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Aberrations in the female reproductive organs and a role of telocytes in a rat model of anorexia nervosa

KAMIL SKOWRON, VERONIKA ALEKSANDROVYCH, MAGDALENA KURNIK-ŁUCKA,
PAULINA STACH, AGNIESZKA BARANOWSKA, BEATA SKOWRON, KRZYSZTOF GIL

Department of Pathophysiology, Jagiellonian University Medical College, Kraków, Poland

Corresponding author: Krzysztof Gil, MD, PhD

Department of Pathophysiology, Jagiellonian University Medical College

ul. Czysta 18, 31-121 Kraków, Poland

Phone: +48 12 633 39 47; Fax: + 48 12 632 90 56; E-mail: mpgil@cyf-kr.edu.pl

Abstract: Background: Anorexia nervosa is a widely prevalent eating disorder that often leads to life-threatening complications. Since it mostly concerns females, many authors have focused on studying the reproductive system in anorexic women. Recently discovered telocytes may give a new insight into the pathophysiology of gynecological complications in these patients.

Material and Methods: We adopted an animal model of anorexia nervosa induced by voluntary physical activity. Sixteen female Wistar rats were divided into two groups: control and activity-based anorexia. When the weight loss of activity-based anorexia (ABA) rats reached 25% animals were euthanized. Size and weight measurements as well as histopathological analysis of the reproductive organs were performed. Additionally, we used immunohistochemical staining for detection of telocytes.

Results: Telocytes were identified in uteri of anorectic rats but no differences were observed when compared to the control group. Nevertheless, in the ABA group the weight of the uteri and the number of follicles in the ovaries decreased significantly.

Conclusions: Our rat model of anorexia nervosa mimics the effects of this eating disorder that occur in the female reproductive system since we reported ovarian dysfunction and uterine involution in the experimental animals. It supports its potential role in the further studies of anorexia pathophysiology and treatment possibilities.

Key words: anorexia nervosa, rat, uterus, ovaries, telocytes.

Introduction

Anorexia nervosa (AN) is a serious, potentially fatal eating disorder that mainly affects young women. Its worldwide prevalence in 2016 has been estimated at around 2,6 million people [1] with a higher mortality rate than any other psychiatric disease. The diagnostic criteria for anorexia are contained in the Diagnostic and Statistical Manual of Mental Disorders. DSM-5 introduces a definition that essentially consists of a restriction of energy intake, intense fear of gaining weight and distortion of the body image. The progressive weight loss results in a vast number of medical complications affecting almost every body's system, including reproductive system. Although an amenorrhea criterion has been eliminated from the latest version of DSM, 66–84% of women with anorexia nervosa suffer from it [2]. This menstrual dysfunction is thought to be secondary to the suppression of the hypothalamic-pituitary-gonadal axis (HPG axis) since the secretion of gonadotropin-releasing hormone (GnRH) is impaired, and therefore luteinizing hormone (LH), follicle-stimulating hormone (FSH) and estradiol serum concentrations are significantly low [3]. As a result, a condition called hypogonadotropic hypogonadism develops, which is characterized by ovarian dysfunction [4].

A rat model of activity-based anorexia was adopted. Rodents show a tendency to self-starvation when exposed to a time-restricted feeding schedule and given the possibility of voluntary physical activity in a running wheel [5]. Since this model has been shown to mimic general concept of AN, we analyzed disturbances in HPG axis and morphology of gonads to assess the usefulness of the model in the reproductive system experiments. Moreover, by the means of immunohistochemistry we investigated the effect of AN on telocytes distribution in uterine horns.

Telocytes (TCs) are newly discovered cells identified in many organs, including uterus and fallopian tubes. The typical telocyte has a small, oval-shaped cellular body, containing nucleus, surrounded by a small amount of cytoplasm. They always have very long prolongations named telopodes (Tps) with thin segments (podomers) and thick segments (podomes). TCs have own unique morphology, demonstrate specific direct (homocellular and heterocellular junctions) and/or indirect (chemical, paracrine/juxtacrine signalling, microvesicles and exosomes, sex hormones and microRNAs) contacts with various surrounding cells, have gene expression and immunohistochemical profiles [6–12]. Their activity can be regulated by sex hormones due to the presence of estrogen and progesterone receptors embedded in the membrane of these cells [13]. Although the exact role of telocytes requires more detailed research, it has been shown that they can affect the contraction of smooth muscles in the uterus [14]. It has also been hypothesized that through intercellular connections or paracrine signaling, these cells can modulate the local immune response [15]. In addition, several experiments have reported that the number of telocytes depends on the reproductive status. During pregnancy, the quantitative measurement of telocytes shows their increase in

endometrium and decrease in myometrium [16]. These features indicate their potential role in the development of various pathological gynecological conditions. Hence, our interest in the influence of anorexia on these cells. The aim of this study was to provide a context for extended research on gynecological complications in patients with AN based on reproductive organs morphology.

Material and Methods

Animals

Wistar rats weighing 170–220 g upon their arrival were housed in groups under controlled conditions — 12 h light/12 h dark cycle and temperature of $22 \pm 2^\circ\text{C}$. They were fed with standard rat chow (Labofeed B, Kcynia, Poland) and tap water *ad libitum*. This study was carried out in accordance with ethical, regulatory and scientific principles (protocol number 65/2017).

Experimental design

After an initial acclimatization period of 5 days, rats ($n = 16$) were randomly assigned to one of two groups ($n = 8$ experimental group, $n = 8$ control group):

1. A control group: no extra activity + *ad libitum* feeding schedule.
2. An ABA group: voluntary activity in a running wheel + restricted feeding schedule.

All cages contained environmental enrichment and bedding material, and were placed adjacent to each other to provide sight, acoustic and odor contact. Body weight, food intake and voluntary activity in a running wheel were monitored daily. The experiment was discontinued and animals euthanized when the body weight loss exceeded 25%. Their gonads were excised for further examination.

Morphometry

After removal of adhering fat, the uteri were weighed and the ratio of uterus weight/body weight was calculated for each of them. Using the calipers, we measured the body of the uterus (length and width), uterine horns (length) and ovaries (the two longest perpendicular diameters). The length of the uterus was evaluated in the long axis from the cervix to the fundus, while the width was defined as the longest transverse diameter.

Tissue processing

Fresh tissue specimens from the body of the uterus, the middle third of the uterine horn and the ovaries were collected and rinsed thoroughly with PBS (phosphate-

-buffered saline, 0.01 M, pH = 7.4), fixed in 4% phosphate-buffered paraformaldehyde, routinely processed and embedded in paraffin. Serial sections were cut and mounted on poly-L-lysine-coated glass slides.

Routine histology

The sections were deparaffinized, rehydrated and stained with hematoxylin–eosin (H&E) to evaluate the gross tissue organization.

Immunofluorescence

Indirect double immunofluorescence after heat-induced epitope retrieval was used to allow the simultaneous visualization of two antigens. After deparaffinization and rehydration the slides were incubated for 30 min in PBS with appropriate normal serum at room temperature, followed by overnight incubation at 4°C in a solution of PBS with appropriate normal serum containing primary antibody (or mixture of primary antibodies) and 0.3% Triton X-100 (Sigma, USA). After 5 washes (10 min each) in PBS, the specimens were incubated for 1 h at room temperature with secondary antibody (or a mixture of secondary antibodies) diluted in PBS. Finally, the slides were washed in two changes (10 min each) of PBS and cover-slipped with Fluorescence Mounting Medium (Dako, Denmark). Labelled specimens were analyzed immediately. The primary and secondary antibodies used for the staining are listed in Table 1.

Table 1. Type, sources and dilution of antibodies.

| Antibody | Catalog number and company | Dilution |
|-----------------------------------|-------------------------------------|----------|
| Primary antibodies | | |
| Polyclonal rabbit anti-c-kit | A4502, Dako | 1 : 100 |
| Monoclonal mouse anti-CD34 | M7165, Dako | 1 : 100 |
| Polyclonal goat anti-PDGFR alpha | AF-307-NA, R&D Systems | 1 : 100 |
| Monoclonal Mouse anti-tryptase | M7052, Dako | 1 : 100 |
| Secondary antibodies | | |
| Alexa Fluor 488 Goat Anti-Mouse | 115-545-146, Jackson ImmunoResearch | 1 : 400 |
| Alexa Fluor 594 Goat Anti-Rabbit | 111-585-144, Jackson ImmunoResearch | 1 : 400 |
| Alexa Fluor 594 Donkey Anti-Goat | 705-585-003, Jackson ImmunoResearch | 1 : 200 |
| Alexa Fluor 488 Rabbit Anti-Mouse | 315-545-045, Jackson ImmunoResearch | 1 : 400 |

Microscopic examination

Slides were examined using an MN800FL epifluorescence microscope (OptaTech, Warszawa, Poland) equipped with a Jenoptik Progress C12^{Plus} color camera. Digital images were collected at either 200× or 400× magnification. The qualitative analysis of cells was provided in 10 consecutive high-power fields of vision (400×) using the computer-based image analysis system Multiscan 18.03 software (CSS, Warszawa, Poland). All samples were assessed by two independent specialists (each blinded to the other) without any knowledge of the clinical parameters or other prognostic factors to avoid bias. The use of mast cell tryptase staining enabled c-kit-positive mast cells to be distinguished from c-kit-positive TCs. TCs were considered cells that were c-kit positive and tryptase negative concurrently, with the characteristic morphology in tissue samples. Additionally, cells double positive for CD34 and PDGFR α with the characteristic morphology and localization were also recognized as TCs. In all sections the immunoreactive cells found were evaluated semi-quantitatively.

Results

Anorexia-induced body weight changing

The rat model of anorexia based on activity leads to a rapid reduction in the body weight. Rats subjected to the activity-based anorexia showed a significant weight loss from the first day of the experiment. After 6–7 days they reached a body weight decrease of about 25%, which was the end point of the experiment. In the control group, half of the rats gained weight during the experimental period and the overall mean weight change was estimated to be $+3.73 \pm 9.04$ g. In the ABA group, the mean weight loss was 41.95 ± 3.23 g (Fig. 1).

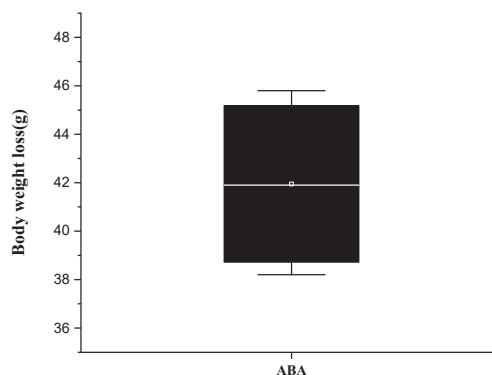


Fig. 1. ABA group. Body weight loss at the end of the experiment in grams. Data expressed as mean \pm standard deviation.

Rats with activity-based anorexia slowly increased food intake, but never reached the level observed in the control group. Despite the progressive weight loss, anorexic rats consumed gradually more food each day during feeding. At the end of the experiment, their food intake reached half of the level of the control group. Rats fed *ad libitum* showed fairly stable eating pattern (Fig. 2).

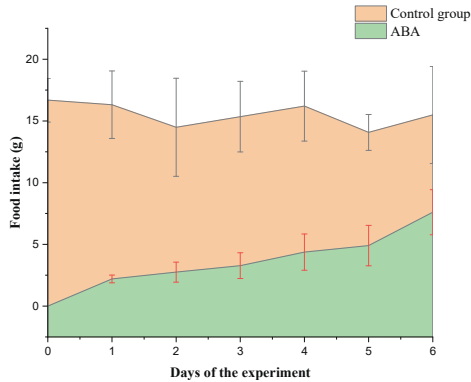
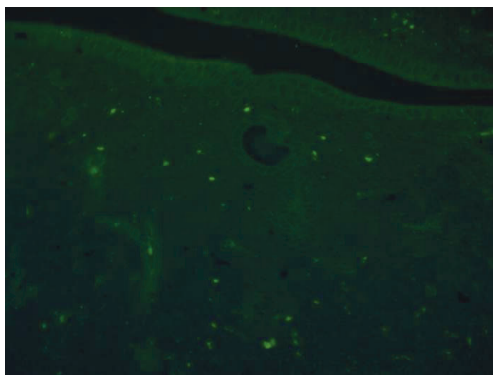


Fig. 2. Food intake over the period of 6 days. Data expressed as mean \pm standard deviation.

Identification of telocytes in rats with anorexia nervosa

We rely on immunohistochemical profile of telocytes used nowadays, and thus in order to confirm a presence of telocytes in rat's uterus double immunofluorescent staining for CD34/PDGFR α was used. Immunolabelling for CD117/c-kit and tryptase was also performed to differentiate telocytes from mast cells. Putative uterine telocytes have been revealed in both experimental groups without any significant difference.



(a)



(b)

Fig. 3. A sample from a horn (a) and the corpus (b) of a uterus in an ABA rat stained for CD34 (green, Alexa Fluor 488) and PDGFR α (red, Alexa Fluor 594). Total magnification: \times 400.

Double immunopositive cells for CD34/PDGFR alpha had an oval shaped body with a little bit elongated contour. They were mostly singly located close to blood vessels in the fundus of a rat's uterus (Fig. 3). However, some of them reflected a direction of myometrial fibers in the horns of a uterus by forming eccentric lines. Important to note, a more intensive immunostaining was common for the corpus of a uterus in comparison with its horns. Double immunopositive cells for CD117/c-kit and tryptase presented mast cells and were dominantly expressed in the corpus of a uterus in both groups, while c-kit-positive cells were detected in horns of a uterus in the majority of samples.

Involution of rat's uterus and ovarian follicle pool

A rat model of an activity-based anorexia induces the involution of the rat's uterus. The gross evaluation of the pathohistological material after section and hysterectomy revealed a huge difference in size and weight of the uterus in both experimental groups. The mean uterine weight of the anorexic rat was almost two times lower than in the control group (Table 2). Additionally, even a macroscopic evaluation revealed the apparent harmful effect of ABA on the structure of the reproductive organs (Fig. 4).

Table 2. The weight of a uterus.

| | Control group | ABA group |
|--------------------|---------------|---------------|
| Uterine weight (g) | 1.085 ± 0.035 | 0.572 ± 0.153 |

Data are expressed as mean ± standard deviation.



Fig. 4. Photos of the rat uteri. Left — an activity-based anorexia rat, right — a control group rat.

Structure of the ovaries

The microscopic analysis of the ovarian tissue samples collected from anorectic rats revealed a significant difference in their histological structure compared to the control group. Although the diameter of follicles was the same in both experimental groups, their number was eight times lower among ABA rats than in the control group (Table 3). A few follicles were detected in anorectic rats but only in the outer edge of ovaries, whereas the healthy ovary had follicles in the central part as well (Fig. 5). Ovaries from non-anorectic rats contained several sizes of follicles including the dominant follicle, while anorexic ovaries were characterized by a poor follicular pool without a leading follicle.

Table 3. The microscopic analysis of rat ovaries.

| | | Ovaries | |
|-----------|----------------------------|---------------|--------------|
| | | Control group | ABA group |
| Follicles | Number | 16 | 2 |
| | Diameter (μm) | 631 ± 219 | 636 ± 21 |

Data are expressed as mean \pm standard deviation.

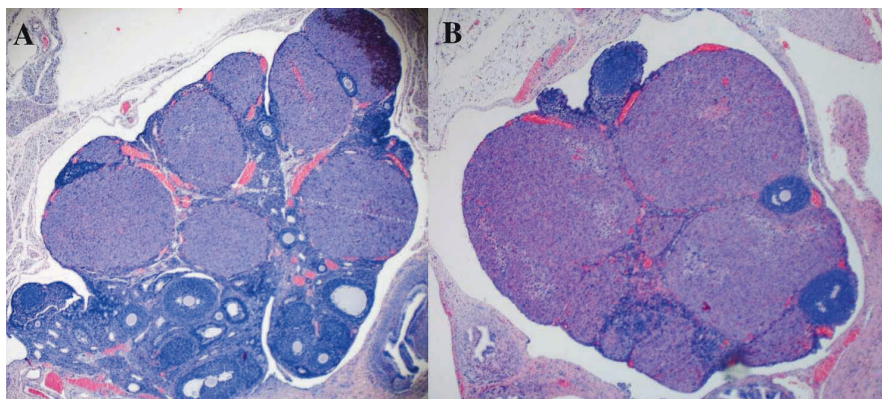


Fig. 5. Hematoxylin-eosin stained sections of a rat's ovaries. A section from the control group (A) compared to a sample from an ABA rat (B). There was a significant difference in the number of follicles between those two ovaries. Total magnification: 40 \times .

Discussion

In order to determine the complex pathophysiology underlying anorexia nervosa there is a need for a thorough analysis of biological, psychological and social factors associated with the disease. In this study, we discovered that the ABA rat model simulates complications in the reproductive system of women suffering from anorexia

nervosa. Brain imaging studies revealed a relationship between appetite fluctuations and the menstrual cycle, which indicates the importance of the female reproductive system in the control of eating habits [17]. We confirmed that rodents show a tendency to self-starvation resulting from a time-limited feeding schedule and free access to a running wheel. Although running itself leads to an increased demand for calories intake, a food restriction paradoxically intensifies this unforced behavior [5]. Rats' excessive running activity was observed even during a meal time. It caused a rapid decrease in the body weight of those animals.

Hypophagy and hyperactivity led to a reduction in the weight and size of the uterus in ABA rats. This reversion to a prepubertal state is attributed to a decreased level of circulating estrogen as the effect of disrupted HPG axis. An important link between the metabolic system and the secretion of sex hormones is played by the hormone produced by adipose tissue called leptin. Various authors have reported that low levels of leptin are characteristic of anorectic patients and leptin insufficiency seems to be the main initiator of the dysregulation in the HPG axis, as well as hyperactivity induced by starvation [18]. These hormonal alterations result in hypogonadotropic hypogonadism, which we determined based on the microscopic examination of the ovaries. The reduced number of follicles is a significant indicator of ovarian dysfunction. Although we have not performed hormonal tests, the gonadotropin as well as estrogen serum levels would be low. Nonetheless, determining the hormonal status is necessary to correlate it with the observed histopathological alterations.

The detected morphological changes in pelvic organs in ABA rats are of a clinical use because they present important diagnostic features that determine reproductive maturity. The size of the uterus, the thickness of the endometrium and the functional status of the ovaries can be used to calculate the desired healthy body weight in patients with AN [19]. Nevertheless, these ultrasound measurements are often inaccurate, which is why we believe that the presented animal model can be possibly used in the future to improve diagnostic criteria. This would require more detailed studies of the sequence of structural changes in the reproductive organs.

The primary identification of TCs in the rat uterus especially with anorexia nervosa might bring a novelty in the context of the uterine and ovarian hormonal regulation. Based on immunohistochemical profile of TCs, their positivity for estrogen and progesterone receptors can be connected with the macroscopic involution of the uterus and ovarian follicles pool because of a fluctuation of LH, FSH and estradiol levels in anorexic rats. On the other hand, close localization to muscle fibers may lead to microscopic changes in collagen and muscle fibers context resulting in a uterine weight loss. Although no difference has been revealed in the observational analysis of TCs in both groups so far, we hypothesize that they play their own role in the pathophysiology of uterine and ovarian disorders. Further observation is required to

describe their possible involvement in the hormonal imbalance that accompanies the cases of anorexia nervosa.

Conclusions

As the current model of activity-based anorexia successfully mimics gynecological complications in the patient population, we believe that it allows us to conduct further reliable research focused on the pathophysiology underlying anorexia nervosa. Reproductive organ morphology in the ABA model shows typical changes observed in women with anorexia such as ovarian dysfunction or uterine involution. However, it requires further research on underlying endocrine mechanisms. Detection of telocytes with their sensitivity to sex steroid hormones might provide additional data for better understanding the pathophysiologic axis influencing anorexia nervosa both in animal models and in humans.

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Conflict of interest

None declared.

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