Simple method for culture of peripheral blood lymphocytes of Testudinidae

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ABSTRACT. We developed and optimized a simple, efficient and inexpensive method for *in vitro* culture of peripheral blood lymphocytes from the Brazilian tortoise *Chelonoidis carbonaria* (Testudinidae), testing various parameters, including culture medium, mitogen concentration, mitotic index, culture volume, incubation time, and mitotic arrest. Peripheral blood samples were obtained from the costal vein of four couples. The conditions that gave a good mitotic index were lymphocytes cultured at 37°C in minimum essential medium (7.5 mL), with phytohemagglutinin as a mitogen (0.375 mL), plus streptomycin/penicillin (0.1 mL), and an incubation period of 72 h. Mitotic arrest was induced by 2-h exposure to colchicine (0.1 mL), 70 h after establishing the culture. After mitotic arrest, the cells were hypotonized with 0.075 M KCl for 2 h and fixed with methanol/acetic acid (3:1). The nonbanded mitotic chromosomes were visualized by Giemsa staining. The diploid chromosome number of *C. carbonaria* was found to be 52 in females and males, and sex chromosomes were not observed. We were

able to culture peripheral blood lymphocytes of a Brazilian tortoise *in vitro*, for the preparation of mitotic chromosomes.

Key words: Testudinidae; *Chelonoidis carbonaria*; Cytogenetic; Lymphocyte culture

INTRODUCTION

Cytogenetic identification is an important parameter for the organism's identification in their natural habitat. The first level of genome analysis involves karyotyping of mitotic chromosomes to determine the organism's genome organization at the cytological level (Singh et al., 1970; Gormam, 1973). The classical methods of chromosome preparation in whole animals involve killing the study specimen or using surgical procedures that are often unsafe for survival of the animal, traumatic and unethical, because *in vivo* inoculation with colchicine solution is required. After killing, cells can be obtained from the spleen, lung, kidney (Medrano et al., 1987) or spinal cord (Kamesaki, 1989). These cells are cultured and stimulated to obtain metaphases (Bickham, 1975; Killebrew, 1975; Bickham and Baker, 1976; Bickham and Carr, 1980, 1983; Medrano et al., 1987; Kamezaki, 1989, 1990). These methods are generally not feasible in endangered species, because of the risk and number of animals available. In such cases, karyotype analysis using cultured blood lymphocytes without animal sacrificing is most desirable and useful. Few reports are available for lymphocyte cultures of tortoises (Maecha, 1998; Ulsh et al., 2000a; Ortiz and Rodríguez, 2003), but the methods previously described did not show satisfactory results, because of cells' differential adaptability, variation in preferred body temperature, lymphocyte population, and immunological responses to mitogen stimulation.

The genus *Chelonoidis* (Fitzinger, 1835) is currently represented by four species in South America: *Chelonoidis carbonaria*, *C. denticulata*, *C. chilensis*, and *C. nigra*. The species *C. carbonaria* is considered to be endangered according to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (IUCN, 2004), which may become extinct if illegal trade and habitat loss are not controlled. *C. carbonaria* occurs in dry forests and areas of forest vegetation in savannas.

We determined the requirements for the growth of peripheral blood cells of *C. carbonaria*, to obtain metaphases and generate information about the karyotypic characteristics of this species, providing the basis for future research in the cytogenetics of this species.

MATERIAL AND METHODS

Experiments were carried out on 8 adult individuals of *C. carbonaria* from the "Reginaldo Uvo Leone" breeding farm in Tabapuã, São Paulo, Brazil (20°59'47.4"S, 49°07'16.6"W). The animal care procedures followed in this study were authorized by the Comissão de Ética em Experimentação Animal (CEEA) of the Universidade Estadual Paulista "Júlio de Mesquita Filho", UNESP/IBILCE (No. 018/09) and approved by IBAMA/SISBIO (No. 19514-1).

A total of 20 experiments were carried out at the Centro de Estudos de Quelônios (CEQ), Universidade Estadual Paulista "Júlio de Mesquita Filho", to determine the conditions

needed to obtain a good mitotic index, such as volume of culture medium, concentrations of phytohemagglutinin and fetal bovine serum and lymphocyte layer volume. We also determined the optimal times for incubation, mitotic arrest and hypotonization (data not shown).

The costal vein, located lateral to the longitudinal axis of the animal, was used as the blood sampling site, which was first cleaned with distilled water and 70% ethanol (Silva et al., 2011).

About 5 mL blood was collected from each animal. The samples were immediately transferred to sterilized tubes containing heparin and stored at 37°C for 2 h. After blood collection, the skin was again swabbed with 70% ethanol to prevent microbial infection. The animals were kept under observation to assure recovery before being released. Cell types such as red blood cells and lymphocytes were separated in a centrifuge tube according to their density. The upper layer lymphocytes were transferred to a sterile centrifuge tube, and incubated at 37°C for complete separation of lymphocytes.

First, 7.5 mL MEM culture medium was placed in a 15-mL Falcon tube. All other components were added serially (see Table 1). These steps were carried out in sterile conditions in a laminar flow hood to avoid microbial contamination. The total volumes used per sample are shown in Table 1.

Table 1. Components of short-term culture of peripheral blood lymphocytes of *Chelonoidis carbonaria*.

Component	Culture volume
MEM (E) with NaHCO$_3$ (CULTILAB)	7.5 mL
Penicillin-streptomycin (10,000 IU/mL-10 mg/mL)	0.1 mL
PHA-P (CULTILAB)	0.375 mL
Fetal bovine serum (CULTILAB)	1.5 mL
Lymphocytes with serum (separated from 5 mL whole blood)	2.0 mL
Colchicine (16 µg/mL)	0.1 mL

The culture was placed in an incubator set at 37°C, and shaken gently every morning to break up cell clumps. After 70 h of incubation, 0.1 mL colchicine was added to a final concentration of 16 µg/mL, and the cells were harvested after 2 h for a good mitotic count (Ulsh et al., 2000a, adapted).

The cells were centrifuged at 900 rpm for 5 min. After discarding the supernatant, the cell pellet was resuspended in a hypotonic solution (1 mL pre-warmed 0.075 M KCl) and the cells incubated at 37°C, for 2 h. At the end of hypotonic treatment, a few drops of fixative solution (one part glacial acetic acid and three parts absolute methanol) were added to the hypotonic solution to prefix the cells, followed by centrifugation at 900 rpm for 5 min. Again, after discarding the supernatant, the cell pellet was resuspended in 1 mL freshly prepared glacial acetic acid-methanol following gentle mixing. The last step (fixation) was repeated twice for proper fixation. The cells were finally suspended in 0.5 mL fixative solution for chromosome preparation.

Chromosomes were prepared by the splash technique (Ulsh et al., 2000b), stained with 6% Giemsa prepared in Sorenson's buffer (Ulsh et al., 2000a), air-dried, and kept in the refrigerator for proper storage. Chromosomes from at least 20 metaphases were counted and the best mitotic spreads were photographed using a light microscope equipped with an image analyzer (AXIOCAM® ICc 3).

Chromosomes were identified according to Bickham (1975) and arranged in three groups: Group A, consisting of large-size macrochromosomes; Group B consisting of medium-size macrochromosomes, and Group C, including all the microchromosomes whose centromeric position was not clearly identifiable.

RESULTS AND DISCUSSION

In this study, the best conditions to obtain a high mitotic index were: MEM culture media (7.5 mL/sample) combined with phytohemagglutinin (0.375 µL/sample), fetal bovine serum (1.5 mL/sample), and 2 mL lymphocyte layer. The cultures were placed in an incubator at 37°C. After 70 h, 0.1 mL colchicine was added, and after further incubation for 2 h, the cells were suspended in 0.075 M KCl for 2 h. Methanol-acetic acid (3:1) was used as the fixative. The mitotic cells were collected after 72 h.

With the method described above, metaphases were identified with a good distribution and number, allowing the identification of sets of chromosomes. The non-banded mitotic chromosomes were visualized by Giemsa staining. *Chelonoidis carbonaria* revealed a diploid number of 2n = 52 chromosomes, in both sexes, divided into three groups (A, B, C). Group A was composed of 28 chromosomes (3 metacentric pairs, one acrocentric and 10 submetacentric pairs), group B consisted of seven pairs of acrocentric chromosomes, and group C showed five pairs of microchromosomes (Figure 1A,B). Sex chromosomes were not observed.
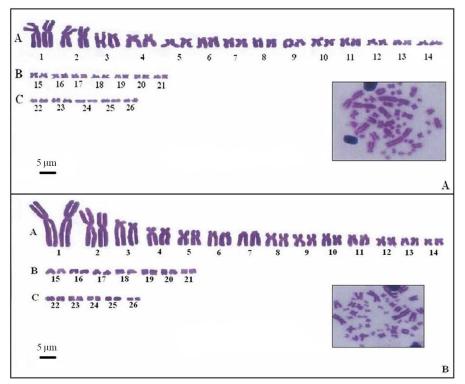

Figure 1. Karyogram of *Chelonoidis carbonaria* by Giemsa staining. **A.** Female. **B.** Male. Bar = 5 µm.

The optimized conditions in this study yielded a good mitotic index of about 10-15 metaphases per slide. Our method showed better results for Testudinidis than those methods described for mammals, including humans and also for other reptiles, resulting in good mitotic index (Ulsh et al., 2000a; Ciofi et al., 2002). The culture technique we optimized can be applied to other tortoise species, also with certain modifications in essential parameters such as incubation temperature, incubation period, serum concentration, and mitogen.

The diploid chromosome number 2n = 52 has been observed in other members of family Testudinidae, including *C. denticulata*, *C. donosobarrosi*, *C. chilensis*, *C. elephantopus*, and *C. gigantea*, and also in members of the families Pelomedusidae and Bataguridae (Forbes, 1966; Sampaio et al., 1971; Goldestein and Lin, 1972; Bickham, 1975; Bickham and Baker, 1976; Benirschke et al., 1976). Our data confirm that some species of the Testudinidae have a relatively smaller number of microchromosomes in relation to the family Trionychidae (Matsuda et al., 2005; Noleto et al., 2006; Rohilla et al., 2006). The mechanisms responsible for reducing the loss of microchromosomes can be numerical, translocation of microchromosomes to acrocentric chromosomes and formation of metacentric chromosomes during the evolution of Testudines (Bickham, 1975).

A simple, efficient and economic method was optimized for *in vitro* culture of peripheral blood lymphocytes from the Brazilian tortoise *Chelonoidis carbonaria* (Testudinidae), through the analysis of parameters including culture medium, mitogen concentration, mitotic index, culture volume, incubation time, duration of culture, and mitotic arrest. The present study was a successful attempt at *in vitro* culture of peripheral blood lymphocytes for the preparation of mitotic chromosomes for this species, and it is important because reveals a viable and economic protocol for Brazilian tortoise cell culture, without harming the animal. This protocol is particularly suited for a modestly equipped laboratory. These data may be useful for more accurate comparative cytogenetic studies regarding the karyotypic evolution in this group.

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