UNIVERSIDADE VILA VELHA – ES

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

ESTABLISHMENT OF HEMATOLOGICAL AND BIOCHEMICAL VALUES FOR *Sicaelis flaveola, Amazona aestiva* (aves) and *Lepidochelys olivacea* (testudines)

FERNANDA ENDRINGER PINTO

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Dissertação apresentada à Universidade Vila Velha, como pré-requisito do Programa de Pós-graduação em Ciências Farmacêuticas, para a obtenção do grau de Mestre em Ciências Farmacêuticas.

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CHAPTER III

Comparative Clinical Pathology (IF:0.338) (Manuscript accepted for publication)

A hematologic and biochemical profile on three months-old hatchlings of *Lepidochelys olivacea*

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Abstract

Background: The olive ridley turtle (*Lepidochelys olivacea*) is globally distributed. However, the species is considered vulnerable. Additionally, hematological and biochemical data for this species are seldom published in the scientific literature.

Aim. The aim of this study was to gather hematological and biochemical data on young *Lepidochelys olivacea*, drawing comparisons with hematological and biochemical data already published for sea turtles.

Methods. Blood samples from 23 olive ridley sea turtles were collected and hematological and biochemical parameters were determined. Mean and standard deviation values were determined for the hematological and biochemical results. From these results, a comparison with the published results for other turtles was performed using a two-way ANOVA and a Bonferroni post-test.

Results. The results of this study are consistent with previous findings in other sea turtles. No differences were found between genders.

Conclusion. The results of the present study could be used as reference values by veterinarians in the treatment of olive ridley turtles as well as other species of sea turtles.

Implication. There is a need to standardize the samples and analyses used to determine the parameters evaluated here to allow the determination of reference values for marine turtles and more comparable results.

Keywords: Biochemistry, hematology, sea turtle, veterinary medicine.

1 Introduction

The olive ridley turtle (*Lepidochelys olivacea*) belongs to the family Cheloniidae, which also includes the green turtle (*Chelonia mydas*), loggerhead (*Caretta caretta*), hawksbill (*Eretmochelys imbricata*), Kemp's Ridley turtle (*Lepidochelys kempii*) and the flatback turtle (*Natator depressa*) (Casal et al. 2009). The olive ridley turtle is considered vulnerable (IUCN2013). It is the smallest species of living sea turtle and is highly migratory. These characteristics may explain its global distribution (Plotkin2003; Castilho and Tiwari 2006). It is carnivorous and is known for its mass nesting behavior (arribada) (Valverdeet al.1999).

For the conservation of marine turtles and reptiles in general, it is important to obtain hematological and biochemical data to assist in medical treatment and in clinical and pathological studies (Casal et al. 2009). Hematological and biochemical studies on turtles are still limited for various reasons (Kakizoe et al. 2007; Kania 2008), and little information of this type is available for marine turtle species. Moreover, the results of different studies of the topic in the scientific literature have not always been consistent. Several studies have cited a lack

of standardized sampling protocols and differences between the units used in different studies (Aguirre and Balazs 2000; Basile et al.2012). Many authors have reported that differences in methodology and instrumentation have produced misleading variation in the results (Boltenet al. 1992; Casal et al. 2009; Basile et al. 2012). Additionally, it has been stated that such inconsistencies could be related to the difficulty of differentiating between various cell types (Aguirre and Balazs 2000; Casal and Orós 2007; Basile et al. 2012).

Accordingly, the aim of this study is to obtain hematological and biochemical data on young *L. olivacea* and to draw quantitative comparisons with the results of other studies on sea turtles in the scientific literature. The authors seek to provide evidence of the advantages of a unique protocol for sampling and analyses to produce data that will be comparable with the results of further studies.

2 Materials and methods

2.1 Collection

The study was approved by the Animal Ethics Committee of the University of Vila Velha (nº. 250/2013) and by the Authorization and Information System on Biodiversity (SISBIO) (37597-1) and have therefore been performed in accordance with the ethical standards. Twenty-three newly laid *L. olivacea* eggs were collected the morning after spawning from five different nests in Santa Isabel Biological Reserve (10° 43'56" S, 36° 50'36" W), located at Pirambú Beach, Sergipe (SE), North-East of Brazil, in January 2013. After collection, the eggs were packed in polystyrene boxes containing moist sand and transported within 12 hours to the University of Vila Velha (UVV) - Espirito Santo (ES), South-East Brazil, where they remained in incubators with temperatures ranging from24 º C to 35 º C (approximately 1 º C variation between incubators). The temperatures were held constant during the incubation. After hatching, the young sea turtles were taken to the National MarineTurtle Conservation Program in Brazil (Projeto TAMAR), located in Vitória (ES) and remained at this location until the time of sample collection. They were kept in an aquarium until the age of three months to complete yolkabsorption and growth to facilitate the visualization of the gonads. Turtles were fed with a commercially available diet. Each animal was tagged to allow subsequent reliable identification.

Sampling for data collection was conducted in August of 2013. Blood was collected by puncturing the jugular vein with disposable insulin syringes, using a disposable small-caliber needle. One blood smear was performed without anticoagulants at the collection site, air dried and stained with hematoxylin and eosin (HE) (Newprov, Pinhais, PR, Brazil). Blood samples were collected and divided into two aliquots. One aliquot was collected in a microtiter Eppendorf tube with sodium heparin anticoagulant (Hemofol/Cristália) for hematological analysis. The other aliquot was stored in a microtiter Eppendorf tube without anticoagulant for biochemical analysis.

Serum was separated by centrifugation of the sample at 2000 g for 10 minutes and frozen (- 20 °C) until

analysis. All samples were identified and deep frozen until the time of analysis. Analyses were performed 15 days after collection.

2.2 Hematological Parameters

Counts of total red blood cells (RBCs), white blood cells (WBCs) and thrombocytes were obtained by manual counting in a Neubauer chamber. The Natt-Herrick diluting solution / dye (Natt and Herrick 1952) was used for the counts, consistent with current practice (Casal et al.2009). A differential leukocyte count was performed on blood smears stained with HE under a light microscope at 100x. The hematocrit (Ht) or packed cell volume (PCV) were determined by the standard micro- hematocrit method and are expressed as percentages.

2.3 Determination of Gender

The animals were euthanized with an intracardiac injection of 0.5 ml of thiopental (Thiopentax/Cristalia) diluted to 25%. The gonads were extracted for the preparation of histological slides and stained with HE. The gender of the young sea turtles was determined from histological analysis following the criteria of Malvasio et al. (2012), who consider immature ovarian follicles diagnostic for females and immature seminiferous tubules diagnostic for males.

2.4 Biochemical Parameters

Analyses were performed in serum with an automated analyzer (LabmaxPlenno-Labtest). The biochemical parameters measured were gamma glutamyltransferase (GGT), aspartate aminotransferase (AST), albumin, total protein (PT), creatinine, cholesterol, urea, calcium and phosphorus.

2.5 Statistical Analysis

The results were expressed in terms of the mean and standard deviation (SD). The mean and SD values obtained in the present study were compared with results previously published for various species of sea turtles. In addition to nonparametric tests, a two-way ANOVA and Bonferroni posttest were used, with a significance level of $p<0.05$. A t-test was used for comparisons between genders. Gender differences were considered significant if $p<0.05$. All statistical analyses were conducted using Graphpad Prism software.

3 Results

In this study we analyzed samples from 23 turtles (17 males and 6 females). No significant differences in hematology and biochemistry were observed between the genders. We did not find basophils or eosinophils. The

hematological data for *L. olivacea* are shown in table 1, where they are compared to hematological data for other sea turtles from the scientific literature. Table 2 shows the biochemical results for *L. olivacea* compared with biochemical data for other sea turtles from the scientific literature.

4 Discussion

The olive ridley turtle is classified as vulnerable in the IUCN Red List of Threatened Animals (IUCN2013). Therefore, it is of substantial importance to obtain data on this species to support its conservation and protection. No significant differences in hematology and biochemistry were observed between the genders. This finding is consistent with the results of previous studies (Innis et al. 2010). Many previous studies analyzed larger samples. However, the relatively low SD values found in the present study are consistent with a normal distribution.

The average found for Ht is similar to those reported by Knotkova et al. (2005) (table 1). A previous study found that the values of Ht increase with age (Kakizoe et al., 2007). This increase may explain the difference between the Ht value found in this study and those previously reported by other studies (table 1).

The RBC count of the present study is comparable with those found by several studies cited in table 1. The same consistency was observed for the WBC count (table 1). In general, the WBC counts reported in the literature are highly similar (table 1).

A differential leukocyte count showed that heterophils were the most frequent, followed by lymphocytes and monocytes. This result is similar to the findings of previous other studies (Knotkova et al. 2005; Deem et al. 2006;Casal et al. 2009;Rossi et al. 2009; Santos et al.2009;Innis et al. 2010; Acevedo et al. 2012). The mean values obtained for heterophils are equivalent to those reported in the literature (table 1). The mean values found for lymphocytes were comparable to those found by previous studies (table 1), and the mean values obtained for monocytes were also similar to those found by previous studies (table 1). Basophils and eosinophils were not found, but several studies have reported a scarcity or absence of basophils in blood smears in turtles (Cannon 1992; Work et al. 1998; Casal et al. 2009; Flint et al. 2010).

Various values for thrombocytes are cited in the literature reviewed for this study (table 1). Such information demonstrates the difficulty of measuring the concentration of thrombocytes in turtles, as noted by Casal et al. (2009), and the difficulty of differentiating between lymphocytes and thrombocytes in reptiles (Alleman et al. 1992; Work et al. 1998). However, the results found for thrombocytes by the present study are similar to those published by Basile and colleagues (2012) (table 1).

The mean biochemical values for creatinine, albumin, AST, PT, calcium and phosphorus were similar to the values reported by Santoro and Meneses (2007) for *L. olivacea* (table 2). These values found by the present study are also similar to those published by Fong et al. (2010) (table 2). The study of Fong et al. (2010) was

performed with the turtle *Chelonia mydas*, a member of the family Cheloniidae, which also includes *L. olivacea*. With the exception of the values for AST, these results are consistent with the findings of previous studies of juvenile and adult sea turtles of various species (table 2). The same pattern can be observed for a study conducted by Casal and Orós (2009) (table 2). AST values vary in studies of sea turtles (table 2). Although the value of AST obtained by the present study was not consistent with the AST values found by most of the studies we reviewed, our results were similar to those of Stewart et al. (2012) and Snoddy et al. (2009) (table 2).

Although the enzyme GGT is infrequently used to assess the health of marine turtles (Wilkinson 2004), this study and several others have measured GGT values, and the mean found in this study is similar to those reported by other studies cited in table 2. The concentration of cholesterol also differs among studies (table 2). The same variation applies for urea; the mean value found in this study is not similar to the mean values reportedby other studies (table 2).

The variations found for several parameters may have been influenced by different methods of analysis, geographical factors, seasonal factors, age, size and diet. Casal and Orós (2007) reported that age, geographical location and the methodology applied can affect the hematological and biochemical values obtained. . Differences to other studies may result from the age as most of the other studies conducted their research in juveniles and adult turtle, whereas the present study was conducted exclusively with three month old hatchlings. Although our data are quite comparable with those of other studies, it is necessary that the samples and analyses be more fully standardized to obtain comparable results and to determine reference values for marine species such as sea turtles.

5 Conclusions

The hematological and biochemical parameters measured in this study are influenced by many factors. These factors produce variation among the results of studies of various sea turtle species. As reference data for *L. olivacea* are scarce, it is difficult to establish normal values for hematological and biochemical parameters for this species. However, the results of this study furnish basic information for veterinarians in investigating treatments for *L. olivacea* as well as other species of sea turtles, as the results of our study were generally similarto those of the other studies reviewed. Note also that there is a need to standardize the samples and analyses usedto determine the parameters evaluated here to allow the determination of reference values for marine turtles.

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7 Conflict of interest statement

The authors declare there is no conflict of interest

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Table 1

Hematological data for *Lepidochelys olivacea* compared with hematological data for other sea turtles reported in the scientific literature.

		Ht (%)	**RBC** ($x10^{12}$/L)	**WBC** ($x10^{9}$/L)	**Heterophils** ($x10^{9}$/L)	**Lymphocytes** ($x10^{9}$/L)	**Monocytes** ($x10^{9}$/L)	**Thrombocytes** ($x10^{9}$/L)
		Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)
Lepidochelys olivacea	Pinto et al. (n=23)	17.2 (3.6)	0.25 (0.06)	6.48 (2.43)	5.51(2.05)	0.87(0.60)	0.10(0.11)	2.98(1.69)
	Acevedo et al., 2012 (n=22)	0.30(0.04)*	0.37(0.12)	4.51(3.03)*	2.87(1.88)	0.63(0.59)	0.06(47.39)	n/a
Dermochelys coriacea	Deem et al., 2006 (n=26)	36(5.4)*	0.38(0.19)	5.9(2.8)	2.4(1.2)	1.6(0.9)	0.2(0.2)	n/a
	Innis et al., 2010 (n=18)	42(7)*	n/a	8.54(6.04)	4.36(2.54)	2.75(1.53)	0.29(0.31)	n/a
Chelonia mydas	Rossi et al., 2009 (n=45)	24.63(2.63)*	0.39(0.052)	5.54(1.15)	4.31(1.04)	0.79(0.16)	0.25(0.05)	16.5(0.003)*
	Santos et al., 2009(n=59)	29(3.9)*	0.39(0.007)	3.55(1.92)	1.92(0.91)	0.71(0.43)	0.33(0.50)	20.53(9.65)*
	Casal and Orós, 2009 (n=77)	0.29(0.07)*	0.18(0.13)	5.8(3.6)	n/a	n/a	n/a	43.8(15.6)*
Caretta caretta	Basile et al., 2012 (n=23)	26(5)*	0.51(0.15)	20.2(6.62)*	n/a	n/a	n/a	2.85(1.74)
	Kazizoe et al., 2007 (n=61)	18.2(2.39)*	0.38(0.83)	7.21(1.52)	2.84(0.38)*	3.59(0.87)*	n/a	n/a
	Casal and Orós, 2007 (n=35)	28(5.77)*	n/a	n/a	n/a	n/a	n/a	n/a
Orlitia borneensis	Knotkova et al., 2005 (n=7)	19(10)	0.36(0.2)	7.4(3.2)	3.4(1.4)	0.7(0.3)	n/a	n/a

*An asterisk indicates a substantial difference from the data of the present study ($P<0.05$).

Table 2

Biochemical data for *Lepidochelys olivacea* compared with biochemical data for other sea turtles reported in the scientific literature.

		Creatinine	Albumin	AST	GGT	Total protein	Cholesterol	Urea	Calcium	Phosphate
		(mg/dL)	(g/dL)	(U/L)	(U/L)	(g/dL)	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)
		Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)	Mean(SD)
Lepidochelys olivacea	Pinto et al. (n=23)	0.3(0.13)	0.7 (0.28)	107.7 (48.4)	4.6(2.8)	2.0(0.4)	102.8(37.5)	121.4(33.5)	5.3(1.7)	6.9(1.9)
	Santoro &Meneses, 2007 (n=21)	0.3(23.2)	0.7(0.9)	73.4(29.3)	n/a	3.9(9)	212.6(1.1)*	n/a	8(0.9)	6.4(0.6)
Caretta caretta	Fazio et al., 2012 (n=52)	0.5(0.1)	0.7(0.34)	26.0(13.36)*	n/a	4.7(1.1)	n/a	59.4(23.8)*	n/a	n/a
	Casal and Orós, 2009 (n=77)	0.4(8.8)	1.1(1)	202.2(268.9)*	n/a	2.8(21)	142.6(3.1)*	n/a	9.7(1.1)	n/a
	Goldberg et al., 2011 (n=28)	0.5(0.1)	1.3(0.4)	151.2(61.86)*	0.7(0.6)	3.9(0.7)	247.7(48.5)*	35.2(13.5)*	9.9(11.1)	7.9(1.8)
Chelonia mydas	Aguirre and Balzs., 2000(n=52)	0.2(0.1)	1.7(0.4)	158.4(41.5)	1.5(12)	4.2(0.6)	140(43)*	n/a	9.1(1.7)	8.2(1.3)
	Montilla et al., 2008 (n=28)	0.1(0.1)	1.5(0.2)	n/a	n/a	4.3(7)	180.7(75.9)*	27.6(17.0)*	7.9(2.6)	5.2(1.3)
	Fong et al., 2010 (n=27)	0.3(1)	2.3(0.4)	142.8(53.2)	n/a	4.7(0.8)	194.8(85)*	n/a	8.8(1.6)	n/a
	Snoddy et al., 2009 (n=12)	n/a	1.5(0.3)	311.6(129.6)*	n/a	3.8(0.7)	n/a	n/a	11.2(2.1)	9.7(2.1)
Dermochelys olivacea	Deem et al., 2006 (n=8)	0.3(0.1)	1.1(0.3)	159.4(49)*	12(2)	n/a	346(96)*	n/a	7.1(1.8)	11(1.5)
	Stewart et al., 2012 (n=12)	n/a	1.54(0.1)	118.4(21.5)	n/a	3.9(1.3)	n/a	n/a	9.7(1.8)	10.6(2.5)
	Innis et al., 2010 (n=18)	0.4(0.5)	1.7(0.3)	286(269)*	n/a	4.6(1)	285(79)*	n/a	6(1.7)	9(1.7)

*An asterisk indicates a substantial difference from the data of the present study ($P<0.05$).