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**UNIVERSIDADE ESTADUAL PAULISTA  
"JÚLIO DE MESQUITA FILHO"  
INSTITUTO DE BIOCÊNCIAS – RIO  
CLARO**



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**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS....  
(BIOLOGIA CELULAR E MOLECULAR)**

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# **CARACTERIZAÇÃO DE FAGÓCITOS MONONUCLEARES DO SANGUE TARTARUGA *PHRYNOPS HILARII* (CHENOLIA;CHELIDADE)**

**Dimitrius Leonardo Pitol**

Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de doutor em Ciências Biológicas (Biologia Celular e Molecular).

**Novembro - 2008**

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**ORIENTADOR: Prof. Dr. Flávio Henrique Caetano**

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## ÍNDICE

	<i>Páginas</i>
<b>1. RESUMO .....</b>	<b>8</b>
<b>2. ABSTRACT.....</b>	<b>10</b>
<b>3. INTRODUÇÃO.....</b>	<b>12</b>
<b>4. OBJETIVOS GERAIS .....</b>	<b>14</b>
<b>5. REVISÃO DA LITERATURA .....</b>	<b>16</b>
5.1. A tartaruga <i>Phrynops hilarii</i> .....	17
5.2. Citologia sanguínea em répteis .....	18
5.3. Fagócitos mononucleares em répteis .....	19
5.4. Fagócitos mononucleares em mamíferos.....	20
<b>6. ARTIGO.1.....</b>	<b>24</b>
Caracterização morfológica de leucócitos circulantes no sangue de tartaruga ( <i>Phrynops Hilarii</i> ).	
<b>7. ARTIGO.2.....</b>	<b>31</b>
Estudo autorradiográfico e distribuição sazonal de leucócitos de tartaruga <i>Phrynops hilarii</i> .	
<b>8. ARTIGO.3.....</b>	<b>38</b>
Caracterização de fagócitos mononucleares de tartaruga <i>Phrynops hilarii</i> .	
<b>9. ARTIGO.4.....</b>	<b>43</b>
Estudo ultraestrutural e citoquímico e atividade Fagocítica de Fagócitos mononucleares de tartaruga <i>Phrynops hilarii</i> .	
<b>10. CONSIDERAÇÕES FINAIS.....</b>	<b>49</b>
<b>11. REFERÊNCIAS BIBLIOGRÁFICAS.....</b>	<b>51</b>



## ***1. Resumo***

O presente estudo teve como objetivo analisar os leucócitos circulantes em microscopia de luz e eletrônica e sua distribuição sazonal, além de procurar estabelecer o período de renovação celular desses leucócitos, e principalmente de caracterizar os fagócitos mononucleares do sangue de tartaruga, e sua capacidade de fagocitose frente a material inerte. Neste trabalho utilizou-se seis tartarugas *Phrynops hilarii*, originárias de ilhas do estuário do rio Guaíba Porto Alegre (RS), que estavam ambientadas em nosso biotério. A coleta de sangue foi realizada em todos os períodos sazonais, por punção de vasos laterais do pescoço e coletados em tubos de ensaio heparinizados. Foram realizados esfregaços sanguíneos, corados com Leishmann e Giemsa, contando-se quinhentas células de cada animal e após a obtenção dos dados, foi aplicado o teste estatístico de Bonferroni. Para a análise autorradiográfica foi injetado 1000 $\mu$ Ci / kg de thymidine-H A. Para microscopia eletrônica processamos a nata leucocitária obtida por meio de centrifugação do sangue, para a análise citoquímica incubamos com citidina-5'-monofosfato, beta-glicerofosfato de sódio, Trimetafosfatase, para averiguar a resposta fagocitária utilizamos 0,01% de carvão coloidal. Os resultados mostram que os leucócitos de *Phrynops hilarii* tem descrição de leucócitos semelhantes às outras espécies, somente os basófilos e linfócitos não sofreram alterações em sua distribuição sazonal. Todos os leucócitos com exceção dos basófilos apresentaram renovação celular após sete dias. Caracterizamos monoblasto, promonócito, monócitos e macrófago no sangue circulante bem como a capacidade dos fagócitos mononucleares de fagocitar células mortas e materiais inerte.

**Palavras-Chave: Fagócito mononuclear, sangue, tartaruga, leucócitos.**

## ***2. Abstract***

The aim of this study was to analyze the leukocytes in the blood using electronic and light microscopy and their seasonal distribution, also to characterize the leukocyte cells replacement and mainly to characterize the mononuclear phagocytes in the blood and their phagocytic capacity. In this study, it was used six turtles (*Phrynops hilarii*), caught at the Guaíba river estuary, Porto Alegre, Rio Grande do Sul, Brazil and lodged for 1 week at the Central Animal House, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. Blood was obtained during the seasonal periods by puncturing the lateral vessels of the neck. The blood samples were stained by Leishmann and Giemsa, counting five hundred cells in each animal. After the obtained data, it was applied the Bonferroni test as statistical method. For the autoradiographic analysis, it was injected in the circulating blood 1000  $\mu\text{Ci/kg}$  of  $^3\text{H}$ -thymidine. For electronic microscopy, it was processed the leukocyte substrate by circulating blood centrifugation. For cytochemical analyses, blood smears were air dried, post-fixed in 4% formalin and submitted to the determination of the following enzyme activities: acid phosphatases ( $\beta$ -glycerophosphatase and citidine-5'-sodium monophosphatase), and trimetaphosphatase. The results showed that the leukocytes of *Phrynops hilarii* have the leukocytes description similar to the other species, only the basophiles and lymphocytes did not suffer alterations in their seasonal distribution. All the leukocytes, in exception of the basophiles showed cells replacement after seven days. It was characterized the monocytes and macrophages in the circulating blood as well as the phagocytes capacity.

**Key-words: mononuclear phagocyte, blood, turtle, leukocytes.**

### ***3. Introdução***

O conhecimento da história natural das espécies de cágados é bastante incipiente. Apesar de estudos conduzidos desde as últimas décadas terem contribuído de maneira significativa para elucidação de vários aspectos da biologia e da ecologia que são de grande importância para o manejo e conservação. Observamos uma carência grande de informações sobre a hematologia dessas espécies, sendo que as informações encontradas na literatura são conflitantes.

Estudos recentes na literatura apontam informações relevantes sobre algumas células sanguíneas como trombócitos, basófilos eosinófilos e heterófilos (neutrófilos), estas células têm suas características e funções bem descritas, entretanto são escassos os estudos envolvendo fagócitos mononucleares. Os fagócitos estão entre as principais células de defesa contra microorganismos e também contra células neoplásicas. Estas células, têm papel importante na resposta inflamatória, secretando uma variedade de citosinas, metabólitos do ácido aracdônico, proteases e antiproteases. Regulam a hemóstasia, a fibrinólise, a replicação dos fibroblastos e das células endoteliais, fagocitando restos de células, neutralizando materiais tóxicos e participando da modulação da resposta imune, por meio da interação com linfócitos T e B. Não resta dúvida que os fagócitos mononucleares são bem descritos em mamíferos, os estágios de diferenciação dessas células e os fatores que regulam essa diferenciação estão escritos e também suas interações nos processos patológicos.

Acreditamos que algumas técnicas citoquímicas aliadas com a análise em microscopia eletrônica de transmissão possam contribuir para uma caracterização desses fagócitos mononucleares no sangue de tartaruga, pois não há estudo na literatura que indique o tempo de renovação celular de leucócitos de tartaruga, portanto espera-se obter tais dados realizando estudos autorradiográfico. Estudos realizados em tartaruga sugerem a possibilidade desses fagócitos serem capazes de realizar fagocitoses de bactérias e fungos, ovos de helmintos e eritrócitos velhos. Neste estudo avaliamos a capacidade de fagocitose dessas células mediante a incubação *in vitro* com material inerte, utilizando-se como modelo experimental a espécie *Phrynops hilarii*.

## ***4. Objetivos Gerais***

Os objetivos deste trabalho foram :

- 1) Descrever os leucócitos presentes no sangue da tartaruga *Phrynops hilarii* em microscopia de luz e também com microscopia eletrônica de transmissão.
- 2) Descrever a sua distribuição sazonal das células do sangue, bem como o tempo renovação dessas células no sangue.
- 3) Caracterizar os fagócitos mononucleares do sangue de tartaruga
- 4) Avaliar a capacidade fagocítica dos fagócitos mononucleares por meio de técnicas citoquímicas ultra-estruturais para enzimas envolvidas no processo de fagocitose e o comportamento dessas células frente material inerte.



## ***5. Revisão da Literatura***

### 5.1 A Tartaruga *Phrynops hilarii*

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Aproximadamente 20% das 278 espécies de quelônios do mundo são encontradas na América do Sul, representando oito famílias (Dermochelyidae, Cheloniidae, Chelydridae, Emydidae, Kinosternidae, Padocheimidae e Chelidae). Dessas famílias, Chelidae, cujos representantes típicos são conhecidos como cágados, é a mais rica contando com 23 espécies, das quais 19 ocorrem no Brasil. Para a família brasileira de cágados, o estudo da história natural das espécies é bastante incipiente, o que significa que muito ainda deve ser feito. Vários fatores podem estar ligados a essa carência de dados, incluindo desde a dificuldade de acesso a algumas áreas de ocorrência das espécies e falta de estímulo para desenvolvimento de pesquisas. A considerável diversidade aliada a uma nítida carência de dados biológicos faz com que os quelônios brasileiros sejam um grupo faunístico muito interessante como fonte de pesquisa (SOUZA, 2004).

*Phrynops hilarii* (DUMÉRIL & BIDRON, 1835) é uma espécie natural do rio Paraná e bacias adjacentes no sul do Brasil, Uruguai e norte da Argentina (IVERSON, 1992), habitando riachos, lagos e brejos (ERNST & BARBOUR 1989; SOUZA 2004) sendo que sua biologia é pouco conhecida. Essa espécie é popularmente conhecida como cágado de lagoa. Possui carapaça achatada com escudos epidérmicos lisos, cuja a coloração é cinza uniforme, contrastando com o plastrão, que é amarelo claro salpicado com pontos e manchas negras. Apresenta cinco unhas nos membros anteriores e quatro nos posteriores; membranas interdigitáveis bem desenvolvidas permitem a este cágado ágeis evoluções aquáticas (BUJES, 1998). Esse cágado pode se alimentar de peixes, moluscos e também carne quando em cativeiro (FREIBERG, 1981). Sua nidificação ocorre preferencialmente em locais de solo arenoso com predomínio de vegetação herbácea e com boa exposição ao sol, a uma distância média de 80 metros do leito d'água (BAGER, 1997; BUJES, 1998).

Essa espécie põe de 10 a 12 ovos esféricos e de casca dura em covas não muito profundas. Essa nidificação pode ocorrer em dois períodos distintos entre fevereiro e maio e entre setembro e dezembro, possivelmente devido ao fato da temperatura ser mais elevada nesses meses. Estudos realizados em cativeiro também sugerem a ocorrência de dois períodos, entretanto um entre outubro e novembro e outro de fevereiro a março (ASTORT, 1984).

*Phrynops hilarii* tem hábitos diurnos, assoalhando durante horas mais quentes do dia sobre troncos, pedras ou ao longo das margens dos rios (MEDEM, 1960; MONTEIRO & DIFENBACH, 1987; MOLINA, 1989; SOUZA, 1999).

## **5.2 CITOLOGIA SANGUÍNEA EM RÉPTEIS**

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As primeiras investigações sobre células do sangue de répteis tiveram início no século XVII, após o advento do microscópio óptico e somente nos meados do século seguinte os resultados dessas pesquisas começaram a ser publicados. Inicialmente trabalhos foram realizados em serpentes (GULLIVER 1842). Em 1879, Hayem estudando sangue de alguns répteis e anfíbios determinou o número e as dimensões de eritrócitos e leucócitos. Já em 1930, surgiu um trabalho melhor elaborado comparando a hematologia de espécies de lagartos, tartaruga e serpentes (BABUDIARI, 1930). Charipper&Davis (1932) estudaram o sangue de *Pseudemys elegans* com enfoque principal nos leucócitos. Em 1943, Ryberson desenvolveu um trabalho que trouxe grande contribuição a morfofisiologia dos eosinófilos de sangue de tartarugas comprovando a diferenciação de duas linhagens de células distintas. Investigações mais detalhadas sobre a morfologia de vários tipos de células foram feitas no início do século. Em muitos grupos de répteis os autores descrevem células de linhagens granulocíticas contendo grânulos que apresentavam em alguns elementos forma esférica e, em outros, fusiforme, dessa forma essas diferenças sugeriam duas hipóteses sobre as suas origens. Alguns autores acreditavam ser um mesmo tipo celular em diferentes estágios de maturação (JORDAM&FLIPPEN, 1943), entretanto, outros autores consideravam as células portadoras de granulações diferentes como entidades celulares distintas (LOWENTHAL, 1928; PIENAAR, 1962; OLIVEIRA, 2000). Posteriormente, esta última idéia encontrou respaldo nos estudos realizados por Ryerson (1943), que demonstrou que os elementos com grânulos fusiformes eram fisiologicamente semelhantes aos heterófilos de aves.

Em 1962, Heady & Rogers, trabalhando com quatro espécies de quelônios, relataram a existência de granulócitos acidófilos grandes, denominados de eosinófilos e não constatarem a presença de basófilos em suas amostras. Estudos realizados em esfregaços sanguíneos de *Chelonia mydas*, não mostraram a presença de monócitos e

classificaram os heterófilos como neutrófilos (WOOD & EBANKS, 1984). Estudos citoquímicos ultra-estruturais realizados na espécie *Chrysemys dorsalis* indicaram que os eosinófilos de tartaruga possuem características morfológicas semelhantes aos eosinófilos de algumas aves e características citoquímicas semelhantes aos eosinófilos de aves e também de mamíferos. Quanto aos heterófilos, suas características morfológicas se assemelham aos de aves, ao passo que as características citoquímicas assemelham-se com de heterófilos de coelho e aves denominado de heterófilo/neutrófilo (AZEVEDO, 1995 ; AZEVEDO & LUNARDI, 2003). Em 1996, Lunardi relata a existência de dois tipos de basófilos sendo o basófilo do tipo I, semelhante aos leucócitos basófilos encontrados no sangue circulante de todos os vertebrados e os basófilos do tipo II com características ultra-estruturais semelhantes aos mastócitos do mesentério deste mesmo animal. Dessa forma, esse autor caracterizou os basófilos do tipo II como mastócitos imaturo.

Também em 1996, Pellizzon caracterizou os trombócitos de tartaruga utilizando como modelo experimental as espécies *Phrynosoma marmoratum* e *Chrysemys dorsalis*, demonstrando a capacidade fagocítica bem como o processo de agregação desses trombócitos

Ainda com auxílio de análise citoquímica ultra-estrutural em fagócitos mononucleares da tartaruga *Phrynosoma marmoratum*, caracterizou-se os fagócitos mononucleares, monoblasto promonócito- monócito e macrófago presentes no sangue circulante desta espécie (PITOL, et al. 2007)

### **5.3 FAGÓCITOS MONONUCLEARES EM RÉPTEIS**

Os répteis representam a ligação entre vertebrados ectotérmicos e endotérmicos, despertando um especial interesse nos estudos das respostas inflamatórias e imunológicas (KIONG, 1971; DAWSON, 1971; SYPEK, 1984; JIA,W.Z, 2003; PELLIZZON,1996 TUCUNDUVA, et al., 2004). Na literatura poucos estudos são realizados em células mononucleares de répteis, incluindo quelônios, quando comparados aos estudos realizados nestas células em mamíferos, tanto em microscópio de luz (WOOD & EBRANKS, 1984; CANNON, 1992), como ultra-estrutural e citoquímica (KELÉNYI & NÉMETH, 1969; ZAPATA et al., 1981; CANNON, 1992; PELLIZZON,1996).

A maioria dos estudos observados na literatura estão relacionados mais com as respostas à patologias do que à caracterização das células propriamente dita.

Assim recentemente, foram realizados estudos de inflamação em *Boa constrictor*, mostrando migração de monócitos depois de quatro horas da inflamação subcutânea que se encontra aumentada após quarenta e oito horas, apresentando macrófagos e células gigante de corpo estranho que permanece durante sete dias no local inflamado (TUCUNDUVA et al., 2004).

São encontrados em lagartos da espécie *Ameiva ameiva*, hematozoários nos monócitos (LAISON et al., 2003). Resposta semelhante também foi observada nesses mesmos lagartos infectados por hematozoários *Lainsonia landau*, cujas alterações nos monócitos, mostram mudanças morfológicas e a presença de “célula gigante de corpo estranho” foram descritas (SILVA et al., 2004).

Células mononucleares como os melanomacrófagos foram removidos do fígado de três famílias de tartarugas e cultivadas *in vitro* e expostas a *E.coli*. Neste estudo os autores sugerem que estas células *in vitro* e, provavelmente, *in vivo* sejam capazes de fagocitar bactérias, fungos, ovos de helmintos e eritrócitos velhos. Johnson et al (1999) e Pasmans et al. (2002) compararam a resposta macrofágica à *Salmonella enterica* de tartarugas *Trachemys scripta* com a resposta observada em pássaros e sugerem não haver diferenças qualitativas na resposta a infecções por salmonela em hospedeiros homeotérmicos e pecilotérmicos. Algumas descrições morfológicas e histoquímicas foram realizadas em tartarugas do deserto *Gopherus agassizii* por Garnner et al (1996) que relataram a dificuldade de identificar células da linhagem monocítica da medula óssea com base na coloração histológica e a necessidade do uso de técnicas histoquímicas para fazer a distinção entre monócitos azurófilos e monoblastos.

#### **5.4 FAGÓCITOS MONONUCLEARES DE MAMÍFEROS**

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Nos mamíferos as células fagocíticas estão distribuídas por vários órgãos e inicialmente foram incluídas em um único sistema denominado, por Metchnikoff (1892), como Sistema Macrofágico. Outras denominações foram propostas, posteriormente, tais como: Sistema Retículo Endotelial (ASCHOFF, 1924) ou Sistema Retículo Histiocitário

(VOLTERA, 1927).

Van Furth et al (1972), baseados em estudos morfológicos funcionais de cinética celular, propuseram a denominação de Sistema Fagocítico Mononuclear (SFM) onde estas células, independente do tecido ou órgão em que se encontravam, mostravam capacidade de fagocitar e pinocitar. A denominação de SFM é utilizada até os dias atuais; tal sistema é composto pelas células precursoras de macrófagos, tais como células tronco, monoblastos, promonócitos e monócitos e pelos macrófagos do tecido conjuntivo, também conhecidos como histiócitos, como por exemplo do fígado (células de Kupffer), do baço, dos linfonodos, do timo, da medula óssea, dos ossos (osteoclasto), das sinóvias (células do tipo A), dos pulmões (macrófagos alveolares) dos tratos gastrintestinal e genitourinário, dos órgãos endócrinos, do sistema nervoso central (células da micróglia), das cavidades corpóreas (macrófagos pleurais e peritoneais) e da pele (células de Langerhans). Participam ainda do SFM os macrófagos encontrados nos processos inflamatórios, tais como os macrófagos exudatos, células epitelióides e células gigantes multinucleadas (VAN FURTH, 1982).

Na vida adulta, as células fagocíticas mononucleares originam-se, principalmente, a partir de monócitos sanguíneos que, por sua vez, originam-se das células tronco (VAN FURTH & COHN, 1968; VAN FURTH, 1972; BAR-SHA VIT et al, 1983, DEXTER & SPOONCER, 1987; METCALF, 1991; COWLING & DEXTER, 1992 ; ABU-AMER; BAR-SHA VIT, 1993; BRUIJN, et al., 1994; ZHAO et al., 2003). O Fator de estimulação de colônia de granulócito está relacionado com a proliferação de granulócitos precursores de monócitos, porém esse mecanismo de regulação ainda não está bem compreendido (NAGATA, et al., 1983; ABRINK, et al., 1994; NICHIDAI, 2003). Estas células têm sido caracterizadas por meio de análises morfológicas incluindo a marcação de proteínas de membrana, como por exemplo CD34 (WATT & VISSER, 1992; CRUSE, & LEWIS, 2003), c-kit, Sca-1 (OKADA et al., 1992) e Flt3 (GABBIANELLI et al., 1995; AMICO, A & WU, 2003). Nas células tronco que são mais indiferenciadas, os receptores CD34, c-kit e Sca-1 estão presentes; enquanto que naquelas mais diferenciadas o marcador Sca-1 não é expresso (OKADA; et al., 1992). O receptor tirosina quinase Flt3 está expresso nas células CD34 da medula óssea humana, e seu ligante (FL) estimula a proliferação das células (GABBIANELLI et al., 1995; RUSTEN et al., 1996; SHAH et al., 1996; ANTONYSAMY,

M.A & THOMSON, .2000; WU, 2003). Os monoblastos foram identificados a partir de cultura de células da medula óssea por meio de análises morfológica e citoquímica, bem como de análise das características funcional e proliferativa (GOUD et al., 1975; MOREIRO, et al., 2004). Monoblastos são considerados células que se proliferam em meio de cultura formando colônias que são os precursores dos promonócitos (GOUD et al; 1975; GOUD & VAN FURTH, 1975). Diferenças morfológicas entre os monoblastos, promonócitos e macrófagos foram detectados, com base em análise citoquímica para detecção da peroxidase celular (VAN FURTH et al., 1970; VAN FURTH & FEDORKO, 1976; NAITO, 1993) ou imuno-citoquímica (NAITO, 1993). Com esses estudos verificou-se que os monoblastos possuíam núcleo grande; nucléolo proeminente; citoplasma escasso; positividade para peroxidase no envoltório nuclear (EN), retículo endoplasmático rugoso (RER) e nos Complexos de Golgi (GC), bem como positividade para o anticorpo monoclonal ER-MP20. À microscopia de luz os monoblastos apresentavam-se como células redondas, com superfície pregueada, núcleo redondo ou denteado, citoplasma fortemente basofílico e escasso, contendo poucos grânulos e vesículas (GOUD et al., 1975). As análises *in vitro* feitas por meio de marcação com isótopo radioativo mostram que os macrófagos têm origem a partir de promonócitos na medula óssea (VAN FURTH & COHN, 1968; VAN FURTH & DIESSELHOFF-DEN DULK, 1970; LIU et al., 1992, ABRINK, et al., 1994). Os promonócitos mostram características citoquímica (peroxidase) e ultra-estrutural que as diferenciam dos monócitos e macrófagos (VAN FURTH et al, 1970; VAN FURTH; FEDORKO,1976; NAITO, 1993). Os monócitos em condições normais migram da medula óssea para a corrente sanguínea onde permanecem, aproximadamente, 17 horas. Após sua adesão às células endoteliais os monócitos deixam o vaso sangüíneo e migram para os tecidos e cavidades serosas onde se diferenciam em macrófagos imaturos e maduros. Os primeiros assemelham-se aos monócitos, exceto que são desprovidos de grânulos positivos para peroxidase. Já, os macrófagos maduros mostram amplo citoplasma com organelas bem desenvolvidas, especialmente os lisossomos, e positividade para a marcação com anticorpo anti-F4-80 e negatividade para o ER-MP20; longas projeções citoplasmáticas são visualizadas nestes macrófagos (MORIOKA et al., 1994; NAITO et al., 1996; BRUIJN, et al 1998). Pela microscopia de luz os macrófagos são diferenciados dos monoblastos e promonócitos por apresentarem membrana intensamente

pregueada, mais distendida, com citoplasma amplo discretamente basofílico contendo numerosos grânulos e vacúolos. Seu núcleo é oval ou denteado, semelhante às células precursoras (GOUD et al., 1975; NAITO, 1993; NAITO, et al, 1996).

A diferenciação celular que ocorre na medula óssea faz-se por meio da ação de fatores estimuladores de colônia. Esses fatores têm papel importante na proliferação, diferenciação e até na ativação das células já em estado diferenciado (NAGATA, et al., 1983; METCALF, 1985; YANG et al., 2000; MINAMINO, et al., 2005; NAKAHARA, et al., 2005). Dentre os vários fatores estimuladores de colônia existentes, o M-CSF, também conhecido como CSF-1, estimula a proliferação e diferenciação do fagócito mononuclear e sua sobrevivência em meio de cultura (GOUD & VAN FURTH, 1975; METCALF, 1989; MEAGER, 1990; PIERCE et al., 1990) Dependendo da concentração o CSF-1 ocasiona alterações morfológicas, tais como: aumento do volume celular; ondulações da membrana plasmática e aumento de vacúolos negativos para fosfatase ácida (TUSHINSKI et al., 1982). O M-CSF é uma glicoproteína encontrada sob a forma dimérica (STANLEY & HEARD, 1977; STANLEY, 1985) que interage com os macrófagos e seus precursores por meio de receptor da família das tirosinas kinases, produto gênico do proto-oncogene (SHERR, 1988; METCALF, 1991; ALBERTS, 1997). Células precursoras não aderentes da medula óssea, em cultura, expressam receptores específicos, interagem com o CSF-1 e adere-se à superfície de plástico (TUSHINSKI et al., 1982; BARTELMEZ & STANLEY, 1985; RYAN et al., 2001; SHERRY, et al 2003). Na reação inflamatória os macrófagos, após saírem do vaso sangüíneo, passam por estágios de diferenciação, tais como macrófago exudato-residente e, por último, macrófago negativo para peroxidase (NAITO, 1993; NAITO et al., 1996). Estímulos como IFN- $\gamma$  e LPS podem determinar a diferenciação dos macrófagos para células epitelióides, cuja morfologia caracteriza-se por núcleo eucromático alongado, nucléolo proeminente, citoplasma abundante com RE evidente, grande número de vacúolos destituídos de fosfatase ácida, sugerindo pouca atividade fagocítica. Os macrófagos podem ainda agregar-se, formando “célula gigante do tipo corpo estranho”, cujos núcleos ficam dispersos aleatoriamente no citoplasma ou, ainda, formar célula gigante de Langerhans, cujos núcleos ficam organizados na periferia dividido, provavelmente, à ação do citoesqueleto (SPECTOR, 1969; MARIANO & SPECTOR, 1974; ADAMS, 1976;)



## ***6. Artigo 1***

## Morphological Characterization of the Leukocytes in Circulating Blood of the Turtle (*Phrynops hilarii*)

Caracterización Morfológica de Leucocitos Circulantes en la Sangre de la Tortuga (*Phrynops hilarii*)

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PITOL, D. L.; ISSA, J. P. M.; CAETANO, F. H. & LUNARDI, L. O. Morphological characterization of the leukocytes in circulating blood of the turtle (*Phrynops hilarii*). *Int. J. Morphol.*, 25(4):677-682, 2007.

**SUMMARY:** The *Phrynops hilarii* specie of turtle has its characterization not well defined in the literature, it was proposed in this study the leukocyte characterization of the blood, stained by Leishman and analyzed under light and transmission electron microscope. It was not observe any cellular type with similar characteristics to neutrophils in mammalian group. We believed, based on the data obtained in this study that the heterophils have a morphofuncional analogy with another neutrophils belonged to mammalian group. This conclusion is being supported in many recent studies found in the literature.

**KEY WORDS:** Turtle; Morphology; Leukocytes; Blood.

### INTRODUCTION

In mammals, leukocytes are easily identified on the basis of their morphological differences and the enzymes and other proteins stored in their cytoplasmic granules (Bainton & Farquhar, 1968, 1970; Bainton *et al.*, 1971; Weller, 1991; Dvorak *et al.*, 1991, 1994). Neutrophils are highly specialized phagocytic cells involved in ingestion, death, and degradation of invading microorganisms (MacCall *et al.*, 1971; Roos *et al.*, 1983; Bainton, 1988). Eosinophils are cells that actively participate in the defense against parasitic infections, in the regulation of hypersensitivity reactions, and in the destruction of cancer cells (Kay, 1985; Dvorak *et al.*, 1991; Weller).

Studies have been conducted on lower vertebrates in order to understand biological roles of leukocytes in defense mechanisms, and to establish phylogenetic studies and new experimental models. Some investigators have demonstrated the existence of 2 forms of eosinophils in the blood of turtles, one of them a mature form and the other an immature form (Jordan & Flippin, 1943; Charipper & Davis, 1932), whereas others have stated that there are 2 distinct cell lineages, i.e. neutrophils and eosinophils (Ryerson, 1943; Taylor *et al.*, 1963; Wood & Ebanks, 1984). Because of the wide morphological variation of these cells in different animal

species, it is impossible to characterize them solely on the basis of morphology.

Veterinary haematology has relied on classical Romanowsky staining (e.g. Leishman, Wright and Giemsa) to identify erythrocytes, thrombocytes and leukocytes, but cellular classification of these leukocytes is not always reliable using classical staining methods. Neutrophils are present in some animals, but this cell types have been reported in a few species (Barber & Westermann, 1978; Tavares-Dias & Moraes). Immature leukocytes also can be present in circulating blood (Meseguer *et al.*, 1994; Tavares-Dias & Moraes, 2004). Thus, cytochemical staining of piscine leukocytes may be particularly useful for identification of cellular lineage and may suggest cell function.

Apart from being useful for identifying cell types in blood and tissues, cytochemical staining is also critical for identifying immunological cell types associated with developmental and pathological processes (Burrows & Fletcher, 1987; Meseguer *et al.*; Lorenzi, 1999; Ueda *et al.*, 2001; Petrie-Hanson & Peterman, 2005). Presence of glycogen (Veiga *et al.*, 2000; Ueda *et al.*; Vale *et al.*, 2002; Rough *et al.*, 2005) and alkaline phosphatase (Meseguer *et*

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*al.*; Burrows *et al.*, 2001) in leukocytes may be associated with phagocytosis. This requires the consumption of energy from both endogenous and exogenous sources (Hayhoe & Quaglino, 1994; Ueda *et al.*). Peroxidase is a lysosomal enzyme, which takes part in intracellular digestion and modulation of phagocytic activity of leukocytes (Hayhoe & Quaglino; Veiga *et al.*; Ueda *et al.*; Vale *et al.*; Azevedo & Lunardi, 2003). Esterases are enzymes also related to cellular defense, facilitating diapedesis, cell migration through tissue, toxic product and microorganism inactivation and tumour cell destruction (Hayhoe & Quaglino; Casaletti-Rosa & Lunardi, 1997; Azevedo & Lunardi). The need to identify these features in leukocytes encouraged numerous studies on turtles (Zinkl *et al.*, 1991; Burrows *et al.*; Ueda *et al.*; Tavares-Dias & Moraes; Palic *et al.*, 2005; Petrie-Hanson & Peterman).

By the fact that the *Phrynops hilarii* specie has its characterization not well defined in the literature, it was proposed in this study the leukocyte characterization of the blood, stained by Leishman and analyzed under light and transmission electron microscope.

## MATERIAL AND METHOD

All the aspects of this research were approved by local ethics committee. It was used in this study six *Phrynops hilarii* turtles, obtained at Guaíba river, Porto Alegre, Rio Grande do Sul, Brazil. The animals were lodged for one week at Central Animals House, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. The blood of these animals was removed by needle aspiration performed on lateral vessels of the neck. The histological process and staining of this blood was performed by Leishmann stain. These histological images were obtained at a photomicroscope using the Leica IM 50 program connected to Leica DMLB2 microscope.

The collected blood was done using heparinized beakers, centrifuged by 15 minutes at 1000rpm at room temperature. The blood plasma was eliminated and the leukocyte suspension was processed by electron microscopy analysis. Samples of the leukocyte suspension were fixed by Karnovsky solution- glutaraldehyde at 2%, paraphormaldehyde at 2% and cacodilat solution at 0.1M pH 7.4, with 0.05% of calcium chlorite, during 2 hours at room temperature. The blood samples were post-fixed in osmium tetroxide at 1% and cacodilat solution 0.1M, during two hours at the same temperature, and later, observed by CM-100 - Philips transmission electron microscope.

## RESULTS

It was found in the present study six types of leukocytes in the turtle blood, *Phrynops hilarii* specie, basophiles, eosinophils, lymphocytes, monocytes, neutrophils and thrombocytes. Basophiles presented spherical conformation with segmented nucleus, spherical granules in cytoplasm (Fig. 1A). Eosinophils were defined as spherical shape and peripheral nucleus, with cytoplasm filled by oval granules (Fig. 1B). Small and spherical lymphocytes, with eccentric nucleus, were observed in almost all cytoplasm (Fig. 1C). Monocytes cells were found in circulating blood, characterized by oval and peripheral nucleus and abundant cytoplasm (Fig. 1D). Neutrophils were found after light microscopy analysis, showing a spherical nucleus and heterophilic aspect, this cellular type was found in electron microscopy analysis, presenting segmented and heterophilic nucleus, and cytoplasm with elongated granules (Fig. 1E). Thrombocytes showed elliptic conformation, with a little cytoplasm and nucleus with elliptic shape too (Fig. 1F).

## DISCUSSION

By the fact that the *Phrynops hilarii* specie has its characterization not well defined in the literature, it was proposed in this study the leukocyte characterization of the blood, stained by Leishman and analyzed under light and transmission electron microscope.

Studies related to leukocytes characterization in turtles do not present a consensus in relation to the description of these leukocytes. Research studies related to ground turtles belonged to the species *Terrapene carolina* and *Gopherus polyphenus*, identified in blood samples the following cellular types, basophiles, eosinophils, lymphocytes, monocytes, neutrophils and thrombocytes (Ryerson, 1943). However, in 1962, Head & Rogers working with four species of turtles did not related in their conclusion, the presence of basophiles in blood samples. In a similar study, performed in blood tissue of the turtle *Chelonia mydas*, it was not observed the presence of monocytes and it was considered the heterophils cells as neutrophils (Wood & Ebanks, 1984). In 2004, Munoz & Fuente, studying lymphoid tissues belonged to *Mauremys caspica* specie of turtle, found heterophils cells after the resultant histological analysis. Studies involving another species aiming to study the leukocytes under light microscopy analysis, showed in the turtles species *Padocnemis expansa* and *Emys orbicularis*, the presence

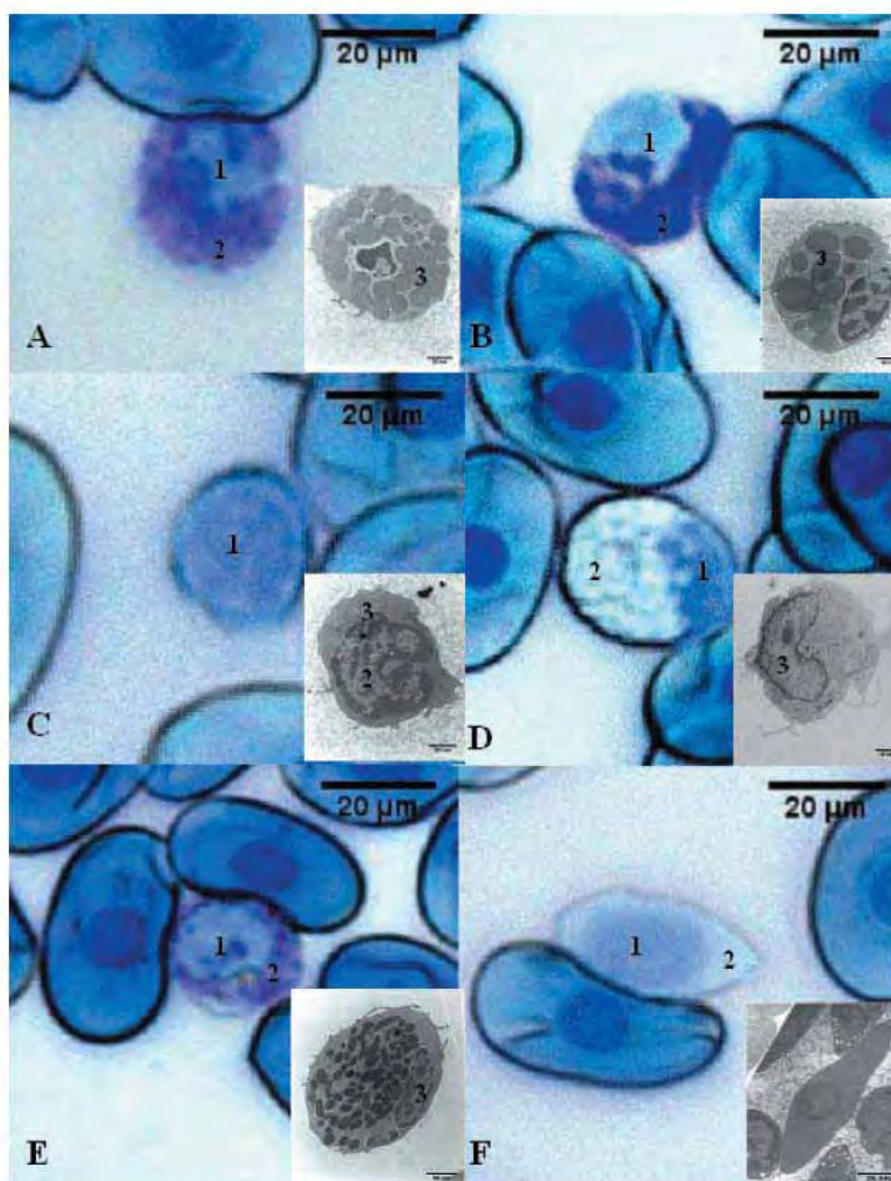


Fig. 1. A. Basophiles showing spherical shape with segmented nucleus (1), cytoplasm filled by many metachromatic granules (2) (400x of original magnification), granules with spherical shape under electron microscopy analysis (3) (2800x of original magnification). B. Eosinophils showing spherical shape and peripheral nucleus (1), cytoplasm filled by large granulus (2) (400x of original magnification). Granules with oval shape (3) (2500x of original magnification). C. Small lymphocytes with spherical shape and eccentric nucleus (1) filling almost all the cytoplasm (2) (400x of original magnification). However, when it is observed under electron microscope, the nucleus presented reniform aspect (3) (2500x of original magnification). D. Monocyte showing spherical shape with peripheral and reniform nucleus (1) and abundant cytoplasm (2) (400x of original magnification). Nucleolus under electron microscope (3) (2500x of original magnification). E. Heterophils (Neutrophils) showing spherical shape, central nucleus (1) and cytoplasm with elongated granules (2) observed under light microscope (400x of original magnification). However, when observed under electron microscope, it was found a peripheral and segmented nucleus (3) (2500x of original magnification). F. Thrombocytes showing an elliptic shape and nucleus with elliptic conformation too (1), and little quantity of cytoplasm (2) (400x of original magnification).



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of basophiles, eosinophils, lymphocytes, monocytes, and heterophils (Oliveira *et al.*, 2000; Metin *et al.*, 2006). Studies involving ultrastructural citochemistry analysis performed in *Chrysemys dorbignih* specie of turtle, confirmed the existence of eosinophils and heterophils in blood samples and different morphological characteristics of these cellular types according to some variations (Azevedo & Lunardi).

The leukocyte identification is based on staining parameters and the morphology showed under light microscopy analysis, but it has some limits when this analysis is performed using leukocytes non granulocytic. Lymphocytes cells are in most cases classified as small, middle and large, and many authors relate the difficulty to analyze the differentiation of large lymphocytes in relation to monocytes or thrombocytes cells (Montali, 1988). Work, in 1998, analyzed the blood of *Chelonia mydas* specie, using sudan black B, periodic acid-schiff and toluidine blue, and cytochemistry reaction for acid phosphatase analysis and later identification of six types of leukocytes under electron microscope apparatus.

The results of the blood sample analysis stained by Leishmann, permitted to observe all leukocytes, being clear

that the differences between monocytes and thrombocytes cells. These results associated to the transmission electron microscopy analysis, permitted to observe the characteristic shape of the basophiles, eosinophils, and heterophils granules in more detail, and to affirm that the leukocyte morphology in *Phrynops hilarii* specie is very similar to the another species and according to morphological descriptions found in the literature (Ryerson; Oliveira *et al.*; Azevedo & Lunardi; Work *et al.*; Deen, 2006.). The Leishmann stain is used in most cases that the objective is to observe the hematological characteristics of the tissues in mammalian groups. Hughes *et al.*, 2003 evidenced very well each cellular type in this group, thus we can affirm that this type of staining is an excellent method for diagnosis in chelonian blood samples.

It was not observe any cellular type with similar characteristics to neutrophils in mammalian group. We believed, based on the data obtained in this study that the heterophils have a morphofuncional analogy with another neutrophils belonged to mammalian group. This conclusion is being supported in many recent studies found in the literature.

PITOL, D. L.; ISSA, J. P. M.; CAETANO, F. H. & LUNARDI, L. O. Caracterización morfológica de leucocitos circulantes en la sangre de la tortuga (*Phrynops hilarii*). *Int. J. Morphol.*, 25(4):677-682, 2007.

**RESUMEN:** La especie de tortuga *Phrynops hilarii* no ha sido aún bien descrita en la literatura. Fue propuesto en este estudio la caracterización de leucocitos de sangre de este animal coloreados con el método de Leishman y analizados con microscopías de luz y electrónica. No fue observado ningún tipo celular con características similares a los neutrófilos de mamíferos. Los resultados indican que los heterófilos tienen analogía morfofuncional con otros neutrófilos presentes en el grupo de los mamíferos. Esta conclusión es sustentada por varios estudios recientes encontrados en la literatura.

**PALABRAS CLAVE:** Tortuga; Morfología; Leucocitos; Sangre.

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## ***7. Artigo 2***





## Radioautographic study of the seasonal distribution of leukocytes in turtles *Phrynops hilarii* (Chelonia Chelidae)

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### Abstract

The aim of this study was to present a morphological description of the leukocytes of *Phrynops hilarii* turtles according to the seasonal distribution of these cells and to show their replacement in the blood circulation using a radioautographic method. Five animals of both sexes weighing 600–1200 g were used. The animal's blood was aspirated, smeared on glass slides, and stained with the Romanowsky stain, and 500 cells of each animal were counted during each season. The results obtained were analyzed statistically by analysis of variance followed by the Bonferroni test (NCSS), with the level of significance set at  $p < 0.05$ . The radioautographic analysis of turtle blood exposed to 1000  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine and developed after 30 days showed a large number of silver grains incorporated into the cells, except for basophils, with cell renewal occurring every seven days. Quantitative data demonstrated a seasonal influence on the distribution of some leukocyte types, with the following “ $p$ ” values: heterophils ( $p = 0.0007$ ), basophils ( $p = 0.0002$ ), monocytes ( $p = 0.0016$ ), eosinophils ( $p = 0.0073$ ). However, using this statistical method, it was not possible to detect a significant difference related to seasonal influence on lymphocytes ( $p = 0.16295$ ) or thrombocytes ( $p = 0.1046$ ). Using this experimental animal model, a seasonal influence on the distribution of some leukocyte types was observed, and the radioautographic method revealed a cell renewal system occurring every seven days, except for basophils.

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**Keywords:** Turtle; Leukocytes; Seasonal; Radioautography; *Phrynops hilarii*

### 1. Introduction

The lymphoid tissue of reptiles represents a well-organized system (Cooper et al., 1985). Reptile leukocytes have been characterized as lymphocytes, monocytes and granulocytes (Zapata et al., 1981; Mead et al., 1983). Similar to mammals, most peripheral blood leukocytes originate in the bone marrow, with the main exception of T-cells, which mature in the thymus (Cooper et al., 1985). The spleen serves as a large reservoir of B- and T-cells (Kroese and Van Rooijen, 1983). These lymphocytes have been reported to produce a vigorous immune response with characteristics similar to those of mammals (Work et al., 2000). The turtle *Phrynops hilarii* is a species native to the Paraná River,

which crosses three countries in South America. The biology of this turtle has not been studied in full.

Seasonal variations seem to affect the morphology and function of the immune system of ectothermic vertebrates, especially reptiles (Zapata et al., 1983; Souza, 2004). Temperature, photoperiod, environmental stressors, chemicals and behavioral factors modify the immune reactivity of teleosts (Avtalion, 1981). Urodeles and anurans also undergo physiological, season-dependent changes affecting hematological parameters (Harris, 1972; Munoz and Fuente, 2004; Souza, 2004) and the structure of lymphoid organs (Plytycz and Bigaj, 1983). Seasonal changes affecting the structure of lymphoid organs, cell viability, proportions of “T-like” and “B-like” cells, antibody titers, numbers of perfluorinated compounds (PFCs), responses to mitogens and mixed lymphocyte reactions (Saad and El Ridi, 1984) have been studied in Egyptian lizards and snakes. In addition, nutritional factors and animal housing in the laboratory as opposed to outdoor conditions modify reptilian immunoreactivity (Worley and Jurd, 1979).

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Radioautography is the technique used to demonstrate the pattern of localization of various compounds labeled with radioactive isotopes in specimens (Nagata, 1992). The specimens used in biology and medicine are usually cells and tissues which contain radioactive substances. They are fixed, sectioned and placed in contact with the radioautographic emulsions, which are exposed and developed to produce metallic silver grains. These specimens are called radioautographs and the patterns of pictures made of silver grains are named radioautograms.

In view of the scarcity of studies on *P. hilarii*, the aim of the present investigation was to describe the morphology of the leukocytes of this species according to their seasonal distribution and to determine cell renewal in the blood circulation using a radioautographic study.

## 2. Material and methods

This study followed the requirements of the Ethics Committee on the Use of Animals in Experimentation of the University of São Paulo, Brazil.

The study was conducted on five adult *P. hilarii* turtles (Chelonia, Chelidae) of both sexes weighing 600–1200 g. The animals were captured from the estuary of the Guaíba River near Porto Alegre, Brazil. The observations were made during the various seasonal periods, with temperatures ranging from 15 to 30 °C.

Blood was aspirated, smeared on glass slides and stained with Romanowsky stain (Fig. 1A and B). A total of 500 cells were counted in the blood samples from each animal during each season. The results obtained were analyzed statistically by analysis of variance followed by the Bonferroni test (NCSS) due to the multiple comparisons, with the level of significance set at  $p < 0.05$ .

To determine the time needed for leukocyte repopulation in blood, 1000  $\mu\text{Ci/kg}$  of  $^3\text{H}$ -thymidine were injected in the circulating blood. The positive reaction to thymidine was identified in the nucleus of the cells that were dividing into other cells. The blood samples were processed for radioautography by covering with a single layer of nuclear emulsion, (NTB2, Kodak) at 4 °C, in a dark room. After 6 days, the material was radiographically exposed to D-170 for 10 min at 10 °C, fixed with 24% sodium thiosulfate for 6 min, and stained with Giemsa. The histological slides were developed at different times (6, 12 and 30 days), as commonly done in this technique. The radioautographic analysis was performed using a point counting method (Weibel et al., 1966), considering the area of the nuclear region of the cell, using an image analysis system (KS300 - Zeiss, Axion Vision, Germany).

## 3. Results

In the present study, six types of leukocytes were detected in the blood of *P. hilarii* turtles, i.e., basophils, eosinophils, lymphocytes, monocytes, heterophils (neutrophils), and thrombocytes. Basophils presented a spherical conformation with a segmented nucleus and spherical granules in the cytoplasm (Fig. 2A). Eosinophils had a spherical shape and a peripheral nucleus, with cytoplasm filled with oval granules (Fig. 2B). Small and spherical lymphocytes with eccentric nuclei were observed in almost the entire cytoplasm (Fig. 2C). Monocytes were found in circulating blood, characterized by an oval and peripheral nucleus and abundant cytoplasm (Fig. 2D). Heterophils (neutrophils) were detected by light microscopy, showing a spherical nucleus, heterophilic aspect and elongated granules (Fig. 2E). Thrombocytes showed elliptic conformation,

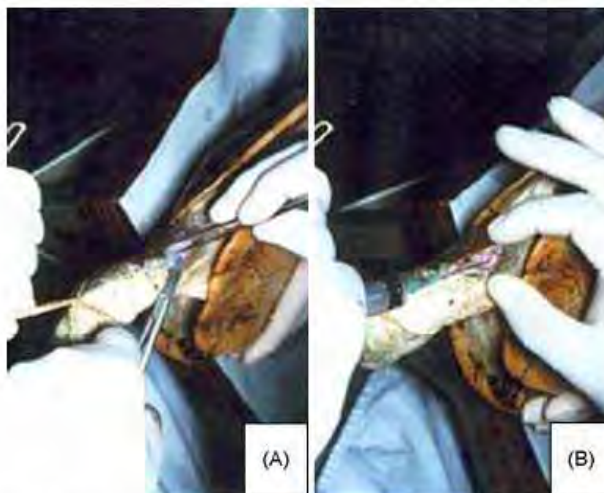


Fig. 1. Experimental animal model used in this study. (A) Image showing the surgical access to the neck aiming to expose the lateral blood vessels. (B) Image showing the aspiration of circulating blood for histological analysis.



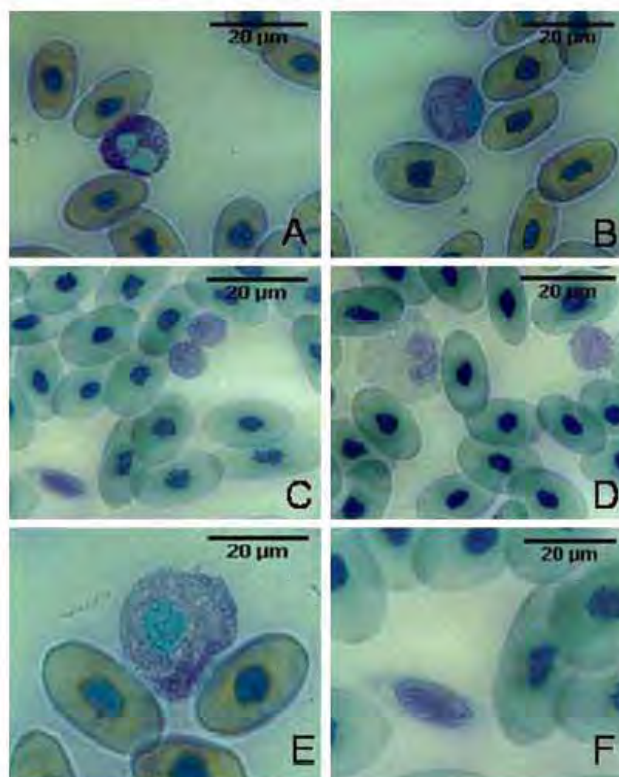


Fig. 2. Photomicrograph showing the leukocytes found in turtle blood. (A) Basophils with a spherical shape with a segmented nucleus (1), and cytoplasm filled with many metachromatic granules (2) (original magnification 400 $\times$ ). (B) Eosinophils with a spherical shape and a peripheral nucleus (1), and cytoplasm filled with large granules (2) (original magnification 400 $\times$ ). (C) Small lymphocytes with a spherical shape and an eccentric nucleus (1) filling almost the entire cytoplasm (2) (original magnification 400 $\times$ ). (D) Monocytes with a spherical shape, a peripheral and reniform nucleus (1) and abundant cytoplasm (2) (original magnification 400 $\times$ ). (E) Heterophils (neutrophils) with a spherical shape, a central nucleus (1) and cytoplasm with elongated granules (2) observed under the light microscope (original magnification 400 $\times$ ). (F) Thrombocytes with an elliptic shape and a nucleus with an elliptic conformation also (1), and a small quantity of cytoplasm (2) (original magnification 400 $\times$ ).

with little cytoplasm and a nucleus also elliptic in shape (Fig. 2F).

Radioautographic analysis of turtle blood exposed to 1000  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine and developed after 30 days showed a large number of silver grains incorporated into the cells, which were identified and quantified using the Analyzer system K3000 – Zeiss Axion Vision. It was possible to observe that cell renewal occurred every seven days, except for basophils (Figs. 3A, B and 4).

The data obtained after differential cell counts clearly demonstrated a seasonal influence on the distribution of some types of leukocytes (Figs. 5 and 6). After statistical analysis, the following “ $p$ ” values were obtained for heterophils ( $p = 0.0007$ ), basophils ( $p = 0.0002$ ), monocytes ( $p = 0.0016$ ), and eosinophils ( $p = 0.0073$ ). However, the statistical method used did not reveal significant differences related to seasonal influence for lymphocytes ( $p = 0.16295$ ) or thrombocytes ( $p = 0.1046$ ).

#### 4. Discussion

This study presents a morphological description of the leukocytes of *P. hilarii* according to seasonal distribution, using a radioautographic method to detect leukocyte replacement in the blood circulation. The classification of blood cells in reptiles is controversial because variable criteria are used to categorize cells or because cellular lineages are uncertain (Work et al., 1998). The reptilian immune system is strikingly affected by the seasonal cycle, which produces changes in the histology of the lymphoid organs and in leukocyte functions as well (Saad et al., 1983; Saad and El Ridi, 1988). Temperature and photoperiod have been related to the changes in the immune reactivity of poikilotherms (Wright and Cooper, 1981).

Few studies are available about seasonal influence on leukocyte distribution in turtles, especially *P. hilarii*. The present study showed a seasonal influence on leukocyte distribution in this turtle. The regulatory agents of these

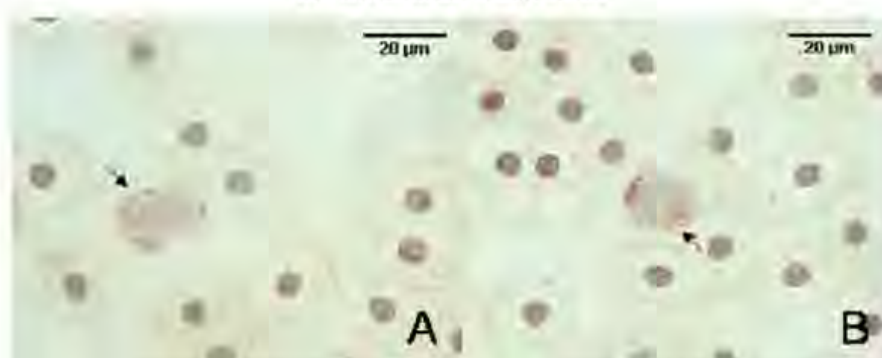


Fig. 3. Images related to the radioautographic analysis performed in this study. (A) Radioautographic monocyte surrounded by hematic tissue (—) with a large number of silver grains incorporated into the cell. (B) Radioautographic basophils surrounded by hematic tissue (—) with a large number of silver grains incorporated into the cell.

seasonal variations are also unclear. Immunosuppressive effects of winter have repeatedly been related to low temperature (El Deeb et al., 1980). However, in a study on *Salmo gairdneri*, Yamaguchi et al. (1981) reported that even when the environmental temperature is kept constant, antibody synthesis is still affected by the seasons. Therefore, other factors have been pointed out as possible causes of the seasonal changes affecting the immune system of ectotherms. Harris (1972), on the basis of the relationships between sex and season, has correlated seasonal changes in the hematology of *Rana pipiens* with the animal's breeding cycle, postulating a control exerted by one or more endocrine factors. In this regard, Saad and El Ridi (1984) have speculated that reptilian immunologic deficiency in winter is related to elevated concentration of glucocorticoids caused by stress. Moreover, according to Plytycz and Bigaj (1983), the seasonal secretory activity of the thymus of *Rana temporaria* can be regulated by the hypothalamic nucleus tuberis-pars tuberalis complex.

We detected six different types of leukocytes in this turtle species (*P. hiliarii*), similar to those described in reptiles by Sypek and Borysenko (1988), in green turtles (Work et al., 1998) and *Chelonia chelidæ* (Pitó et al., 2007). Studies related to leukocyte characterization in turtles do not present a consensus

regarding the description of these leukocytes. Studies on land turtles of the species *Terrapene carolina* and *Gopherus polyphemus* identified the following cell types in blood samples: basophils, eosinophils, lymphocytes, monocytes, neutrophils, and thrombocytes (Ryerson, 1943). However, Head and Rogers (1962), working with four species of turtles, did not detect basophils in their blood. In a similar study performed on blood tissue of the turtle *Chelonia mydas*, monocytes were not observed and heterophils were considered to be neutrophils (Wood and Ebanks, 1984). Munoz and Fuente (2004) detected heterophils in lymphoid tissues of *Mauremys caspica*, specie of turtles. Light microscopy studies on the leukocytes of other species have shown the presence of basophils, eosinophils, lymphocytes, monocytes, and heterophils in the turtles species *Podocnemis expansa* and *Emys orbicularis*, (Oliveira et al., 2000; Melin et al., 2006). Ultrastructural cytochemistry studies performed on *Chrysemys dohertyi* turtles confirmed the existence of eosinophils and heterophils in blood samples and different morphological characteristics of these cell types according to seasonal period of time (Azevedo and Lunardi, 2003).

Radioautographic analysis of turtle blood exposed to 1000 µCi of  $^3\text{H}$ -thymidine and developed after 30 days showed a large number of silver grains incorporated into the cells. Various kinds of image analyzers are now commercially available for the quantitative analysis of these radioautograms (Nagata, 1993, 1995). The number of nuclei per total cell population labelled with  $^3\text{H}$ -thymidine is determined to calculate the labelling index, or the number of silver grains per cell body or per unit area labeled with other macromolecular precursors is determined to calculate the relative incorporation rates. This technique demonstrates the sites of incorporation, synthesis and discharge of various substances in living organisms by macroscopic and microscopic radioautography, permitting the localization of intracellular sites of metabolism at the cellular and organelle levels in various objects.

On the basis of the results obtained in the present study on *P. hiliarii* using the differential cell count method, we may conclude that there is a seasonal influence on the distribution of

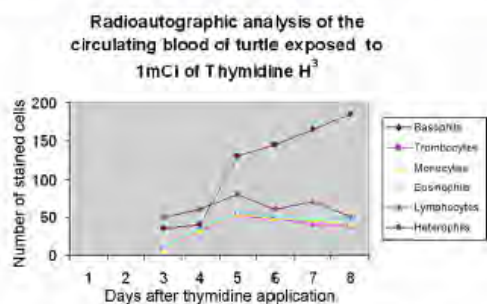


Fig. 4. Quantitative data of the radioautographic analysis.



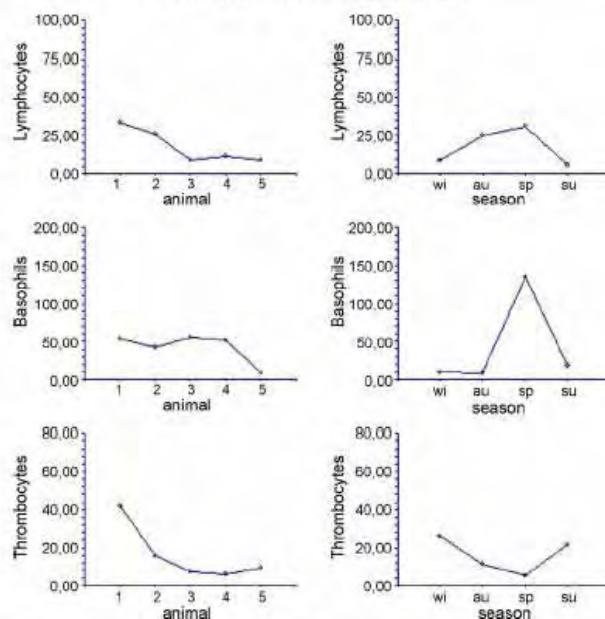


Fig. 5. Quantitative data regarding lymphocytes, basophils and thrombocytes for each animal and each season (Wi, winter; Au, autumn; Sp, spring; Su, summer).

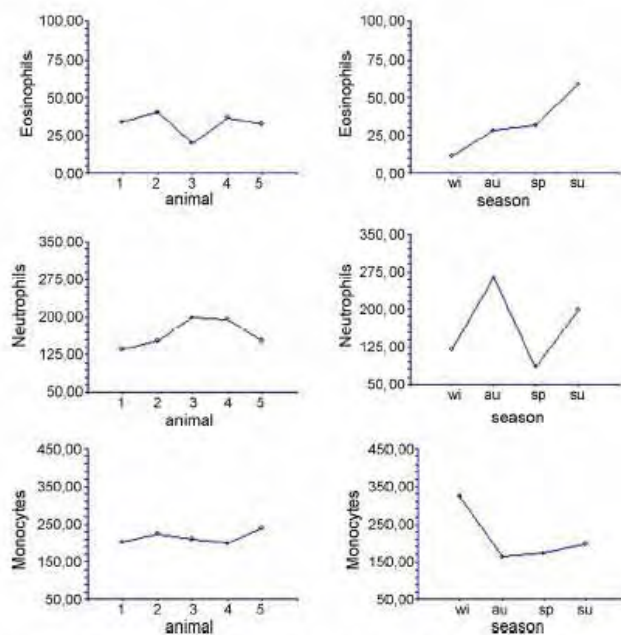


Fig. 6. Quantitative data regarding eosinophils, heterophils and monocytes for each animal and each season (Wi, winter; Au, autumn; Sp, spring; Su, summer).

some types of leukocytes, perhaps due to hibernation and to the reproductive period. Using the radioautographic method, a cell renewal system occurring every 7 days was detected, except for basophil cells.

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## **8. *Artigo 3***

*Int. J. Morphol.*,  
25(2):363-366, 2007.

## Characterization of Blood Mononuclear Phagocytes in *Phrynops hilarii* (Chelonia Chelidae)

Caracterización de los Fagocitos Mononucleares en la Sangre de *Phrynops hilarii* (Chelonia Chelidae)

\*Dimitrius Leonardo Pitol; \*\*João Paulo Mardegan Issa; \*\*\*Flávio Henrique Caetano & \*\*\*\*Laurelúcia Orive Lunardi

PITOL, D. L.; ISSA, J. P. M.; CAETANO, F. H. & LUNARDI, L. O. Characterization of blood mononuclear phagocytes in *Phrynops hilarii* (Chelonia Chelidae). *Int. J. Morphol.*, 25(2):363-366, 2007.

**SUMMARY:** The localization of peroxidase activity in different cell regions is used as a criterion for the classification of the stage of maturation of mammalian mononuclear phagocytes with a positive peroxidase reaction indicating the presence of monoblasts, promonocytes, monocytes and macrophages. In this study it was evaluated the peroxidase activity of blood mononuclear phagocytes of this turtle detected at different stages of differentiation. The present observations suggest that, in turtles, the differentiation of mononuclear phagocytes occur in the blood circulation, in contrast to animals, where only are monocytes in circulating blood and macrophage differentiation occurs in other body compartments.

**KEY WORDS:** Mononuclear phagocytes; Blood; Turtle.

### INTRODUCTION

Mononuclear phagocytes represent a cell lineage consisting of monoblasts, promonocytes, monocytes, and macrophages. The similarities of the morphological, cytochemical, and functional characteristics of these cells have led to the concept of a mononuclear phagocyte system (Van Furth *et al.*, 1972; Pellizzon *et al.*, 2002). One of the major functions of mononuclear phagocytes is phagocytosis and the killing of microorganisms (Langermans *et al.*, 1994; Halliwell, 2006).

In mammals, mononuclear phagocytes differentiate in bone marrow from stem cells- monoblasts, promonocytes and monocytes. Monocytes enter the circulation and migrate to body cavities and or tissue, where they differentiate into macrophages as growth factors, interleukins and others (Van Furth *et al.*, 1972; Beelen & Fuitsma, 1982; Beelen *et al.*, 1989; Metcalf, 1989; Metcalf & Nicola, 1992; Naito, 1993; Sudhakaran *et al.*, 2007). Mononuclear phagocytes in the different stages of development have been well characterized in mammals on the basis of ultrastructural observation of peroxidase activity and distribution (Beelen *et al.*, 1978, 1979; Beelen, 1981; Beelen & Fuitsma).

Mononuclear phagocytes have not been fully characterized in turtles. The turtle (*Phrynops hilarii*) has special

interest because of its easy breeding, rapid development, and easy access and lodging. In this study it was evaluated the peroxidase activity of blood mononuclear phagocytes of this turtle detected at different stages of differentiation.

### MATERIAL AND METHOD

This study followed the requirements of the Ethics Committee on the Use of Animals in Experimentation at the University of São Paulo, Brazil.

The study was conducted on five adult *Phrynops hilarii* turtles (Chelonia, Chelidae) of both sexes weighing 600-1200g. The animals were captured around the estuary of the Guaíba River, near Porto Alegre, Brazil. All the observations were made during the spring and early summer at a temperature ranging from 20 to 30°C. Three mL of blood obtained by puncturing the jugular vein of the neck were collected into a heparinized glass tube and centrifuged for 20 min at 1000 rpm. The coat buffer was fixed for 10 min in 1% glutaraldehyde in 0.1M Na-cacodylate-HCL buffer, pH 7.4, at 4°C. This was then washed in the same buffer and pre-incubated in 0.1% 3,3-diaminobenzidine-tetrahydrochloride (Polyscience,

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PITOL, D. L.; ISSA, J. P. M.; CAETANO, F. H. & LUNARDI, L. O.

Warrington, PA) in 0.1M Na-cacodylate-HCL buffer, pH 6.5, in the absence the H<sub>2</sub>O<sub>2</sub> at room temperature for 45 min in the dark.

The coat buffer was divided into two aliquots, one of which was incubated in freshly prepared medium in the presence of 0.01% H<sub>2</sub>O<sub>2</sub> for 30 min at room temperature, while the other (control) was incubated in fresh medium in the absence of H<sub>2</sub>O<sub>2</sub> (Beelen & Fuitsma). The cells were washed in 0.1M Na-cacodylate, pH 7.5, and post-fixed in 0.1 % OsO<sub>4</sub> in the same buffer for 30 min at 40C, then rinsed in distilled water, dehydrated and embedded in epoxy resin. Thin sections were observed with a transmission electron microscope (Jeol 100C).

## RESULTS

Different stages of mononuclear phagocyte differentiation are observed in the circulating blood of turtle, as defined by ultrastructural peroxidase cytochemistry.

In this study, we identified cells with monoblast characteristics, i.e., a large nucleus with prominent nucleoli, a nucleocytoplasmatic ratio greater than 1, and the peroxidase reaction was localized in the cytoplasmic granules (Fig. 1A). Promonocytes, with an indented nucleus and a nucleocytoplasmatic ratio of less than 1, clearly visible cytoplasmic prolongations, peroxidase reaction in the nuclear envelope, rough endoplasmatic reticulum, and cytoplasmic granules can be seen in Fig. 1B. Monocytes have a positive peroxidase reaction in the cytoplasmic granules and peripheral nucleus (Fig. 1C). A circulating macrophage with a positive peroxidase reaction in cytoplasmic granules, nuclear envelope, and granular endoplasmic reticulum is illustrated in Fig. 1D.

## DISCUSSION

Peroxidase activity was observed ultrastructurally in the circulating blood of *Phrynops hilarii*, identifying monoblasts, promonocytes, monocytes and macrophages.

In mammals, the monoblast-promonocyte-monocyte differentiation occurs in bone marrow, with monoblasts and promonocytes being detected in circulating blood only in specific pathological situations (Van Furth, 1989, 1992). In *Phrynops hilarii* this cell differentiation was observed in circulating blood under normal conditions. The distribution of peroxidase activity in these mononuclear phagocytes was similar to that observed in the bone marrow, peritoneal cavity,

and inflammatory sites of mammals (Bellen *et al.*, 1978, 1979; Beelen, 1981; Van Furth, 1989; Pellizzon *et al.*).

In mammals, it has been suggested that the monocytes production is controlled by different growth factors as CFU-GEMM, CFU-GM e M-CSF (Metcalf; Metcalf & Nicola) or 11-6 (Otsuka *et al.*, 1991). These cells in the bone marrow move to circulating blood, 24h after its differentiation. They are spread off in the circulating and endothelial vasa borders, posteriorly they move into tissues and serum cavities and differentiate into exudates and resident macrophages (Van Furth, 1989; Sudhakaran *et al.*). In the inflammatory responses, the circulating monocytes influx are mediated by different chemotactic factors as the protein-1 (MCP-1), and in the inflammatory focus differentiate into exudates macrophage (Bodel *et al.*, 1977; Beelen *et al.*, 1978, 1981; Van Furth, 1989; Leonard & Yoshimura, 1990; Issekutz *et al.*, 1981; Jandhl, 1991; Wiktor-Jedrzejczak *et al.*, 1992). Monocytes, macrophages with peroxidatic activity characteristic of resident macrophages, and negative peroxidase macrophages, were observed in this study in the turtles circulating blood suggesting that these cells move to the tissues probably in a differentiated stage.

It was observed in this study macrophages exhibiting a great phagocytose in the turtles circulating blood. These cells showed two forms, one of these is the morphological form characterized as mature macrophages and others very similar as the observed in mouse during the fetal development (Takahashi *et al.*, 1989; Naito *et al.*, 1990).

Naito defined these primitive macrophages as "fetal macrophages", we prefer to define as primitive macrophage, considering that the studied animals were in reproductive phase, thus adult animals. The mononuclear phagocytes differentiation in mammalians is dependent of the local environment conditions and the specific conditions of the specific tissues. These conditions are mediated by CSF and cytokines (Geisler *et al.*, 1989; Falk & Vogel, 1990; Naito *et al.*, 1991, 1993). Naito *et al.*, 1991 work, using bone marrow culture, showed that M-CSF presence is fundamental for monocyte differentiation into mature macrophage. The results of this study showed that mononuclear phagocytes differentiation into monoblasts-promonocytes-macrophages are not similar to mononuclear phagocytes in vertebrates because apparently they do not depend of the tissues environment conditions, and there is not necessary the monocytes migration into tissue with the objective to promote the macrophages differentiation, because this process occurs during the period that these cells are circulating. The significance of this defensive process in turtles, as well as, the necessity to understand the regulatory mechanism of the macrophages differentiation in these animals, especially the growth factors participation (CSF) in this process, must study in future researches.

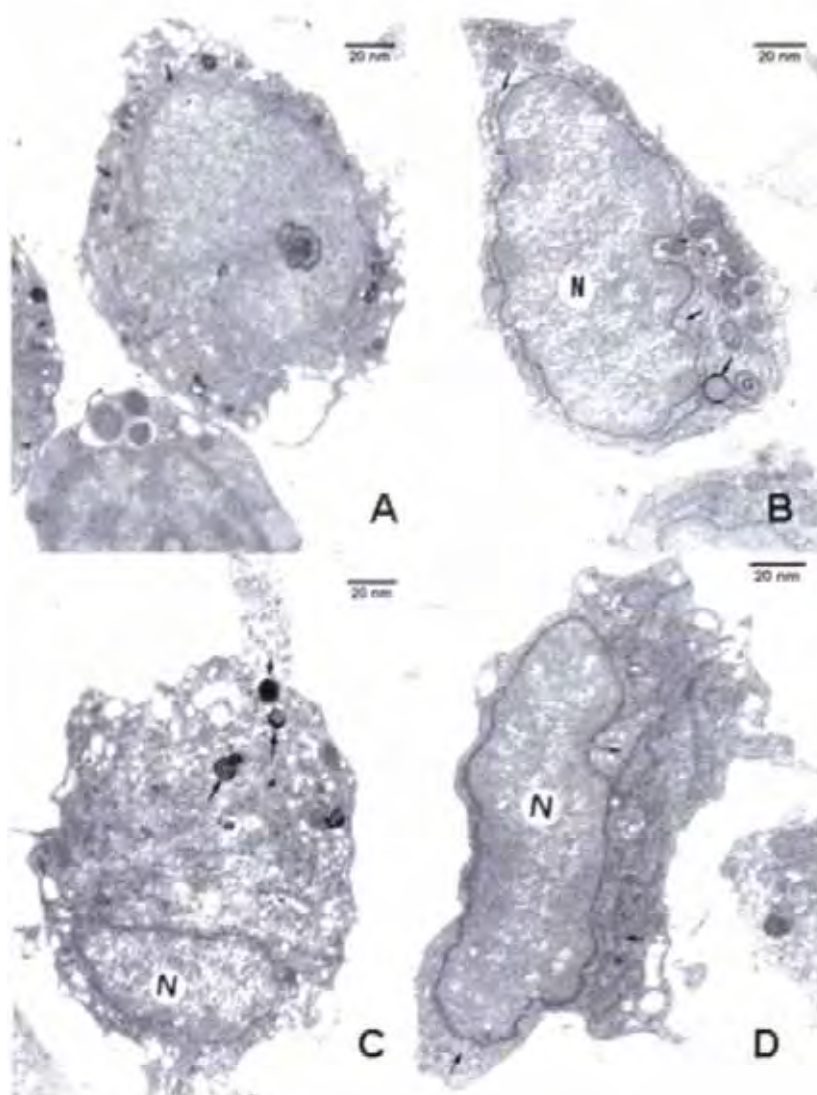


Fig. 1A. Monoblast showing positive reaction for peroxidase observed in the cytoplasmic granules (arrow) of the rough endoplasmic reticulum.

Fig. 1B. Promonocyte with positive reaction for peroxidase in the cytoplasmic granules (arrow), in the rough endoplasmic reticulum (arrow) and in the nuclear envelope (arrow).

Fig. 1C. Monocyte showing a few cytoplasmic granules with positive reaction for peroxidase in the nuclear envelope (arrow).

Fig. 1D. Macrophage circulating in blood showing peroxidase activity in the rough endoplasmic reticulum (arrow), and in the nuclear envelope (arrow).

PITOL, D. L.; ISSA, J. P. M.; CAETANO, F. H. & LUNARDI, L. O. Caracterización de los fagocitos mononucleares en la sangre *Phrynops hilarii* (Chelonii: Chelidae). *Int. J. Morphol.*, 25(2):363-366, 2007.

**RESUMEN:** La localización de la actividad de la peroxidasa en diversas regiones de la célula se utiliza como criterio para la clasificación de la etapa de maduración de fagocitos mononucleares. Una reacción positiva de peroxidasa indica la presencia de monoblastos, promonocitos, monocitos y macrófagos. En este estudio fue evaluada la actividad de la peroxidasa de los fagocitos mononucleares de la sangre de la tortuga *Phrynops hilarii* detectada en diversas etapas de la diferenciación. Las actuales observaciones sugieren que, en tortugas, la diferenciación de fagocitos mononucleares ocurre en la circulación de la sangre, en contraste a los mamíferos, donde están solamente los monocitos en la sangre circulante y la diferenciación de los macrófagos ocurre en otras partes del cuerpo.

**PALABRAS CLAVE:** Fagocitos mononucleares; Sangre; Tortuga.

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## ***9. Artigo 4***



## Mononuclear phagocytes in the blood of turtles characterized by ultrastructural and cytochemical analyses and by phagocytic activity

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### ABSTRACT

Ultrastructural and cytochemical characteristics of mononuclear phagocyte cells in turtles are not well described in the literature, especially in *Phrynosoma hilarii*. Thus, the aim of this study was to evaluate these characteristics in the mononuclear phagocyte cells and their phagocytic activity “in vitro” using the turtle *P. hilarii* as an experimental animal model. The six turtles used in the study were observed in two seasons, spring and summer. Results showed that mononuclear phagocytes incubated only in diluted solution or with colloidal charcoal have cytoplasm phagolysosomes. The cells incubated with colloidal charcoal and further exposed to the cytochemical reaction for acid  $\beta$ -glycerophosphatase, showed cytoplasm phagolysosomes filled by charcoal particles being digested and some positively stained lysosomes. Acid  $\beta$ -glycerophosphatase positive reaction was present in lysosomes and inside the phagolysosomes, while acid cytidine 5-monophosphatase staining occurred in lysosome surroundings. A positive reaction for trimetaphosphatase was also found inside phagolysosomes. In conclusion, the presence of lysosomal enzymes like trimetaphosphatase and cytidine-5'-sodium monophosphate, in the circulating blood of *P. hilarii* indicate that mononuclear phagocytes participate in the phagocytic process by gathering many phagocytic cells and forming multinucleated giant cells, which probably have a role in the blood “clearance” process.

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### 1. Introduction

Erythrocytes and leukocytes are two classes of vertebrate blood cells. Non-mammalian erythrocytes are nucleated and easily recognized morphologically. However, there is marked ambiguity in the classification of non-mammalian granulocytes mainly due to the diverse terminology applied, lack of functional studies (Meseguer et al., 1994) and morphological similarities to the mammalian granulocytes (Sypek and Borysenko, 1988). Recently, this terminology has been tentatively standardized and leukocyte counts of some reptile species determined by light microscopy (Alleman et al., 1999; Harr et al., 2001). Nevertheless, some ambiguity still exists due to species differences (Cannon et al., 1996; Bounous et al., 1996; Alleman et al., 1999). Thus,

ultrastructural and functional studies may help to clarify this classification problem.

Chelonian leukocyte is the reptilian cell best characterized at the ultrastructural level. Two granulocytes named heterophils (round granules) and eosinophils (ellipsoid granules) were found in two species of turtles (Ryerson, 1943). Taylor et al. (1963) also found eosinophils (one granule type) and heterophils (three granule types). Recently, it was demonstrated by electron microscope cytochemistry that eosinophils and heterophils in the blood of a species of turtle are two distinct blood cell lineages (Azevedo et al., 2002; Azevedo and Lunardi, 2003). Studies regarding phagocytic capacity of these blood cells are still scarce. Phagocytic capacity of thrombocyte cells is well characterized in *Phrynosoma hilarii* turtles (Pellizzon and Lunardi, 2000), but not in other cells like mononuclear phagocytes, monoblasts, promonocytes, monocytes and macrophages (Pitol et al., 2007). In addition, few studies found in the literature show the phagocytic capacity of these cells in *P. hilarii* as an experimental animal model. Morphological analysis is not the best parameter to characterize these cells, due to their great structural diversity in different groups of animals but cytochemical methods are important tools in

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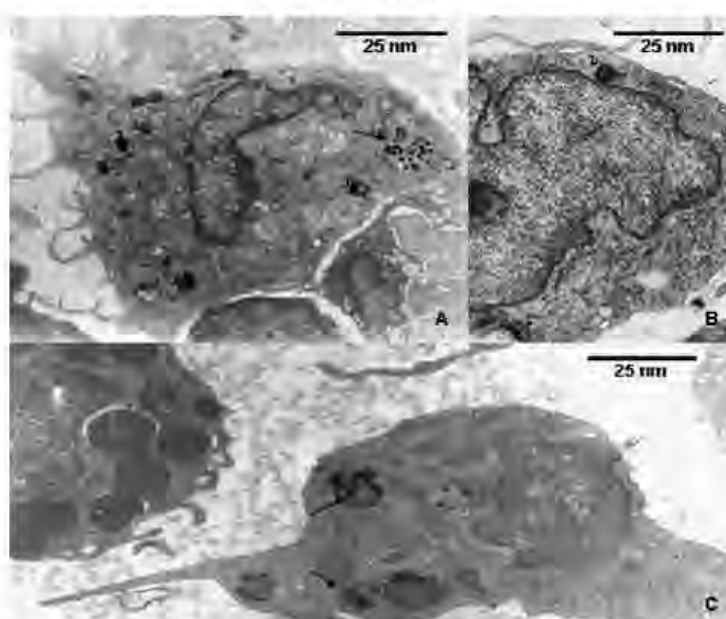


Fig. 1. Lysosomal enzymes detected by cytochemical analysis in mononuclear phagocytes of the circulating blood in *P. hiliarii* turtles: (A)  $\beta$ -acid glycerophosphatase reaction in lysosomes (–) and interior of phagolysosomes (\*), original magnification 5500 $\times$ ; (B) acid cytidine-5-monophosphatase reaction around lysosomal cells (–), original magnification 12,500 $\times$ ; (C) trimetaphosphatase reaction inside the phagolysosomes (–), original magnification 7500 $\times$ .

this task. Thus, the aim of this study was to evaluate the ultrastructural and cytochemical characteristics of the mononuclear phagocyte cells and characterize their phagocytic activity (*in vitro*) using the turtle *P. hiliarii* as the experimental model.

## 2. Materials and methods

All procedures in this study were approved by the local ethics committee. Six *P. hiliarii* turtles were caught at the Guaíba river estuary, Porto Alegre, Rio Grande do Sul, Brazil and lodged for 1 week at the Central Animal House, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. Blood was obtained by puncturing the lateral vessels of the neck, which were surgically exposed in the spring and summer, at a mean temperature of 37 °C. For ultrastructural analyses, approximately 1.5 mL of blood was obtained from each animal and placed in heparinized test tubes. The heparinized blood was centrifuged for 15 min at 1000 rpm at room temperature for analysis by transmission electron microscopy. Plasma was discarded and the leukocyte buff was fixed in 3% glutaraldehyde in 0.1 M phosphate buffer solution, pH 7.4, for 2 h at 4 °C, postfixed in 1% osmium tetroxide for 2 h at 4 °C, dehydrated in a growing acetone series, and embedded in Araldite resin (Reynolds, 1963). Thin sections were analyzed with a JEOL 100C transmission electron microscope.

For cytochemical analyses, blood smears were air dried, post-fixed in 4% formalin and submitted to the determination of the following enzyme activities: acid phosphatases ( $\beta$ -glycerophosphatase and citidine-5'-sodium monophosphatase), and trimetaphosphatase. The substrate was trimetaphosphate, plumb acetate the acceptor and incubation was for 30 min at 37 °C in a water-bath (Zugibe, 1970).

Aiming to determine the *in vitro* endocytosis activity, total heparinized blood was incubated with 0.01% colloidal charcoal (São Paulo, Brazil) for 30 min at 37 °C and centrifuged. The leukocyte buff was removed and divided into samples for transmission electron microscopy.

## 3. Results

The mononuclear phagocytes in the circulating blood of the *P. hiliarii* turtle showed positive reactions for lysosomal enzymes like acid phosphatases and trimetaphosphatase. Cytochemical analyses indicated that acid  $\beta$ -glycerophosphatase was present in lysosomes and inside the phagolysosomes (Fig. 1A). Cytidine 5-monophosphatase acid staining occurred in lysosome surroundings (Fig. 1B). It was also possible to find a positive reaction for trimetaphosphatase inside phagolysosomes (Fig. 1C). Aspects of phagocytic activity in the turtle mononuclear phagocytes are shown in Fig. 2. In Fig. 2A, cell cytoplasm shows lysosomes, mitochondria and phagolysosomes containing particles being digested. Mononuclear phagocytes containing particulate samples being digested and phagolysosomes with *Corpus mielinicus* are depicted in Fig. 2B. Fig. 2C shows mononuclear phagolysosomes phagocytosing an immature cell with a positive reaction for trimetaphosphatase in lysosomes and inside phagolysosomes (Fig. 2C). In a higher magnification, it is possible to see lysosomes release in the space between the cells (Fig. 2C).

Fig. 3A and B represents ultrastructural aspects of control mononuclear phagocytes incubated only in diluted solution or with colloidal charcoal, respectively, both showing cytoplasm phagolysosomes. Cells incubated in colloidal charcoal and submitted to the cytochemical reaction for betaglycerophosphatase

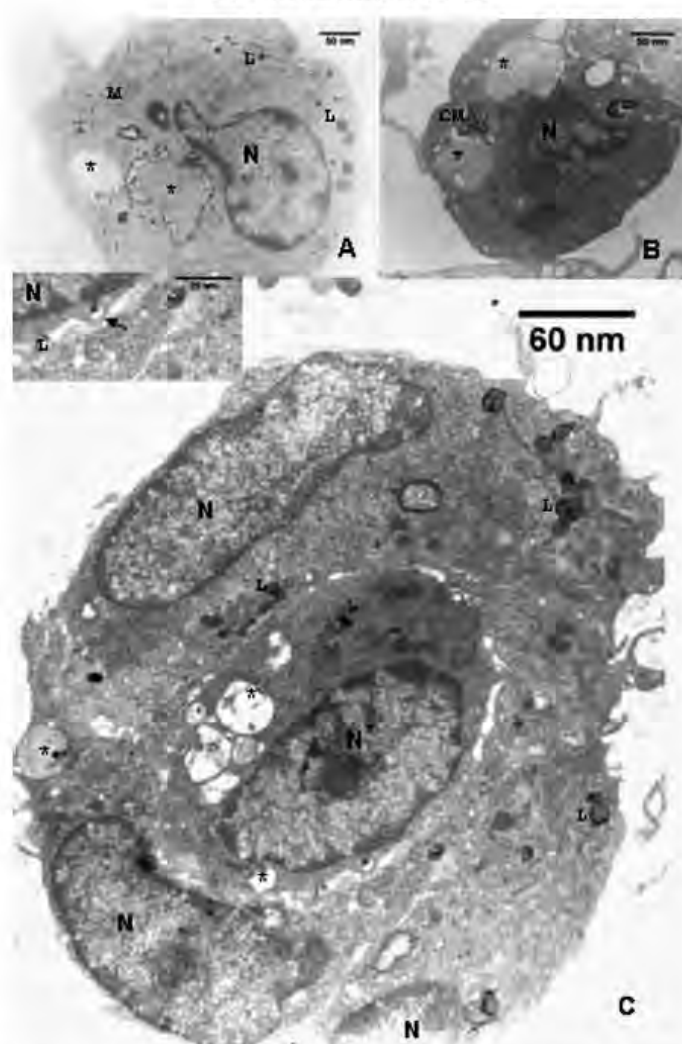


Fig. 2. Mononuclear phagocytes in the circulating blood of the turtle *P. hilarii* in phagocytic activity during spring and summer: (A) mononuclear cell showing cytoplasmic lysosomes (L), mitochondria (M) and phagolysosomes (\*) containing particles being digested, original magnification 4200 $\times$ ; (B) mononuclear cell showing cytoplasmic phagolysosomes (\*) containing particles being digested and phagolysosomes (CM), and the presence of *C. melnicus*, original magnification 5400 $\times$ ; (C) mononuclear cells phagocytosing an immature cell (N\*). Positive reaction for trimetaphosphatase in the lysosomal cells (L), and in the interior of the phagolysosomes (\*). Insert figure showing lysosomal cells released in the space between the cells (→), original magnification 6600 $\times$ .

acid show cytoplasm phagolysosomes filled with charcoal particles being digested and some positively stained lysosomes (Fig. 3C).

#### 4. Discussion

The hematology of some reptiles of different species is described in the literature but classification of different cellular types is still controversial. Few studies were found regarding the *P. hilarii* turtle. Thus, the aim of this study was to evaluate ultrastructural and cytochemical characteristics of mononuclear

phagocyte cells and characterize their phagocytic activity using the turtle *P. hilarii* as an experimental animal model.

It was observed in this study that ultrastructural analysis is important to characterize leukocytes in the circulating blood. As mentioned before, these studies in chelonians are few, but the cells are well characterized in other reptilians. It was shown in the *Caiman crocodilus* that eosinophils and heterophils are separate cell lineages (Moura et al., 1997). Alleman et al. (1999) found only heterophils in the blood of the snake *Crotalus adamanteus*. Kelényi and Németh (1969) suggested that in squamata the two described granulocytes



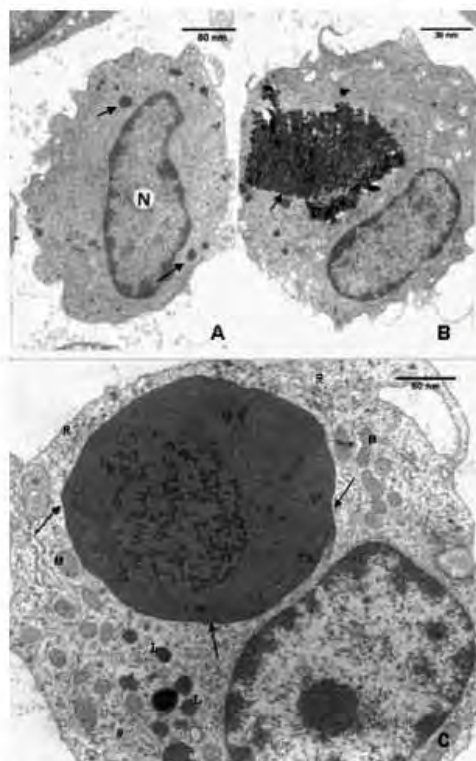


Fig. 3. Mononuclear phagocytes in the blood of the *P. hiliarii* turtle as controls and incubated with colloidal charcoal: (A) mononuclear blood cells incubated only with the diluted solution, without the colloidal charcoal showing cytoplasm phagolysosomes (→), original magnification, 3300 $\times$ ; (B) mononuclear blood cells incubated with colloidal charcoal showing cytoplasm phagolysosomes (→) filled by colloidal charcoal, original magnification, 5200 $\times$ ; (C) mononuclear blood cells incubated with colloidal charcoal and submitted to the cytochemical reaction for acid betaglycerophosphatase showing cytoplasm phagolysosomes (→) phagocytosing colloidal charcoal. Endoplasmatic reticulum (R), Mitochondria (M) and lysosome cells (L) are stained positively in the cytoplasm (L), original magnification 7400 $\times$ .

correspond to the same cell at different stages of maturation. Zapata et al. (1981) characterized two granulocytes with different types of granules, which were classified as heterophils.

Ultrastructural analysis in this study described monocytes with the usual organelles distributed through the cytoplasm. This finding is in accordance to descriptions in other reptiles where the monocyte cytoplasm is full of organelles (Taylor et al., 1963; Sypek and Borysenko, 1988) but in contrast to the results described by Alberio et al. (2005).

Cytidine 5-monophosphatase acid staining occurred in the surroundings of lysosomes and in the inner of the phagolysosomes, and a positive reaction was found for trimetaphosphatase. This finding is important, considering that these enzymes have a role in the phagocytic process. Promonocytes show cytochemical and ultrastructural characteristics different of the monocyte and macrophage cells (Naito, 1993). In the adult mononuclear phagocytic cells originate from stem cells. Macrophage-colony stimulating factor (M-CSF) is associated with granulocyte proliferation, but this mechanism is not fully understood.

The present study shows many phagocytic cells forming multinucleated giant cells, a process explained by the fact that macrophages can gather and compose multinucleated giant cells with the nucleus spread out in the cytoplasm or compose Langerhans giant cells. The nucleus stay divided and organized in the periphery of the cell due to cytoskeleton action (Baron et al., 1993; Brito and Franco, 1994).

Mononuclear phagocytes containing particle samples being digested were observed in this study. The phagocytic process may occur at any site along the plasma membrane (Zucher-Franklin, 1981) starting from the recognition of the particle to be phagocytized. This recognition occurs through specific membrane receptors, usually glycoconjugates (Gruenberg and Maxfield, 1995), as well as by an increase in the energetic activity of the cell (McRipley and Sbarra, 1967). Phagocytic activity was also observed in the thrombocytes of amphibians (Carlson et al., 1968; Daimon and Uchida, 1985), birds (Sweeny and Carlson, 1968; DaMatta et al., 1998) and in turtles (Pelizzon and Lunardi, 2000). Pelizzon and Lunardi showed that thrombocyte cells can participate not only in the plasma aggregation process, but also may be present in the phagocytic process. Zucher-Franklin (1981), detected phagocytic activity in human platelets *in vitro*, which internalized latex particles. The particles were first engulfed by the plasma membrane and afterwards detected in the surface-linked open-channel system.

Mononuclear phagolysosomes phagocytizing an immature cell were found in this study. This finding is very important because, among the many consequences of phagocytosis, the most important may be the ability to kill pathogens. Macrophage phagocytic activity has been used as an immunologic parameter to evaluate the health/immune function of different species under different biotic and abiotic factors such as pollutants (Weeks and Warinner, 1986), diets (Blazer, 1991), temperature (Hardie et al., 1994), pathogens (Ainsworth and Dexiang, 1990) and genetic variation (Sarder et al., 2001).

In the present study, phagocytic activity was evaluated only in the spring and summer thus, without great variations in temperature. Studies involving phagocytosis in ectotherms depend in part on incubation time, immune stimulant used, stress, feeding level and temperature (Sakai, 1999). The temperature used to lodge the *P. hiliarii* in this experiment may have had an effect on the phagocytic activity. The animal comes from a region where temperatures range from 20 to 38 °C. Perhaps, the temperature in this study (37 °C) reflects the results found. It would be very interesting to study the effect of the temperature, in all seasonal periods and not only in the spring and summer, with the aim to verify if there are differences between winter and summer in the macrophage phagocytosis of *P. hiliarii*.

In summary, the presence of lysosomal enzymes like trimetaphosphatase and cytidine-5'-sodium monophosphate, was observed in mononuclear phagocytes in the circulating blood of the turtle *P. hiliarii* indicating that these cells are able to participate in the phagocytic process gathering many phagocytic cells and forming multinucleated giant cells, possibly having a role in the blood "clearance" process.

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## ***10. Considerações Finais***

A caracterização dos leucócitos circulantes de *Phrynops hilarii* analisados por meio de microscopia de luz, após coloração com Leishman, e com a microscopia eletrônica de transmissão, não indicou nenhum tipo celular semelhante aos neutrófilos mamíferos. Baseando nos dados obtidos neste estudo, sugerimos que os heterófilos têm uma analogia de morfofuncional com o neutrófilo pertencente ao grupo dos mamíferos. Esta conclusão está sendo apoiada em muitos recentes estudos achados na literatura. Podemos concluir que as células sanguíneas de *Phrynops hilarii* seguem o mesmo padrão de descrição de outras espécies: eritrócitos, trombócitos, leucócitos, monócitos, linfócitos, eosinófilos, basófilos e heterófilos.

Fatores sazonais interferem nos processos de reprodução, regulação hormonal, com alterações histológicas em órgãos linfóides. Em *Phrynops hilarii*, constatou-se que existe influência sazonal para monócitos, basófilos, eosinófilos e heterófilos por meio da análise estatística, sendo que não obtivemos resultado significativo para linfócitos e basófilos, o estudo autorradiográfico mostrou que as células do sangue de tartaruga se renovam a cada sete dias, com exceção dos basófilos.

Em mamíferos, a produção de monócitos é controlada por meio de fatores de crescimento, sendo que estas células são produzidas na medula óssea e, após 24 horas, já diferenciadas, migram através do endotélio para os tecidos, finalmente diferenciando-se em macrófagos. Nossos resultados mostram que a diferenciação de fagócitos mononucleares de tartaruga é diferente de outros vertebrados monoblasto, promonócito, monócito e macrófago que ocorrem ainda no sangue circulante, não dependendo da característica de cada tecido para a diferenciação em macrófagos.

Em nossos resultados encontramos fagócitos mononucleares fagocitando uma célula imatura e também detectamos a presença de enzimas importantes para o processo de fagocitose, podemos afirmar que esses mononucleares podem participar do processo de fagocitose, inclusive formando células gigantes multinucleadas, possivelmente participando do processo de clarence celular.

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