

## Effect of thymocytes on reproductive parameters of nude female mice

## Efeito dos timócitos sobre a reprodução de fêmeas de camundongos "nude"

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### SUMMARY

Heterozygous and homozygous nude female mice with the genetic background BALB/c were housed in a clean conventional colony at the Laboratory Animal Facilities of Instituto Butantan. Nude animals are known to have severe deficiencies in reproductive function. Nude female is usually sterile where only 8.8% of them present spontaneous fertility. In order to study the involvement of the thymus for these reproductive defects, athymic nude female at different stages of development were treated with thymocytes suspensions and mated with heterozygous males. The results obtained for nude females at 10 and 30 days of age were respectively: a) number of pregnant = 48% and 12%; b) number offspring per female = 5.0 and 4.0; c) natality rates were 2.40 and 0.48; and d) under conditions used in this study the life span for nude animals was prolonged until 7 months. The use of this treatment would increase the animal numbers, which are important research models for biomedical investigations. Considering that nude mice are difficult to raise, an obtainment of these animals in higher number by this method appear to be an essential event, and might offer to grow better for the animals, which are used for breeding.

**UNITERMS:** Nude mice; Homozygotes; Heterozygotes; Thymocytes.

### INTRODUCTION

Immunodeficient mice are indispensable research models for biomedical investigators for studies in oncology, immunology and infectious diseases. The mutant was mentioned in 1962, by Isaacson and Cattanach<sup>15</sup> and described in detail in 1966, by Flanagan<sup>8</sup>. The "nu" mutation occurred in a closed but not deliberately inbred stock of albino in the Virus Laboratory Ruchill Hospital, Glasgow, Scotland. The mutation produces a hairless state, generating the name "nude" (nu/nu). Even under clean breeding conditions there are weight differences between nu/nu and heterozygous nu/+, the first weighing 60 to 80% less at any age. In addition, nude mice present a failure of the thymus to develop normally to maturity, generating the name athymic. In nude mice aplasia of the thymus leads to a distinctive syndrome of pleiotropic effects, including deficiencies in reproductive function<sup>3,26</sup> and in the competence and development of the immune system<sup>20,21,22</sup>.

Thymus plays a critical role in the generation of thymocytes to induce the differentiation pre-T-cells into active T-cells<sup>17</sup> by cell-to-cell contacts as well as by secreting growth factors or hormones<sup>31</sup>. The thymic hormones characterized as

thymosin alpha 1, thymulin and thymopoietin<sup>2,11</sup> have been reported to circulate and do not act identically on all T-cell subsets; they alter preferentially post-thymic precursor cells, and mature cytotoxic and suppressor T-cells.

Evidence that the thymus can also function as an endocrine gland has mainly been derived from immunological investigations<sup>2,10,29,30</sup>. As endocrine glands do not function independently, it is likely that thymus is involved in a number of endocrine functions<sup>11</sup>. Studies of either neonatally thymectomized mice<sup>3,27</sup> or congenitally athymic nude mice<sup>27,28</sup> indicate that the thymus gland is involved in sexual maturation and protective in the female. Nude females are known to have severe deficiencies in reproductive function. They are infertile whilst males are only rarely fertile<sup>8</sup>. Sexual maturity in nude female mice is delayed and the reproductive organs, including the reduced ovaries weight and size<sup>28</sup>, increased follicular atresia and decreased fertility<sup>26</sup>.

The objective of this study was to investigate the effect of treatment with thymocytes suspensions on the fertility and life span of nude mice.

### MATERIAL AND METHOD

## Animals

Homozygous (nu/nu) and heterozygous (nu/+) BALB/c mice were kindly provided by Dr. L. Denaro, Instituto Butantan (SP, Brasil) and housed under conventional conditions in the Laboratory Animal Facilities of Instituto Butantan.

## Environmental control

Temperature in the mouse room was kept at 27°C. The ventilation rate was between 3 and 6 changes of outside air per hour. Mice were maintained in a quiet, isolated, controlled environment with a uniform light-dark cycle of 12 hours of light and 12 hours of dark. The mice were maintained in plastic cages wrapped with one piece of filter paper, and bedding consisted of micaceo granulate<sup>18,24</sup>. The water was acidified with hydrochloric acid to pH of 2.5 - 2.8<sup>16,19,24</sup>. All cages with bedding and water were autoclaved prior to use. The nutrient requirements for the nude are similar to those of other mouse strain. The diet for all mice was commercially pelleted Nuvilab (PR, Brasil) and irradiated<sup>24</sup>. The laboratory personnel worn special covering suits, head covers, masks, gloves, and shoe covers. Gloves were sterilized with alcohol between the handling of each animal.

## Breeding system

The mice were paired monogamously for 15 days. For each heterozygous male one nude female was provided<sup>12</sup>. Nude females were mated at 6 to 10 weeks old.

## Weaning

Nude offspring derived from nude females were transferred to other cages, containing heterozygous females. The weaning of the homozygous nu/nu was realized as described by Petricevich *et al.*<sup>24</sup>.

## Sexual maturation

To examine the influence of adult males urine on the rate of sexual maturation in homozygous and heterozygous females, 500 virgin nude female mice or 500 heterozygous females were divided at random into control and stimulated groups. Before female mice were mated, they were kept for 12 h in soiled cages recently occupied by adult males. In these cages the females were kept for 12 h, and after this time, they were mating with heterozygous or homozygous males<sup>6</sup>.

## Thymocytes suspensions

Thymus were obtained from heterozygous females at 3 and 5 days old, by method described by Besedovsky and Sorokin<sup>3</sup>. In brief: animals were anaesthetized and thymus lobes were removed by suction through a midline anterior incision in the lower neck-upper thorax region. Thymus cells suspensions were obtained by teasing apart thymus in

minimum essential medium (MEM) purchased from Flow Laboratories (IR, UK). The thymocytes were obtained by centrifugation at 1,500 rpm for 15 minutes and resuspended in the same medium. One hundred of these suspensions containing  $1 \times 10^6$  cells were injected intraperitoneously (i.p.) in nude males and females. Three separate groups of nude females tested the transferring of thymocytes in the effectiveness for sexual maturation. Control group and two groups of 250 females or males each. One mice group at 10 days old and the other at 30 days old. Animals were observed daily and the appearance of fertility, natality and treated noted. The viability of cells was estimated by the trypan blue exclusion method.

## Statistical analysis

The indicators used for evaluation were: a) natality rate = (number of mice born/number of female mated) and b) fertility rate = (number of female pregnant/number of female mated). Statistical significance were analyzed by "X<sup>2</sup>" test<sup>7</sup>.

## RESULTS

### 1. Effect of adult male urine on the rate of sexual maturation in female

Experiment was designed to examine the influence of adult male's urine on the rate of sexual maturation in homozygous and heterozygous females. After this kind of stimulation, it became possible to observe that maturation of nude and heterozygous female as measured by age vaginal opening and first oestrus was accelerated due adult mice urine. On the other hand, an increase of the number of athymic female pregnant was observed (Tab. 1). The results obtained demonstrate the fertility rates for nu/nu female were 8.8% and 10% for control and stimulated group, respectively. Although it was observed an increase around 1.2% in the fertility rate in stimulated females groups, it was not significant. In addition, Table 1 shows that no significant difference was observed between both groups concerning offspring number per female and nude offspring.

Similar experiments were realized and the results summarized in Tab. 2 that showed nu/+ females were also distributed in two groups. One group nu/+ females were used as control, while in another, they were stimulated. The results showed that the fertility rate was increased up to 20% ( $p > 0.001$ ). The number of offspring per female was similar in both groups (Tab. 2). While the age of the nude female, which presented pregnancy, was 8 weeks old, the groups of nu/+ females presented pregnancy between 5 and 20 weeks-old (Data not showed).

### 2. Effect of thymocytes transfer on the fertility rate of nude female

**Table 1**

Mating between nude females and heterozygous (nu/+) males. Instituto Butantan, São Paulo, 1990-1992.

nu/nu females	Pregnancy Females	Fertility Rate (%)	Total of Offspring	Offspring per Female	Natality Rate	Nude Offspring
Control group 250	22	8.8	92	4.2	0.36	45
Stimulated group 250	25	10.0	100	4.0	0.40	46

**Table 2**

Mating between heterozygous (nu/+) females and nude (nu/nu) males. Instituto Butantan, São Paulo, 1990-1992.

nu/+ females	Pregnancy Females	Fertility Rate (%)	Total of Offspring	Offspring per Female	Natality Rate	Nude Offspring
Control group 250	190	76.00	1577	8.3	6.30	786
Stimulated group 250	240	96.0*	2074	8.6	8.29	1037

(\*)  $p < 0.001$ .

**Table 3**

Effect of thymocytes transfer on nude (nu/nu) females mice. Instituto Butantan, São Paulo, 1990-1992.

nu/nu females	Pregnancy Females	Fertility Rate (%)	Total of Offspring	Offspring per Female	Natality Rate	Nude
Control group 250	20	8.0	80	4.0	0.32	40
10 days group 250	120	48.0*	600	5.0	2.40	280
30 days group 250	30	12.0	120	4.0	0.48	70

(\*)  $p < 0.001$ .

**Table 4**

Effect thymocytes transfer on the survival of nude mice at the Instituto Butantan, São Paulo, 1990-1992.

	Female Survival		Male Survival	
	n*	(%)	n*	(%)
Non treated	250	48	500	6
Transfer 10 days	250	76*	500	30
Transfer 30 days	250	60*	500	20

n\* (number of total animals observed up to 210 days);  $p < 0.001$ .

Separated groups of nude females tested the transferring of thymocytes in the effectiveness for sexual maturation, and the results obtained are shown in Tab. 3. After this treatment, nu/nu females groups at 10 days old, presented high levels in the natality and fertility rates 2.40 and 48.0%, respectively. In contrast, low natality and fertility rates 0.48 and 12.0%, respectively were obtained in nu/nu female group treated at 30 days old with thymocytes suspensions. Significant levels ( $p < 0.001$ ) were observed in 10-days-old female groups. Natality and fertility rates were around 6 times higher than when we compared with those obtained for control groups.

The birth weight of nude mice was comparable with that of phenotypically normal littermates. With time the nude display progressively poorer weight gain and by this reason these animals might to be maintained separately from those normal littermates. Moreover, none nude female could suckle the littermates; consequently, the nude

newborn were transferred to cages containing nu/+ females. The nude newborn was kept with these females until 45 days of age.

The survival of nu/nu male and female is shown in Tab. 4. After treatment with thymocytes suspensions, we observed that the life span of nu/nu mice was prolonged. The survival rates observed for nude female at 10 days and 30 days old were 76% and 60% respectively; whereas in groups of nude males the rates were 30% and 20%, respectively. Although the survival rates have been markedly increased among treated groups with thymocytes suspensions, this increasing was better observed in 10-days-old groups (Tab.4). The peak of mortality obtained among control nu/nu mice was observed in the period between 10-60 days of age. In control group the maximum life span obtained in conditions used for this study was 90 days. On the other hand, in groups which the animals received thymocytes suspensions, the life span of these animals was obtained with 150-210 days, for both males and females. After the treatment with thymocytes suspensions the number of death among nude mice was decreased, consequently, the life span of nude mice was prolonged.

## DISCUSSION

In nude mice, while some young mice are totally nude, the others show some hairless, even at a very early age, but head and shoulders are always nude. Nudes can be distinguished in a new litter within the first week, definitely within 8 or 10

days. The nude is small and is possible to observe a progressively poorer weight gain, and this weight is around 60% less than heterozygous mice. Nude female and male young mice have been weaned and separated at approximately 45 days of age. In the homozygous females the fertility is not normal, only 8.8% of them presented pregnancy, make them difficult to breed<sup>8</sup>. The pre-treatment designed to examine the influence of adult male urine on the rate of sexual maturation in nude and heterozygous females showed that the vaginal oestrus was accelerated for both groups. After this treatment the females from both groups were mated with nude and heterozygous males. The results of this study have shown that 10% and 96% for nude and heterozygous female, respectively, were pregnant.

Thymus functions have been comprise as reviewed by several investigators<sup>1,4,5,10,13,14,25</sup>. Thymus reaches its maximum development soon after the birth and suffers accentuate involution after the puberty and is postulated to play a fundamental role on the deterioration of the immunological as well as the endocrine functions. These endocrine influences decline with age and are associated with "thymic menopause", and cellular immune senescence, contributing to the development of diseases during ageing<sup>25</sup>.

Thymus is involved in a number of endocrine functions that is characterized by the action of various hormones<sup>10</sup>, consisting of thymocytes epithelial cells and thymic stromal cells. Experimental evidences that the thymus also influences the maturation of female sexual function during a critical period of development. Abnormalities were observed in the morphology and function of endocrine glands in the nude mice, including pituitary gland degranulation and enlargement of endoplasmic reticulum in somatotropin-producing cells.

The thymic epithelial cells that are responsible for thymic hormone secretion include morphologically and antigenically distinct subpopulations<sup>31</sup>. Studies in congenitally athymic nude mice indicated that the thymus gland is involved in sexual maturation and reproductive in the female<sup>3</sup>. There are data in the literature indicating differences in the concentrations of

hormones in athymic mice model<sup>26</sup> and the postnatal development of the ovary<sup>1</sup>. Normal mice thymectomized at 3 days of age in congenitally athymic mice thymic transplants from the 5<sup>th</sup> day of life<sup>10</sup>. The data demonstrated that abnormal function of the adrenals and testes in nude mice is related to the absence of a functionally active thymus during early ontogenesis. Treatment with thymocytes during the first ten days of life increases the fertility rate up to 48% in nude female. Although there was an increase of the pregnancy number in nude female treated at 30 days old, it was observed this increase was less than effective when compared to those obtained for 10-days-old nude female. In this study, we also show that the nude female treated with thymocytes suspensions increased on the fertility rate. Heterozygous and homozygous females produced litter size were 8 and 4, respectively, and each litter segregating nude homozygous generally contained 1 - 4 and 2 nude, respectively. Soon after birth the nude was transferred to cages containing heterozygous female with littermates of nude, during lactation period. Extrathymic hormonal influences include pituitary-derived hormones, such as prolactin and indirectly by ACTH via hydrocortisone from the adrenal, by thyroid-stimulating hormone via thyroid hormones from the, and the by luteinizing hormone via sex steroids from gonads and adrenal<sup>10,11</sup>.

On the basis of current knowledge reproductive defects in mice models<sup>1,10,26</sup>, our results suggest the involvement of thymus during period of development. In addition, nude mice are very difficult to raise, these animals presented average span being less than 3 months<sup>8,9</sup>. However, it was found in this study that after thymocytes treatment the life span for the nude females and males were prolonged until 7 months. These results, combined with the observation that grafting a thymus into nude mice restores their immunocompetence<sup>20,21,22,23</sup> as well as prevents or minimizes other pleiotropic effects of the mutant, would suggest that the thymocytes treatment confers a longer life span and better reproduction in nude female. The use of this treatment would increase the animal numbers that are indispensable as research models for biomedical investigations.

## RESUMO

A colônia de camundongos da linhagem BALB/c, heterozigotos e homozigotos, foi mantida em condições convencionais no Biotério Experimental do Instituto Butantan. Os camundongos "nude" apresentam severa deficiência no sistema reprodutor. As fêmeas de camundongos "nude" são geralmente estéreis; apenas 8,8% apresentam fertilidade espontânea. Para estudar o envolvimento do timo sobre os defeitos na reprodução em diferentes estágios de desenvolvimento, as fêmeas de camundongos "nude" foram tratadas com suspensões de timócitos, e, em seguida, acasaladas com machos heterozigotos. Os resultados obtidos para fêmeas "nude" com 10 e 30 dias de idade foram respectivamente: a) número de fêmeas prenhes = 48% e 12%; b) número de filhotes por fêmea = 5,0 e 4,0; c) as taxas de natalidade foram 2,40 e 0,48; e d) nas condições utilizadas neste trabalho, a vida média dos camundongos "nude" foi prolongada até 7 meses. Este método descreve o aumento do número de animais "nude", importantes em estudos biológicos. Considerando a dificuldade de obtenção de camundongos "nude" em grande número, o uso deste método poderia contribuir para o melhoramento das condições de vida dos animais utilizados nos acasalamentos.

**UNITERMOS:** Camundongos nude; Homozigotos; Heterozigotos; Timócitos.

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