1101-1114.
3. Nekaris, K.A.I., Campbell, N., Coggins, T.G., Rode, E.J. and Nijman, V., 2013 (A). *Tickled to death: analysing public perceptions of 'cute'videos of threatened species (slow lorises–Nycticebus spp.) on Web 2.0 Sites*. PloS one, 8(7), p.e69215.
4. Nekaris, K.A.I., Moore, R.S., Rode, E.J. and Fry, B.G., 2013. *Mad, bad and dangerous to know: the biochemistry, ecology and evolution of slow loris venom*. Journal of Venomous Animals and Toxins including Tropical Diseases, 19(1), p.21
5. Alterman, L., 1995. Toxins and toothcombs: potential allospecific chemical defenses in Nycticebus and Perodicticus. In *Creatures of the Dark* (pp. 413-424). Springer US
6. Krane, S., Itagaki, Y., Nakanishi, K. and Weldon, P.J., 2003. " *Venom" of the slow loris: sequence similarity of prosimian skin gland protein and Fel d 1 cat allergen*. Naturwissenschaften, 90(2), pp.60-62.
7. Nekaris, K. A. I., Pimley, E. R., and Ablard, K. M. (2007). Predator defense by slender lorises and pottos. In 'Primate Anti-Predator Strategies'. (Eds S. Gursky and K.A.I. Nekaris.) pp. 220–238. (Springer: New York.)
8. Grow, N.B. and Nekaris, K.A.I., 2015. *Does toxic defence in Nycticebus spp. relate to ectoparasites? The lethal effects of slow loris venom on arthropods.* Toxicon, 95, pp.1-5
9. Rode-Margono, J. E., & Nekaris, K. (2015). Cabinet of curiosities: Venom systems and their ecological function in mammals, with a focus on primates. *Toxins, 7*(7), 2639-2658.
10. Fuller, G., Lukas, K. E., Kuhar, C. W., & Dennis, P. M. (2014). A retrospective review of mortality in lorises and pottos in North American zoos, 1980-2010. *Endangered Species Research, 23*, 205-217.
11. Madani, G. and Nekaris, K.A.I., 2014. *Anaphylactic shock following the bite of a wild Kayan slow loris (Nycticebus kayan): implications for slow loris conservation*. Journal of venomous animals and toxins including tropical diseases, 20(1)
12. Wilde, H., 1972. Anaphylactic shock following bite by a slow loris, Nycticebus coucang. *Am J Trop Med Hyg*, *21*(5), pp.592-594.
13. Mosmann, Tim. "*Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay"* Journal of immunological methods 65, no. 1-2 (1983): 55-63.
14. Hussain, R.F., Nouri, A.M.E. and Oliver, R.T.D., 1993. *A new approach for measurement of cytotoxicity using colorimetric assay*. Journal of immunological methods, *160*(1), pp.89-96.
15. Moorhouse TP, Balaskas MB, D'Cruze N, Macdonald DW (2016) *Information could reduce consumer demand for exotic pets.* Conservation Letters. doi: 10.1111/conl.12270
16. Nekaris KAI, Campbell N (2012) *Media attention promotes conservation of threatened Asian slow lorises*. Oryx 46: 169–170.

Background image: adapted from Williams, P . (2014) '*Rescued Javan Slow Loris in a holding cage, Jakarta, Indonesia*'. Iron Ammonite photography. Available at: http://www.ironammonite.com/2014_03_01_archive.html