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Studies on preventive and improvement effects of a kampo medicine on the decline in reproductive functions and peripheral blood flow in aged female rats

(漢方薬の老齢雌ラットの生殖機能ならびに末梢血流量低下に対する予防および改善効果に関する研究)

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Preface

Aging is the accumulation of changes in an organism or object over time (Bowen and Atwood, 2004). In biology, senescence is the state or process of aging. Cellular senescence is a phenomenon where isolated cells demonstrate a limited ability to divide in culture (Hayflick and Moorhead, 1961), while organismal senescence is the aging of organisms. The organismal senescence is characterized by the declining ability to respond to stress, increasing homeostatic imbalance and increased risk of disease. This irreversible series of changes inevitably ends in death. Although the age-related changes are obvious from the molecular to the physiological and behavior levels, there is no one unifying theory to explain them. The mechanisms of aging could be quite complex and have diversity in organisms, tissues, and cells (Troen, 2003). There are many environmental factors including stress influence the age-related changes. For example, it is reported that the increase in oxidative stress, an imbalance between endogenous levels of oxygen radicals and antioxidative defense, is one of the causes of aging (Sasaki et al., 2008). These factors also lead to complicate elucidations of primary mechanisms of the aging. It is also difficult to evaluate the progression of aging, because an appropriate marker for it is lacking.

Aging and longevity sciences need appropriate animal models for understanding and capturing the physiological aging process individually. Small laboratory rodents such as rats and mice are used extensively in the
researches (Sone et al., 2007; Sasaki et al., 2008). It is also reported that senescence marker protein-30 (SMP30) knockout mice which having an increased mortality rate at the 3 months of age, being attribute to highly susceptible to various harmful reagents, may be a useful tool for aging and biological monitoring (Maruyama et al., 2004). Because progression of aging is influenced not by the one gene function, but by multiple genetical and environmental factors described as above, it is not clear whether SMP30 KO mice represent whole of the accelerated aging process as an model of biological dysfunctions. The further investigations are needed to reveal them.

Reproduction is one of the most fundamental biological characteristics common to all mammals. The aging researches on reproduction have characterized the reproductive aging of female laboratory rats using fertility and fecundity on Long-Evans (Matt et al., 1986; Matt et al., 1987) and estrous cycles on Sprague-Dawley (Clements and Meites, 1971; Quadri et al., 1973; Everett, 1980), since the primary mechanism is mostly identical between rats and humans. The estrous cycle well reflects ovarian activity, which is regulated through the hypothalamus-anterior pituitary axis. The estrous cycle is thus a convenient and useful parameter for monitoring individual reproductive aging throughout the lifespan of the rats. Nowadays, both the observations of the vaginal smear and the measurements of the electrical impedance in the vagina (EIV) are used for classification of stages of the estrous cycle. Vaginal cytology is used for classification of female rat estrous cycle stages which can be reliably identified from vaginal smears.
(Montes and Luque, 1988). Recently, Rezac (2008) described that the measurement of EIV is convenient and reliable to explore various reproductive processes. Although female rodents do not experience menopause, they undergo a transition from regular ovulatory cycles to irregular cycles to acyclicity. The process of reproductive aging appears to be divided into three sequential phases based on the cyclicity: the cyclic disturbance, the cycle cessation, and the postcyclic vaginal state. The mechanisms for these changes are largely unknown, but it is believed that these biological dysfunctions are affected by multiple factors.

The treatment and prevention of the age-related changes has been examined from epochs beyond historical reach. It has been described that there are many approaches to prevent the progression of senescence. In these days, caloric restriction (CR) with adequate nutrition, nutritional supplements and hormonal therapy and kampo therapy are getting attention. CR has been shown to reduce oxidative stress, improve insulin sensitivity, and alter neuroendocrine responses and central nervous system function in animals (Martin et al., 2009). It has particularly profound and complex actions upon reproductive health (Martin et al., 2008). The anti-oxidant supplements include vitamins A, C and E were also used for the prevention of progressive and irreversible accumulation of oxidative damage (Kondo et al., 2008), since oxidative stress the primary causal factor underlying aging-associated declines in physiological function (Kregel and Zhang, 2007). Unfortunately, there is little evidence any of these products actually works. Hormone replacement therapy is commonly prescribed to postmenopausal
women for symptoms of aging, but side effects such as increase the risk for heart attack, strokes and breast cancer have also been reported (O'Connor et al., 1998).

A number of kampo medicines have been used for many centuries in East Asia, including China and Japan, for the treatment of menstrual disorders, infertility and undefined symptoms, e.g. hot flushes and chilly sensation. In general, the traditional Chinese herbal prescriptions are rather inexpensive and safe with little side effects, and have properties for normalizing biological balances. Also, the characteristics of medicinal herbs, especially their applications in different disease stages (prevention and intervention) and multi-targets properties, allow them to be potential anti-aging intervention in prevention and treatment of the aging-associated disorders. However, animal studies that evaluating the effects of the administration of kampo medicines on the age-related biological dysfunctions have little been performed. It is considered that examining evidence to support the effectiveness of the kampo medicines may provide useful information for clinical applications for various age-related symptoms in human.

The objects of the present study were to examine the preventive and improvement effects of Nanpao, a kampo medicine, on biological dysfunctions in aged female rats. In Chapter 1, the author examined the correlation between the value of EIV and morphological conditions of the epithelial cell layer of vaginal mucosa in the aged Sprague-Dawley rats exhibiting abnormal estrous cycles. In Chapter 2, the author performed to actually investigate the utility of the method for the measurement of vaginal
impedance in the study of the progression of reproductive aging in female rats and to evaluate the time-dependent effects of the administration of Nanpao on the age-related changes in the estrous cycle. In Chapter 3, the author examined the effects on a decline in various reproductive functions of administration of Nanpao (Chapter 3-1). Moreover, the effects on cold constitution of Nanpao were examined quantitatively and objectively using measurements of peripheral blood flow and surface skin temperature in aged female rats (Chapter 3-2).
Chapter 1

A correlation between the value in electrical impedance and the conditions of the epithelial cell layer of vaginal mucosa in aged female rats
Summary

Age-related changes, including loss of regular estrous cycles in female rats are well known phenomenon (Meites and Huang, 1976; Lu et al., 1979). The estrous cycle is thus a convenient and useful parameter for monitoring individual reproductive aging throughout the lifespan of the rats.

The measurement of electrical impedance in the vagina (EIV) can be used for monitoring various reproductive events in female mammalian. In this Chapter, the author examined the correlation between the value of EIV and the morphological conditions of the epithelial cell layer of vaginal mucosa in the aged Sprague-Dawley rats exhibiting abnormal estrous cycles. The value of EIV was considered to correlate with vaginal cytology in aged rats as well as in young rats. Moreover, the values of EIV both in persistent estrus and continual diestrus rats reflected the histological conditions of the epithelial cell layer of vaginal mucosa. In conclusion, the author describes that the measurement of EIV is useful for studying the progression of reproductive aging.
Introduction

The average rat lifespan is approximately 2 to 3 years. By 2 months of age, young female rats are reproductively mature and exhibit estrous cycles and ovulation every 4 to 5 days (Kohn and Clifford, 2002). The rat estrous cycles can be further divided into four stages: proestrus, estrus, metestrus, and diestrus. The estrous cycle is characterized by cyclical changes in the uterus, ovaries, vaginal mucosa, behavior, and hormone levels (Maeda et al. 2000; Nicholas, 1949).

The loss of a regular estrous cycle is well known as one of the representative declines in reproductive performance in aged female rats (Meites and Huang, 1976; Lu et al. 1979; Ichihashi et al., 2008). Beginning at middle age (at the age of 6 to 10 months), many females display prolonged, irregular estrous cycles instead of regular cyclicity (Aschheim, 1976; Meites and Huang, 1976; Lu et al., 1979; Westwood, 2008). Shortly thereafter, aging females become persistently estrus, a condition resulting from chronic anovulation. This may be followed by state in which cycles are ten to fourteen days long, suggestive of prolonged luteal phase, which termed repetitive pseudopregnancy (Nelson and Felicio, 1985). The final stage of reproductive senescence is persistent diestrus (vom Saal et al., 1994).

Vaginal cytology is used for classification of female rat estrous cycle stages which can be reliably identified from vaginal smears (Montes and Luque, 1988). In addition to the vaginal smears, the measurement of electrical impedance in the vagina (EIV) can be used to identify estrous in several species (Bartos, 1977; Taradach, 1982). Consequently, the elevation (over 3.0
kΩ) of EIV accompanying cornification of the epithelial cell layer in the proestrus stage indicates the optimum day for mating in rats and mice (Koto et al., 1987 a; Koto et al., 1987 b). A transient increase in EIV correlated strongly with changes in estradiol levels (Bartos, 1977; Koto et al., 1987 b).

Rezac (2008) described that the measurement of EIV may be useful to explore various reproductive processes. However, age-related EIV characteristics are not well known. Recently, longitudinal studies, such as the effect of chronic atrazine on estrous cycle patterns (Eldridge et al., 1999) and changes of the F344/N estrous cycle with aging (Sone et al., 2007) were performed using female rats in the field of reproductive toxicology, physiology and theriogenology. Since it is not easy to purchase aged rats from breeders, aged rats are very valuable for executing studies and there is a need for these research fields to collect data without autopsy. Therefore, if the present study were to demonstrate that the value of EIV reflect the morphological conditions of vaginal mucosa in rats exhibiting abnormal estrous cycles, the measurement of EIV is helpful to predict temporal vaginal histology without autopsy. Moreover, since these measurements are technically easy, their detection may allow us to improve efficiency for studying progression of reproductive aging.

In this Chapter, the author examined the correlation among the value of EIV, the cytological and histological conditions of the epithelial cell layer of vaginal mucosa in 13-months (expected age that rats exhibit the cessation of regular or extended cycles) and 19-months (expected age that rats exhibit completely lose cyclicity) aged rats. This study was evaluated using SD rats,
which was reported a correlation between EIV and the hormone levels in the proestrus stage (Taradach, 1982).
Materials and Methods

Animals

Female rats of the Sprague-Dawley (Crl: CD [SD]) strain were purchased from Charles River Japan, Inc. at the age of 5 months (n=12) or at the age of 14 weeks (n=10), and used in this study. Eight of 12 and remaining four rats purchased at the age of 5 months were examined at the age of 13 months (13 M) and at the age of 19 months (19 M), respectively. During the experimental period, the animals were housed individually in stainless steel wire cages (W: 430 × D: 450 × H: 170 mm) in a barrier-sustained animal room (temperature: 23 ± 1°C; relative humidity: 55 ± 5%; fresh-air ventilation: 12 changes/hr; lighting: 12h/12h light/dark cycle). The animals were allowed free access to food (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. All animal studies were approved by the Institutional Animal Care and Use Committee of Mitsubishi Tanabe Pharma Corporation in accordance with the “Guidelines for Animal Studies”.

General study design and sampling

Intact young rats (n=10) in 15 weeks of age were examined once daily (between 13:00 and 15:00) for 14 days for vaginal impedance and cytology measurements.

Intact aged rats in 13 M (n=8) and 19 M (n=4) of age were examined for vaginal impedance and cytology measurements in the same manner as in young rats. All aged rats were sacrificed immediately after final sampling of
day 14. They were anesthetized with ether, and then sacrificed by exsanguination by cutting abdominal artery and vein under the deep ether anesthesia, and the vaginas were taken.

**Vaginal cytology**

The vaginal smears were taken by wiping the vaginal wall lightly with a water-moistened cotton swab, Giemsa-stained on glass slides. Slides were examined for the following features: cornified epithelial cells, nucleated epithelial cells, leukocytes, and mucus. Estrous cycle stage then was determined using the following criteria (Maeda et al., 2000; Sharp et al., 1998; Weiss et al., 2000): 1) proestrus-predominantly nucleated epithelial cells, few cornified epithelial cells, and few leukocytes; 2) estrus-predominantly cornified epithelial cells; 3) metestrus-predominantly leukocytes and cornified epithelial cells; and 4) diestrus-predominantly leukocytes, some nucleated epithelial cells, and mucus. In the observation of vaginal smears of each animal, the estrous cycles were defined as “regular estrous cycles” when a regular cycle of 4- or 5-days was observed in at least 2 consecutive periods, as “irregular estrous cycles” when one estrous cycle was prolonged, respectively. Aged rats that had exhibited persistent vaginal cornifications continuously for 2 weeks were considered to be in persistent estrus (Lu et al., 1979), whereas rats that had displayed diestrus smears for 2 weeks were considered to be in continual diestrus (repetitive pseudopregnancy or persistent diestrus (Westwood, 2008)).
**Vaginal impedance measurements**

Vaginal impedance measurements were using the Impedance Checker MK-10B (Muromachi Kikai Co., LTD, Tokyo, Japan). The vaginal probe was inserted until taking constant impedance measurements. All impedance measurements were done immediately before collection of vaginal smears. The vaginal impedance probe was cleaned with 70 % alcohol before each measurement.

**Vaginal histology**

The vagina of each rat was dissected and fixed in 10 % neutral buffered formalin and processed for histology using routine paraffin-embedding. Sections were prepared at 5-6 μm and stained with hematoxylin and eosin by the routine method for microscopy.
Results

Observations of estrous cycles by cytology

All young animals exhibited regular estrous cycles (data not shown). Rats classified in proestrus (4.0 ± 1.5 kΩ, n=31 samples) by vaginal cytology had higher EIV than in the other estrous stages (estrus, 2.2 ± 1.1 kΩ, n=35; metestrus, 1.2 ± 0.1 kΩ, n=33; diestrus, 1.1 ± 0.2 kΩ, n=41).

Figure 1-1 summarizes the estrous cycles in individual rats from 13 M and 19 M groups. All aged rats exhibited irregular estrous cycles.

In the 13 M group, one rat (13M-g) showed both regular estrous cycle and intermittent prolonged diestrus. Two rats (13M-d and 13M-h) showed persistent estrus after transition from proestrus to estrus. One rat (13M-a) showed a persistent estrus characterized by chronic anovulation and persistent vaginal cornification. During the persistent vaginal cornification, the vaginal smear consisted of clumped-to-densely packed fields of cornified epithelial cells. Nucleated epithelial cells were present in spare to slight number (Figure 1-2-A). The remaining four rats (13M-b, 13M-c, 13M-e and 13M-f) showed persistent diestrus characterized by a number of leukocyte and some epithelial cells. Moreover, the mucin was observed sporadically in the smear during persistent diestrus (Figure 1-2-B).

In the 19 M group, all rats displayed irregular estrous cycle. One rat (19M-b) showed persistent estrus and the remaining three rats (19M-a, 19M-c and 19M-d) showed persistent diestrus.
Relationship between vaginal cytology and vaginal impedance value

One rat (13M-g) that had showed almost regular estrous cycle had displayed enough elevation (over 3.0 kΩ) of EIV in proestrus stage (Figure 1-3-A). All animals except 13M-g had not displayed enough elevation of EIV in all stages. Among these rats, two rats (13M-d and 13M-h) that had shown persistent estrus after transition from proestrus to estrus had not displayed enough elevation (under 3.0 kΩ) of EIV even in proestrus stage (Figure 1-3-B). In addition, the average values of EIV in two rats (13M-a and 19M-b) classified in persistent estrus by vaginal cytology had higher than those of seven rats (13M-b, 13M-c, 13M-e, 13M-f, 19M-a, 19M-c, and 19M-d) classified in persistent diestrus for 2 weeks (Figure 1-4).

Relationship between vaginal histology and vaginal cytology

The histological appearance of rats classified in persistent estrus by vaginal cytology is shown Figure 1-5-A. The epithelium in the right side was composed both of a stratum germinativum that was similar to day of estrus and a stratum germinativum and a stratum granulosum that was similar to day of proestrus. Moreover, stratum corneum-like structure was observed in the part of epithelial cell layer.

The histological appearance of rats classified in persistent diestrus by vaginal cytology is shown Figure 1-5-B. The epithelium consisted of a stratum germinativum and a stratum mucification with cuboidal to ovoid cells. A few cells of the epithelium cell layer contained a mucin vacuole. The
stratum corneum-like structure was not observed in the epithelial cell layer.
Discussion

In this Chapter, the author examined the correlation between the value of EIV and the cytological and histological conditions of the epithelial cell layer of vaginal mucosa in the aged rats exhibiting the decline in the reproductive functions.

All young animals exhibited regular estrous cycles. Rats classified in proestrus by vaginal cytology had higher EIV than in the other estrous stages. These results were consistent with those from previous reports (Koto et al., 1987 a; Taradach, 1982). In contrast to these findings, Singletary et al. (2005) indicated that estrus Wistar rats had higher EIV than did nonestrous rats, that no correlation occurred between EIV and hormone levels in proestrus rats. However, correlation between EIV and hormone levels in their study was not examined at estrous stage associated with increase in impedance. These findings suggest that the association of EIV with respect to estrous cycle stage and hormone levels may vary between rodents stocks and strain.

All aged rats exhibited irregular estrous cycles. There are many reports concerning of the age-related loss of regular estrous cycles in rats (Ingram, 1959; Mandl, 1961; Meites and Huang, 1976).

In the 13 M group, one rat (13M·g) showed both regular estrous cyclicity and intermittent prolonged diestrous. This animal displayed enough elevation (over 3.0 kΩ) of EIV in only proestrus stage, and it was lower (under 3.0 kΩ) at other stage of estrous cycle. These results suggested that the cornification of the epithelial cell layer of the vaginal mucosa in the proestrus stage of
13M-g rat was sufficient since it is reported that the vaginal cornification occurred concurrently the elevation of EIV in the proestrus stage of rats exhibiting regular estrous cycles (Koto et al., 1987 b). In addition, since the vaginal cornification was caused by estrogen (Montes and Luque, 1988; Allen, 1922; Evans et al., 1990; Boutin and Cunha, 1997), it was presumed that secretion of hormone in 13M-g rat was almost regular. On the other hand, two rats (13M-d and 13M-h) that had shown persistent estrus after transition from proestrus to estrus had not displayed enough elevation of EIV even in proestrus stage. From these results, though estrous cycle stage was judged to be proestrus by vaginal cytology, the cornification of the epithelial cell layer of the vaginal mucosa was considered to be not sufficient in these rats. The insufficient cornification of the epithelial cell layer in these rats was considered to be due to age-related decrease in serum estrogen levels. The determination of the hormone levels and the vaginal histological appearance in the proestrus rats might be needed to further define and confirm these results.

In the observation of estrous cycle of 13 and 19 M groups, two rats (13M-a and 19M-b) and seven rats (13M-b, 13M-c, 13M-e, 13M-f, 19M-a, 19M-c, and 19M-d) showed persistent estrus and persistent diestrus, respectively. The vaginal histological appearance of rats classified in persistent estrus was a mixture of findings observed in proestrus and estrous stages of rats exhibiting regular estrous cycle, not typical smear appearance observed in estrus rats. In the vaginal histology, the stratum corneum-like structure was confirmed in the part of epithelial cell layer in the persistent estrus rat. In
addition, the average of impedance values in persistent estrus rats was approximately-constant for 2 weeks. The previous studies have reported that the noncyclic patterns of estrogen and progesterone secretion in aging persistent estrus rats resulted in persistent vaginal cornifications (Lu et al., 1979). Nagaoka et al. (1995) reported that the histological changes, such as vaginal epithelium cornifications, observed in persistent estrus rats at the age of 10 to 12 months are considered to be signs of estrogenization, and in fact the results coincided with increased estrogen: progesterone ratios during the period. On the other hand, the vaginal histological appearance of rats classified in persistent diestrus was almost the same findings observed in diestrus of rats exhibiting regular estrous cycle. The stratum corneum-like structure was not observed in the vaginal mucosa of persistent diestrus rats. The average of impedance values in persistent diestrus rats was approximately-constant, but were lower impedance values than those in persistent estrus throughout 2 weeks. These results suggested that the continuous high value of EIV in persistent estrus rats was considered to be due to continuous a slight stratum corneum-like structure in the epithelial cell layer. Since the serum levels of estrogen were greater in persistent estrus than in persistent diestrus rats (Lu et al., 1979), the difference between the absence and presence of the stratum corneum-like structure was considered to be related to the amount of serum estrogen, which caused the vaginal cornification.

In conclusion, this Chapter showed that the value of EIV was considered to correlate with results from vaginal cytology in aged rats exhibiting abnormal
estrous cycles as well as in young rats. Moreover, the values of EIV both in persistent estrus and continual diestrus rats reflected the histological conditions of the epithelial cell layer of vaginal mucosa. These results indicated that the measurement of EIV is useful for predicting temporal vaginal histology without autopsy in the fields of reproductive physiology and toxicology and might be worthwhile approach to study more precisely the age-related reproductive processes in rats.
Fig. 1-1. The estrous cycles for 2 weeks in individual rats from 13 M and 19 M groups. Black, stippled and white blocks represent proestrus phase, estrus phase and diestrus phase, respectively.
Fig. 1-2. Photomicrographs of typical vaginal smears taken from 13M rats. (A) Feature of rats classified in persistent estrus by vaginal cytology. (B) Feature of rats classified in continual diestrus by vaginal cytology. Giemsa stain.
Fig. 1-3. Relationship between vaginal cytology and the value of EIV for 14 days (A) in 13M-g rat and (B) in 13M-d rat, respectively. Black, stippled and white blocks represent proestrus phase, estrus phase and diestrus phase, respectively.
Fig. 1-4. Comparison of the average values of EIV between persistent estrus and continual dioestrus rats for 14 days. Points are means, and vertical lines represent SD.
Fig. 1-5. Histological features of vaginal mucosa.

(A) Photomicrographs of rats classified in persistent estrus by vaginal cytology. The stratum corneum-like structure (←) was observed in the part of epithelial cell layer in the right side. Hematoxylin-eosin (HE) stain. (B) Photomicrographs of rats classified in continual diestrus by vaginal cytology. A few cells of the epithelium cell layer contained a mucin vacuole (→). The stratum corneum-like structure was not observed in the epithelial cell layer. HE stain.
Chapter 2

Effects of Nanpao®, a kampo medicine, on the decline in estrous cyclicity with advancing age in female rats, as measured by vaginal impedance methods
Summary

In this Chapter, the author performed to evaluate the time-dependent effects of Nanpao, a kampo medicine, on age-related changes in the estrous cycle of female rats, and to investigate the utility of measuring EIV for studying transitional changes in the estrous cycle. Rats were allocated to 3 groups: control, Nanpao 30 mg/kg/day, and 100 mg/kg/day groups. EIV measurements and cytology samples were taken for 14 days at the age of 6 months before the initial treatment. After the start of the treatment, these data were collected at about monthly intervals until the age of 10 months in the same manner. Observations at the ages of 7 (weeks 2–3 of dosing) and 8 months (weeks 6–7 of dosing) showed that loss of a regular estrous cycle in the 100 mg/kg/day group was inhibited as compared to the control group. Moreover, at the ages of 9 (weeks 11–12 of dosing) and 10 months (weeks 17–18 of dosing), these effects were identified not only in the 100 mg/kg/day group, but also in the 30 mg/kg/day group. Since vaginal cytology and EIV gave almost concordant results as indicators of estrous cyclicity, the measurement of EIV was capable of detecting time-dependent changes in the estrous cycle as well as observations of vaginal smears. In conclusion, Nanpao administration inhibited loss of regular estrous cycles, and the EIV method was a worthwhile approach to a more precise study of estrous cyclicity in rats exhibiting abnormal estrous cycles.
Introduction

A number of kampo medicines have been used for many centuries in East Asia, including China and Japan, for the treatment of menstrual disorders, infertility and undefined symptoms, e.g. hot flushes and chilly sensation. In general, the traditional Chinese herbal prescriptions are rather inexpensive and safe with little side effects, and have properties for normalizing biological balances. Nanpao, the test drug used in the present study, is an over-the-counter kampo medicine for humans. It is a mixture of 31 Chinese crude drugs (Table 2-1) and exerts a nourishing and revitalizing effect. The test drug is reported to be clinically effective in treating various age-related symptoms such as fatigue, excessive sensitivity to cold feeling and feeling of weakness (Kumahara et al., 1989). On the other hand, it is reported that a long-term administration of the test drug improves decreases in sexual behaviors (grooming and mounting), copulation rate and insemination rate in male rats (Kobayashi et al., 1996). However, the effects of Nanpao on reproductive functions in female rats are not examined.

The stages of the estrous cycle of the rat can be reliably identified cytologically from vaginal smears (Montes and Luque, 1988). In addition, measurement of the EIV is one of the methods that can be used to monitor various reproductive events in several species, including the rat (Rezac, 2008; Taradach, 1982). In Chapter 1, the author showed that the value of EIV correlate with vaginal cytological changes in aged rats exhibiting abnormal estrous cycles as well as in young rats. Moreover, the values of EIV both in persistent estrus and continual diestrus rats reflect the histological
conditions of the epithelial cell layer of vaginal mucosa. Therefore, EIV measurement allows us to improve efficiency for studying progression of reproductive aging.

In this Chapter, the author performed to actually investigate the utility of the method for the measurement of vaginal impedance in the study of the progression of reproductive aging in female rats and to evaluate the time-dependent therapeutic effects of the administration of Nanpao on the aged-related changes in the estrous cycle.
Materials and Methods

Animals

Female rats of the Sprague–Dawley (Crl: CD [SD]) strain were purchased from Charles River Japan, Inc. at the age of 5 months (n = 36). During the experimental period, the animals were housed in the same manner as described in Chapter 1.

All animal studies were approved by the Institutional Animal Care and Use Committee of Mitsubishi Tanabe Pharma Corporation as described in Chapter 1.

Group composition and treatment

Female rats (n = 36) were stratified by body weight at the end of the quarantine/acclimation period and randomly allocated to three groups. The control group (n = 12) received distilled water alone; a second group (n = 12) was given the test drug at 30 mg/kg/day (equivalent to the clinical dose for adult humans); and a third group (n = 12) was given the test drug at 100 mg/kg/day.

The test drug or distilled water was administered to the animals by oral gavage once a day for 5 days a week from the age of 6 months onwards. The test drug (Nanpao powder extract, Lot No. 69044; Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) was dissolved in distilled water just before daily dosing. The volume administered was set at 5.0 mL/kg bodyweight. The week at which administration was initiated was defined as
week 0 of dosing.

**General study design and sampling**

All the rats were sampled once daily (between 13:00 and 15:00) for 14 days for vaginal impedance and cytology measurements at the age of 6 months, before the initial treatment (pretest measurements). After the start of treatment, sampling for vaginal impedance and cytology measurements was performed at the ages of 7 (weeks 2–3 of dosing), 8 (weeks 6–7 of dosing), 9 (weeks 11–12 of dosing) and 10 months (weeks 17–18 of dosing) in the same manner as that used for the pretest sampling. All rats were sacrificed immediately after the final sampling by exsanguination by cutting the abdominal artery and vein under deep ether anesthesia.

**Vaginal cytology**

Vaginal smears were taken in the same manner, and the stage of the estrous cycle was determined in the same criteria as described in Chapter 1. The length of each cycle and the total number of cycles per animal were also analyzed in the control and treated groups.

**Vaginal impedance measurements**

Vaginal impedance was measured in the same manner as described in Chapter 1.
**Statistical analysis**

Mean values for the drug-treated group and control group were compared by Dunnett’s multiple comparison test, and pre- and post-treatment comparisons in each group were made using the paired *t*-test. Values are presented as mean ± S.D. Statistical analysis was performed with EXSAS version 7.50 (Arm Corp., Osaka, Japan), and *P* values less than 0.05 were considered statistically significant.
Results

The results of the observations of the estrous cycle and measurements of EIV at the ages of 6–10 months are shown in Table 2-2. Time-dependent changes in animals exhibiting typical estrous cycle patterns in each group are shown in Figure 2-1.

At the age of 6 months (before initiation of daily treatment with water or Nanpao), the rats in each group exhibited various patterns of estrous cycles. Regular estrous cycles (three or more cycles in 2 weeks) were observed in 17 of the 36 (47.2%) animals. The remaining 19 animals exhibited abnormal estrous cycles. Among these 19 animals, the incidence of animals showing two cycles, one cycle, and persistent estrus was 12/36 (33.3%), 6/36 (16.7%), and 1/36 (2.8%), respectively. Animals with each of these estrous cycle patterns were distributed almost equally across the three groups. There were no differences in mean estrous cycle length and the mean number of cycles per animal among the three groups. The mean frequency of proestrus by vaginal cytology and the mean number of days with EIV over 3.0 kΩ were similar in all groups (Table 2-2).

At the age of 7 months (weeks 2–3 of dosing), the number of animals exhibiting regular estrous cycles was almost the same; however, the number of animals with only one cycle was higher in the control group than in 6-month-old-animals. With the cessation of regular or extended cycles in the control group, the mean frequencies of days in proestrus and with EIV over 3.0 kΩ declined to 2.0 ± 1.1 days ($P < 0.05$ vs. pretreatment) and 1.5 ± 1.2 days, respectively, while the mean estrous cycle length increased to 8.4 ± 5.0
days ($P < 0.05$ vs. pretreatment). The mean number of cycles per animal
decreased to $2.0 \pm 1.1$. In the 30 mg/kg/day group, the pattern of estrous
cycles was the same as that observed in the same animals at 6 months of age.
In addition, changes in other parameters were not observed, with no
difference between the control and 30 mg/kg/day groups. On the other hand,
a progressive decline in estrous cyclicity was not observed in the 100
mg/kg/day group. In this group, the mean frequency of days in proestrus and
with EIV over 3.0 kΩ was $2.6 \pm 1.2$ days and $2.2 \pm 1.1$ days, respectively,
higher than in the control group. Mean estrous cycle length in the 100
mg/kg/day group was $6.8 \pm 4.5$ days, which was shorter than in the control
group; the mean number of cycles per animal was $2.4 \pm 0.9$, which was also
higher than in the control group.

At the age of 8 months (weeks 6–7 of dosing), estrous cycle patterns in the
control and 30 mg/kg/day groups were almost the same as those observed at
7 months. Changes in other parameters were not observed in the control and
the 30 mg/kg/day groups, and there was little difference between the control
and the 30 mg/kg/day groups. On the other hand, the frequency of animals
with regular estrous cycles was higher in the 100 mg/kg/day group than in
the control group. Moreover, mean estrous cycle length and the mean
number of estrous cycles per animal in the 100 mg/kg/day group was $6.2 \pm
3.8$ days and $2.3 \pm 1.1$ times, respectively, with differences between the
control group and the 100 mg/kg/day group. There were differences in the
mean frequency of proestrus by vaginal cytology and the mean number of
days with EIV over 3.0 kΩ between the control and 100 mg/kg/day groups.
At the age of 9 months (weeks 11–12 of dosing), the number of animals showing regular estrous cycles was lower than in 8-month-old animals. In particular, persistent estrus was observed in many animals in the control group. In the control group, the mean estrous cycle length (11.8 ± 4.1 days) was significantly longer than in the pretreatment period ($P < 0.01$). In this group, there were also significant decreases in the mean frequency of days in proestrus and with EIV over 3.0 kΩ and the mean number of cycles per animal. On the other hand, more than half of the animals in the treated groups had estrous cycles with at least two cycles in 2 weeks. The mean estrous cycle length was 8.9 ± 4.6 days in the 30 mg/kg/day group and 8.8 ± 4.0 days in the 100 mg/kg/day group, shorter than in the control group. The mean frequency of days in proestrus and the mean number of days with EIV over 3.0 kΩ were higher in the treated groups than in the control group.

At the age of 10 months (weeks 17–18 of dosing), only 1 of the 12 animals in the control group exhibited regular estrous cyclicity. Most animals had completely lost cyclicity. Moreover, the mean frequency of days in proestrus and the mean number of days with EIV over 3.0 kΩ were markedly lower than the values in this group at 9 months. In the treated groups, changes in the distribution of estrous cycle pattern and other parameters compared with values at 9 months were not observed. The mean frequency of days in proestrus in the 100 mg/kg/day group was significantly higher than that in the control group.

Throughout this experiment, the mean frequency of days in proestrus judged by vaginal smears and the mean number of estrous cycles were
similar regardless of administration of the test drug. In addition, the concordance rate of days in proestrus by vaginal cytology and by days with EIV over 3.0 kΩ was approximately the same (63.2–85.7%) at each measurement point. In most cases, the EIV value of animals with a prolonged estrous cycle did not exceed 3.0 kΩ even in the proestrus stage, although the value tended to be higher in proestrus than in other stages of the cycle.
Discussion

In this Chapter, the author performed to investigate the utility of vaginal impedance as a method for detecting the progression of reproductive aging in female rats and to evaluate the time-dependent effects of the administration of the test drug, Nanpao, on age-related changes in the estrous cycle.

Before the initiation of daily treatment with water or Nanpao, abnormal estrous cycles were observed in about one half of the animals. Subsequently, the number of animals with abnormal estrous cycles and the degree of abnormality of the estrous cycle increased gradually in the control group from 7 to 10 months of age. In particular, persistent estrus was observed in about half of the 10-month-old animals in the control group. There are many reports of age-related loss of regular estrous cycles in mammalian species (Jones and Krohn, 1961; Talbert, 1968; Meites and Huang, 1976; Finch, 1978). It is also known that the estrous cycles become irregular in rats between the ages of 6 and 10 months (Aschheim, 1976; Meites and Huang, 1976; Lu et al., 1979; Westwood, 2008). Changes in the volume of hormones secreted by the hypothalamic region, the pituitary gland, and the ovary resulting from the effect of aging on the hypothalamic–pituitary control system are thought to be responsible for such abnormalities of the estrous cycle (Wise et al., 1989). Moreover, the loss of a regular estrous cycle has been correlated with depletion of the oocyte pool (ovarian levels), or a combination of both processes (Acuna et al., 2009). There are reports that the cessation of the estrous cycle in aged animals is related to a decrease in the luteinizing hormone (LH) response to the feedback stimulus of estrogen.

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(Nass et al., 1984; Wise, 1984; Lu et al., 1985), decreases in the progesterone and LH surges in proestrus (Gray et al., 1980; Lu et al., 1980; Wise, 1982), and a deficiency in progesterone secretion (Tsai et al., 2004). In most cases, cessation of cyclicity was followed by persistent estrus in SD rats (Finch et al., 1984). Felicio et al. (1984) indicated that the appearance of persistent estrus and its duration depended on the age at cycle cessation. Persistent estrus is considered to be an estrogenic state dependent on continued secretion of estrogens by the ovaries, and its duration is therefore limited by the size of the oocyte reserves in the ovaries (Finch et al., 1984). In the present study, the age-related changes in the estrous cycle observed in the control group had a course similar to those described in the reports discussed above.

In the present study, observations of the estrous cycle and measurement of EIV at the ages of 7 (weeks 2–3 of dosing) and 8 months (weeks 6–7 of dosing) demonstrated nearly normal estrous cycles in many of the animals in the 100 mg/kg/day group, and the mean estrous cycle length in this group was shorter than that of the control group. The mean frequency of days with EIV over 3.0 kΩ in proestrus and the mean number of cycles in 2 weeks were higher in the 100 mg/kg/day group than in the control group. Moreover, at the ages of 9 months (weeks 11–12 of dosing) and 10 months (weeks 17–18 of dosing), an increase in the number of animals with abnormal estrous cycles was prevented not only in the 100 mg/kg/day group, but also in the 30 mg/kg/day group. These results indicate that a short period of administration of the test drug prevented the age-related loss of regular estrous cycles.
Studies on the effects of other herbal medicines have indicated that herbs can improve estrogen status and have pro-fertility effects (Qian et al., 1998). In addition, studies on the effects of herbal formulas in androgen-sterilized rats showed that these formulas restored regular estrous cycles and induced ovulation, possibly by regulating the expression of hypothalamic neuropeptide Y and leptin receptor mRNA (Sun and Yu, 1999; Sun et al., 2000). Although the mechanism of the effect of the test drug on the decline in reproductive function in female animals was not clear in the present study, the author consider the mechanism by which Nanpao produced its effects involved multiple effects on the pituitary–ovarian axis and the adrenal glands (Wise et al., 1989).

Since the concordance rates of days in proestrus by vaginal cytology and EIV over 3.0 kΩ in the present study were high and approximately constant, we suggest that the measurement of EIV, as well as the observation of the estrous cycle based on vaginal smears, can be used to detect the progression of reproductive aging in female rats. In a review, Rezac (2008) reported that electrical impedance methods may be used not only to predict ovulation, but also to explore various other reproductive processes. The present results illustrate that the measurement of EIV could be a worthwhile approach to the more precise study of age-related changes in the reproductive processes in the fields of physiology and theriogenology.

The number of days with EIV over 3.0 kΩ did not necessarily correspond to the proestrus stage indicated by vaginal cytology, because the EIV of animals in which the estrous cycle was prolonged tended not to be over 3.0 kΩ even in
the proestrus stage. We do not think cornification of the epithelial cell layer of the vaginal mucosa is an adequate explanation of this finding in rats exhibiting prolongation of the estrous cycle. In this regard, the disturbance in hormone balance seen in animals exhibiting abnormal estrous cycles is considered to affect the histology of the vagina (Nass et al., 1984; Wise, 1984). Although changes in EIV in young animals exhibiting regular estrous cycles are physiologically sound, the physical and biological factors that may affect the variation in impedance in the female reproductive system are still poorly understood. Investigations of these questions may provide information that will enable vaginal impedance methods to reflect more accurately the histology of the epithelial cell layer of the vaginal mucosa and to provide useful information for studies of reproductive functions.

In conclusion, administration of Nanpao inhibited the age-related loss of regular estrous cycles in female rats. Moreover, the author showed that the measurement of EIV is a worthwhile approach to the examination of estrous cyclicity and the histological condition of the vaginal mucosa in studying the progression of reproductive aging in female rats.
Table 2-1. Components and their contents in daily dose of Nanpao for human use

<table>
<thead>
<tr>
<th>Linnean Classification</th>
<th>Components Pin Yin Name</th>
<th>Amount (mg)</th>
<th>Linnean Classification</th>
<th>Components Pin Yin Name</th>
<th>Amount (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervi Parvum Comu</td>
<td>Lurong</td>
<td>67</td>
<td>Cistanchis Herba</td>
<td>Roucongrong</td>
<td>67</td>
</tr>
<tr>
<td>Canis Penis et Testis</td>
<td>Guanggoushen</td>
<td>53</td>
<td>Cynomorii Herba</td>
<td>Suoyang</td>
<td>67</td>
</tr>
<tr>
<td>Donkey Penis et Testis</td>
<td>Heilvshen</td>
<td>235</td>
<td>Dipsaci Radix</td>
<td>Chuanjiduan</td>
<td>33</td>
</tr>
<tr>
<td>Angelicae Radix</td>
<td>Danggui</td>
<td>133</td>
<td>Rehrmanniae Radix</td>
<td>Shudihuang</td>
<td>133</td>
</tr>
<tr>
<td>Ginseng Radix</td>
<td>Renshen</td>
<td>133</td>
<td>Rubi Fructus</td>
<td>Fupenzi</td>
<td>67</td>
</tr>
<tr>
<td>Moutan Cortex</td>
<td>Mudanpi</td>
<td>33</td>
<td>Aconiti Tuber</td>
<td>Paofuzi</td>
<td>67</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Haima</td>
<td>67</td>
<td>Lycii Fructus</td>
<td>Gouqiao</td>
<td>133</td>
</tr>
<tr>
<td>Eucommiae Cortex</td>
<td>Duzhong</td>
<td>67</td>
<td>Scrophulariae Radix</td>
<td>Xuanshen</td>
<td>67</td>
</tr>
<tr>
<td>Asini Gelatinum</td>
<td>Ajiao</td>
<td>67</td>
<td>Astragali Radix</td>
<td>Huangqi</td>
<td>133</td>
</tr>
<tr>
<td>Cinnamomi Cortex</td>
<td>Guipi</td>
<td>53</td>
<td>Attacltylis Rhizoma</td>
<td>Baizhu</td>
<td>67</td>
</tr>
<tr>
<td>Curculginis Rhizoma</td>
<td>Xianmao</td>
<td>67</td>
<td>Comi Fructus</td>
<td>Shanzhuyu</td>
<td>67</td>
</tr>
<tr>
<td>Cuscutae Semen</td>
<td>Tusizi</td>
<td>67</td>
<td>Hoelen</td>
<td>Fuling</td>
<td>133</td>
</tr>
<tr>
<td>Psoraliae Semen</td>
<td>Buguzhi</td>
<td>67</td>
<td>Ophiopogonis Tuber</td>
<td>Maimendong</td>
<td>67</td>
</tr>
<tr>
<td>Epimedi Herba</td>
<td>Yinyanghuo</td>
<td>133</td>
<td>Achiyranthis Radix</td>
<td>Nuxi</td>
<td>33</td>
</tr>
<tr>
<td>Trigonellae Semen</td>
<td>Huluba</td>
<td>67</td>
<td>Glycyrrhiza Radix</td>
<td>Gancao</td>
<td>33</td>
</tr>
<tr>
<td>Morindae Radix</td>
<td>Bajitian</td>
<td>67</td>
<td></td>
<td></td>
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</tbody>
</table>
Table 2.2: Estrous cycle observations and measurements of EIV for 2 weeks in female rats at the age of 6 - 10 months

<table>
<thead>
<tr>
<th></th>
<th>6 months of age (prior to treatment)</th>
<th>7 months of age (at weeks 2–3 of dosing)</th>
<th>8 months of age (at weeks 6–7 of dosing)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Nanook 30 mg/kg/day</td>
<td>Nanook 100 mg/kg/day</td>
</tr>
<tr>
<td>No. of animals examined</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>No. of days in proestrus (mean ± SD)</td>
<td>32 (2.7 ± 1.2)</td>
<td>24 (2.0 ± 1.2)</td>
<td>20 (2.4 ± 1.3)</td>
</tr>
<tr>
<td>No. of days with EIV of over 3.0 kD (mean ± SD)</td>
<td>23 (1.9 ± 1.0)</td>
<td>16 (1.3 ± 1.3)</td>
<td>20 (1.7 ± 1.4)</td>
</tr>
<tr>
<td>Concordance rate between vaginal cytology and EIV, % a)</td>
<td>71.8</td>
<td>66.6</td>
<td>69.0</td>
</tr>
<tr>
<td>Estrous cycle length, day, mean ± SD</td>
<td>6.4 ± 3.8</td>
<td>7.8 ± 4.0</td>
<td>6.7 ± 3.7</td>
</tr>
<tr>
<td>Cycle per animal, n, mean ± SD</td>
<td>2.3 ± 1.0</td>
<td>2.1 ± 0.8</td>
<td>2.3 ± 0.8</td>
</tr>
<tr>
<td>No. of animals with 3 cycles or more</td>
<td>7</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>No. of animals with 2 cycle</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>No. of animals with 1 cycle</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>No. of animals with persistent estrus</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>9 months of age (at weeks 8–12 of dosing)</th>
<th>10 months of age (at weeks 17–18 of dosing)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Nanook 30 mg/kg/day</td>
</tr>
<tr>
<td>No. of animals examined</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>No. of days in proestrus (mean ± SD)</td>
<td>12 (0.0 ± 1.0)***</td>
<td>19 (1.6 ± 1.2)</td>
</tr>
<tr>
<td>No. of days with EIV of over 3.0 kD (mean ± SD)</td>
<td>8 (0.7 ± 0.8)**</td>
<td>12 (1.0 ± 1.0)</td>
</tr>
<tr>
<td>Concordance rate between vaginal cytology and EIV, % a)</td>
<td>66.6</td>
<td>63.2</td>
</tr>
<tr>
<td>Estrous cycle length, day, mean ± SD</td>
<td>11.8 ± 4.1***</td>
<td>8.9 ± 4.6</td>
</tr>
<tr>
<td>Cycle per animal, n, mean ± SD</td>
<td>1.6 ± 1.3**</td>
<td>1.8 ± 1.1</td>
</tr>
<tr>
<td>No. of animals with 3 cycles or more</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>No. of animals with 2 cycles</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>No. of animals with 1 cycle</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>No. of animals with persistent estrus</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

a) Total number of days with EIV of over 3.0 kD / Total number of days in proestrus by vaginal cytology ≥ 100
*, *P < 0.05; **, *P < 0.01; ***P < 0.001 vs. pre-treatment (6 months of age) by paired t-test
##P < 0.05 vs. the control group by Dunnett method multiple comparison
Fig. 2-1. Time-dependent changes in vaginal cytology and the value of EIV in animals exhibiting typical estrous cycle patterns each group. Black, stippled, diagonal and white blocks represent proestrus, estrus, metestrus, and diestrus phase, respectively. Correlation between vaginal cytology and the value of EIV for 14 days (a) at 6 months of age (prior to treatment), (b) 8 months of age (at weeks 6 – 7 of dosing), and (c) 10 months of age (at weeks 17 – 18 of dosing), respectively.
Chapter 3

Preventive and improvement effects of Nanpao® on biological dysfunctions in aged female rats
Summary

In this Chapter, the author evaluated the improvement effect of Nanpao on the age-related decline in reproductive function (Chapter 3-1) and cold constitution (Chapter 3-2) in female rats given the test drug for a long-term period. In Chapter 3-1, young rats were allocated to the cesarean section and natural delivery groups to examine reproductive performance (young rat groups). Five-month-old rats were allocated to the 3 groups (aged rat groups): 1 control and 2 Nanpao-treated groups. Five-month-old rats were given orally in a dose of 0, 30 or 100 mg/kg/day of the test drug. In aged rats, the first mating experiment was initiated at Week 21 of dosing to evaluate reproductive performance by natural delivery and the second mating experiment at Week 31 of dosing to evaluate them by cesarean section. In the first and second mating experiments, various reproductive functions decreased in aged rats as compared with young rats. On the other hand, loss of regular estrous cycles, decreases in delivery and pregnancy rates and mean fetal weights were inhibited in the treated groups as compared with the control group. In addition, decreases in the numbers of mean live offspring and fetuses were inhibited in the 100 mg/kg/day group. It was suggested that Nanpao maintained normal embryo-fetal development in female rats.

In Chapter 3-2, five-month-old rats were administered Nanpao orally at dose of 0, 30, or 100 mg/kg/day. The peripheral blood flow and surface skin temperature in the hind paws was measured using laser Doppler blood flow meter and infrared thermography, respectively. In animals treated with
Nanpao, the peripheral blood flow increased dose-dependently compared to that in the control group. Moreover, the surface skin temperature after immersion in ice-cold water was higher in the treated groups than in the control group at all measurement points. These results suggest that Nanpao has the potential to improve cold constitution associated with decreased peripheral blood flow in women. In this Chapter, the author indicated that Nanpao had the preventive and improvement effect on the age-related biological dysfunctions in female rats.
Introduction

A decline in reproductive performance is well known as one of the representative biological dysfunctions associated with aging. Some reports described age-related changes, including loss of regular estrous cycles in female rats (Meites and Huang, 1976; Lu et al., 1979). Simultaneously, a decline in uterine function (Miller and Riegle, 1980), a decrease in fertility (Ingram et al., 1958; Miller et al., 1979; Matt et al., 1986) and an increase in developmental defect of embryos (Matt et al., 1987) are reported the age-related changes.

Some kampo medicine has been reported to be effective in the treatment of pituitary-ovarian dysfunction in young women, the treatment of infertility in women during the reproductive age and the treatment of several undefined symptoms in peri-and postmenopausal women (Ushiroyama, 2007). In Chapter 2, the author indicated that administration of Nanpao inhibited the age-related loss of regular estrous cycles in female rats. It is study of interest research in assessing the anti-aging effects on reproductive performance and embryo-fetal development of Nanpao.

The existence of cold constitution in women was first reported in 1956 (Kushima and Saitoh, 1956), and its relation to various diseases was reported recently (Aomine and Yamato, 2002; Hosono, 2003). Sadakata and Yamada (2007) have suggested that cold constitution might be caused by an abnormality in the distribution of thermal receptors, which are located in the subcutaneous tissue and are responsible for temperature sensation, and by chronically reduced peripheral blood flow.
Nowadays, majority of women suffer from cold constitution; therefore, the potential of Nanpao for improvement of cold constitution has important implications for enhancing the quality of life in women. As part of the elucidation of the mechanism of the effects of Nanpao on cold constitution, the author performed with a focus on improving hemodynamics and maintaining surface temperature, since Nanpao contains herbs with vasodilatory effects.

In this Chapter, the author examined the effects on a decline in various reproductive functions of administration of Nanpao (Chapter 3-1). Moreover, the effects on cold constitution of Nanpao were examined quantitatively and objectively using measurements of peripheral blood flow and surface skin temperature in aged female rats (Chapter 3-2).
3-1 Anti-aging effects of Nanpao® on reproductive functions in aged female rats

Materials and Methods

Animals

Female rats of the Sprague-Dawley (Crl: CD [SD]) strain were purchased from Charles River Japan, Inc. at the age of 5 months (n=49) or at the age of 11 weeks (n=32), and used in this study. During the experimental period, the animals were housed in the same manner as described in previous Chapter. From Day 21 of gestation to Day 21 of lactation, the animals were housed individually in plastic cages (W: 275 × D: 425 × H: 204 mm). In addition, necessary numbers of 12-week-old fertile males of the same strain were purchased and subjected to mating.

All animal studies were approved by the Institutional Animal Care and Use Committee of Mitsubishi Tanabe Pharma Corporation as described in previous Chapters.

Group composition and treatment

Female rats (n=32) purchased at the age of 11 weeks were used for mating at the age of 14 weeks. The animals in which the copulation was confirmed were randomly allocated to the following two groups: the cesarean section group (n=16) and natural delivery group (n=14). These young animals were
not given the test drug and their reproductive performances were compared with those in the aged rats which were not given the test drug (control group).

Aged female rats (n=49) purchased at the age of 5 months were stratified by body weights determined at the end of the quarantine/acclimation period and randomly allocated to the 3 groups: the control group (n=16) given distilled water alone, the 30 mg/kg/day group (n=17) (30 mg/kg/day: equivalent to the clinical dose of adult humans per day), and the 100 mg/kg/day group (n=16).

The test drug or distilled water was administered to the animals by oral gavage once a day for 5 days a week from the age of 5 months. The animals in which the copulation was confirmed were given the test drug once a day during the gestation and lactation periods. The test drug (Nanpao powder extract, Lot No.: 58025, Tanabe Seiyaku Co., Ltd., Osaka, Japan) was prepared by in the same manner as described in Chapter 2.

The week at which the administration was initiated was defined as Week 0 of dosing, and in the reproductive function tests, the day when the copulation was confirmed was considered as Day 0 of gestation and the day showing the end of delivery was defined as Day 0 of delivery (Day 0 of lactation).

**General signs, body weights and food consumptions**

The animals were observed daily for general signs by inspection and palpation. Body weights were measured once a week and food consumptions
twice a week except the mating period. Body weights and food consumptions of females with the confirmed copulation were measured on Days 0, 4, 9, 14, 17 and 21 of gestation. During the lactation period, body weights and food consumptions of dams were measured on Days 0, 4, 9, 14, 17 and 21 of lactation.

**Reproductive and developmental function tests**

The mating in young animals was initiated at the age of 14 weeks. In aged animals, the first mating was initiated at Week 21 of dosing (at the age of 10 months) and the second mating at Week 31 (at the age of 12 months) to examine the respective reproductive and developmental functions.

**Methods of mating and estrous cycle observations**

The animals were mated on a one-to-one basis from the evening to the following morning during the mating period. Copulation was regarded as established when sperm was found in the vaginal smears taken the following morning. The females in which copulation could not be confirmed were mated repeatedly with a same partner in the same group and the copulation was examined for a maximum of 14 days. The number of days required for copulation was calculated as the mating days from the starting of mating to the confirmation of copulation.

For 14 days prior to mating, vaginal smears were taken by wiping the vaginal wall lightly with a water-moistened cotton swab, Giemsa-stained on
glass slides, and examined for assessment of the estrous cycles. In the observation of vaginal smears of each animal, the estrous cycles were defined as “regular estrous cycles” when a regular cycle of 4- or 5-days was observed in at least 2 consecutive periods, as “irregular estrous cycles” when one estrous cycle was prolonged, and as “persistent estrous” when persistent vaginal cornification was observed continuously for 2 weeks (Lu et al., 1979), respectively.

Mating experiment in young rats

As mentioned above, young female animals were used for mating at the age of 14 weeks. The animals with confirmed copulation were randomly allocated to the following 2 groups: the cesarean section group and natural delivery group.

The animals of the caesarean section group were anesthetized with ether on Day 21 of gestation and the abdomen was incised to confirm the pregnancy by the presence or absence of implantations in the uterus. In pregnant animals, the number of corpora lutea in the ovaries and the number of implantations in the uterus were counted to calculate the implantation rate. All the fetuses removed from each dam were checked for survival. The surviving fetuses were examined for the external abnormalities, sexed and weighed. Deaths and absorbed fetuses (embryos) were classified into early or late resorption embryos and macerated fetuses.

The animals of the natural delivery group were examined carefully for
parturition twice a day (at about 10:00 and 16:00) from the morning on Day 21 to Day 23 of gestation. The gestation period and number of days required for parturition were calculated as follows: The morning on Day 0 of gestation was defined as Day 0.0 of gestation and the afternoon on Day 0 of gestation as Day 0.5 of gestation. The gestation period was regarded as the period from Day 0.0 of gestation to the day of gestation when the parturition was started, and the number of days required for parturition was regarded as the period from the day of gestation (when the parturition was started) to the day of gestation when the completion of delivery was confirmed.

On Day 0 of lactation, the number of newborns (offspring and stillborns) was counted and offspring were sexed by the genital-anal distance. General signs (including external and behavior abnormalities) were observed and body weights were measured. Thereafter general signs were observed daily in individual offspring until Day 21 of lactation. All the offspring were bled to death under ether anesthesia by cutting the axillary artery on Day 21 of lactation. In addition, the dams in which the parturition was completed and the animals in which the copulation could not be confirmed were all euthanized in the same manner.

**The first mating experiment in aged rats**

As mentioned above, the first mating experiment was performed at Week 21 of dosing (at the age of 10 months). The animals in which copulation was confirmed were examined for parturition twice a day (at about 10:00 and
16:00) from the morning on Day 21 to Day 28 of gestation. The calculation of the gestation period and the number of days required for parturition and observation of newborns (offspring and stillborns) on Day 0 of lactation were examined in the same manner as in the young rats of the natural delivery group. Thereafter, general signs were observed in individual offspring once a day up to Day 21 of lactation and body weights were examined on Days 4, 9, 14, 18, and 21 of lactation. All the offspring were bled to death under ether anesthesia by cutting the axillary artery on Day 21 of lactation. However, since the number of offspring for observation and measurement was not sufficient, data for body weights, sex ratio and weaning rate of offspring were excluded from the evaluation data.

In the first mating experiment, dams in which parturition was completed and the individual animals which did not give birth even after Day 28 of gestation and the animals with unsuccessful copulation were given the test drug continuously and used for the second mating experiment.

**The second mating experiment in aged rats**

The second mating test was performed at Week 31 of dosing (at the age of 12 months). The animals with confirmed copulation were anesthetized with ether and euthanized by exsanguination on Day 21 of gestation and the caesarean section was performed in the same manner as in the young animals of the caesarean section group. The pregnancy was judged based on the presence or absence of implantations in the uterus, and the number of
corpora lutea in the ovaries and the number of implantations were counted
to calculate the implantation rate. All fetuses were removed from each dam
and checked for survival. The surviving fetuses were examined for external
abnormalities, sexed and weighed. Deaths and absorbed fetuses (embryos)
were classified into early or late resorption embryos and macerated fetuses.

**Statistical analysis**

The homogeneity of variance was analyzed by Bartlett’s test for data on
body weights, food consumptions, estrous cycles (frequency of estrous phase),
numbers of days required for copulation (the number of mating days for
copulation), numbers of corpora lutea, numbers of implantations,
implantation rates, pre- and post-implantation losses of embryos (fetuses),
numbers of live fetuses and mean fetal weights (level of significance: 5%).
Multiple comparison was carried out using the parametric Dunnett method
when a set of variance was homogenous and using the non-parametric
Dunnett method when a set of variance was not homogenous to test the
difference in mean between the treated groups and control group (level of
significance: 5% and 1%). The copulation rate, pregnancy rate and delivery
rate in the treated groups were compared one-to-one with those in the
control group using Fisher’s exact test (level of significance: 5 % and 1 %).
Results

General signs, body weights and food consumptions

No noteworthy changes were observed in general signs in young female animals. On the other hand, eye discharge, chromodacryorrhea and loss of fur were noted sporadically in the aged animals during the observation period, regardless of administration of the test drug.

No abnormal changes were observed in body weights and food consumptions in the young animals (data not shown). The body weights of aged animals in the treated groups increased during the measurement period as compared with the control group (Figure 3-1-1). In addition, the body weights of animals in the 30 and 100 mg/kg/day groups increased significantly from Week 27 and Week 11 of dosing onwards, respectively. Changes in food consumptions of aged animals were similar to those in body weights (Figure 3-1-2).

In the reproductive function tests at the age of 10 and 12 months, body weights and food consumptions of pregnant animals increased significantly in the treated groups as compared with the control group (data not shown).

Reproductive and developmental functions

The mating experiment in young rats

The results of the reproductive function in the young animals are shown in Tables 3-1-1, 3-1-2 and 3-1-3. These results in the young animals are shown below, comparing with those in the aged animals.
The first mating experiment in aged rats

In the observation of the estrous cycles for 2 weeks before mating, regular estrous cycles were observed in all young animals (Table 3-1-1), but in 4 of the 16 aged animals (25.0%) in the control group (Figure 3-1-3). On the other hand, the incidences of regular estrous cycles were 52.9% (9/17) in the 30 mg/kg/day group and 56.3% (9/16) in the 100 mg/kg/day group (Figure 3-1-3).

The mean frequency of estrous phase was 3.0 times in the young animals (Table 3-1-1), whereas it was 1.8 times in the aged animals of the control group (Table 3-1-4). On the other hand, the mean frequency of estrus phase was higher in the treated groups than in the control group (Table 3-1-4). In particular, the mean frequency of estrus phase was significantly higher in the 30 mg/kg/day group than in the control group, with no great differences between the aged animals of the 30 mg/kg/day group and the young animals (Tables 3-1-1, 3-1-4). The copulation rate and the number of days required for copulation were 93.8% and 3.4 days, respectively, in the young females (Table 3-1-1) and 81.3% and 3.6 days, respectively, in the aged animals of the control group (Table 3-1-4), with little differences between the young and the aged animals. In addition, there were no differences in these parameters between the control group and treated groups of aged animals (Table 3-1-4).

The delivery rate was 100.0% in the young animals (Table 3-1-2), but was clearly low, 15.4% in the aged animals of the control group (Table 3-1-5). On the other hand, the delivery rate was higher in the treated groups than in the control group, and the number of newborns and offspring per dam was
significantly higher in the 100 mg/kg/day group than in the control group. Also, the ratio of live newborns to total newborns was higher in the treated groups than in the control group (Table 3-1-5). The gestation period and the number of days required for delivery were 22.0 days and 0.5 days, respectively, in young animals (Table 3-1-2), whereas they were 25.8 days and 1.8 days, respectively, in aged animals of the control group (Table 3-1-5). On the other hand, they were short in the 100 mg/kg/day group, as compared with the control group (Table 3-1-5). In addition, no abnormal changes in the lactation state of dams or in general signs were observed in offspring during the lactation period.

The second mating experiment in aged rats

In the observation of the estrous cycle for 2 weeks before mating, the number of animals showing irregular estrous cycles increased in the aged animals of each group (the control and treated groups), as compared with that in the first mating experiment (Tables 3-1-4, 3-1-6). Persistent estrus characterized by chronic anovulation and persistent vaginal cornification was observed in some aged animals. The incidences of regular estrous cycles in the control, 30 and 100 mg/kg/day groups were 6.3% (1/16), 29.4% (5/17) and 37.5% (6/16), respectively (Figure 3-1-4).

The mean frequency of estrus phase was slightly higher in the 100 mg/kg/day group than in the control group (Table 3-1-6). The copulation rate in the aged animals of each group (the control and treated groups) was
clearly lower than that in the young animals (93.8%), and that in the aged animals in the first mating experiment (Tables 3-1-1, 3-1-4, 3-1-6). The number of days required for copulation tended to be slightly longer in the 100 mg/kg/day group than in the control group (Table 3-1-6). The pregnancy rate was 100.0% in young animals (Table 3-1-3), whereas it was clearly low, 25.0% in the aged animals of the control group (Table 3-1-6). On the other hand, the pregnancy rate was higher in the treated groups than in the control group (Table 3-1-6).

There were no differences between the young and the aged animals of each group in the mean number of corpora lutea, and the test drug did not affect the number of corpora lutea (Tables 3-1-3, 3-1-7). On the other hand, the implantation rate was 93.4% in the young animals but was clearly low, 41.1% in the aged animals of the control group (Tables 3-1-3, 3-1-7). In addition, the implantation rate was higher in the 100 mg/kg/day group than in the control group (Table 3-1-7). The post-implantation loss rate was 6.5% in the young animals but was clearly high, 50.0% in the aged animals of the control group (Tables 3-1-3, 3-1-7). On the other hand, the post-implantation loss rate was lower in the treated groups than in the control group (Table 3-1-7). The mean fetal weights were 5.54 g in the young animals but 4.75 g in the aged animals of the control group, but the mean fetal weights of the aged animals in the treated groups were about 1 g higher than that in the control group, and were also higher than fetal weights of young animals (Tables 3-1-3, 3-1-7). In the external observation of fetuses, there were cranioschisis in 1 fetus of the young animals and omphalocere in 1 fetus of the aged
animals of the 30 mg/kg/day group. No abnormal changes were noted in the other fetuses (Tables 3·1·3, 3·1·7).
Discussion

In this section, the author performed to compare reproductive and developmental functions of the young female rats and those of the aged female rats in detail and to investigate the effect of the test drug, Nanpao, on aged-related changes in reproductive performances.

Body weights of the aged animals were significantly higher in the treated groups than in the control group throughout the experiment period. This was considered to be due to an increase in food consumption reflecting the orexigenic effect of the test drug.

In the observation of estrous cycles of the young and the aged animals in the control group during the pre-mating period of 2-weeks, the estrous cycles were regular in all the young animals, while irregular in many aged animals. The number of animals with irregular estrous cycles increased in the 12-month-old animals as compared with the 10-month-old animals. Persistent estrus was observed in about one half of the 12-month-old groups. There are many reports of aged-related loss of regular estrous cycles in mammalian species (Jones and Krohn, 1961; Talbert, 1968; Meites and Huang, 1976; Finch, 1978). In addition, it is also known that the estrous cycles become irregular in rats at the age of 10 to 12 months (Aschheim, 1976; Meites and Huang, 1976; Lu et al., 1979). Change in hormone volume secreted from the hypothalamic region, pituitary gland and ovary by the effect of aging on the hypothalamic-pituitary control system are thought to be responsible for such abnormality in estrous cycle (Wise et al., 1989). In addition, there are some reports that the cessation of the estrous cycles seen
In aged animals is related to a decrease in luteinizing hormone (LH) response to the feedback stimulation of estrogen (Nass et al., 1984; Wise, 1984; Lu et al., 1985), decreases in progesterone and LH surge in the proestrus (Gray et al., 1980; Lu et al., 1980; Wise, 1982) and a deficiency of progesterone secretion (Tsai et al., 2004).

In the observation of estrous cycles in the 10-month-old animals, regular estrous cycles were observed in a large number of animals of the treated groups as compared with the control group. The increase in the number of the 12-month-old animals showing abnormal estrous cycle was inhibited in the treated groups as compared with the control group. Although the effect of the test drug on hormone production in the female animals was not clear in the present study, there were no large differences in the frequency of estrus phase between the treated groups at the age of 10 months and the young animals. These results suggested that the test drug maintained the regular estrous cycles.

The copulation rate was lower in aged animals of the control group than in the young animals, and it was lower at the age of 12 months than at the age of 10 months. In addition, the delivery and pregnancy rates and the number of mean live newborns and fetuses per dam were lower in the aged animals than in the young animals and there were age-related decreases in fertility and litter size. One of the causes of decreases in litter size and fertility is considered to be the aging of the ova and blastocysts, since there were no changes in the numbers of corpora lutea in the aged animals, although the numbers of pre-/post-implantation losses and fetal death rate were markedly
high. In this regard, it is reported that the fertility rate and the ability to maintain pregnancy decrease more greatly in animals showing irregular estrous cycles than in animals showing regular estrous cycles, and that the percentage of retarded zygote and ovum (without developmental capacity) increase in animals at the age of 8 to 12 months (Matt et al., 1987; Fugo and Butcher, 1966, 1971).

There were no differences between the treated and the control groups in copulation rates of animals at the age of 10 and 12 months, but the delivery rate at the age of 10 months and the pregnancy rate at the age of 12 months were clearly higher in the treated groups than in the control group. The decrease in fertility was inhibited in the treated groups. Moreover, the numbers of mean total newborns and live newborns in the delivery observation at the age of 10 months and the numbers of implantations and live fetuses at the time of cesarean section at the age of 12 months were greater in the 100 mg/kg/day group than in the control group. These results suggest that the inhibitory effect of the test drug on the decrease in fertility may contribute not only to the maintenance of the regular estrous cycle but also to the prevention of aging of the ovum.

The mean fetal weights at cesarean section were lower in the aged animals of the control group than in the young animals. Differences in environment of embryonic and fetal development between the aged and the young animals might be a cause of such differences in fetal weights. On the other hand, fetal weights of animals were higher in the treated groups than in the control group, indicating that the test drug not only reduces embryonic and fetal
death but also promotes fetal growth.

There are reports of age-related prolongation of gestation period in Long-Evans rats (Miller et al., 1960), mice (Holinka et al., 1978) and hamsters (Soderwell et al., 1960). In this study, the gestation period and the number of days required for delivery were longer in the aged animals of the control group than in the young animals. On the other hand, prolongation of the gestation and delivery periods was shorter in the 100 mg/kg/day group than in the control group. Prolongation of gestation and delivery periods suggested an age-related decrease in myometrial tissues (Soriero, 1978) together with a disappearance of the fetus-derived delivery signaling caused by intrauterine fetal death (Schriefer et al., 1982). Moreover, the test drug might inhibit the age-related decrease in uterine contraction at the time of delivery.

In conclusion, it was clarified in the present study that various reproductive functions decreased in aged female rats as compared with young female rats and that Nanpao inhibited an age-related loss of regular estrous cycles, decreases in fertility and the number of litter size. Nanpao maintained normal embryo-fetal development in mature female rats.
Table 3·1·1. Mating performances in young female rats.

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of females paired</td>
<td>32</td>
</tr>
<tr>
<td>No. of estrus before pairing, mean±SD</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>Estrous cycle observations</td>
<td></td>
</tr>
<tr>
<td>No. of females with regular cycle</td>
<td>32</td>
</tr>
<tr>
<td>No. of females with irregular cycle</td>
<td>0</td>
</tr>
<tr>
<td>No. of females with successful copulation</td>
<td></td>
</tr>
<tr>
<td>1st week</td>
<td>29</td>
</tr>
<tr>
<td>2nd week</td>
<td>1</td>
</tr>
<tr>
<td>Total (%) a)</td>
<td>30 (93.8)</td>
</tr>
<tr>
<td>Duration until copulation, day, mean±SD</td>
<td>3.4 ± 1.4</td>
</tr>
</tbody>
</table>

a) (Number of females with successful copulation / Number of females paired) x 100
Table 3-1-2. Reproductive performances in young pregnant rats of natural delivery group and developmental findings in newborns.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of females examined</td>
<td>14</td>
</tr>
<tr>
<td>No. of females with delivery</td>
<td>14</td>
</tr>
<tr>
<td>Delivery rate, % a)</td>
<td>100.0</td>
</tr>
<tr>
<td>Gestation length, day, mean±SD</td>
<td>22.0 ± 0.4</td>
</tr>
<tr>
<td>Delivery length, day, mean±SD</td>
<td>0.5</td>
</tr>
<tr>
<td>No. of newborns (mean±SD)</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>(13.6 ± 2.7)</td>
</tr>
<tr>
<td>No. of still newborns (mean±SD)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(0.1 ± 0.5)</td>
</tr>
<tr>
<td>No. of live newborns (mean±SD)</td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>(13.4 ± 2.6)</td>
</tr>
<tr>
<td>Live/still born ratio, mean±SD b)</td>
<td>0.99 ± 0.03</td>
</tr>
<tr>
<td>Sex ratio of live newborns, mean±SD c)</td>
<td>0.50 ± 0.14</td>
</tr>
</tbody>
</table>

a) (Number of females with delivery / Number of females with successful copulation) x 100
b) (Number of live borns / Number of newborns)
c) (Number of males / Number of live newborns)
Table 3-1-3. Developmental observations of fetuses from young pregnant rats of cesarean section group.

<table>
<thead>
<tr>
<th>No. of females examined</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of pregnant females</td>
<td>16</td>
</tr>
<tr>
<td>No. of corpora lutea, mean±SD</td>
<td>16.1 ± 1.5</td>
</tr>
<tr>
<td>No. of pre-implantation loss, mean±SD</td>
<td>1.1 ± 1.3</td>
</tr>
<tr>
<td>No. of implantations, mean±SD</td>
<td>14.9 ± 1.1</td>
</tr>
<tr>
<td>Implantation rate, %, mean±SD a)</td>
<td>93.4 ± 7.2</td>
</tr>
<tr>
<td>Pre-implantation loss rate, %, mean±SD b)</td>
<td>6.5 ± 7.1</td>
</tr>
<tr>
<td>No. of deaths and resorption (%), mean±SD c)</td>
<td>15 (6.5 ± 7.1)</td>
</tr>
<tr>
<td>Implantation scars</td>
<td>0</td>
</tr>
<tr>
<td>Early resorptions</td>
<td>15</td>
</tr>
<tr>
<td>No. of live fetuses (mean±SD)</td>
<td>224 (14.0 ± 1.7)</td>
</tr>
<tr>
<td>Sex ratio, mean±SD d)</td>
<td>0.50 ± 0.12</td>
</tr>
<tr>
<td>Fetal weight, g, mean±SD</td>
<td>5.54 ± 0.44</td>
</tr>
<tr>
<td>No. of fetuses with external malformation (%), mean±SD e)</td>
<td>1 (0.1 ± 0.3)</td>
</tr>
<tr>
<td>Cranioschisis</td>
<td>1 (0.1 ± 0.3)</td>
</tr>
</tbody>
</table>

a) (Number of implantations / Number of corpora lutea) x 100
b) (Number of pre-implantation loss / Number of corpora lutea) x 100
c) (Number of deaths and resorptions / Number of corpora lutea) x 100
d) (Number of males / Number of live fetuses)
e) (Number of live fetuses with external malformation / Number of live fetuses) x 100
Table 3-1-4. Mating performances in female rats at the age of 10 months.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nanpao 30 mg/kg/day</th>
<th>Nanpao 100 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of females paired</td>
<td>16</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>No. of estrus before pairing, mean±SD</td>
<td>1.8 ± 0.9</td>
<td>2.6 ± 0.9 *</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>No. of females with successful copulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st week</td>
<td>10</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>2nd week</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total (%) a</td>
<td>13 (81.3)</td>
<td>14 (82.4)</td>
<td>14 (87.5)</td>
</tr>
<tr>
<td>Duration until copulation, day, mean±SD</td>
<td>3.6 ± 3.1</td>
<td>4.1 ± 1.9</td>
<td>4.1 ± 3.8</td>
</tr>
</tbody>
</table>

a) (Number of females with successful copulation / Number of females paired) x 100

*: Significantly different from the control (P<0.05) by Dunnett method multiple comparison.
Table 3-1-5. Reproductive performances in pregnant rats at the age of 10 months and developmental findings in newborns.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nanpao 30 mg/kg/day</th>
<th>Nanpao 100 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of females examined</td>
<td>13</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>No. of females with delivery</td>
<td>2</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Delivery rate, % a)</td>
<td>15.4</td>
<td>42.9</td>
<td>35.7</td>
</tr>
<tr>
<td>Gestation length, day, mean±SD</td>
<td>25.8 ± 2.5</td>
<td>25.3 ± 1.6</td>
<td>24.4 ± 1.6</td>
</tr>
<tr>
<td>Delivery length, day, mean±SD</td>
<td>1.8 ± 1.1</td>
<td>2.1 ± 1.6</td>
<td>1.2 ± 1.0</td>
</tr>
<tr>
<td>No. of newborns (mean±SD)</td>
<td>7 (3.5 ± 3.5)</td>
<td>22 (3.7 ± 3.9)</td>
<td>34 (6.8 ± 5.0)</td>
</tr>
<tr>
<td>No. of stillborns (mean±SD)</td>
<td>1 (0.5 ± 0.7)</td>
<td>6 (1.0 ± 1.3)</td>
<td>13 (2.6 ± 4.2)</td>
</tr>
<tr>
<td>No. of live newborns (mean±SD)</td>
<td>6 (3.0 ± 4.2)</td>
<td>16 (2.7 ± 4.2)</td>
<td>21 (4.2 ± 5.8)</td>
</tr>
<tr>
<td>Live/still born ratio, mean±SD b)</td>
<td>0.50 ± 0.71</td>
<td>0.57 ± 0.5</td>
<td>0.60 ± 0.55</td>
</tr>
<tr>
<td>Sex ratio of live newborns, mean±SD c)</td>
<td>0.50</td>
<td>0.65 ± 0.47</td>
<td>0.37 ± 0.28</td>
</tr>
</tbody>
</table>

a) (Number of females with delivery / Number of females with successful copulation) x 100

b) (Number of live borns / Number of newborns)

c) (Number of males / Number of live newborns)
Table 3-1-6. Mating performances in female rats at the age of 12 months.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nanpao 30 mg/kg/day</th>
<th>Nanpao 100 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of females paired</td>
<td>16</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>No. of estrus before pairing, mean±SD</td>
<td>1.3 ± 0.9</td>
<td>1.4 ± 1.3</td>
<td>1.8 ± 1.1</td>
</tr>
<tr>
<td>No. of females with successful copulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st week</td>
<td>5</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>2nd week</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total (%) a)</td>
<td>8 (50.0)</td>
<td>8 (47.0)</td>
<td>6 (37.5)</td>
</tr>
<tr>
<td>No. of females with successful pregnancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st week</td>
<td>1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>2nd week</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Total (%) b)</td>
<td>2 (25.0)</td>
<td>5 (62.5)</td>
<td>4 (66.7)</td>
</tr>
<tr>
<td>Duration until copulation, day, mean±SD</td>
<td>3.7 ± 4.1</td>
<td>4.4 ± 4.0</td>
<td>7.5 ± 5.0</td>
</tr>
</tbody>
</table>

a) (Number of females with successful copulation / Number of females paired) x 100
b) (Number of females with successful pregnancy / Number of females with successful copulation) x 100
Table 3-1-7. Developmental observations of fetuses from pregnant rats at the age of 12 months in cesarean section.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nanpao 30 mg/kg/day</th>
<th>Nanpao 100 mg/kg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of females examined</td>
<td>2</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>No. of corpora lutea, mean±SD</td>
<td>15.0 ± 1.4</td>
<td>14.4 ± 1.5</td>
<td>13.0 ± 2.2</td>
</tr>
<tr>
<td>No. of pre-implantation loss, mean±SD</td>
<td>9.0 ± 4.2</td>
<td>8.8 ± 5.1</td>
<td>4.8 ± 2.9</td>
</tr>
<tr>
<td>No. of implantations, mean±SD</td>
<td>6.0 ± 2.8</td>
<td>5.6 ± 4.3</td>
<td>8.3 ± 4.6</td>
</tr>
<tr>
<td>Implantation rate, %, mean±SD a)</td>
<td>41.1 ± 22.7</td>
<td>40.1 ± 31.0</td>
<td>61.3 ± 26.3</td>
</tr>
<tr>
<td>Pre-implantation loss rate, %, mean±SD b)</td>
<td>59.0 ± 22.7</td>
<td>59.9 ± 31.0</td>
<td>38.7 ± 26.3</td>
</tr>
<tr>
<td>No. of deaths and resorption (%, mean±SD c)</td>
<td>5 ( 50.0 ± 35.4 )</td>
<td>6 ( 21.1 ± 23.0 )</td>
<td>4 ( 10.7 ± 13.7 )</td>
</tr>
<tr>
<td>Implantation scars</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Early resorptions</td>
<td>5</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>No. of live fetuses (mean±SD)</td>
<td>7 ( 3.5 ± 3.5 )</td>
<td>22 ( 4.4 ± 3.8 )</td>
<td>29 ( 7.3 ± 4.0 )</td>
</tr>
<tr>
<td>Sex ratio, mean±SD d)</td>
<td>0.67 ± 0.47</td>
<td>0.46 ± 0.27</td>
<td>0.26 ± 0.20</td>
</tr>
<tr>
<td>Fetal weight, g, mean±SD</td>
<td>4.75 ± 0.77</td>
<td>5.74 ± 1.07</td>
<td>5.68 ± 0.49</td>
</tr>
<tr>
<td>No. of fetuses with external malformation (% , mean±SD e)</td>
<td>0 ( 0.0 ± 0.0 )</td>
<td>1 ( 1.8 ± 4.1 )</td>
<td>0 ( 0.0 ± 0.0 )</td>
</tr>
<tr>
<td>Omphalocele</td>
<td>0 ( 0.0 ± 0.0 )</td>
<td>1 ( 1.8 ± 4.1 )</td>
<td>0 ( 0.0 ± 0.0 )</td>
</tr>
</tbody>
</table>

a) (Number of implantations / Number of corpora lutea) x 100
b) (Number of pre-implantation loss / Number of corpora lutea) x 100
c) (Number of deaths and resorptions / Number of implantations) x 100
d) (Number of males / Number of live fetuses)
e) (Number of live fetuses with external malformation / Number of live fetuses) x 100
Fig. 3-1-1. Body weight changes of aged females treated orally with Nanpao until the first mating period. *: Significantly different from the control ($P<0.05$) by Dunnett method multiple comparison.
Fig. 3-1-2. Food consumption changes of aged females treated orally with Nanpao until the first mating period. *: Significantly different from the control ($P < 0.05$) by Dunnett method multiple comparison. **: Significantly different from the control ($P < 0.01$) by Dunnett method multiple comparison.
Fig. 3-1-3. Incidence of aged females that showed a regular and irregular cycle in the first mating at the age of 10 months.
Fig. 3-1-4. Incidence of aged females that showed a regular and irregular cycle in the second mating at the age of 12 months.
3-2 Effects of Nanpao® on peripheral blood flow and surface skin temperature in aged female rats

Materials and Methods

Animals

Forty-eight female Sprague-Dawley (Crl: CD [SD]) rats were purchased from Charles River Japan, Inc., at the age of 5 months. During the experimental period, the animals were housed in the same manner as described in previous Chapters. The measurements of peripheral blood flow and surface skin temperature were performed in the animal room.

All animal studies were approved by the Institutional Animal Care and Use Committee of Mitsubishi Tanabe Pharma Corporation as described in previous Chapters.

Group composition and treatment

The female rats were stratified by body weights determined at the end of the quarantine/acclimation period and 16 females were randomly allocated to 3 groups. One group was administered 30 mg/kg/day (equivalent to the clinical daily dose for adult humans). The second group was administered 100 mg/kg/day of Nanpao. And the third group, which served as the control, was given distilled water.

The test drug (Nanpao powder extract, Lot No.; 58025, Mitsubishi Tanabe
Pharma Corporation, Japan) was prepared by in the same manner as described in previous Chapters. The test drug or distilled water was administered to the animals by oral gavage once a day for 5 days a week from the age of 5 months. The dosing volume was set at 5.0 mL/kg/bodyweight. The week in which administration began was defined as week 0 of dosing.

The peripheral blood flow volume measurement

The peripheral blood flow volume measurement was performed using a laser Doppler blood flow meter (Omega Flow FLO-N1, Omegawave, Japan). The Doppler signals are received by light-receiving fibers and recorded by a detector as frequency spectra, the mean frequency which is proportional to the mean flow rate of erythrocytes, and the mean amplitude which is proportional to the density of erythrocytes.

Processing of these signals can yield tissue blood flow, tissue blood volume, and blood velocity. Skin blood flow is measured continuously using this principle. Tissue blood flow was calculated using the following equation:

\[ \text{Tissue blood flow} = k_1 \int_0^\infty P(\omega) \omega \, d\omega / l^2 \]

where \( k_1 \) is proportionally constant, \( \omega \) denotes the angular frequency (2\( \pi f \)), \( P(\omega) \) is the power spectrum of signal, and \( l \) is the amount of light received.

The measurement was performed about 5 h after dosing at week 20 (corresponding to an age of 10 months) in all 48 animals in the control and treated groups. The blood flow volume was measured in the right hind paw.
using a non-contact probe (ST-N, Omegawave, Japan). Data were transferred to a data analysis system (Ver. 4.1, NOTOCORD-hem, Primetech, Japan) and a 15-s average value representing the steady blood flow volume was measured and computed.

The surface skin temperature measurement

The surface skin temperature in the right hind paw was monitored using an infrared thermography device (Handy Thermography TH6200, NEC San-ei, Japan). The measurement was performed about 5 h after dosing at week 33 (at the age of 13 months) in 8 females chosen at random from each of the 3 groups (24 total). Real-time thermographic images and surface temperature data from the right hind paw surface were obtained using an infrared camera before and at 3, 6, 9, and 12 min after immersion in ice-cold water. The mean surface temperature on a fixed hind paw surface was calculated using an analysis software (Sat-Report, NEC, Japan) for each measurement point.

Statistical analysis

Statistical analysis was carried out as below. The homogeneity of variance was analyzed by Bartlett’s test for data on the peripheral blood flow volume and surface temperature in the hind paws (level of significance: 5%). Multiple comparisons were carried out, using the parametric Dunnett method when a set of variance was homogenous and the non-parametric
Dunnett method when a set of variance was heterogeneous, to test the difference in mean between the treated groups and the control group (level of significance: 5% and 1%). Moreover, the decreasing rates of surface temperature at each measurement point were analyzed and compared with the surface temperature before cooling (level of significance: 5% and 1%).
Results

In the peripheral blood flow volume measurement experiment, the peripheral blood flow volume in the right hind paw of the animals was 10.9, 11.8, and 13.0 ml/min/100g, respectively, in the control, 30, and 100 mg/kg/day groups. In the treated groups, the mean blood flow volume tended to increase dose-dependently compared to that in the control group (Figure 3-2-1).

In the surface skin temperature measurement experiment, the surface skin temperature in the right hind paw of the animals was comparable in each group before immersion in ice-cold water. However, the surface skin temperature in the treated groups was significantly higher than that in the control group immediately after cooling, and the decrease rates of the surface skin temperature were 50.3%, 45.9%, and 40.5%, respectively, in the control, 30, and 100 mg/kg/day groups (Figure 3-2-2). Moreover, the surface skin temperatures in the treated groups were higher than those of the control group at all measurement points, which were 3, 6, 9, and 12 min after cooling (Figure 3-2-2). The thermographic images are shown in Figure 3-2-3.
Discussion

In this section, the effects of Nanpao on cold constitution were examined quantitatively and objectively by measuring peripheral blood flow and surface skin temperature in aged female rats.

The peripheral blood flow volume in the treated groups measured with a laser Doppler blood flow meter showed a tendency to increase dose-dependently compared to that in the control group. The surface skin temperature in the hind paws monitored using thermography was comparable in each group before cooling. However, the surface skin temperature in the treated groups at all measurement points after immersion in ice-cold water was significantly higher than that in the control group. These results suggest that long-term administration of Nanpao may protect against diminished peripheral circulatory function and accelerate recovery from disturbances in peripheral blood flow produced by cooling, and improve the surface skin temperature in aged female rats.

Cold constitution occurs more frequently in women than in men, and it is believed to be caused because of a disturbance in peripheral circulation involving arterial, venous, and/or microcirculation (e.g., capillaries). The balance between vascular vasodilation and contraction is precisely regulated by various mechanisms; for instance, estrogen acts not only as a direct vasodilator but also as a secondary vasodilator through the promotion of the enzyme nitric oxide synthase (Orimo et al., 1993; Tostes et al., 2003).

Some of the constituent herbs of Nanpao, such as Angelicae radix, Cinnamomi cortex, and Rehmanniae radix, are known to have
pharmacological actions on peripheral vasodilation, as evidenced by increased blood flow and elevated skin temperature in the tails of rats and mice (Zhao et al., 1990; Hayashi, 1977; Harada and Yano, 1975; Matsuda et al., 1995). Furthermore, aqueous extracts of Astragali radix and Ginseng radix induce nitric oxide generation in cultured rat vascular smooth muscle cells (Fukuda et al., 1994 and 1995), and 50% ethanolic extracts of Rehmanniae radix increase the amount of the blood flow in the veins and peripheral circulation (Matsuda et al., 1995). Moreover, Danggui buxue tang, which contains only Astragali radix and Angelicae radix, induces estrogen-driven promoter activity in vitro and stimulates blood flow in vivo (Gao et al., 2007). Therefore, the inhibitory effect of Nanpao on decreased peripheral blood flow and skin surface temperature after cooling may be due to additive or synergistic pharmacologic effects of the various herbs contained in Nanpao.

The results of the present study strongly support the clinical effects of Nanpao on cold constitution (Kumahara et al., 1989), and that Nanpao has the potential to produce improvement in the surface skin temperature of women with cold constitution.
Fig. 3-2-1. Peripheral blood flow volume measured in the hind paw using a laser Doppler blood flow meter at week 20 of dosing (at the age of 10 months). Vertical lines represent SD.
Fig. 3-2-2. Surface skin temperature in the hind paw before and after immersion in ice-cold water at week 33 of dosing (at the age of 13 months).

*: Significantly different from the control ($P < 0.05$).

**: Significantly different from the control ($P < 0.01$).

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Fig. 3-2-3. Thermograph of hind paw before and after immersion in ice-cold water at week 33 of dosing (at the age of 13 months).
(a) Thermograph of hind paw before immersion. (b) Thermograph of hind paw immediately after immersion.
(c) Thermograph of hind paw 6 minutes after immersion. (d) Thermograph of hind paw 12 minutes after immersion.
Conclusion

In these studies, the author tried to investigate the utility of measuring electrical impedance in the vagina (EIV) for studying transitional changes in the estrous cycle, and examine on preventive and improvement effects of a kampo medicine on the decline in reproductive functions and peripheral blood flow in aged female rats.

1. The correlation between the value of EIV and morphological conditions of the epithelial cell layer of vaginal mucosa was examined in the aged Sprague-Dawley rats exhibiting abnormal estrus cycles in Chapter 1. The value of EIV was considered to correlate with vaginal cytology in the aged rats as well as in the young rats. Moreover, the values of EIV both in persistent estrus and continual diestrus rats reflected the histological conditions of the epithelial cell layer of vaginal mucosa. The measurement of EIV is useful approach for studying the progression of reproductive aging.

2. The preventive and improvement effects of Nanpao, a kampo medicine, on biological dysfunctions in the aged female rats was evaluated on the basis of the age-related changes in the estrous cycle, and to investigate the utility of measuring EIV for studying transitional changes in the estrous cycle in Chapter 2. Since vaginal cytology and EIV gave almost concordant results as indicators of estrous cyclicity, the measurement of
EIV was capable of detecting time-dependent changes in the estrous cycle. In addition, Nanpao administration inhibited loss of regular estrous cycles.

3. The effects on a decline in various reproductive functions of administration of Nanpao were evaluated in Chapter 3-1. Moreover, the effects on cold constitution of Nanpao were examined quantitatively and objectively using measurements of peripheral blood flow and surface skin temperature in the aged female rats in Chapter 3-2. It was able to demonstrate that Nanpao maintained normal embryo-fetal development in the mature female rats, and Nanpao had the potential to improve cold constitution associated with decreased peripheral blood flow in the female rats.

Based on these studies, the measurement of EIV was capable of detecting time-dependent changes in the estrous cycle as well as observations of vaginal smears. The EIV method was a worthwhile approach to a more precise study of estrous cyclicity in the fields of physiology and theriogenology. Moreover, it was indicated that Nanpao, a kampo medicine, maintained normal reproductive functions in the female rats. A part of mechanism of the effects of Nanpao on the reproductive functions is considered to be due to increasing blood flow volume in the uterus and ovaries, since Nanpao has the potential to improve peripheral blood flow and subsequently surface skin temperature.

These results indicates that Nanpao have preventive and improvement
effects on biological dysfunctions in aged female rats, and have possibilities of clinical applications for various age-related symptoms in human.
List of Publications


References


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