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Concise Review

Practical approaches for evaluating adrenal toxicity in nonclinical safety assessment

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Abstract: The adrenal gland has characteristic morphological and biochemical features that render it particularly susceptible to the actions of xenobiotics. As is the case with other endocrine organs, the adrenal gland is under the control of upstream organs (hypothalamic-pituitary system) *in vivo*, often making it difficult to elucidate the mode of toxicity of a test article. It is very important, especially for pharmaceuticals, to determine whether a test article-related change is caused by a direct effect or other associated factors. In addition, antemortem data, including clinical signs, body weight, food consumption and clinical pathology, and postmortem data, including gross pathology, organ weight and histopathologic examination of the adrenal glands and other related organs, should be carefully monitored and evaluated. During evaluation, the following should also be taken into account: (1) species, sex and age of animals used, (2) metabolic activation by a cytochrome P450 enzyme(s) and (3) physicochemical properties and the metabolic pathway of the test article. In this review, we describe the following crucial points for toxicologic pathologists to consider when evaluating adrenal toxicity: functional anatomy, blood supply, hormone production in each compartment, steroid biosynthesis, potential medulla-cortex interaction, and species and gender differences in anatomical features and other features of the adrenal gland which could affect vulnerability to toxic effects. Finally practical approaches for evaluating adrenal toxicity in nonclinical safety studies are discussed. (DOI: 10.1293/tox.2015-0025; J Toxicol Pathol 2015; 28: 125–132)

Key words: adrenal cortex, adrenal medulla, nonclinical toxicity, species differences, steroidogenesis

Introduction

Adrenal glands, as endocrine glands, work cooperatively in responding to internal and/or external stimuli in order to maintain homeostasis of the whole body. Therefore, functional modulation in one single endocrine gland could possibly result in various pathophysiological responses not only in target tissues but also in various other organs.

The effect of a xenobiotic on the adrenal glands is first recognized at necropsy as a change in size, color and/or organ weight and subsequently can be observed during histopathologic examination, which still serves as the gold standard for evaluation of adrenal changes. In addition, measurement of serum/plasma hormone levels, special staining or immunohistochemical analysis can also provide pivotal information; however, due to their labor-intensive or expensive nature, these examinations are by no means recommended in routine toxicity studies. Therefore, examination of the adrenal glands in a step-by-step fashion, adding additional investigations as required, is reasonable and is proposed as the best approach toward understanding the effects of xenobiotics on the adrenal glands. In these studies, antemortem data, including clinical signs, body weight, food consumption and clinical pathology, and postmortem data, including gross pathology, organ weight and histopathologic examination of the adrenal glands and other related organs, should be carefully monitored and evaluated. During evaluation, the following should also be taken into account: (1) species, sex and age of the animals used, (2) metabolic activation by a cytochrome P450 (CYP) enzyme(s) and (3) physicochemical properties and the metabolic pathway of the test article.

In this review, we describe the following crucial points for toxicologic pathologists in evaluation of adrenal toxicity: functional anatomy, blood supply, hormone production in each compartment, steroid biosynthesis, potential medulla-cortex interaction, species and gender differences in anatomical features and characteristic features of the adrenal gland that could affect vulnerability to toxic effects. Finally, practical approaches for evaluating adrenal toxicity in nonclinical safety studies are discussed.

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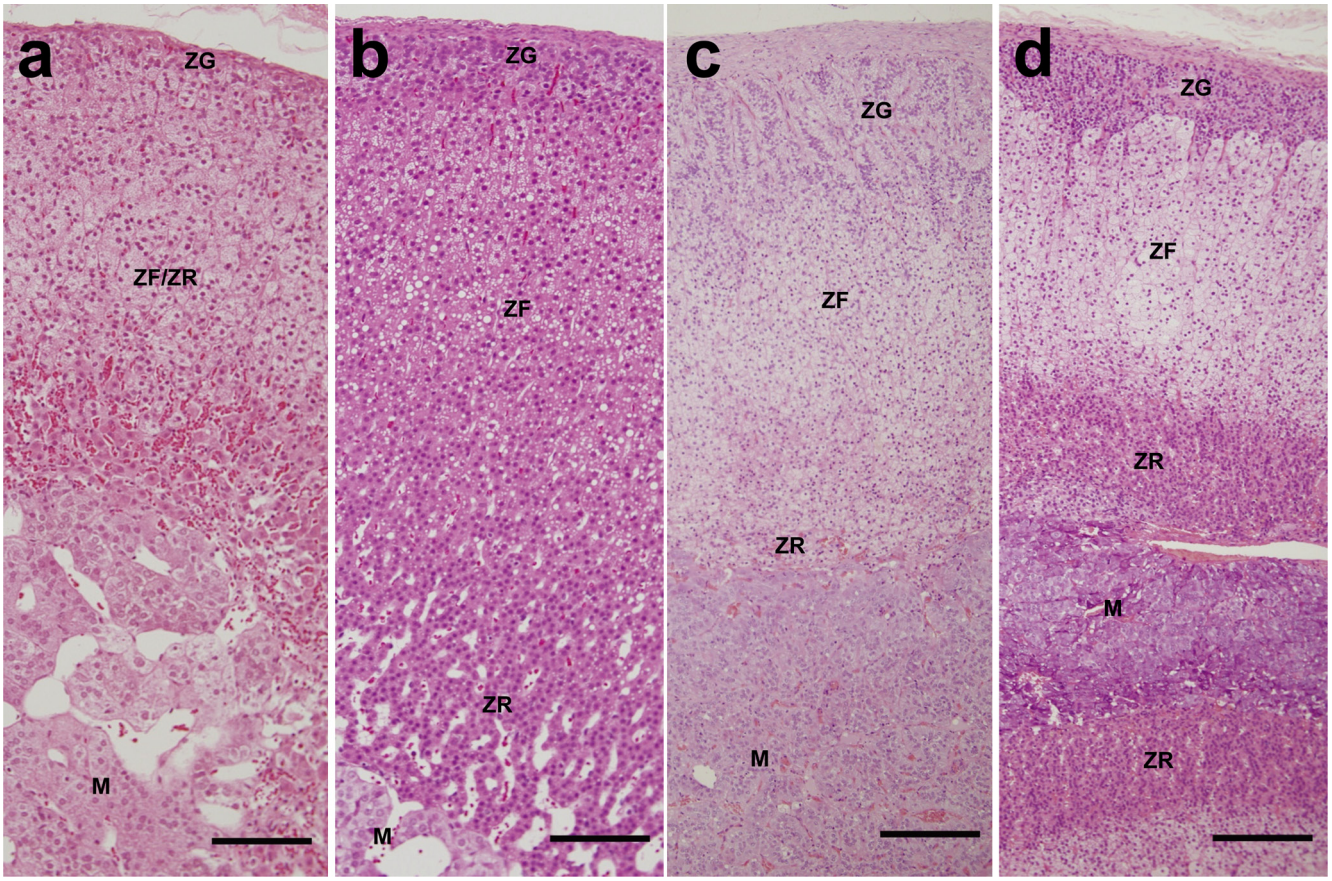

Fig. 1. Cross sections of normal adrenal glands from a a) mouse, b) rat, c) dog and d) monkey. The adrenal glands are divided into two distinct endocrine tissues, the cortex and medulla. The cortex is characterized histologically by three layers, namely, the zona glomerulosa, zona fasciculata and zona reticularis. Note that the zona glomerulosa in the dog has a very different appearance compared with other species and consists of relatively large, flattened cells, which stain palely and are stacked in large loops (Fig. 1c). The zona reticularis is not clearly distinguishable in some rodents, particularly in the mouse. This zone is more distinct in rats compared with mice (Figs. 1a and b). ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; M, medulla. HE stain. Bars = 100 µm (a, b), 200 µm (c, d).

Functional Anatomy of the Adrenal Gland

In mammals, the adrenal glands are paired organs, each located close to the anterior pole of the kidneys. The adrenal glands are comprised of two embryologically and functionally distinct endocrine tissues: 1) the adrenal cortex, which is derived from the mesoderm and secretes steroid hormones derived from cholesterol, and 2) the adrenal medulla, which is derived from the neural crest and secretes catecholamines produced by metabolism of the amino acid tyrosine[1]. Cross sections of adrenal glands from a mouse, rat, dog and monkey are shown in Fig. 1.

Blood Supply of the Adrenal Gland

The adrenal gland is a highly vascular tissue, receiving a large proportion of the cardiac output relative to its size. The arterial blood supply to the adrenal glands is mainly from the dorsal aorta via several small arteries that form a subcapsular sinusoidal vascular plexus. Three sets of branches emerge from the plexus: one set supplies the capsule, while the second set enters the cortex, forming sinusoids percolating between the zona glomerulosa and fasciculata that coalesce and form a capillary network in the zona reticularis before entering the medulla. The third set generates medullary arteries that travel along connective tissue trabeculae of the cortex without branching and supply blood only to the medulla[2, 3]. The blood flows centripetally into the large medullar sinusoids that drain into a central vein (Fig. 2). This dual blood supply to the medulla results in the transport of glucocorticoids necessary for phenylethanolamine-*N*-methyltransferase (PNMT) activation and the supply of fresh blood to the medulla, which is required for rapid response to stress. These unique anatomical features of the adrenal blood supply are important for its function and conversely also contribute to the development of lesions.

Adrenal Cortex and Hormones

The adrenal cortex is characterized histologically by three layers, namely, the zona glomerulosa, zona fasciculata and zona reticularis. The outermost layer, located immedi-

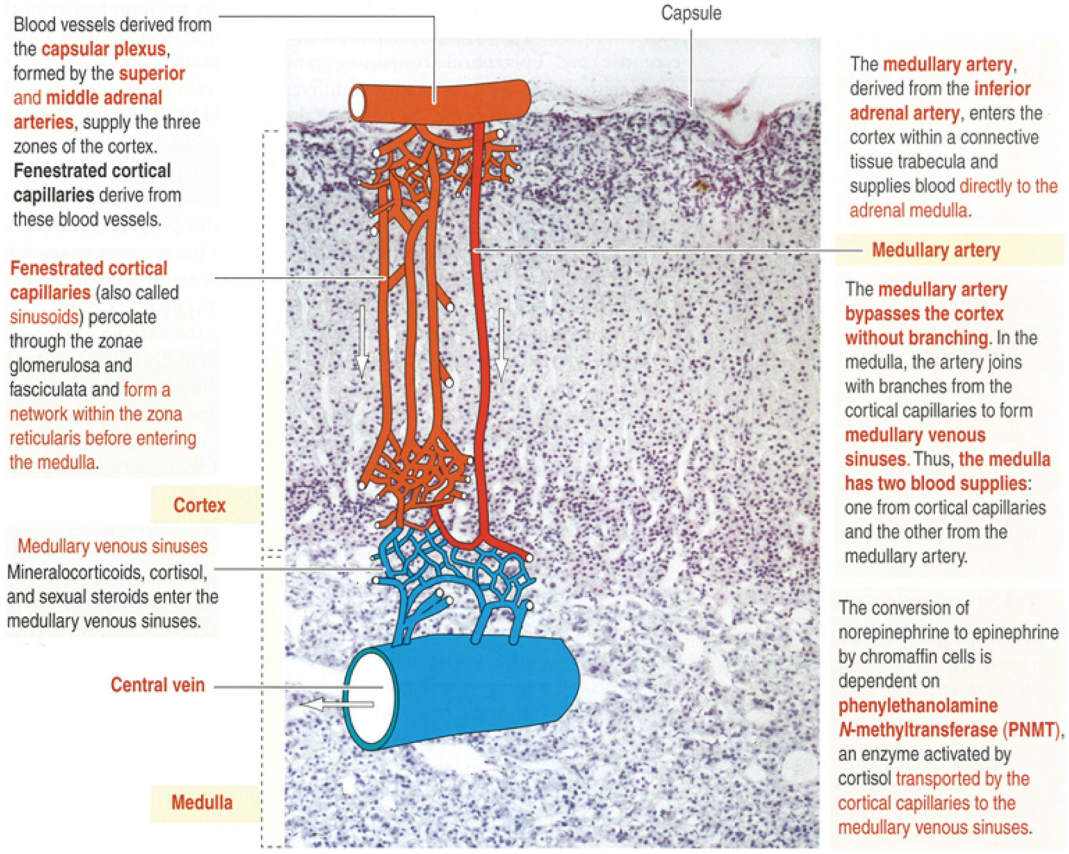

Fig. 2. Blood supply to the adrenal gland. Note that the dual blood supply to the medulla results in the transport of glucocorticoids necessary for PNMT activation and the supply of fresh blood to the medulla, which is required for rapid response to stress. These unique anatomical features of the adrenal blood supply are important for its function and conversely also contribute to the development of lesions. From Kierszenbaum AL[3] with permission of Elsevier Ltd.

ately below the thin fibrous capsule, is the zona glomerulosa. The appearance of this zone and its percentage of the whole adrenal gland vary considerably between species, generally being larger in dogs and monkeys[2]. The zona glomerulosa exclusively produces mineralocorticoids. The zona fasciculata comprises the greatest part of the cortex in all species and demonstrates a similar appearance in most species. This zone produces the glucocorticoids class of steroid hormones. The cells of this zone are arranged in radial cords separated by small capillaries and are larger than those of the zona glomerulosa, and under non-stress conditions, they contain abundant lipid droplets. In humans, the cells of this zone are termed clear cells. The amount of lipid droplets containing cholesterol varies according to the physiological status of the individual. The zona reticularis is the innermost zone. The cells of this zone are smaller than those of the zona fasciculata and are arranged in clusters. The zona reticularis produces glucocorticoids and small amounts of androgens in some species.

Not all steroids produced in the cortex are stored in significant amounts. Because of this, steroid synthesis by adrenal cells must be continuous in order to maintain whole body homeostasis. In addition, blood concentrations of specific steroid hormones are usually considered to reflect the rates of synthesis of these steroid hormones. It has been hypothesized that adrenocortical cells produce a gradient of a substance or substances in the bloodstream that alters adrenocortical cell function and morphology to create the zonation of the adrenal cortex[4, 5].

The stem or undifferentiated cell zone located in the innermost portion of the zona glomerulosa and the outermost portion of the zona fasciculata is the site for cell replication (Fig. 3). A BrdU pulse-chase analysis and PCNA staining revealed S-phase cells in and around the periphery of the undifferentiated cell zone, suggesting that this population may provide a pool of progenitors that differentiate into the neighboring zones[6].

Electron microscopic examination of the adrenal cortex shows that, ultrastructurally, the cells have numerous lipid droplets that contain cholesterol, the basic substrate for steroid biosynthesis, and are close to the smooth endoplasmic reticulum (SER) and mitochondria. Ultrastructural features of the cortical cells can be very useful for their identification; a detailed description of the ultrastructure of the adrenal cortex has been published[7, 8].

Steroid Biosynthesis in the Adrenal Cortex

The adrenal cortex secretes three main types of hormones: mineralocorticoids (aldosterone and deoxycorticosterone), glucocorticoids (cortisol and corticosterone) and the sex steroids (mainly the androgen precursors dehydroepiandrosterone [DHEA] and androstenedione). The most physiologically important of these corticosteroids are the mineralocorticoids, which tightly regulate the Na+/K+ balance in extracellular fluids and also impact blood pressure homeostasis. Glucocorticoids are important in glucose homeostasis, the response of the organism to stressors, immune modulation and other important functions. The adrenal androgens are formed by CYP17, a single enzyme with both 17α-hydroxylase and 17,20-lyase activities. CYP17 hydroxylates pregnenolone and progesterone to form the respective 17α-hydroxysteroids, a process that occurs in the zona fasciculata and reticularis but not in the zona glomerulosa[9]. CYP17 immunoreactivity in the adrenal glands of an adult male cynomolgus monkey is illustrated in Fig. 4a; there is robust immunoreactivity in the zona fasciculata and reticularis. The immunoreactivity of dehydroepiandrosterone sulfotransferase (DHEA-ST), which catalyzes the conversion of DHEA to dehydroepiandrosterone sulfate (DHEA-S) in the adrenal gland[10, 11], is detected in the zona reticularis in the monkey adrenal gland (Fig. 4b and c). In rats, mice and rabbits, corticosterone is the major glucocorticoid, and the zona fasciculata and reticularis do not secrete a significant amount of either androgen or cortisol. This is because the adrenal glands of these species lack CYP17[12]. Resulting from the use of molecular oxygen in corticosteroid biosynthesis mediated by CYP enzymes, cells become particularly susceptible to the toxic effects of free radicals, such as lipid peroxidation[13]. In order to protect the cells or CYP enzymes from the toxic effects of oxygen radicals, adrenocortical cells contain high concentrations of biological antioxidants including superoxide dismutase (SOD), catalase, α-tocopherol, glutathione and ascorbic acid[14, 15].

The cholesterol used in steroid biosynthesis is derived from two sources: *de novo* synthesis from acetate in the adrenal gland or receptor-mediated uptake of plasma lipoproteins. In the human, bovine and most other species, most cholesterol in plasma is bound with low-density lipoproteins (LDLs), while in the rat and other rodents, cholesterol is predominantly associated with high-density lipoproteins (HDLs)[16]. Most cholesterol is stored in lipid droplets in an esterified form that is rapidly accessible in response to acute stimulation of steroidogenesis and is then replenished[2].

The biosynthesis of cortisol is regulated by adrenocorticotropic hormone (ACTH). The first steroid hormone produced by cortical cells from cholesterol is pregnenolone. Pregnenolone is created by the action of mitochondrial CYP11A1 (20α, 22R-hydroxylase cholesterol side-chain cleavage)[8]. This reaction is the rate-limiting step of steroid hormone biosynthesis (Fig. 5). ACTH binds to cell membrane receptors linked to G-proteins and stimulates increased cytoplasmic cAMP as well as increased availability of cholesterol to CYP11A1, resulting in increased pregnenolone synthesis. In rodents, synthesis of glucocorticoids continues in the mitochondria and the smooth endoplasmic reticulum after synthesis of pregnenolone, resulting in the formation of corticosterone. This is the principal glucocorticoid in rats, mice and rabbits, as described previously. In other species, such as guinea pigs, dogs, cats, nonhuman primates and humans, the smooth endoplasmic reticulum contains additional hydroxylases that are responsible for synthesis of cortisol. Cortisol is produced in greater amounts compared with corticosterone in these species and represents approximately 80% of the glucocorticoid production. The androgens produced by the zona reticularis can be metabolized to testosterone or estrogens by the cortical cells themselves or by metabolic pathways in other organs, such as the gonads. Aldosterone is the principal mineralocorticoid produced in the zona glomerulosa, since CYP11B2 is found only in this zone. Angiotensin II acts as a trophic hormone to increase aldosterone production, which acts on target cells in the kidney to conserve sodium, excrete potassium and increase blood volume.

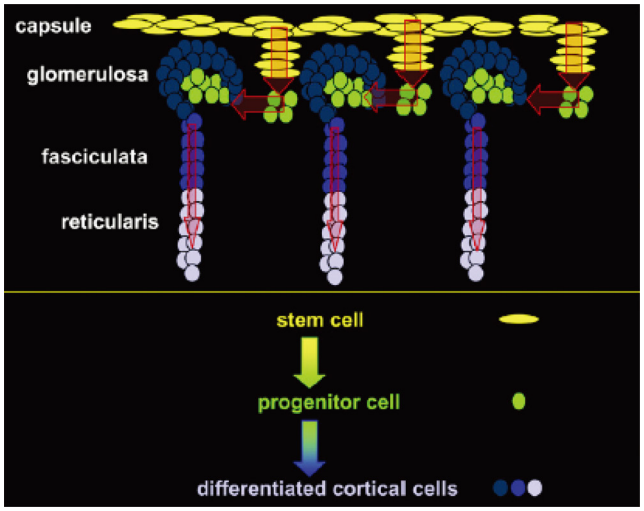

Fig. 3. Hypothetical model of stem and progenitor cell centripetal migration and differentiation into steroidogenically competent adrenocortical cells. The stem or undifferentiated cell zone located in the innermost portion of the zona glomerulosa and the outermost portion of the zona fasciculata is the site for cell replication. This population may provide a pool of progenitors that differentiate into the neighboring zones. From Kim AC and Hammer GD[6] with permission of Elsevier Ltd.

Adrenal Medulla and Hormones

The adrenal medulla consists of three types of cells: chromaffin, neuronal (ganglion-like) and sustentacular cells[17, 18]. The chromaffin cells are the sites of synthesis and storage of catecholamines, and the major secretory products of the medulla are catecholamines, adrenaline and, to a lesser extent, noradrenaline. Their release is stimulated pre-

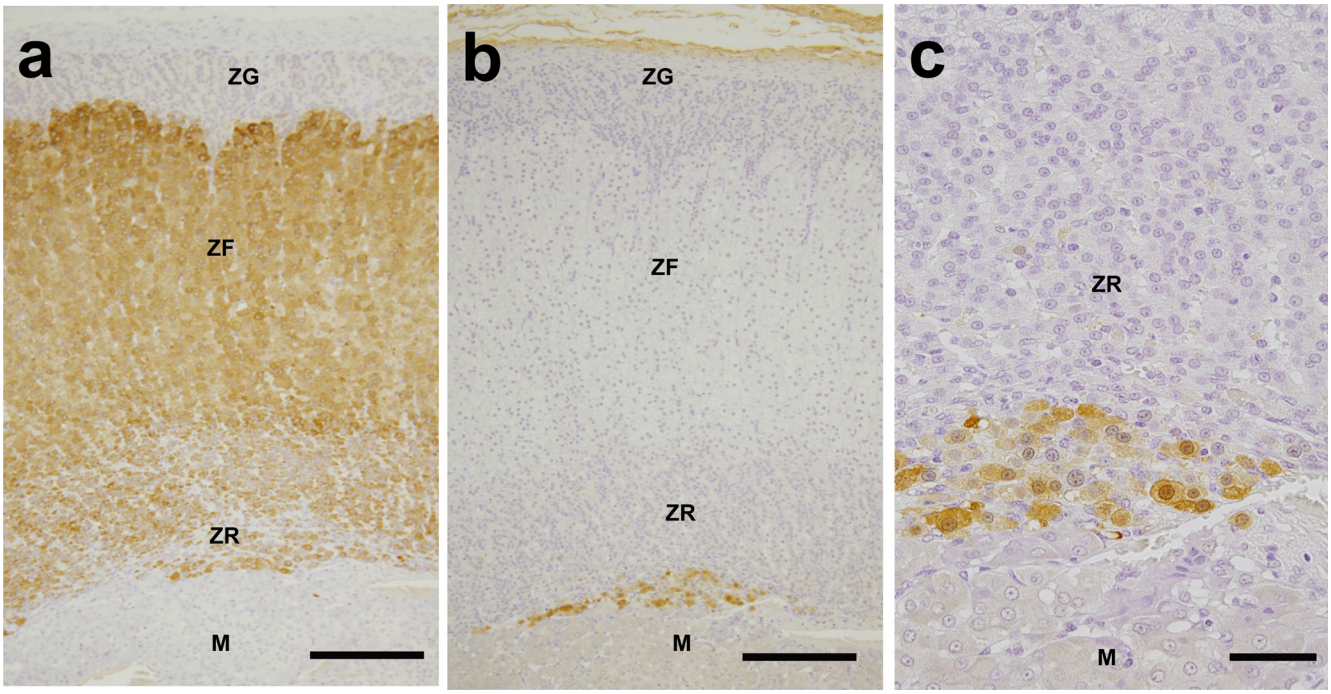

Fig. 4. Cross sections of a normal adrenal gland from a monkey. Immunohistochemistry of (a) CYP17 and (b) low- and (c) high-magnification images of immunohistochemistry of DHEA-ST. The immunoreactivity of CYP17 is robust in the zona fasciculata and reticularis (Fig. 4a). The immunoreactivity of DHEA-ST is detected in the zona reticularis (Fig. 4b and c). ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; M, medulla. Bars = 200 µm (a, b), 50 µm (c).

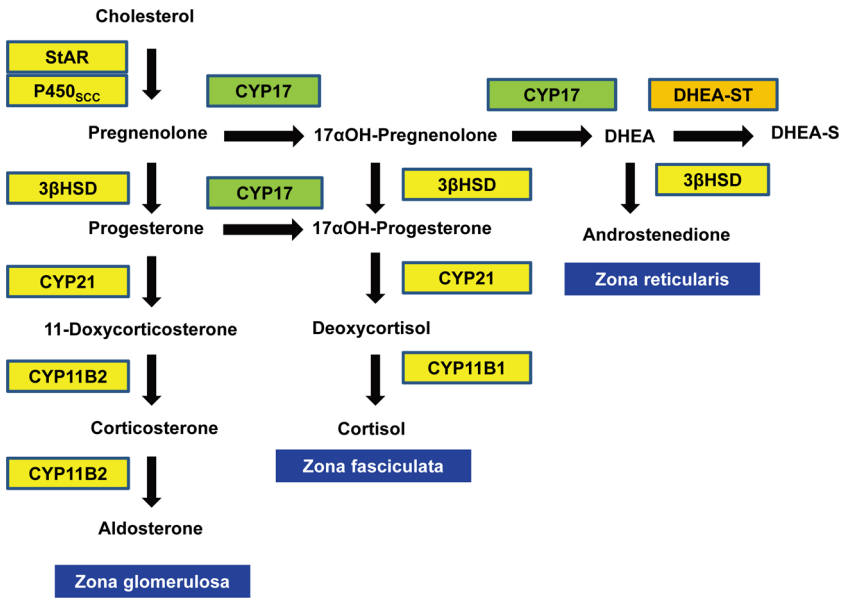

Fig. 5. Pathways of adrenal steroid biosynthesis. StAR, steroid acute regulatory protein; $P450_{SCC}$, P450 side chain cleavage enzyme; 3βHSD, 3β-hydroxysteroid dehydrogenase; DHEA, dehydroepiandrosterone; DHEA-ST, dehydroepiandrosterone sulfotransferase; DHEA-S, dehydroepiandrosterone sulphate.

dominantly by cholinergic innervation through the splanchnic nerve. Noradrenaline leaves the granule to be converted into adrenaline in the cytosol by PNMT, and subsequently adrenaline reenters the granule for storage in the cell[8]. The secretion of catecholamines is controlled by sympathetic innervation. Production and secretion of catecholamines are

triggered by acute events such as stress, trauma and shock, as well as by fasting, hypoxia or hypoglycemia.

Medulla-Cortex Interaction

The anatomical relationship between two embryologically distinct endocrine tissue types united under one organ capsule has to be synchronized. As an example, the response of the endocrine system to stress is characterized by the concomitant release of catecholamines from the adrenal medulla and glucocorticoids from the adrenal cortex. *In vitro* studies conclusively demonstrated that the expression of PNMT, and consequently the biosynthesis of adrenaline in adrenomedullary chromaffin cells, is induced by the high local concentration of glucocorticoids in sinusoidal blood from the adrenal cortex[19]. The involvement of intra-adrenal interactions in this coordination of the body's response to stress has been well documented by Ehrhart-Bornstein and Bornstein[20].

Species and Gender Differences in Anatomical Features of the Adrenal Glands

In mice, growth and function of the adrenal glands are markedly influenced by gender and age. Female mice usually have heavier adrenal glands, the zona fasciculata of which has a higher volume, and a correspondingly higher level of total circulating corticosterone between weeks 5 and 11 as compared with males[21]. In rats, the adrenal gland of the female is significantly larger than that of the male, although the relative difference varies among different strains. Adult female rats generally demonstrate increased sizes for all zones of the adrenal cortex, which may be attributed to the effects of estrogen[22]. This sex difference is not detected in either the dog or human adrenal cortex.

The zona glomerulosa in the dog has a very different appearance compared with other species and consists of relatively large, flattened cells, which stain palely and are stacked in large loops (Fig. 1c). The zona reticularis is prominent in humans, but not clearly distinguishable in some rodents, particularly in the mouse. This zone is more distinct in rats compared with mice (Figs. 1a and b).

A specific feature of the mouse adrenal cortex is the so-called X-zone, a putative postpartal remnant of the fetal adrenal zone located at the junction of the cortex and medulla (Fig. 6). In males, this zone disappears rapidly with the approach of puberty (approximately 5 weeks old)[23]. In unbred females, this particular zone undergoes slow regression and degeneration. In pregnant females, it undergoes vacuolar degeneration during their first pregnancy. However, its precise function remains unknown, though it may be similar to the fetal zone in primates.

Adrenal Vulnerability to Toxicants

Chemically induced endocrine lesions have been reported to be most commonly encountered in the adrenal glands, followed in descending order by the testes, thyroid, ovaries, pancreas, pituitary and parathyroid glands[24, 25]. There are characteristic morphological and biochemical features of the adrenal gland that render it particularly susceptible to the actions of toxins[26]. These features are summarized in Table 1. However, it is not within the scope of this review to describe the known adrenal toxins in detail; these have been thoroughly reviewed elsewhere[7, 8, 24, 27].

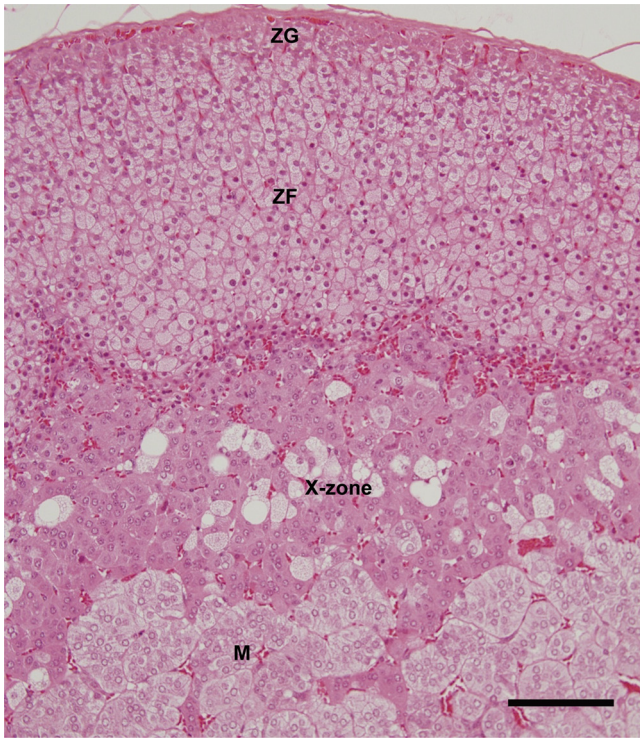

Fig. 6. X-zone in a cross section of the normal adrenal gland from a female mouse. The X-zone is a specific feature of the mouse adrenal cortex, a putative postpartal remnant of the fetal adrenal zone located at the junction of the cortex and medulla. Its precise function remains unknown, though it may be similar to the fetal zone in primates. ZG, zona glomerulosa; ZF, zona fasciculata; M, medulla. HE stain. Bar = 100 µm.

Practical Approaches for Assessing Adrenal Toxicity in Nonclinical Safety Studies

Points for toxicologic pathologists to consider and crucial examination items to be taken into account when evaluating adrenal toxicity are summarized in Table 2. In general, the effect of a test article on the adrenal glands is first recognized as a change in size, color or organ weight at necropsy or upon histopathologic examination. Measurement of serum/plasma hormone levels, special staining or immunohistochemical analysis using antibodies to hormonal peptides can also provide pivotal information; however, it would be inadvisable to perform these examinations in routine toxicity studies in terms of time and cost. It would be more practical to examine the adrenal glands in a step-by-step manner

Table 1 Characteristic Features of the Adrenal Gland that Render it Vulnerable to Toxicants[26]

1. Highly vascular gland with high rate of blood flow
2. Mechanisms for uptake and storage of lipoproteins (and associated lipophilic toxins)
3. Free radical generation during steroid biosynthesis
4. Potential for bioactivation of toxicants by cytochrome P450 enzymes
5. High membrane content of unsaturated fatty acids (substrates for lipid peroxidation)
6. Multiple sites at which function may be influenced

Table 2 Points to Consider and Crucial Examination Items for Evaluating Adrenal Toxicity

Points to consider
Direct and/or indirect effect of a test article
Species, sex, and age of animals used
Metabolic activation by a cytochrome P450 enzyme(s)
Physicochemical properties of a test article

Examination items	
Antemortem	Postmortem
General	*General*
Clinical signs	Gross pathology: color, size
Body weight	Organ weight including related organs
Food consumption	Histopathology
Clinical pathology	Points for toxicologic pathologists to consider
Hematology	Functional anatomy
Blood chemistry: glucose, sodium, potassium, etc.	Blood supply
	Hormone production in each compartment
	Steroid biosynthesis
	Medulla-cortex interaction
	Species/gender differences in anatomical features
	Related organs (pituitary, kidneys, thymus, etc.)
Optional	*Optional*
Serum/plasma hormone levels	Immunohistochemistry
ACTH, corticosterone/cortisol, aldosterone, etc.	Hormonal peptide(s), CYP enzyme(s)
ACTH stimulation test	Electron microscopic examination

from early studies and to add test parameters as needed and as suggested by the accumulated data. In these studies, antemortem data, including clinical signs, body weight, food consumption and clinical pathology (serum sodium, potassium, glucose levels, etc.), and postmortem data, including gross pathology, organ weight and histopathologic examination of the adrenal glands and other related organs (thymus, pituitary, kidney, etc.), should be carefully evaluated.

When a test article-related change in the adrenal glands is observed, it is very important, especially for pharmaceuticals, to determine whether the change is caused by a direct effect or by other associated factors. As an example, adrenocortical cell hypertrophy can be induced not only by an inhibitory effect of a test article on steroid biosynthesis but also by a nonspecific stressful condition subsequent to severe anorexia due to another primary toxicity at the high dose of a test article in toxicity studies. Thus, careful interpretation of the data would provide useful information that would help in designing a clinical trial protocol and in choosing whether or not to include particular measurement criteria.

In order to explain the mode of toxicity of a test article, measurements of hormone levels (ACTH, corticosterone/cortisol, aldosterone) or CYP enzyme activities could provide crucial information. An ACTH stimulation test could also be helpful in evaluating the ability of the adrenal gland to increase the plasma corticosterone/cortisol concentration in response to ACTH stimulation[28]. If, for some reason(s), it is difficult to examine these additional parameters in main groups, a satellite group could optionally be added to the study, or a separate study focused on a target organ could be conducted. The adrenal glands are under the control of upstream organs (the hypothalamic-pituitary system) *in vivo*, and it often becomes difficult to explain the mode of toxicity. In such cases, an in vitro system using a specified cell line or primary culture independent from the control of associated organs or hormones or a reporter gene assay may be informative. For future toxicity evaluations, it may be supportive to obtain some information regarding the structure-activity correlation or receptor binding activity by *in silico* systems prior to conducting *in vivo* studies.

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