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Effect of environmental and physiological variables on saliva production in *Hirudinaria granulosa* (Indian cattle leech)

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Abstract

Rhythmicity in the saliva production of leech *Hirudinaria granulosa* has not been evaluated. This is, possibly, the first effort to evaluate the amount of saliva production under different environmental and physiological variables. A total of 90 leeches were divided into six groups (n=15) to elucidate the variation in saliva production based on temperature, body weight, and time period. Significantly ($P \leq 0.01$) higher volume of saliva was produced (2.23 ± 0.10) at a temperature of 37°C compared to that at 25°C (1.53 ± 0.11). Based on body weight, a significantly ($P \leq 0.01$) higher amount of saliva (2.52 ± 0.05) was produced by the leeches of the large group ($\geq 0.4\text{g}$ weight) as compared to the smaller group (1.66 ± 0.08) with a bodyweight of ($\leq 0.3\text{g}$ weight). In addition, the Pre-feeding and post feeding body weight of leeches (n=30) with saliva production revealed significantly high correlation of 0.962 and 0.870. Evaluation based on periodicity revealed a significantly higher amount of saliva production in the morning hours (2.32 ± 0.09) compared to evening hours (2.01 ± 0.10). Thus, our study concluded a relatively more elevated amount of saliva production in the morning hours at a temperature of 37°C with higher body weight. Therefore, our study infers biological rhythms in the leech *Hirudinaria granulosa*.

Keywords: *Hirudinaria granulosa*, temperature, body weight, periodicity

1. Introduction

Hirudotherapy or leech therapy is one of the astounding noninvasive alternative treatments in medicine that employs medicinal leeches for various medical ailments (Whitaker *et al.*, 2004) [15]. Bloodletting and the therapeutic use of medicinal leeches have been reported in ancient Egypt and is currently practised in dermatology, neurology, gynaecology, and surgery with splendid results (Mory *et al.*, 2000) [11]. Medicinal properties have been attributed to leech saliva that contains more than 100 bioactive substances (Fort 2001; Abdullah *et al.*, 2012) [7, 3]. For studying the medicinal properties of leech's efforts are being made to harvest leech saliva. Saliva production in humans and animals is under the control of circadian rhythm (Zheng *et al.* 2012) [16]. Circadian rhythms of motor behaviour are exhibited by a wide range of organisms and these regular rhythms assist animals in synchronizing their activities with changing environment including light and temperature fluctuations and availability of the prey (Rivkees 2007) [14]. Studies have revealed higher salivary flow rates on the elevation of body temperature in rats (Kanosue *et al.* 1990) [9]. Leeches also exhibit rhythmicity concerning some behaviour like swimming pattern (Angstadt and Moore 1997) [4], but circadian rhythm-based studies on saliva production in leeches are lacking. In case of leeches, feeding behaviour is also believed to be influenced by external factors (Abdualkader *et al.* 2011, 2013) [1, 2] and the amount of blood sucked by leeches depends on their body weight (Dickinson and Lent 1984) [6]. This study evaluated saliva production in leeches as influenced by physiological and environmental variables, including temperature, body weight, and periodicity.

2. Materials and Methods

This study was carried out in the Division of Clinical Veterinary Medicine, Ethics and Jurisprudence, Faculty of Veterinary Sciences and Animal Husbandry SKUAST Kashmir, Srinagar. All leeches were handled and studied adequately after getting approval from the Institute's animal ethics committee (Faculty of Veterinary Sciences and Animal Husbandry SKUAST Kashmir, Srinagar).

2.1 Procurement and maintenance of leeches

A total of 90 (N=15) medicinal leeches *Hirudinaria granulosa* (Fig 1) starved for three months were purchased from Regional Research Institute of Unani Medicine, Hazratbal Srinagar and were housed in well-aerated plastic containers filled with fresh water under 12h:12h light and dark cycle at the room temperature (25°C). Freshwater (dechlorinated) was regularly changed every two days. All leeches were divided into six groups of 15 leeches each.



Fig 1: Dorsal view of *Hirudinaria granulosa* (Indian cattle leech)

2.2 Preparation of phagostimulatory solution and leech feeding

To harvest leech saliva, leeches were fed a phagostimulatory solution containing 0.001 M Arginine in 0.15M sodium

chloride (Abdualkader *et al.* 2011) [1]. For saliva collection, the leeches were allowed to bite the parafilm wrapped around the open end of a glass funnel filled with the phagostimulatory solution (Fig 2a, 2b). The leeches were allowed to continue with the sucking of the phagostimulatory solution till they got satiated and dropped down. After feeding all leeches were transferred to clean polythene bags and kept in ice for 15-20 minutes to force them to vomit all the sucked solution (Fig 3).

Then, the leeches were squeezed from the posterior end forwards to get out the remaining sucked solution. The collected fluid was then centrifuged at 4°C for 10 min at 9000 rpm. The resulted fluid (crude leech saliva) was measured and lyophilized within 24 hours and stored at -80 °C for future scientific studies. After saliva collection, the leeches were put back in well-aerated plastic containers. For evaluating the effect of body weight on the amount of saliva harvested, the leeches were divided into two groups of 15 leeches each. Group I (n=15) included leeches with a bodyweight (≤ 0.3 g) and the group II had leeches with a bodyweight of (≥ 0.4 g). For evaluating the impact of temperature and rhythmicity on the amount of saliva harvested, the leeches were randomly divided into four groups of 15 leeches each. Group III and group IV were fed at two different temperatures (25 °C and 37 °C) whereas group V and Group VI were fed at morning and evening hours.



Fig 2a: Leech feeding at temperature 37 °C



Fig 2b: Leech feeding at temperature 25°C



Fig 3: Leeches kept in well closed plastic bags immersed in an ice container

2.3 Statistical analysis

Data were analyzed by SPSS Software for mean, standard error and significance by applying independent t-test. The pearson correlation was also analyzed using the same software. Values with $P \leq 0.05$ and $P \leq 0.01$ were considered as statistically significant.

3. Results

The effect of body weight on saliva production (mean \pm SE) is shown in Table 1. Pre feeding and post-feeding body weight revealed the relatively higher amount of saliva production (2.52 ± 0.05) in the leeches of large group (≥ 0.4 g weight) as compared to the leeches of a smaller group (≤ 0.3 g weight) (1.66 ± 0.08), and the difference was statistically significant ($P \leq 0.01$). This difference was further analyzed statistically for determining correlation between variables of body weight and saliva production. Pre-feeding bodyweight of leeches (n=30)

revealed a high correlation with a correlation coefficient of 0.962 whereas Correlation of post-feeding bodyweight of leeches (n=30) with saliva production revealed the values of 0.87 with strong correlation between two variables (Table 2). The mean amount of saliva production (mean±SE) based on temperature is given in Table 1. There was highly significant ($P \leq 0.01$) difference in the amount of saliva produced between two groups of leeches with one group (n=15) at temperature 37°C (2.23±0.10) as compared to another group (n=8) at 25°C (1.53±0.11).

Feeding behaviour revealed, all leeches (n=15) in Group II consumed phagostimulatory solution (0.001 M Arginine in 0.15 M sodium chloride) at a temperature of 37°C. At 25°C, out of 15 leeches only eight leeches sucked the phagostimulatory solution, as shown in Figure 1. Effect of the time on saliva production (mean±SE) is shown in Table 1. While feeding at morning hours, a higher amount of saliva

production was recorded (2.32 ±0.09) compared to evening hours (2.01±0.10) at a uniform temperature of 37 °C, the difference of means was statistically significant ($P \leq 0.05$).

Table 1: Saliva production based on environmental and physiological variables in the Indian cattle leech (*Hirudinaria granulosa*)

Variable	N	Mean ±SE	P Value
Temperature	25°C	1.53±0.11 ^a	0.00
	37°C	2.23±0.10 ^b	(≤0.01)
Body weight	Small (≤ 0.3 g)	1.66±0.08 ^a	0.00
	Large (≥ 0.4 g)	2.52±0.05 ^b	(≤0.01)
Periodicity	Morning	2.32 ±0.09 ^A	0.04
	Evening	2.01±0.10 ^B	(≤0.05)

Superscripts a, b shows significant difference for temperature and body weight variable at significance level of ≤0.01 and superscripts A, B show significant difference for temperature and body weight variable at significance level of ≤0.05.

Table 2: Pearson Correlation of body weights (pre-feeding and post-feeding) of Indian cattle leech (*Hirudinaria granulosa*) with their saliva production

Parameters	Mean ±Sd	Correlation		
		Pre-feeding	Post-feeding	Saliva production
Pre-feeding body weight (n=30)	0.454±0.18	1	0.948	0.962**
Post-feeding body weight (n=30)	3.084±1.74	0.948**	1	0.870**
Saliva production	2.090±0.51	0.962**	0.870**	1

**Correlation is significant at the level 0.01 (2-tailed)

4. Discussion

Leeches have traditionally been used for medicinal purpose for ages (Mory *et al.* 2000) [11]. The medicinal properties have been attributed to the bioactive principles present in the saliva of leeches. Efforts have been made to harvest the leech saliva for scientific studies and to evaluate its therapeutic potential in human and animal medicine. The present study aims at assessing some environmental and physiological parameters for their impact on harvesting leech saliva.

Circadian rhythms are endogenously generated fluctuations that occur within 24 hours (Zheng *et al.* 2012) [16]. External environmental indicators (day and night cycle) are the chief determiner of biological rhythms (Reinberg and Ashkenazi, 2003; Benstaali *et al.* 2001) [13, 5]. In annelids like leeches, biological rhythm studies are still in the early phase with some minor studies on its swimming pattern (Friesen and Kristan Jr 2007) [8]. Our present study demonstrated the influence of environmental and physiological factors on rhythmicity of saliva secretion in the medicinal leech (*Hirudinaria granulosa*). The higher amount of saliva secretion recorded at 37°C may be due to the simulation of the temperature of the phagostimulatory solution to that of body temperature of the host. This result corroborated with one study that shows leeches prefer feeding at warmer temperatures (Peterson *et al.* 2011) [12]. Hence, our trial also revealed that the Indian cattle leeches usually bite repeatedly upon warm parafilm surfaces. Our study also observed that biting frequency decreased when the temperature of the phagostimulatory solution decreased from 37°C to 25 °C, and when the temperature exceeds 40°C. For this, the phagostimulatory solution was again warmed to 37°C to increase the biting frequency.

Increased saliva production recorded in morning hours may be due to the freshness of leeches and non-agitated behaviour. Therefore, morning hours are preferred for hirudotherapy (Kumar *et al.* 2012) [10]. Leeches of higher body weight produce a higher volume of saliva that may be due to the higher capacity of storing the phagostimulatory solution and

subsequently its excretion from the body. The amount of phagostimulatory solution sucked by the leeches depends on their body weight since it has been described that medicinal leeches can gulp down 8.9±0.2 times of their preliminary body weight (Dickinson and Lent 1984) [6].

Hence this study provides baseline insights about the biological rhythms of the leech *Hirudinaria granulosa* (Indian cattle leech) with prospects for the exploration and further medicinal applications.

5. Conclusion

Biological rhythms are inevitable processes in the physiology of organisms. They reflect the wellbeing and normal functioning of the various biological systems in the organisms. Saliva production in leeches represents one such rhythmicity. Our study indicated a relatively higher amount of saliva production in the morning hours at a temperature of 37 °C in *Hirudinaria Granulosa*. Our analysis also revealed higher saliva production by leeches with higher body weight.

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7. Disclosure of interest

Authors declare no conflict of interest.

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