



# An immunohistochemical study on testicular steroidogenesis in the Sunda porcupine (*Hystrix javanica*)

Anni NURLIANI<sup>1-3</sup>), Motoki SASAKI<sup>1,2)\*</sup>, Teguh BUDIPITOJO<sup>4</sup>), Toshio TSUBOTA<sup>5</sup>), Masatsugu SUZUKI<sup>2,6</sup>) and Nobuo KITAMURA<sup>1,2</sup>)

<sup>1</sup>)Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

<sup>2</sup>)United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

<sup>3</sup>)Department of Biology, Faculty of Mathematics and Natural Sciences, Lambung Mangkurat University, South Kalimantan 70714, Indonesia

<sup>4</sup>)Department of Anatomy, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia

<sup>5</sup>)Laboratory of Wildlife Biology and Medicine, Department of Environmental Veterinary Science, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido 060-0818, Japan

<sup>6</sup>)Laboratory of Zoo and Wildlife Medicine, Faculty of Applied Biological Science, Gifu University, Gifu 501-1193, Japan

**ABSTRACT.** In the testes of the Sunda porcupine (*Hystrix javanica*), the expression of the steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes, such as cytochrome P450 side chain cleavage (P450scc), 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD), cytochrome P450 17 $\alpha$ -hydroxylase (P450c17) and cytochrome P450 aromatase (P450arom), was immunohistochemically examined to clarify the location of steroidogenesis. In this study, complete spermatogenesis (spermiogenesis) was observed in the testes of the examined Sunda porcupine, and spermatozoa of the Sunda porcupine had a spatulate sperm head unlike that of rats and mice which has an apical hook. On immunostaining of StAR, P450scc, 3 $\beta$ -HSD, P450c17 and P450arom, immunoreactivity for all proteins was only detected in the Leydig cells and not observed within the seminiferous tubules, suggesting that the Leydig cells can synthesize both androgen and estrogen from cholesterol in the Sunda porcupine testes.

**KEY WORDS:** Leydig cell, porcupine, sperm head, steroidogenesis, testis

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The Sunda porcupine (*Hystrix javanica*), which is a species of rodent in the family Hystricidae, is endemic to Indonesia and distributed in Java, Bali, Sumbawa, Flores, Lombok, Madura, Tonahdjampea and South Sulawesi [50]. *H. javanica* was previously considered to belong to same species as *H. brachyura* [10], but Van Weers [47] examined the cranial and external characters of *H. javanica*, and demonstrated it to be a separate species from *H. brachyura*. Based on the Law of the Republic of Indonesia number 7 of 1999 concerning the list of protected animals in Indonesia, only *H. brachyura* is listed as a protected animal, whereas *H. javanica* is not listed. However, from June 2018, *H. javanica* became a protected animal based on the Law of Ministry of Environment and Forestry, Republic of Indonesia (Number P.20/MENLHK/SETJEN/KUM.1/6/2018) related to decline of their population. Although, on the IUCN Red List of Threatened Species, this species is currently listed as being of least concern by International Union for Conservation of Nature [27].

Historically, people in Indonesia have hunted and consumed porcupine meat because they believed it to have medicinal properties. The meat from the porcupine tail was cooked and consumed as an aphrodisiac [3]. The continuous hunting of the Sunda porcupine without considering conservation principles may significantly reduce their population. Therefore, reproductive strategies must be designed for conservation of this species, and knowledge of aspects related to the reproduction of the Sunda porcupine is essential to support its successful conservation.

The *in vitro* fertilization (IVF) is one of the assisted reproductive technologies (ARTs) which could be applied as reproductive strategies to increase the number of population of the Sunda porcupine. Regarding this point, the knowledge about morphology of sperm head is important to predict the outcome of IVF. The significance of morphology of sperm head as a marker of successful fertilization has been reported previously [14]. Information about the sperm head shape has not been available yet in the Sunda

\*Correspondence to: Sasaki, M.: sasakim@obihiro.ac.jp

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porcupine and the findings in this study were provided as the leading information.

Male reproductive function is regulated by steroid hormones such as androgen and estrogen. In the Sunda porcupine, the testicular dynamics of steroid production are not fully understood. Information on testicular steroidogenesis is needed to improve our understanding of testicular systems regulated by steroid hormones.

Gonadal steroidogenesis is controlled by changes in the steroidogenic acute regulatory protein (StAR). StAR mediates the transfer of cholesterol from the outer to the inner mitochondrial membrane. Cholesterol is then converted to androgen via catalysis activity of steroidogenic enzymes such as cytochrome P450 side chain cleavage (P450<sub>sc</sub>), 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) and cytochrome P450 17 $\alpha$ -hydroxylase (P450<sub>c17</sub>) [28]. Then, another biosynthetic enzyme, cytochrome P450 aromatase (P450<sub>arom</sub>), converts androgen into estrogen [20]. This study also investigated the production site of steroid hormones in the testis of the Sunda porcupine by examining the localization of these steroidogenic enzymes immunohistochemically to clarify the reproductive process.

## MATERIALS AND METHODS

### *Animals and tissue collection*

Four wild male Sunda porcupines were obtained from Central Java, Indonesia. The animals were euthanized by an overdose injection of ketamin HCl (10–15 mg/kg) and xylazine HCl (0.10–0.15 mg/kg) administrated intramuscularly before taking organ samples. The removed testes were immediately fixed in Bouin's fluid for 24 hr. After fixation, the tissue samples were dehydrated in a graded series of ethanol, cleared in xylene and embedded in paraffin. The samples were cut serially at 4  $\mu$ m thickness and mounted on aminopropyl-triethoxy-silane-coated slides (S9226, Matsunami Glass, Osaka, Japan). After deparaffinization, the testicular tissue sections were stained with hematoxylin and eosin (HE) to examine the general structure. All experimental procedures were authorized by the Ethics Committee for the Use of Animals at Gadjah Mada University (Number: 0014/EC-FKH/Eks/2017). Collection permit (number: SK.56/KSDAE/SET/KSA.2/2/2018) was issued by the Directorate General of Natural Resources and Ecosystem Conservation (Direktorat Jenderal Konservasi Sumber Daya Alam dan Ekosistem, Jakarta, Indonesia) under the Ministry of Environment and Forestry of the Republic of Indonesia.

### *Immunohistochemistry*

To examine the localization of StAR and steroidogenic enzymes in testes, the sections were stained immunohistochemically using an avidin-biotin peroxidase complex (ABC) method. The primary antibodies used in this study are summarized in Table 1. For the ABC method, the sections were deparaffinized in xylene, rehydrated in graded alcohols and washed with tap water. After microwaving in high pH target retrieval solution (1:10, S3307; Dako Cytomation, Inc., Carpinteria, CA, U.S.A.) for antigen retrieval, the endogenous peroxidase activity was blocked with methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature (RT). Then, the sections were incubated with normal goat serum for 30 min at RT to prevent nonspecific staining. The samples were incubated with each primary antibody overnight at 4°C. The biotinylated anti-rabbit IgG (1:200 dilution, BA-1000, Vector Laboratories, Burlingame, CA, U.S.A.) as the secondary antibody was applied for 30 min at RT, and the sections were then incubated with the Vectastain *Elite* ABC Kit (1:2 dilution, PK-6100, Vector Laboratories) for 30 min at RT. The immunoreactive sites were visualized using Tris HCl buffer containing 0.02% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 0.006% H<sub>2</sub>O<sub>2</sub>. The negative control sections were prepared without the primary antibody.

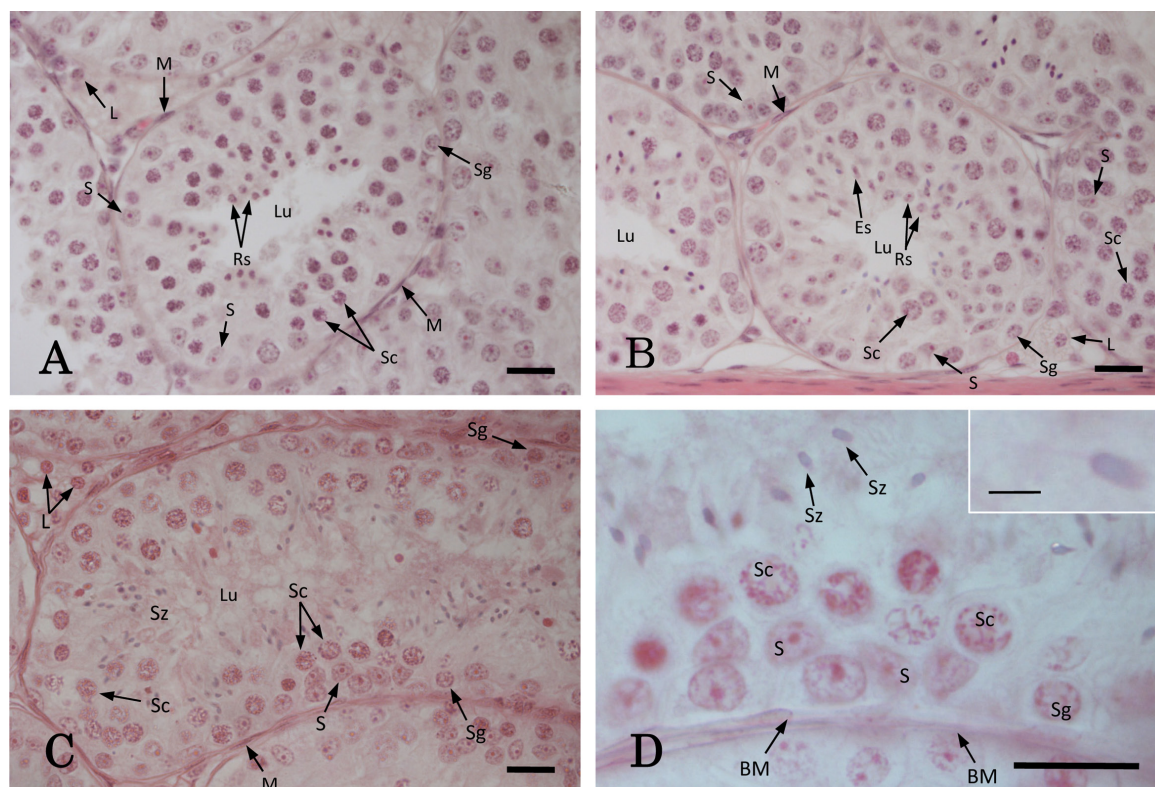
## RESULTS

On histological observation of testes of the Sunda porcupine, the seminiferous tubules with several stages of spermatogenesis were observed, although the stage number of the spermatogenetic (seminiferous epithelium) cycle was not identified in this study (Fig. 1). Spermatozoa of the Sunda porcupine have a spatulate sperm head (Fig. 1C, 1D). Among the seminiferous tubules, the interstitial tissue, including the Leydig cells exhibiting a round nucleus, was identified (Fig. 1A–C).

The localization of StAR and steroidogenic enzymes in the testes is shown in Fig. 2. Immunoreactivities for StAR, P450<sub>sc</sub>, P450<sub>c17</sub>, 3 $\beta$ HSD and P450<sub>arom</sub> intensely were detected only in the cytoplasm of the Leydig cells.

**Table 1.** Characteristics and dilutions of primary antibodies

Antigen	Host	Type	Dilution	Catalog No. or Source
Steroidogenic acute regulatory protein (StAR)	Rabbit	Polyclonal	1:1,000	PAI-560, Affinity Bioreagents, Golden, CO, U.S.A.
Rat cytochrome P450 side-chain cleavage enzyme (P450 <sub>sc</sub> )	Rabbit	Polyclonal	1:200	AB-1244, Chemicon International, Temecula, CA, U.S.A.
Porcine cytochrome P450 17 $\alpha$ -hydroxylase (P450 <sub>c17</sub> )	Rabbit	Polyclonal	1:500	[19]
Human 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD)	Rabbit	Polyclonal	1:500	[12]
Human cytochrome P450 aromatase (P450 <sub>arom</sub> )	Rabbit	Polyclonal	1:10	GTX18995, Gene Tex, Irvine, CA, U.S.A.



**Fig. 1.** Histological features of the testes of the Sunda porcupine. Several stages of the spermatogenic cycle were observed within the seminiferous tubules. Spermatogenic cells until round spermatids were noted in some seminiferous tubules (A). The stage with round and elongated spermatids (B), and the stage including spermatozoa with a spatulate head (C, D) were also observed in the testis. D is magnified sections of C. Inset of D shows enlarged spermatozoon. BM, basal membrane; Es, elongated spermatid; L, Leydig cell; Lu, lumen; M, peritubular myoid cell; Rs, round spermatid; S, Sertoli cell; Sc, spermatocyte; Sg, spermatogonia; Sz, spermatozoon. Bar=20  $\mu$ m (A–D), 3  $\mu$ m (D inset). HE staining.

## DISCUSSION

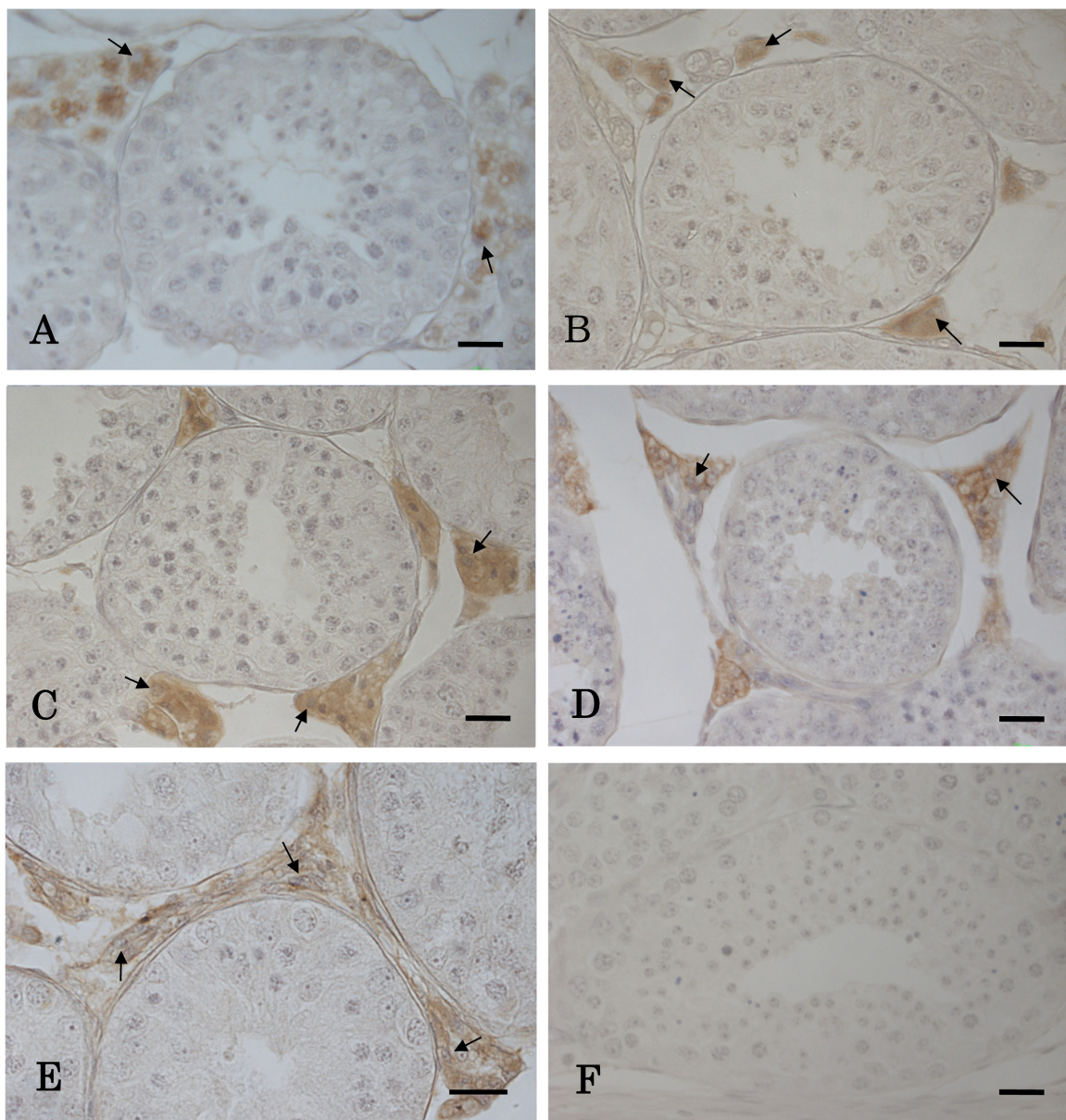
Little is known about the reproductive process of the Sunda porcupine. In our histological observations of Sunda porcupine testes, round and elongated spermatids, and spermatozoa were found in the seminiferous tubules. The presence of spermatozoa in the lumen demonstrated that the animals undergo complete spermatogenesis (spermiogenesis).

In the Sunda porcupine, spermatozoa had a sperm head lacking the apical hook. This is different from the common apical hook of the sperm of most rodents, including experimental rodents such as rats and mice [15, 17, 25, 34, 35]. This sperm head type found in the Sunda porcupine is known as a spatulate sperm head or pear-shaped sperm head and is also observed in other several rodents such as Mediterranean pine vole (*Microtus duodecimcostatus*), bastard big-footed mouse (*Macrotarsomys bastardy*), Dormouse tufted-tailed rat (*Eliurus myoxinus*) and African greater cane rat (*Thryonomys swinderianus*) [11, 17, 40]. It suggested that the sperm head type is not related to rodent phylogeny because this sperm head type was randomly noted among different genera.

Importance of sperm head morphology for successful fertilization in IVF has been described in previous studies [14, 29]. Sperm head morphology enable to be used as a strategy to elect the heterogenous spermatozoon that are suitable for fertilization. Variation in their head shape is associated with differences in fertilization ability [36]. Thus, preliminary information about sperm head of the Sunda porcupine in the current study will be a basic information to support and encourage reproductive technologies in this species.

In this study, we examined the location of steroidogenesis in the Sunda porcupine testes. Immunoreactivity for StAR in the testis of the Sunda porcupines was only noted in the Leydig cells. Similarly, in muskrats [51] and Thai red jungle fowl [24], immunoreactivity for StAR was only detected in the Leydig cells. Moreover, in humans, strong immunoreactivity for StAR was restricted to the Leydig cells, with little to no staining of the Sertoli cells [42]. StAR is the primary regulator of steroidogenesis and acts as a rate-limiting factor [48]. This protein is located in the inner mitochondrial membrane where it facilitates cholesterol import from the outer to inner mitochondrial membrane, and the transported cholesterol is subsequently converted into pregnenolone by P450scc on the inner membrane as the first step of steroidogenesis [2, 4, 48].

In the testes of the Sunda porcupine, expression of P450scc, P450c17 and 3 $\beta$ HSD was also detected only in the Leydig cells, suggesting that the Leydig cells can synthesize androgen from cholesterol. The present result is similar to that reported for



**Fig. 2.** Immunostaining of steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes in the testes of the Sunda porcupine. Immunoreactivity for StAR (A), cytochrome P450 side chain cleavage (P450scc) (B), cytochrome P450 17 $\alpha$ -hydroxylase (P450c17) (C), 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ HSD) (D) and cytochrome P450 aromatase (P450arom) (E) intensely was only positive in the Leydig cells (arrows). No signal was detected in control sections (F). Bar=20  $\mu$ m.

muskrats [31, 51], sika deer [21, 22], horses [1], shiba goat [49], rats [41] and humans [5]. In other species, such as raccoons [39], raccoon dogs [43], Japanese black bears [38], Hokkaido brown bears [44], American black bears [45] and monkeys [33], these steroidogenic enzymes were also found in Sertoli cells and/or germ cells in addition to the Leydig cells.

The present study revealed that aromatase enzyme was only expressed in the Leydig cells. Thus, the Leydig cells can synthesize estrogen and androgen. This result is similar to that of previous studies on other animals such as South American plains vizcachas [18], sika deer [21, 22], humans [7, 8, 26], stallions [13], rams [6], boars [16] and shiba goat [49]. Many previous studies identified localization of this enzyme in other testicular cells, such as Sertoli cells and/or germ cells, in raccoons [39], wild ground squirrels [32], Japanese black bears [38], Japanese raccoon dogs [43], Hokkaido brown bears [44], American black bears [45], northern fur seals [46], mice [37] and rats [30].

Therefore, in mammalian testes, the production site of steroid hormones differs among species. Notwithstanding, comparison among porcupine species could not be served due to lacking of information about the presence of steroidogenic enzymes as well as sperm morphology in the testis of the other porcupine species. In addition, it has been reported that the localization of testicular steroidogenic cells varies during development. For example, in prepubertal horses, immunoreactivity for P450arom was observed

in the Leydig cells and within the seminiferous tubules [1, 23]. However, in postpubertal horses, P450arom expression within the seminiferous tubules disappeared and was detected only in the Leydig cells [1, 13, 23]. Under abnormal conditions, the localization of steroidogenic cells changed. In human testicular tumors, strong reactivity for P450arom was noted in Sertoli cells [7], whereas in normal human testes, P450arom was only expressed in the Leydig cells [7, 8, 26].

Intense expression of steroidogenic enzymes in the Leydig cells of the Sunda porcupine observed in this study proposed that the production of androgen and estrogen was occurring highly. Generally testosterone was much required for the maintenance of spermatogenesis [39] and estrogen was essential for development of spermatozoa [9]. In this study, however, the changes of immunoreactivities of steroidogenic enzymes could not be examined through the year, although we could reveal immunohistochemically the possibility of strong synthesis of androgen and estrogen in the the Leydig cells. To understand annual reproductive strategy and make use of the knowledge for conservation, therefore, we need to examine the dynamics of spermatogenesis, expression of steroidogenic enzymes and blood concentration of steroid hormones through the year in further studies.

In conclusion, the present study revealed that the Sunda porcupine has spermatozoa with a spatulate sperm head, and Leydig cells are the only one production site for androgen and estrogen in the testis of the Sunda porcupine. To clarify the testicular dynamics of steroidogenesis during the development of the Sunda porcupine, the localization of StAR and steroidogenic enzymes in fetal and early postnatal testes needs to be examined. Combination data from this study associated to other reproductive characteristics of the Sunda porcupine is extremely required to design the reproductive strategies in this animal.

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