

A Study on the Biological Characteristics of Kidney Yin Deficiency and Kidney Yang Deficiency Models in SPF-grade Kunming Mice

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Abstract : Objective: To investigate the establishment and biological characteristics of Kidney Yin Deficiency (KYD) and Kidney Yang Deficiency (KYangD) models in Specific Pathogen Free (SPF)-grade Kunming mice. Methods: Seventy-two SPF-grade Kunming mice were randomly divided into KYD, KYangD, and blank control groups. The KYD group received subcutaneous injections of hydrocortisone at 50 mg/kg for 5 days, while the KYangD group received 25 mg/kg for 10 days. Various physiological parameters were measured after modeling. Results: Compared to the control group, the KYD and KYangD groups showed significant differences in body weight gain, food intake, urine volume, cold water swimming time, grasping irritability, active grid crossings, epididymal sperm count, and active sperm count ($P < 0.05$). The KYD group had higher isolation-induced aggression scores ($P < 0.05$), while the KYangD group had lower rectal and tail temperatures ($P < 0.05$). Compared to the KYangD group, the KYD group had higher tail and rectal temperatures and increased active grid crossings ($P < 0.05$). Conclusions: The hydrocortisone-induced KYD and KYangD models in mice exhibited corresponding symptoms and biological characteristics, reflecting the features of these syndromes in Traditional Chinese Medicine. This study provides a foundation for further research on Kidney deficiency models.

1. Introduction

The KYangD model is the earliest animal model of Traditional Chinese Medicine (TCM) syndromes, first established in the early 1960s using hydrocortisone^[1]. KYD and KYangD are among the most common pathological states encountered in clinical TCM practice^[2, 3]. The establishment of these models employs an integrative approach combining Western and Chinese medicine, bridging the gap between Western medical diseases and TCM syndromes^[4]. This approach lays the foundation for exploring the essence of TCM syndromes and the substantive properties of Chinese medicines^[5], providing a valuable tool for researching KYD and KYangD diseases and evaluating the efficacy of kidney-tonifying drugs. Over the past several decades, numerous methods for establishing KYD and KYangD animal models have been developed, and significant progress has been made in systematically understanding KYD and KYangD syndromes^[6]. However, studies on the biological characteristics of these models often remain conceptual, leading to ambiguity in the models' biological properties. There is a paucity of reports comparing the macroscopic biological characteristics of KYD and KYangD model animals^[2]. To address this gap, the present study employs the classic hydrocortisone-induced

KYD and KYangD syndromes to investigate the establishment and biological characteristics of these models, aiming to provide a scientific reference for related experimental research.

2. Materials and Methods

2.1 Materials

2.1.1 Animals

72 healthy adult Specific Pathogen Free (SPF) grade Kunming (KM) mice, equally divided between males and females, weighing 20 ± 2 g, were purchased from the Experimental Animal Center of Youjiang Medical University for Nationalities. Certificate of conformity: SCXK (Gui) 2022-0003.

2.1.2 Drugs

Hydrocortisone injection (batch number: H12020886, specification: 25mg/5ml) was purchased from Tianjin Jinyao Pharmaceutical Co., Ltd. Normal saline (batch number: H13022576, specification: 4.5g/500ml) was purchased from Hebei Tiancheng Pharmaceutical Co., Ltd.

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2.2 Methods

2.2.1 Grouping and administration

The 72 SPF-grade KM mice were randomly divided into 3 groups: KYD group (13 females, 13 males, n=26), KYangD group (13 females, 13 males, n=26), and blank control group (10 females, 10 males, n=20). The KYD model group received subcutaneous injections of hydrocortisone at 50 mg/kg body weight for 5 days, while the KYangD model group received hydrocortisone at 25 mg/kg body weight for 10 days (0.1ml, subcutaneous on the back of the neck). The blank control group was administered an equal volume of normal saline once daily.

2.2.2 Animal housing and feeding

Mice were housed in standard conditions and fed a standard diet. Feed was provided at noon daily, and mice had ad libitum access to tap water. The activity and mortality of the experimental animals were monitored throughout the study.

2.3 Observation indicators

During the modeling process, clinical symptoms of mice were observed, including coat condition, presence and extent of hair loss, sweating, movement, mental state, sleepiness, and fear of humans.

2.4 Measurement indicators

2.4.1 Body weight

Measured using an electronic balance, each mouse was weighed once before and after the experiment. The average daily body weight gain was calculated.

2.4.2 Water and food intake

After the last injection, each group of mice was placed in metabolic cages for 5 days. Water and feed were provided daily, and the daily water and food intake of each mouse was calculated using the reduction method. The daily water intake per mouse = (the amount of water provided - the remaining amount) / (number of mice × number of days). The daily food intake per mouse = (the amount of feed provided - the remaining amount) / (number of mice × number of days). Measurements were recorded continuously for 5 days, and the average value was calculated.

2.4.3 Urine volume

Urine was collected using metabolic cages, and the volume was recorded continuously for 5 days. The average value was calculated, and the color of the urine was observed.

2.4.4 Rectal temperature

A thermometer probe was inserted into the anus to a depth of approximately 0.5 cm and held in place for 1 min. The stable temperature value on the thermometer was recorded. Measurements were taken continuously for 3 days, and the average value was calculated.

2.4.5 Tail temperature

An infrared thermometer was used to measure the skin temperature of the tail 3 cm from the tail root. Measurements were taken continuously for 3 days, and the average value was calculated.

2.4.6 Grasping irritability test

This test reflects the stress response and mental state of the animals. The scoring criteria are as follows:

Score 1: No struggle when grasped, mentally depressed.

Score 2: Vocalizes when grasped, but no obvious resistance, slow response.

Score 3: Slight resistance when grasped, relatively good mental state.

Score 4: Strong resistance when grasped, but still can be caught.

Score 5: Sensitive response when the skin on the back of the neck is touched, quickly escapes, extremely resistant, tries to bite, difficult to catch.

2.4.7 Isolation-induced aggression test

Mice were isolated and housed in individual cages for 5 days. After modeling, two animals from each group were paired and placed in a cage, where they would fight and attack each other. Observations were made for 5 minutes, and scores were recorded based on the degree of aggressive behavior. The degree of irritability was scored as follows:

Score 0: No attack.

Score 25: Mutual sniffing, tail wagging, occasional attacks on each other, but the number of attacks does not exceed 1.

Score 50: Attempts to actively attack, squeaks, strongly attacks the other, but the number of attacks does not exceed 2.

Score 75: In addition to the above manifestations, mutual tearing and biting occur, or the number of attacks exceeds 3.

Score 100: Fierce biting, fighting in a ball, biting each other, or biting the other until bleeding.

2.4.8 Cold water swimming time

Mice were placed in a water pool (50cm×50cm×40cm) with a water temperature of $6 \pm 1^\circ\text{C}$ and a water depth of 30 cm. The time from the start of swimming to sinking into the water for 30 s without being able to swim up was recorded.

2.4.9 Open field test

Mice were placed in a square uncovered box with the bottom divided into 36 equal grids. A 150 W light bulb was hung approximately 100 cm above the box. The experiment was conducted with the light on, and the number of grids the mouse traversed within 4 min after being placed in the box was recorded. This test reflects the autonomous activity of the animals.

2.4.10 Sperm quality assessment

The unilateral epididymis of 36 male mice was removed and placed on a culture dish. A few drops of normal saline were added, and the epididymis was quickly minced. A drop of the suspension was placed on a slide, covered with a coverslip, and the sperm motility was observed under a microscope. The total sperm count, active sperm count, and abnormal sperm count were recorded. This experiment reflects the sperm quality of male mice.

2.4.11 Organ coefficient calculation

After completing the modeling, the mice were sacrificed by cervical dislocation. The bilateral testes of 36 male mice and the kidneys of each group of mice were removed and immediately weighed on a precision balance. The kidney and testis organ coefficients were calculated using the formula: Organ coefficient = organ weight (g) / body weight (g) × 100%.

2.5 Statistical analysis

Data were analyzed using SPSS 19.0 statistical software. Differences were considered statistically significant at $p < 0.05$. Data were expressed as mean ± standard error ($X \pm SE$).

3. Results

3.1 General condition of mice

In this study, mice in the blank control group exhibited dry and shiny fur, no hair loss, good mental state, normal eating habits, and no abnormal phenomena. On the 4th day after the injection of hydrocortisone, a small number

of mice in the KYD and KYangD groups immediately exhibited weakness in the limbs on the side of the injection site, moved slowly, and in severe cases, had difficulty crawling, similar to limb paralysis. These mice were unable to move, appeared mentally depressed, and displayed squinting eyes. With continued hydrocortisone injection, most mice in the KYD and KYangD groups exhibited raised fur, lack of luster, no obvious sweating, lack of abdominal wall elasticity, sunken abdominal wall, small areas of hair loss at the injection site, rough skin surface, and even scabbing after skin shedding. Some mice showed obvious thoracolumbar kyphosis and died after several occurrences of these symptoms. The KYD group mice exhibited symptoms such as increased activity, easily startled behavior, hair loss, dry and hard stools, and yellow urine, which were relatively consistent with the symptoms of KYD in TCM. During the modeling period, the KYangD group mice exhibited reduced activity, hunched posture, slow movement, deep and slow breathing, sluggish reactions, curled up posture, cold aversion, huddling, preference for lying down, loose stools, and clear, long urination, which were relatively consistent with the symptoms of KYangD in TCM.

3.2 Effects of KYD and KYangD on physiological parameters

Compared to the blank control group, the KYD and KYangD groups showed significant differences in daily average body weight gain, food intake, urine volume, cold water swimming time, grasping irritability score, and the number of active grid crossings ($P < 0.05$). However, there were no significant differences in water intake and kidney coefficient between the control group and the KYD and KYangD groups ($P > 0.05$). The KYD group mice showed no significant differences in rectal and tail temperatures compared to the control group ($P > 0.05$) but had significantly higher isolation-induced aggression scores ($P < 0.05$). In contrast, the KYangD group mice had significantly lower rectal and tail temperatures ($P < 0.05$) but no significant difference in isolation-induced aggression compared to the control group ($P > 0.05$). When comparing the KYD and KYangD groups, the KYD group mice had significantly higher tail and rectal temperatures ($P < 0.05$) and significantly increased active grid crossings ($P < 0.05$) (shown in Table 1).

Table 1. The measurement results of various indicators in mice

Index	KYD (n=26)	KYangD (n=26)	Control (n=20)
Daily average body weight gain (g)	0.98±0.04 ^a	0.89±0.04 ^a	1.54±0.08
Daily average water intake (ml)	1.80±0.10	1.67±0.09	2.15±0.20
Daily average food intake (g)	2.24±0.12 ^a	2.34±0.09 ^a	3.57±0.19
Daily average urine volume (ml)	0.11±0.02 ^a	0.11±0.03 ^a	0.12±0.03
Tail temperature (°C)	20.43±0.16 ^b	19.81±0.12 ^a	20.66±0.21
Rectal temperature (°C)	37.37±0.15 ^b	36.29±0.14 ^a	37.45±0.09
Cold water swimming time (min)	1.16±0.05 ^a	1.17±0.06 ^a	2.29±0.94
Grasping irritability test	2.85±0.20 ^a	2.85±0.21 ^a	3.85±0.13

Isolation-induced aggression test	68.27±7.01 ^a	56.73±6.44	35.00±7.78
Number of active grid crossings	132.12±8.62 ^b	111.42±4.92 ^a	230.95±8.38
Kidney organ coefficient (%)	1.51±0.09	1.56±0.01	1.77±0.15

Note: P<0.05 indicates a significant difference; “a” indicates a significant difference compared with the blank control group; “b” indicates a significant difference compared with the Kidney Yang Deficiency (KYangD) group.

3.3 The results of testis and sperm quality in SPF mice

In table 2, in comparison with the 10 male mice in the blank control group, the 13 male mice in both the KYD and KYangD groups showed no significant difference in testis coefficient (P >0.05). However, both the KYD and KYangD groups had significantly decreased epididymal sperm count and active sperm count (P <0.05) compared to the control group. There was no significant difference

in abnormal sperm count between the control group and the KYD and KYangD groups (P >0.05).

These findings suggest that the hydrocortisone-induced KYD and KYangD models in mice exhibited symptoms and biological characteristics that were consistent with the respective TCM syndromes. The observed differences in various physiological parameters between the KYD, KYangD, and control groups provide evidence for the successful establishment of these models and their potential use in further studies investigating the mechanisms and treatments of KYD and KYangD.

Table 2. The measurement results of testis and sperm quality in SPF mice

Index	KYD (n=13)	KYangD (n=13)	Control (n=10)
Testis organ coefficient (%)	0.75±0.06	0.77±0.05	0.70±0.05
Epididymal sperm count	2.31±5.63 ^a	58.31±7.00 ^a	94.20±4.89
Active sperm count	21.38±5.29 ^a	19.54±3.38 ^a	43.60±7.14
Abnormal sperm count	7.31±0.67	6.77±1.07	5.70±1.00

Note: P<0.05 indicates a significant difference; “a” indicates a significant difference compared with the blank control group; “b” indicates a significant difference compared with the KYangD group.

4. Discussion

Over the past half-century, China has made significant achievements in the research of animal models for TCM syndromes. Among these, the KYangD model established using hydrocortisone has had a profound impact on subsequent research^[1]. In the present study, we replicated the KYD and KYangD models by injecting mice with hydrocortisone. By observing the animals' external appearance, behavior, and measuring relevant indicators, we explored the establishment of KYD and KYangD models and their corresponding biological characteristics.

According to traditional Chinese medicine, the kidney stores essence and is considered the "root of congenital constitution" and the "house of water and fire" ^[7]. The main pathologies of kidney diseases are dysfunctions in storing essence, governing water metabolism, and receiving “Qi”. Common clinical symptoms include lumbar and knee soreness or pain, tinnitus, tooth loosening and loss, impotence, spermatorrhea, oligospermia, menstrual disorders, infertility, edema, excessive sweating, and abnormal urination and defecation^[8, 9]. Kidney deficiency syndromes are divided into KYD and KYangD. KYD refers to the deficiency of kidney “Yin”, leading to a lack of nourishment and the generation of internal heat, resulting in yin deficiency symptoms such as scanty dark urine and dry stools^[10]. KYangD refers to the decline of kidney “Yang”, leading to a lack of “warmth” and “Qi” transformation, resulting in cold deficiency symptoms such as clear, long urination and loose stools^[2].

During the study, some mice in the model groups died. The modeling mechanism of the hydrocortisone-induced kidney deficiency model involves using a certain amount of exogenous glucocorticoids to inhibit the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, thereby reducing the secretion of corticosteroids from the adrenal cortex^[11]. When corticosteroids are suddenly discontinued, the suppressed state of the hypothalamic-pituitary-adrenal axis is immediately exposed^[11]. Previous studies have reported that due to the injection of large amounts of exogenous corticosteroids, liver glycogen increases, and the glucocorticoid receptors in the liver cell cytoplasm increase accordingly, causing hydrocortisone to accumulate in large quantities in the mice, resulting in subacute toxic reactions^[12]. This may be the reason for the observed mortality in the model groups.

The experimental results showed that compared with the blank control group, the KYD and KYangD mice had slower body weight gain, reduced food intake, decreased urine volume, significantly reduced cold water swimming time, decreased grasping irritability, reduced autonomous activity, and decreased sperm quality (all P <0.05). Kidney qi deficiency hinders growth and development, leading to emaciation, reduced food intake, decreased exercise endurance, and mental fatigue. The kidney governs water metabolism, and the qi transformation function of the kidney essence plays an important role in regulating the balance of body fluid metabolism^[13]. Reduced urine output suggests that KYD and KYangD mice have a certain degree of water metabolism disorder. The grasping irritability test reflects

the mental state of animals and their response to external stimuli. Previous studies have shown that KYD animals have significantly increased irritability^[4]. In this experiment, due to the injection of large amounts of exogenous glucocorticoids, the KYD and KYangD animals experienced kidney qi deficiency and exhaustion of essence and qi, resulting in poor mental state and weakened response to external stimuli, which may explain the observed differences. As the kidney stores essence and governs reproduction, sperm production depends on the nourishment of kidney yin and the warmth of kidney yang^[10]. KYD and KYangD can lead to a decline in sperm quality and reproductive ability. Interestingly, in this experiment, there were no significant differences in water intake, kidney coefficient, and testis coefficient between the KYD and KYangD groups ($P > 0.05$). Previous reports have shown that KYD mice have increased water intake, elevated body temperature, and increased overall basal metabolic rate, while KYangD mice have decreased water intake^[14]. Additionally, both KYD and KYangD mice have been reported to have decreased kidney and testis organ coefficients^[14]. The inconsistency between our results and these findings may be related to factors such as the choice of experimental animals, experimental conditions, modeling duration, modeling dose, and observation time. Further research is needed to clarify these discrepancies.

Compared with the blank control group, the KYD mice showed no significant differences in rectal and tail temperatures ($P > 0.05$) but significantly increased isolation-induced aggression ($P < 0.05$). The KYangD mice had significantly lower rectal and tail temperatures ($P < 0.05$) but no significant difference in isolation-induced aggression ($P > 0.05$). TCM theory postulates that yin deficiency generates internal heat, while yang deficiency leads to cold^[15]. In the isolation-induced aggression test, KYD animals were easily irritated, suggesting that the nervous system of this group of mice was disturbed, and their ability to regulate and adapt to the environment was reduced. The "Treatise on the Origin and Symptoms of Diseases" by Chao Yuanfang from the Sui Dynasty described the understanding of KYD syndrome, stating that "when yin qi is insufficient and yang qi is excessive, there will be irritability and restlessness"^[3]. When comparing the KYD and KYangD groups, the KYD mice had significantly higher tail and rectal temperatures ($P < 0.05$) and significantly increased active grid crossings ($P < 0.05$). It has been reported that yin deficiency generates internal heat, leading to increased body temperature and basal metabolic rate^[16]. The ancient book "Huangdi Neijing" mentions that "yang governs movement, and yin governs stillness"^[14]. Therefore, the autonomous activity of KYD mice is slightly stronger than that of KYangD mice.

The differentiation and classification of TCM syndromes are the results of long-term experience and summarization. Symptom diagnosis is indispensable in the diagnosis and treatment of diseases in TCM^[17]. In animal experiments, TCM syndrome differentiation and treatment are mainly achieved by observing the corresponding external appearance, behavior, and measuring relevant indicators. However, there are

significant differences between humans and animals, and the symptoms and signs they exhibit are not exactly the same. The manifestations of kidney deficiency syndrome in the human body involve multiple systems such as the nervous, endocrine, and immune systems, affecting multiple levels, including organs, tissues, cells, and even gene and protein expression^[18, 19]. Therefore, during the modeling process, it is necessary to combine the latest advances in existing research in both Chinese and Western medicine to improve the design of modeling methods.

It is important to acknowledge the potential limitations of using hydrocortisone to induce kidney deficiency syndromes^[20]. First, while hydrocortisone administration can induce symptoms and biological characteristics that resemble KYD and KYangD syndromes, it may not fully capture the complexity and specificity of these TCM syndromes^[9]. Other factors, such as diet, environment, and constitution, also play important roles in the development of kidney deficiency syndromes in clinical practice. However, the current study provides a valuable starting point for investigating the biological basis of these syndromes and lays the foundation for future research that can incorporate additional factors to better mimic the clinical presentation of KYD and KYangD.

Second, hydrocortisone is a glucocorticoid that can affect multiple organ systems beyond the kidney, such as the endocrine, immune, and metabolic systems. These systemic effects may influence the observed symptoms and biological characteristics in the animal models, making it important to interpret the results with caution^[12]. However, the use of hydrocortisone to induce kidney deficiency syndromes is a well-established method in TCM research, and the current study employs a rigorous experimental design and appropriate statistical analyses to minimize the impact of potential confounding factors. Future studies could further validate the specificity of the observed changes to the kidney using additional histological, biochemical, and molecular analyses^[21].

The potential clinical implications of our findings are significant. By establishing and characterizing the KYD and KYangD models using hydrocortisone, we have provided a valuable tool for understanding the biological basis of kidney deficiency syndromes in TCM. These models can be used to investigate the underlying mechanisms of KYD and KYangD, which may lead to the identification of new therapeutic targets and the development of novel treatment strategies.

One promising avenue for future research is the use of these models to screen and evaluate the efficacy of TCM herbal formulas and other natural products in the treatment of kidney deficiency syndromes. Many TCM herbal formulas have been used clinically for centuries to treat KYD and KYangD, but their mechanisms of action and therapeutic effects have not been fully elucidated^[3]. By applying these formulas to the hydrocortisone-induced KYD and KYangD models, researchers can assess their ability to alleviate the symptoms and biological characteristics of these syndromes, as well as explore their potential mechanisms of action at the molecular and cellular levels.

Furthermore, these models can be used to develop and test new therapeutic strategies that integrate the principles of TCM with modern pharmacology and drug discovery^[4]. For example, researchers could use these models to identify the active compounds in TCM herbal formulas that are responsible for their therapeutic effects on kidney deficiency syndromes. These compounds could then be isolated, purified, and optimized for drug development, leading to the creation of novel pharmaceuticals that combine the best of both TCM and Western medicine.

In addition to drug discovery, the KYD and KYangD models can also be used to investigate the role of lifestyle factors, such as diet and exercise, in the prevention and treatment of kidney deficiency syndromes. By manipulating these factors in the models, researchers can gain insights into how they influence the development and progression of KYD and KYangD, and develop evidence-based recommendations for patients with these syndromes.

Finally, the establishment of these models provides a platform for further research into the genetic and epigenetic basis of kidney deficiency syndromes^[10]. By comparing the gene expression profiles and epigenetic modifications between the KYD, KYangD, and control groups, researchers can identify the key genes and pathways that are involved in the pathogenesis of these syndromes. This knowledge could lead to the development of personalized medicine approaches that tailor treatment strategies based on an individual's genetic and epigenetic profile.

5. Conclusion

The hydrocortisone-induced KYangD model, despite limitations, reflects characteristics of KYD and KYangD syndromes in TCM. This study provides insights into the biological basis of kidney deficiency syndromes and lays the foundation for future research. Combining TCM principles with modern medicine can lead to novel approaches for prevention, diagnosis, and treatment. Future studies should focus on validating these models, exploring mechanisms, and translating findings into clinical practice to improve patient health and well-being.

Ethics statement

The studies involving animals were approved by the Ethical Committee for Animal Study of the Youjiang Medical University for Nationalities, Baise, PR China. Animal ethics approval (Study Code: YMUN-2021-09172/Research ID: YMUN-2021-09172, 09 October 2021) was obtained before the commencement of study.

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