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Prenatal Development of Gonads in Siamese Crocodile Embryos (*Crocodylus siamensis*)

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ABSTRACT

In crocodiles, differentaition of gonads to testis or ovary was influenced by incubation temperatures. To manipulate sexual outcome of the offspring, the information of gonadal development in terms of timing and stages of development is crucial. However, there has been no reports in Siamese crocodiles (*Crocodylus siamensis*), indigenous fresh water crocodiles of Thailand. This study aims to investigate gonadal development in Siamese crocodile embryos at 35, 48, 63 and 69 days of incubation which belonged to the second half of incubation. Microscopic study of gonad from Siamese crocodile embryos showed the presence of gonadal ridges in embryos at 35 days of incubation, Migration of primordial germ cells was found within gonads at day 35 of incubation and during the same period sex cords were developed. Bipotential gonads were detected at 48 days of incubation and sex cords extensively developed at 63 days and at 69 days of incubation. The data suggested that development of testis and ovary may occur after hatching. Expressions of sex steroid hormones and receptors for sex hormones in Siamese crocodile are needed to be elucidated.

Key words: Siamese crocodile, prenatal development, gonads

INTRODUCTION

In farm animal production, gender preference occurs when either male or female animals are required for certain purposes of production. In Thailand Siamese crocodiles (*Crocodylus siamensis*), Thai fresh-water crocodiles once inhabited in nature but now are reared and bred in farms. Main products of crocodile farms in Thailand are skin and meat. Male crocodiles would be preferred because of their larger sizes than females. Breeding farms need both sexes to produce offspring. There has been showed the influence of incubation temperatures on sex differentiation in reptiles (Ferguson and Joanen, 1982). The advantage of this knowledge is to manipulate gender of preference in reptiles. The information regarding the time of gonadal formation is essential for determining of critical period for sex differentiation in Siamese crocodiles. However, the description of gonadal development in Siamese crocodiles has not been reported.

In nature, the incubation temperatures of Siamese crocodiles range between 28-35 °C. The temperatures for commercial production are between 31-32 °C and approximately 99% humidity. The incubation period in Siamese crocodiles is approximately 70-77 days. (Kaewchaum *et al.,* 2006).

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In mammals, birds and reptiles the gonadogenesis contains three phases: formation of gonadal ridge, the development of bipotential gonads, and the formation of testis and ovary. Gonadal ridges are composed of mesenchymal cells and covered with germinal epithelium. They subsequently differentiate to bipotential gonads which are characterized with the presence of sex cords and the migration of primordial germ cells underneath germinal epithelium. The stage of bipotential gonads is a critical point for sex determination when gonads are induced by incubation temperatures to differentiate to testis or ovary (Bystov and Høyer, 1988). Testis formation was marked by the presence of seminiferous tubules and Sertoli cells.

In reptiles, the onset of gonadal development occurred approximately second quarter of incubation periods. Studies in *Alligator mississipensis* revealed that the gonadal ridges were first observed at days 25-27 of 64-day incubation period in male embryos and days 31-34 of 72-day incubation period in female embryos. The early formation of testis was detected at the beginning of third quarter, approximately 32-34 days of incubation. The early formation of ovarian follicles was detected after of 44-49 days of incubation. By the end of the incubation the testes were well-developed (Smith and Joss, 1993). In salt water crocodiles, *Crocodylus porosus* gonadal differentiation took place between days 58-70 of 80-85-day incubation period (Smith and Joss, 1994).

The purpose of this study was to investigate the period of gonadal development in Siamese crocodile embryos. The finding from this study will help understand the critical developmental period of this organ and provide the basic knowledge of gonadal development in Siamese crocodiles for further studies on the process of sex determination as well as controlling mechanisms of gonadal development in Siamese crocodiles.

MATERIALS AND METHODS

Sample collection and incubation

Forty fertilized eggs were divided into two groups. Eggs were incubated at incubation temperature between 31°C and 32°C. Ten eggs from each time point were randomly collected at days 35, 48, 63 and 69 of incubation. The eggs and incubators are the courtesy of Sri Racha Tiger Zoo, Chonburi.

Histological study of gonads in Siamese crocodile embryos

Embryos were obtained from eggs at incubation periods of 35, 48, 63 and 69 days. The gonad and kidney were examined and dissected except the embryo from day 35 of incubation period. The embryos of day 35 of incubation and kidneys and gonads of the embryos from the remaining periods were fixed with 10% neutral buffer formalin or Bouin's solution overnight.

Specimens underwent the tissue processing. Each paraffin block was prepared from individual specimens. The sections were serially cut at 10 microns thick, mounted onto glass slides and stained with hematoxylin & eosin. The histology of gonads was examined using a light microscope.

RESULTS

Histology of developing gonads in Siamese crocodile embryos

The morphology of gonads in Siamese crocodile embryos at days 35, 48, 63 and 69 of incubation was described as followed. At day 35 of incubation, gonadal ridge was detected at the ventromedial surface of metanephros (Figure 1a). The germinal epithelium covering the gonadal ridges was pseudostratified columnar epithelium. The stroma contained mesenchymal cells, which were characterized as round cells with round nucleus and nucleolus. Primordial germ cells, large

round cells with vesicular nucleus and eosinophilic cytoplasm were located in the area immediately below the germinal epithelium (Figure1b).

At day 45 of incubation, bipotential gonad was developed from gonadal ridge. The cross sections of gonad showed the formation of sex cords. Sex cords consisted of mesenchymal cells aggregating to form cords which arranged perpendicularly to germinal epithelium (Figure 2a and b). Mesenchymal cells surrounding sex cords

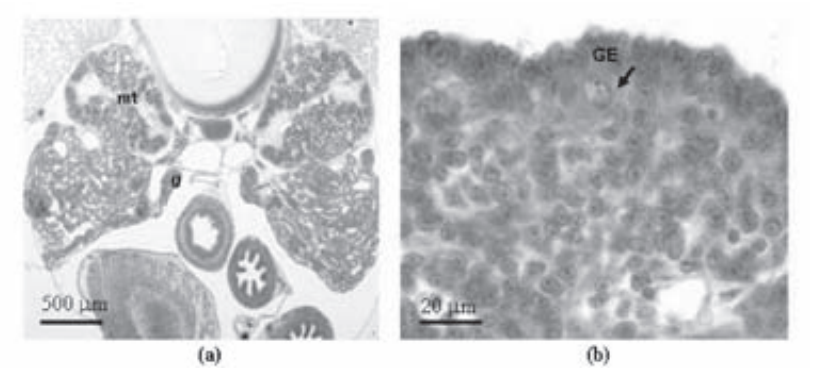

Figure 1 Photomicrographs of cross sections of crocodile embryo at 35 days of incubation.
(a) Gonadal ridges (g) were situated medially to ventromedially to metanephros (mt).
(b) Primordial germ cells (arrow) were located beneath the germinal epithelium (GE).

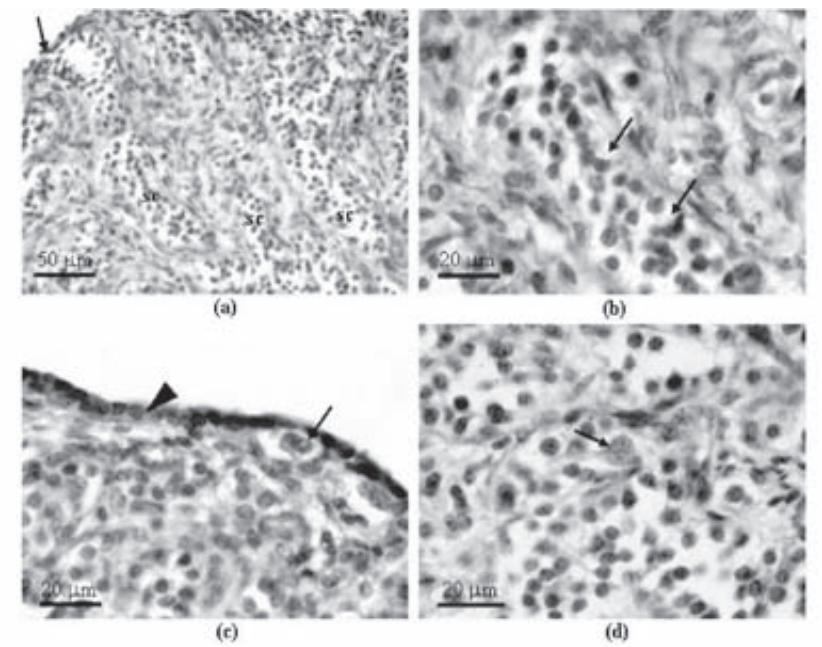

Figure 2 Photomicrographs of cross sections of crocodile embryo at day 48 of incubation
(a) Sex cords (sc) lied perpendicular to germinal epithelium (arrows).
(b) Sex cords contained numerous mesenchymal cells (arrows) packing together.
(c) Primordial germ cell (arrow) was present under the germinal epithelium (solid arrowhead).
(d) Some primordial germ cells (arrow) were located within sex cords.

differentiated into spindle-shaped cells (Figure 2b and d). Interstitial areas were composed of mixed population of spherical and spindle-shaped mesenchymal cells, and collagen fibers. Primordial germ cells were found in areas underneath the germinal epithelium and within the sex cords (Figure 2c and d). The germinal epithelial cells in this stage of gonadal development decreased their

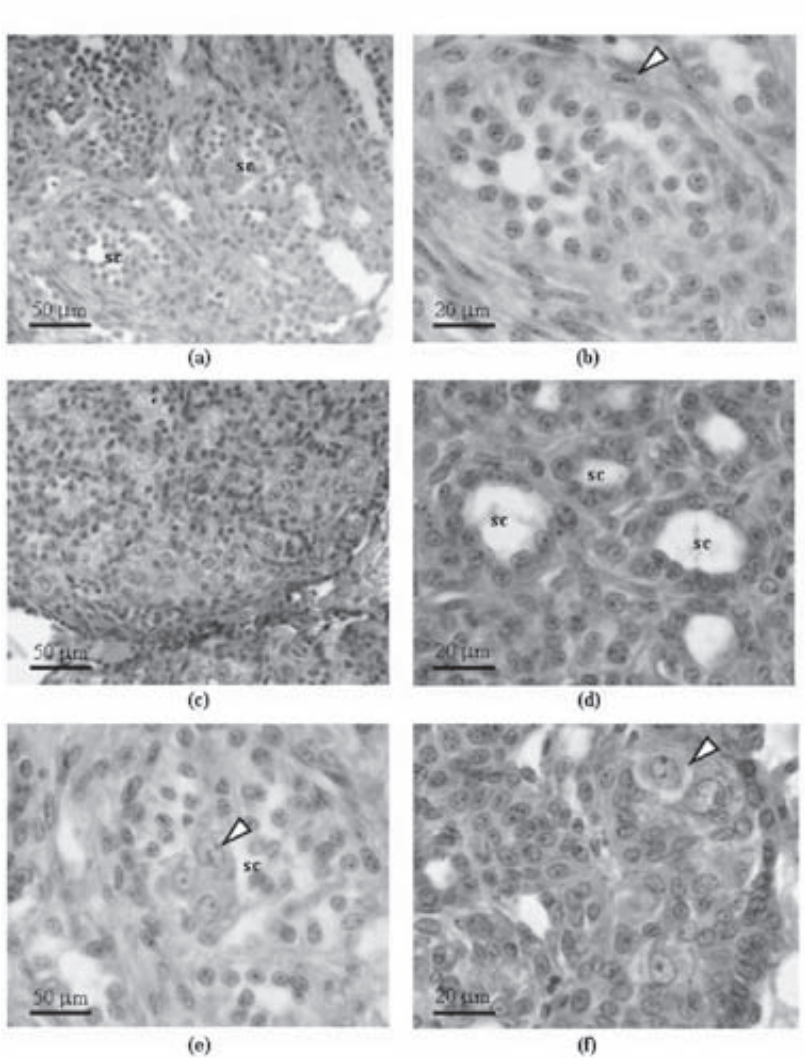

Figure 3 Photomicrographs of cross sections of crocodile embryo of 63 days of incubation

(a) The cranial lobe had sex cords (sc) which were thick-walled cords extending from germinal epithelium into stroma.

(b) Spindle-shaped mesenchymal cells (hollow arrowhead) surrounded sex cords.

(c) The caudal lobe contained numerous sex cords with simple cuboidal epithelium

(d) Sex cords were lined with simple cuboidal epithelium.

(e) In the cranial lobe of gonads, primordial germ cells (hollow arrowhead) were accumulated within a lumen of a sex cord.

(f) In the caudal lobe of gonads, primordial germ cells (hollow arrowhead) were found under the germinal epithelium and surrounded by mesenchymal cells (arrow).

height and the epithelium was simple cuboidal epithelium. The basal lamina was developed (Figures 2c). Dense mass of mesonephric cells was found at the dorsolateral border of the gonads.

At day 63 of incubation, bipotential gonads became larger in size. Longitudinal sections showed that the gonads were divided in to cranial and caudal lobes. The cranial lobe was covered with simple cuboidal epithelium. Sex cords developed into thick walled cords surrounded by spindle-shaped mesenchymal cells and collagen fibers (Figure 3a and b). Caudal lobe was composed of sex cords lined with simple cuboidal epithelial cells. The germinal epithelium was simple columnar epithelium. The basal lamina was not detected (Figure 3f). Different locations of primordial germ cells were presented between cranial and caudal lobes of bipotential gonads. In the cranial lobe, two or three germ cells accumulated together within sex cords (Figure 3e) whereas the aggregation of primordial germ cells occured mostly in the areas beneath the germinal epithelium. Some primordial germ cells were surrounded by a group of mesenchymal cells (Figure 3f).

At day 69 of incubation, embryo had bipotential gonads which were histologically similar to those found in day 63 embryo. The cranial lobe contained sex cords lined with stratified epithelium and some cords had primordial germ cells inside. Small blood vessels invaded into interstitial areas (Figure 4a). The caudal lobe contained the smaller sex cords lined with simple cuboidal epithelium. The primordial germ cells were restricted to areas underneath the germinal epithelium (Figure 4b).

DISCUSSION

Here, we reported the presence of gonadal ridge which was the first feature of gonadal development after 35 days of incubation. The gonadal ridge gradually became bipotential gonad whose main characteristic was formation of sex cords after 48 days of incubation. At 63 and 69 days of incubation, bipotential gonads morphologically changed as they formed cranial and caudal lobes. The cranial lobe contained well-developed sex cords and distribution of fibroblastic-like cells within interstitial areas whereas the caudal lobe showed accumulation of primordial germ cells underneath germinal epithelium. The histology of the cranial lobe of the gonads carried characteristics of testis and the caudal lobe showed the sign of ovarian differentiation (Pieau *et al.*, 1995). It suggested that bipotential gonads in Siamese crocodiles started differentiating at the final quarter of

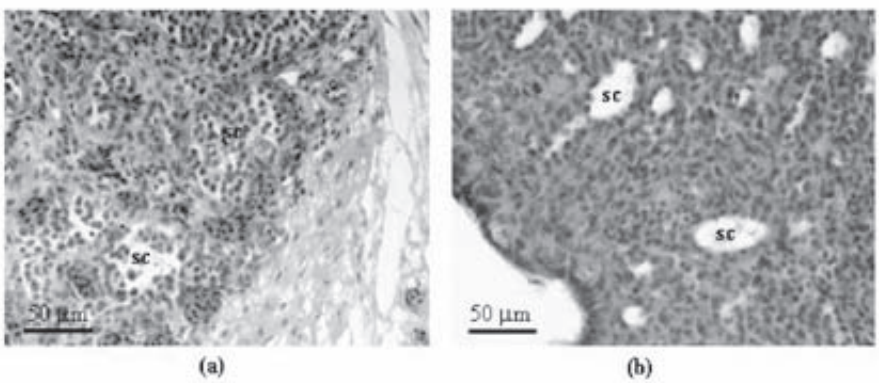

Figure 4 Photomicrographs of cross sections of crocodile embryo at 69 days of incubation.
(a) The cranial lobe consisted the thick-walled sex cords (sc).
(b) In the caudal lobe, sex cords (sc) were lined by simple cuboidal epithelium.

incubation period. This finding was in contrast to reports in alligators and salt water crocodiles whose gonadal differentiation took place between second to third quarter of incubation periods (Smith and Joss, 1993; 1994). Gonadal differentiation in reptile embryos has been shown to be controlled by the expression of several genes that control differentiation of steroid-producing cells such as SOX-9, DMRT1 (Western *et al.*, 1999; Maldonado *et al.*, 2002) as well as the production of sex steroid hormones including 17-β estradiol, testosterone (Ramaswami and Jacob, 1965; Merchant-Larios, *et al.*, 1997) and steroid-related enzymes including aromatase (Fleming *et al.*, 1999; Pieau and Dorizzi, 2004). The late beginning of gonadal differentiation of *Crocodylus siamensis* might be related to expressions of such molecules which were responded to incubation temperatures. Therefore, the thermosensitive period in *Crocodylus siamensis* may fall between days 48-62 of incubation period.

The appearance of early testicular features in the cranial lobe and early ovarian feature in the caudal lobe within developing gonads of Siamese crocodile embryos has not been reported. However, the finding of tissues of both sexes has been reported on the gonad in immature female alligators (Fox, 1977). The shift of sexes to either male or female in Siamese crocodiles would happen and be completed after hatching. However, the molecules influence and control the sexual differentiation in young crocodiles are needed for further study.

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