Tropical Natural History 16(1):33-42, April 2016

Distribution of Body Fluid in Reticulated Python, *Broghammerus reticulatus* (Schneider, 1801), During Subcutaneous Administration with Ringer's Acetate Solution

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Received: 18 August 2015; Accepted: 17 March 2016

Abstract.– Alterations of the distribution of body fluid were studied in Reticulated Python, *Broghammerus reticulatus*, (Schneider, 1801), which were subcutaneously injected with 20 ml per kg body weight of Ringer's acetate (RA) solution. The distribution of body fluid was observed by monitoring plasma volume (PV), blood volume (BV), extracellular fluid (ECF) and intracellular fluid (ICF) volumes including total body water (TBW) and water turnover (WTO) rate prior to treatment and treatment periods. The Reticulated Python erythrocyte as a representative of a cell model was performed *in vitro* to determine intracellular sodium (Na^+) and potassium (K^+) ion concentrations including the mean corpuscular fragility (MCF) index during fluid loading. The results demonstrated that the PV, BV, ECF and TBW significantly increased at 60 minutes after administration of RA solution, while as ICF volume, WTO rate, and Na^+ and K^+ concentrations were not significantly disturbed. The Na^+ and K^+ concentrations in erythrocytes were higher in ICF than in the plasma in both pretreatment and treatment periods. MCF index showed that 0.26% of NaCl solution caused erythrocyte to be lysed. This study suggests that only ECF volume was increased during fluid loading with RA solution in the Reticulated Python. However, the ICF volume was not disturbed with such treatment due to high resistance of erythrocyte membrane to water permeability.

KEY WORDS: Body fluid distribution, reticulated python, Ringer's acetate solution

INTRODUCTION

There are a number of biodiversities of both animals and plants are investigated in the tropical countries. Reptiles are usually considered as the first vertebrates capable of completing their life cycle on dry land. Reptiles, particularly, snake species, have fascinated to scientists for many decades. In Thailand, at least 196 species of snakes have been reported with 137 species of non-venomous snakes and 59 species of venomous snake (Chanhome, et al., 2001; 2011). Snakes are known to involve in both extensive morphological and physiological adaptation. Most snakes evolve a suite of radical adaptations to consume large prey relative to their body size, including the ability to endure extreme adverse physiological fluctuation. Additionally, a few data are available on the physiological phenomenon of body water economy in snakes.

The Reticulated Python, *Broghammerus reticulatus*, (Schneider, 1801), is the largest and longest group of snake species in the world. It is a terrestrial snake usually having habitat near water in the wild and sometimes is found at the riverside of cities like Bangkok. Reticulated Python was distributed widely in tropical zones of Southeast Asia, from Laos southward to the

Malay Archipelago, Philippine island and Indonesia. (Auliya, et al., 2002). The Reticulated Python is believed to involve in the balance of ecological system with the high consumer of terrestrial food chain of small animals. The natural diets of the Reticulated Python is largely made up of birds and small mammals especially rodents (Auliya, et al., 2002; Fredriksson, 2005). It maintains body fluids more than amphibians and less dependent on the availability of fresh water and humid environments. The ways of water gain in the snake are drinking, feeding and metabolism, including an uptake across the skin, while the ways of water loss occur via urine, feces and evaporation via respiration. Cutaneous water loss is low in comparison to all other vertebrates species (Bennett and Licht 1975). However, the mechanism responsible for alteration in body fluid during feed intake, especially in the reticulated python, has not been determined, although feed consumption with large prey relative to their body size has been observed in reticulated pythons. Information on body fluid distribution in reticulated pythons is limited, although the most common type of dehydration in reptile is isotonic dehydration and some incidence of fluid retention was observed in sick snake after subcutaneous normal saline administration (Taksa Vasaruchaponga, unpublished data). Whether the regulation of body fluid in reticulated pythons is different from other vertebrate species, should be investigated. As part of a study aimed to elucidate the parameters responsible for the regulation of body fluids variables in the reticulated python in order to determine whether any alteration could be identified which might themselves be factors responsible for body fluid retention. Moreover, since dehydration and starvation may occur in wild animals under natural conditions in the harsh environment, the results may be of wider interest. To provide some of this information, an investigation has been carried out by exogenous loading body fluid with subcutaneous injection of Ringer's acetate solution. The distribution of fluid in different compartments of in extracellular fluid and intracellular fluid, including water turnover rate in reticulated python during fluid loading were carried out. To obtain a more complete picture on water and electrolyte exchanged between body fluid compartments, measurements of other parameters relevant to the process of body fluids regulation, i.e. concentrations of electrolytes in both plasma and erythrocytes including the mean corpuscular fragility of erythrocytes were also made during subcutaneous administration of Ringer's acetate solution in reticulated python.

MATERIALS AND METHODS

Animals

Eight reticulated pythons, body weight ranging from 3.90 to 12.00 kg, were employed in this study. They were housed at the Snake Farm of Queen Saovabha Memorial Institute (QSMI), The Thai Red Cross Society, Bangkok. Animals were kept in outdoor cage 2m×4m×1.8m and acclimatized for 3 months prior to study. The ambient temperature ranged between 27 and 29°C, with relative humidity between 70 and 80%. Laboratory rats were fed once a week to a snake in the amount of 10% of its body weight and clean water was supplied ad libitum each morning?. The protocol of the present study was submitted to the QSMI Animal Ethics Committee for principal and guideline in compliance with the National Research Council of Thailand guidelines for care and use of animals in research.

Experimental procedures

Eight Reticulated Pythons were allocated into two groups: Group I, comprising of five snakes, were used to study the distribution of body fluid and ICF Na+ and K+ concentrations in erythrocyte. Each snake was subcutaneously injected with 20 ml of Ringer's acetate solution (RA) per kg body weight. Group II, comprising of three reticulated pythons, were used to study MCF index which indicates the level of saline solution that causes erythrocyte fragility of 50%.

On the specific day of study, each snake was immobilized with 5% isoflurane (Abbott, Thailand) with a flow rate of 1 L/min. Polyethylene sterile catheter sized 20g (1.0×0.80×32mm); Terumo Corporation, Tokyo, Japan was inserted into dorsal palatine vein to facilitate marker injection. Blood samples were collected via coccygeal vein by puncture percutaneous with disposable needle (#21) into the heparinized tubes containing of 20 iu per ml whole blood for determinating TBW, ECF, PV, PCV and Na^+ and K^+ ion concentration in both plasma and erythrocyte. The measurement of these parameters was performed both in the pre-treatment period as a control period and the treatment period with loading of RA solution.

Determination of body fluid

At the beginning of either pretreatment or treatment period, single dose of 0.25 ml per kg body weight of 0.5% Evans blue marker, 0.5 ml per kg body weight of 15% sodium thiocyanate (NaSCN) marker and 0.1 ml per kg body weight of 15% urea marker were injected into each snake via intravenous route for determinations of PV, ECF and TBW, respectively on the basis of diluting principle. It has been demonstrated that volume of urea space is equivalent to total body water (Moore et al., 1956). The actual dose given of each marker was determined by subtracting the volume of the dead-space from the weight of solution delivered from the syringe. Blood samples for determination of body fluid were withdrawn at 20, 40, 60, 80 and 100 minutes via coccygeal vein and followed by injection of such markers in each period of study. 1.5 ml of heparinized blood sample was centrifuged and plasma sample was kept at -20°C for further analysis. Hematocrit, Hct, was measured after centrifugation of the blood in a micro-capillary tube.

Determinations of PV, ECF and TBW

The blood samples were centrifuged at 2300 ×g for 15 min to separate plasma layer. PV was measured at the wave length of 625 nm with a spectrophotometer. Plasma NaSCN and urea concentrations were determined by the methods of Medway and Kare (1959) and Fawcett and Scoott (1960), respectively. The concentration of marker was plotted on a semi logarithmic paper against time course (min) after dosing, and the dilution of marker was determined by extrapolation for concentration at theoretical zero time of complete mixing of marker in determination of PV, ECF and TBW as the following equation: The respective zero-time values were extrapolated for the plasma concentrations of Evans blue, NaSCN and urea found at 10, 20, 30, 40, 60 and 80 min following injection of each snake. PV, ECF and TBW were calculated as following:

$$V = \frac{\text{mg of marker injected}}{\text{sample concentration (mg per ml) at zero time}}$$

$$TBW = \frac{\text{dose of urea injected}}{\text{urea concentraton at zero time}}$$

$$BV = \frac{PV}{(1 - Hct)}$$

$$ICF = TBW - ECF$$

$$WTO = \frac{0.693 \text{ x } TBW}{\text{Half - life of urea}}$$

Determination of sodium, potassium ions concentrations in plasma and erythrocytes

At either pretreatment or treatment period, 2 ml of blood sample was collected from coccygeal vein into a microtube containing heparin at a concentration of 20 iu per ml whole blood. From each sample, 1 ml of blood was pipetted out into a centrifuged tube and centrifuged at 15000 ×g for 5 min to separate plasma layer. The other part of whole blood was frozen at −20°C to induce red cell hemolysis. Determination of the Na^+ and K^+ concentrations of' both whole blood and plasma were performed by flame photometer (Flam photometer 410c, Corning). Na^+ and K^+ concentrations in mEq per L red blood cell were calculated using the following formula (Chaiyabutr et *al.*, 1993):

$$(C_x)rbc = \frac{[(C_x)_w\ 100 - (C_x)_P\ (100 - PCV)]}{PCV}$$

where $(C_x)rbc$, $(C_x)_W$ and $(C_x)_P$ representatives for concentrations of electrolytes in red blood cells (mEq/l), hemolyzed whole blood and plasma, respectively.

Determination of MCF index of erythrocyte

5 mL of various concentrations of NaCl solution, namely 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and distilled water for 100% hemolysis were used in determination of MCF index. Two ml of whole blood was collected via coccygeal vein into a heparinized tube. 50 µl of Blood was added to each concentration and incubated at room temperature for 30 min and then centrifuged at 5000 ×g for 3 min. The supernatant was measured with spectrophotometer at a wavelength of 515nm for hemolytic content with distilled water serving as the blank. The percentage of hemolysis in each NaCl concentration was evaluated taking the tube with maximum hemolysis as 100%. The mean corpuscular fragility (MCF) index was analyzed for 50% of red cells hemolysis.

Statistical analysis

All data were presented as the means ± SD. Statistical significant difference between pre-treatment and treatment periods in the same group was determined by the paired t-test.

RESULTS

Changes in TBW, ECF, ICF, IF (interstitial fluid), WTO rate and water half-life.

The data in Table 1 showed that both absolute values and relative values per body weight of TBW, ECF and IF of the Reticulated Python were increased significantly at 60 min after subcutaneous injection of RA solution as compared to those values at the pre-treatment period. After administration of RA solution, there was no significant difference in ICF, WTO rate and water half-life ($T^{1/2}$) between pre-treatment and treatment period.

TABLE 1. Changes in the measured parameters of body fluids for total body water (TBW), extracellular fluid (ECF), intracellular fluid (ICF), interstitial fluid (IF), water turnover rate (WTO) and water half-life ($T^{1/2}$) of reticulated pythons treated with subcutaneous injection of Ringer acetate solution. (Values are mean ± SD, n=5)

Parameter	Pre-treatment	Treatment	P-value*
TBW (L)	4.83±3.00	5.02±3.02	0.002
TBW (L/100Kg)	78.97±7.44	82.71±8.49	0.014
ECF (L)	3.41±2.43	3.56±2.48	0.027
ECF (L/100Kg)	54.37±7.13	55.97±7.17	0.003
ICF (L)	1.41±0.60	1.46±0.59	NS
ICF (L/100Kg)	24.69±5.51	25.98±7.37	NS
IF (L)	3.17±2.32	3.29±2.36	0.050
IF (L/100Kg)	50.19±7.70	52.16±7.97	0.048
WTO rate (ml/hr)	61.3±28.9	64.3±-32.9	NS
T ½ (hr)	89.2±18.80	89.2±31.30	NS

*By paired t- test; significant differences at level P< 0.05 between pre-treatment and treatment periods; NS, not significant

Changes in PV, BV, Hct and plasma, erythrocyte Na^+ and K^+ of reticulated pythons

Changes in intravascular fluid were shown in Table 2. The levels of both absolute value and relative value per body weight of PV were significantly increased after administration of RA solution, which coincided with a marked increase in BV. However, there was no significant difference in Hct.

The concentrations of Na^+ and K^+ in plasma and intracellular of erythrocyte of the Reticulated Python were shown in Table 3. Na^+ and K^+ concentrations in plasma and intracellular of erythrocyte were not affected by the administration of RA solution. Intracellular Na^+ and K^+ concentrations in erythrocytes appeared to be markedly higher than those in plasma.

Mean corpuscular fragility index of erythrocyte of the Reticulated Python

The osmotic fragility curve of reticulated python erythrocytes was illustrated in Figure 1. An initial hemolysis of erythrocytes was observed at 0.46 % NaCl and complete hemolysis was observed at 0.23% NaCl. The mean corpuscular fragility (MCF) index for the concentration of NaCl producing 50% lysis of the reticulated python erythrocytes was 0.26% NaCl.

DISCUSSION

The result demonstrated that at the pretreatment period, the mean TBW of the Reticulated Python was 78.97% body weight. Such value corresponded well with that of other two species of snakes: 77.17% of the Black Rat Snake, *Elaphe obsolete obsolete*, and 77.2% of the Pacific Rattle Snake, *Crotalus viridis helleri* (Smits and

TABLE 2. Changes in the measured parameters of fluids in vascular compartment for plasma volume (PV), blood volume (BV) and hematocrit (Hct) of reticulated pythons treated with subcutaneous injection of Ringer's acetate solution.(Values are mean ± SD, n=5).

Parameter	Pre-treatment	Treatment	P-value*
Hct (%)	22.6±2.51	23.2±2.17	NS
PV (ml)	243±13	267±14	0.006
PV (L/100Kg)	4.14±1.36	4.62±1.32	0.018
BV (ml)	314±17	350±17	0.007
BV (L/100Kg)	5.35±1.71	6.01±1.69	0.017

*By paired t- test; significant differences at level P< 0.05 between pre-treatment and treatment periods; NS, not significant

Illywhite, 1985). However, the ECF of 54.37% body weight of the Reticulated Python was higher than those both in the Black Rat Snake and the Pacific Rattle Snake (41.2 and 42.9%, respectively) (Smits and Illywhite, 1985), including in lizard (Nagy, 1972). It is indicated that the relatively expanded ECF of the Reticulated Python may reflect on the common incidence of hydration relating to its existence on living nearly water in the tropical environment. The results showed that the relative values of both PV (4.1% body weight) and BV (5.4% body weight) of the Reticulated Pythons at pretreatment period were in the range as reported in other reptiles and mammals (Illywhite and Smits, 1984), whereas the relative value of ICF was 24.7% body weight, which was less than that reported in other snakes (ICF 35.2% of body weight) (Smits and Illywhite, 1985). These results can be explained in term of the large extracellular fluid volume that may distribute to interstitial space than that to vascular space in the Reticulated Python. The Reticulated python showed sign of fluid retention with high volume of ECF, whether are a part of the process of homeostasis for maintenance of body water for effort in tolerance when confronted with conditions of high environmental temperature and/or dehydration, which should be further investigated.

It was found that the PV, BV, IF, ECF

TABLE 3. The concentrations of sodium ion and potassium ion in plasma and intracellular of erythrocytes (RBC) of reticulated pythons treated with subcutaneous injection of Ringer's acetate solution. (Values are mean ± SD, n=5)

Parameter	Pre-treatment	Treatment	P-value*
Plasma Na^+(mEq/l)	153.88±9.58	149.80±6.05	NS
Plasma K^+ (mEq/l)	3.84±1.10	3.28±0.60	NS
RBC Na^+ (mEq/l rbc)	217.41±59.68	274.38±18.72	NS
RBC K^+ (mEq/l rbc)	72.61±18.72	66.62±20.41	NS

*By paired t- test; significant differences at level P< 0.05 between pre-treatment and treatment periods; NS, not significant

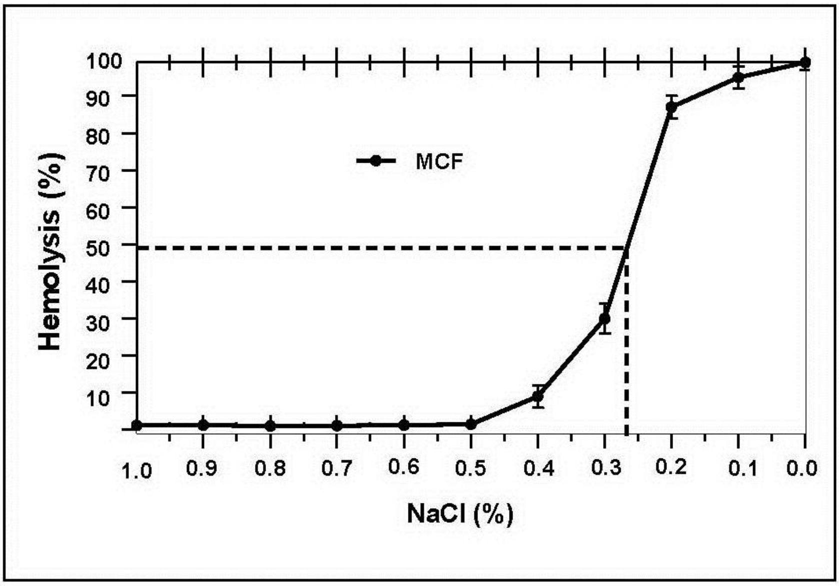

FIGURE 1. Osmotic fragility curve; mean corpuscular fragility (MCF) index for the concentration of 0.26% NaCl producing 50% hemolysis of 3 reticulated python erythrocytes.

and TBW significantly increased at 60 min after subcutaneous administration of RA solution. Fluid loading with RA solution in the Reticulated Python would affect primarily to increase in ECF volume especially in both plasma and IF compartments with less affecting to ICF compartment. This finding showed that the loading fluid entering ECF space was entirely transferred from subcutaneous to plasma and IF spaces, consequently, contributed to the expansion of ECF space, in other word, the Reticulated Python is capable of withstanding fluid retention. Low responsiveness of an increase in ICF space following RA loading probably involved in several factors, such as membrane integrity, Na^+ and K^+ concentrations and protein property in exerting on colloidal osmotic pressure between IF and ECF space, which augment water transfer from ECF to ICF compartment. It is well known that body water plays an essential role in the process in which heat dissipates via evaporative mechanism in vertebrates, especially in mammal. WTO rate in the Reticulated Python was not affected by a short period of RA solution loading. The magnitude of the WTO rate in this study was markedly less the range as compared with reported in mammals (Chaiyabutr et al., 1987). A number of studies in mammals indicated a high relationship between WTO rate to energy metabolism (MacFarlane and Howard, 1970) or food intake (King et al., 1982). In this study food intake on the WTO rate in the Reticulated Python was not expected to be affected due to an increase in energy metabolism as food was withheld prior to the period of study. Changes in WTO rate could not simply reflect a part of the process of adaptation to maintain normal body temperature during a rise in heat from exogenous sources.

The Na^+ and K^+ concentrations were not affected by RA solution loading (Table 3).

ICF Na^+ and K^+ concentrations in red blood cell appeared to be markedly higher than those in ECF in which the plasma Na^+ and K^+ concentrations were shown in normal physiological ranges as reported in other vertebrate species. It is known that influx of water and other substances is occurred by both the diffusion and permeability of the cell membrane. The asymmetric Na^+ and K^+ concentrations probably resulted from the activity of Na^+-K^+ ATPase that induced the Na^+, K^+- pump in mediating active transport of sodium out of the cell and potassium into the cell (Brown, 1986; Clausen and Flatman, 1987). Therefore, it is possible that a low number of membrane pumps and/or low efficiency of Na-K pump activity in python erythrocytes can lead to accumulation of both Na^+ and K^+ ions in cell reached at higher concentrations. During RA solution loading, no change in Na^+ and K^+ concentration depended on the permeability characteristic of the cell membrane of the Reticulated Python erythrocyte, which reduced a shift of osmotic water flow from ECF into ICF. However, few data was available on the control mechanism for equilibrium of the extremely high concentration of electrolytes in nucleated red blood cell of the Reticulated Python and whether it will relate to cell volume regulation will required to be explored, although a possible mechanism for Na^+ and K^+ concentration the cell has been noted. (Moore and Morrill 1976).

In the present study, the determination of the erythrocyte osmotic fragility of the Reticulated Python was also correlated to the permeability characteristic of the cell membrane during exogenous fluids loading. The result showed that mean corpuscular fragility (MCF) causing 50% hemolysis of the Reticulated Python erythrocyte was 0.26% NaCl which was lower than those of various mammalian erythrocytes: 0.53% NaCl in buffalo and 0.51% NaCl in cattle (Chaiyabutr et al., 1982). The low MCF suggests that the nucleated erythrocyte of the Reticulated Python was more resistant to hypotonic solution, although a high accumulation of Na^+ and K^+ concentrations was apparent in the cell. The high level of Na^+ and K^+ concentration in its erythrocyte remained constant in period of RA loading. It would be an adaptive advantage for the Reticulated Python in term of maintaining cell volume and stabilizing the integrity of cell membrane. This finding supports the report of Aldrich et al. (2006) that the nucleated erythrocyte of ectoderms possessed a greater osmotic resistance than the endoderm of the non-nucleated mammalian erythrocyte. Although the high ICF Na^+ and K^+ concentration was apparent in its erythrocytes, there was no indication whether such high ICF Na^+ and K^+ concentration directly affected to cell membrane capacity in resistance to cell swelling and maintained cell volume or not, which is still open to speculation. However, the high resistance to lysis of the nucleated erythrocyte has been shown to be involved in intrinsic membrane formation in which nucleus is anchored for cytoskeleton binding together with cell membrane (Centonze, 1986). Therefore, non-significant change in ICF volume following RA solution administration might be attributed to a high resistance of cell membrane which leads to low permeability of the cell membrane to water transport into the cell.

In conclusion**,** the data presented here show profound and relatively rapid effects on changes in PV, BV, ECF and TBW in the Reticulated Python at 60 min after subcutaneous injection with RA solution. These changes were affected primarily to increase in ECF volume with less affecting to ICF volume. Na^+ and K^+ concentrations in erythrocytes remained constant with the low MCF index between pretreatment and treatment period. Maintenance on erythrocyte integrity in low hypotonic solution demonstrates that simply reflect a part of a process of adaptation in term of maintaining cell volume and stabilizing the integrity of cell membrane. No alteration in ICF volume after RA loading would be attributed to the high resistance of cell membrane to permeability of the membrane for water transport into the cell. However, there is still for more information on physiological adaptive mechanism in the process of cell volume regulation in the Reticulated Python, particularly under natural conditions in harsh environments.

ACKNOWLEDGEMENTS

The authors would like to thank the staff of Queen Saovabha Memorial Institute (QSMI), The Thai Red Cross Society for devoting time and animals support for research collaboration.

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