Correction of the functional state of female rats after unilateral ovariectomy using a succinate containing composition

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Abstract
Unilateral ovariectomy (UOE) of female rats caused an increase in duration of the non-reproductive period (NRP) – metestrus and diestrus phases, and shortening of the reproductive period (RP) – proestrus and estrus phases of the estrous cycle (a symptom of hormonal deficiency). Signs of stress persisted 5 weeks after UOE: reduction of the ratio of thymus weight to the weight of adrenal glands (T/A). A two-week course of the succinate-containing composition with added B vitamins (SCCV) administered to the control female rats and those upon UOE has increased the T/A ratio as compared to that of intact animals, which is an indication of “activation” according to Garkavi et al. [1] and restored the NRP/RP ratio. Cytobiochemical analysis found a significant increase in nitroblue tetrazolium reduction during succinate oxidation in lymphocytes according to reading of animal blood smears.

Keywords
Unilateral ovariectomy, Succinate containing composition, Estrous cycle, Stress, Cytobiochemical analysis

Introduction
Amber acid has been traditionally viewed as an intermediate of the tricarboxylic acid cycle in the energy metabolism. However, studies conducted in the last several decades revealed its new role as a signaling molecule that participates in functional regulations at the cellular level and that of the whole organism [2, 3]. These new views formed thanks to a discovery by He et al. [2] of ligand properties of small succinate concentrations (about 50 mkM) acting on the SUCNR1 receptor found in many animal tissues [2, 3]. Activation of the SUCNR1 receptor by the ligand stimulates release of calcium ions from intracellular depot, which leads to significant changes in the activity of cells, tissues, and the whole organism. Small succinate concentrations also inhibit prolyl hydroxylase – a molecule that promotes degradation of the labile subunit of the hypoxia inducible factor 1 (HIF1) [4]. As the result of HIF1 stabilization, formation of the functionally active HIF dimer takes place, and the adaptation to oxygen deficit begins by transcriptional activation of the genes responsible for angiogenesis, erythropoietin synthesis, genes encoding glucose caries and multienzyme glycolysis complex.

The new data on the regulatory role of succinate requires re-evaluation of the effects of succinate-based compositions (SBC) on the functional state of animals and humans, and actualizes the need to control effects of SBC on the organism as a whole. Here, we investigated effects of two versions of substrate composition – SBC and SBC enriched with vitamins (SBCV) – on the functional state of intact female rats and animals, which have undergone unilateral ovariectomy (UOE).

The SBC’s effects on the hormonal status of female rats after UOE were assessed by the estrous cycle phases. In order to quantify stress development and the effects of the SBC course on the stress, we relied on the classical description of stress by Selye [5]:
decrease in the thymus weight and concurrent hypertrophy of the adrenal cortex.

In order to assess the state of live organisms, we turned to the cytotobiochemical (CBC) method developed by M. N. Kondrashova. The method allows one to measure the amount of nitro blue tetrazolium (NBT) converted to formazan during redox reactions that are stimulated by exogenous substrates [6] in the immobilized peripheral blood cells of specially-treated blood smears.

According to M. N. Kondrashova, the activation of succinate oxidation detected by the CBC method is reflective of the sympathicotonic changes in the body that lead to the mobilization of the energy metabolism. Because the CBC method uses enzymatic intermediates and inhibitors of the tricarboxylic acid cycle, which takes place in mitochondria, lymphocytes, having more mitochondria compared to other blood elements, are the main contributors to the formazan formation. NBT is a highly electrophilic molecule, and using it as an acceptor of reducing equivalents in the CBC test allows us to assess not only the levels of coupled electrons coming from the carries of the mitochondrial respiratory chain, but also to detect the presence of single electron leaks that occur during reactive oxygen species formation. According to the developers of the CBC method, the offered analysis of the mitochondrial reactions in lymphocytes can be informative and reflect hormonal and energy state of the body [6]. Specifically, in case with UOE, as with any stressful state, there is an increase in the blood levels of glucocorticoids that causes not only thymus degradation, but also degradation and suppression of the circulating lymphocytes’ function and significant modifications in energy metabolism, which can substantially change the entire spectrum of the NBT reactions.

The goal of the study was to analyze the effects made by SBC on the functional state of the animals, who have undergone unilateral ovariectomy, and to assess the potential of using the cytotobiochemical method of reading of the peripheral blood smears to evaluate the respective changes in live organisms.

**Materials and methods**

The study was approved by the Commission on Bioethics and Humane Treatment of Animals at the Institute of Theoretical and Experimental Biophysics of Russian Academy of Sciences (Protocol No.14 dd.17 December, 2013).

The experimental studies were carried out in 43 fertile female rates (18 months old), S-D line, weighing 350–400 g. The animals were divided into 8 groups as follows: 1) intact control (INT), n = 5; 2) control after a course of SBC (SBC) n = 5; 3) control, after a course of vitamins B (VIT), n = 5; 4) control after a course of SBC with vitamins (SBCV), n = 5; 5) female rats upon unilateral ovariectomy (UOE), n = 5; 6) female rats, who received a 2-week course of SBC 3 weeks after UOE (UOE+SBC), n = 6; 7) female rats, who received a vitamin course after UOE (UOE +VIT), n = 6; and 8) female rats which received a vitamin-enriched SBC course after (UOE + SBCV), n = 6.

Unilateral ovariectomy was performed according to the generally accepted methodology [7]. Three weeks after UOE, SBC was administered in the amount of 34.4 mg per kg of animal body mass, perorally, once every 24 hours, for 2 weeks. The said dose is 6x of the human dose as per the Guidelines for testing new pharmacological compounds.

The succinate-based composition contained 50 % ammonium succinate with the rest of the ingredients present in equal amounts: calcium disuccinate, magnesium disuccinate, sodium glutamate, and glycine. Vitamin composition contained vitamins B1, B2 and B6 in equal amounts and was administered perorally; it was 6x of the half daily prophylactic human dose, together or in combination with SBC.

The estrous cycle (EC) phases – proestrus, estrus, metestrus, and diestrus – were determined daily during the entire experiment and were based on the cytology of the vaginal smears, taken at 9:00 AM [8]. Fractions of round nucleated epithelial cells, irregularly shaped anuclear cornified cells, and leukocytes were calculated in the total cell pool.

Changes in the animals’ functional state were evaluated based on the integral parameter: ratio of thymus weight to adrenal glands weight (T/A). Thymus and adrenal glands were taken from decapitated animals and weighed to the 0.1 mg accuracy. An increase in the T/A ratio is an evidence of the increase in thymus mass, and thus points to the development of “activation” [1]. A decrease in the T/A ratio usually depends on the degradation of thymus and hypertrophy of the adrenal cortex and points to the development of stress [1, 5].

For the CBC analysis [6], blood smears were prepared using a special device in order to get replicable mono-
layer samples. The smears were dried and then fixed for exactly 30 sec in 60% acetone (in 10mM HEPES, pH 5.2 aqueous solution), then rinsed and dried. Fixed smears were incubated at 370 °C for 1 hour in a medium which contained 125 mM KCl, 10 mM HEPES (pH 7.2), and 1.22 mM NBT under oxidation of added potassium succinate. Potassium malonate was added to the incubation medium in order to inhibit succinate dehydrogenase (SDG).

A probe without substrates served as the control one; it allowed evaluation of the endogenous substrates (ES) contribution. After incubation with NBT, the smears were rinsed and a nuclear stain was added (0.5 % neutral red) for 8 min. Specific formazan color during NBT reaction was determined with a video camera built into the Leica DM1000 light microscope connected to PC. Intensity of formazan staining, normalized to the background levels of unstained cells, was expressed in arbitrary units (AU) calculated using BioImages program. 30 stained cells were evaluated in each smear. The following probes were used for the experimental studies: ES – oxidation of exogenous substrates, MAL – 5mM potassium malonate, SUC – 5mM potassium succinate.

Statistical analysis was applied to the collected data, with significance calculation utilizing Excel and OriginPro.

**Results and discussion**

**Effects of partial ovariectomy and succinate-based compositions on the functional state of the animals**

UOE produced distortions of the estrous cycle pattern in female rats. Usually, to quantify changes in the estrous cycle, the changes in the lengths of each of the 4 phases are considered separately. However, in our case, evaluation of the statistical significance of the postoperative changes turned out to be overly complex because of the small samples of control and intact animals; the animals were included in the experiment at the same time, and it was almost impossible to get uniform groups of females with synchronized estrous cycle phases. Conducting the surgery and administering the substrate compositions during the same phase of the cycle in all animals would have significantly lengthened the study, and some...
factors which are difficult to control would be at play: time and age trends, fluctuations in the weather and temperature conditions, food etc.

In order to get more homogenous data, which can be statistically analyzed, instead of comparing the length of each estrous cycle phase, we grouped the phases in pairs, separating non-reproductive and reproductive periods (NRP and RP), and used the ratio of the NRP to RP length as a quantitative parameter. NRP was made up of total lengths of metestrus and diestrus, RP – proestrus and estrus. The NRP/RP length ratio turned to be a significantly more uniform parameter.

Among the female rates in the 3 groups, which have not undergone UOE, the NRP/RP ratios were similar to those in the intact animals, regardless of which substrate composition was administered (left 4 columns in Figure 1 above herein).

UOE induced an increase in the NRP/RP ratio as the result of longer NRP and shorter RP. Such a change in the estrous cycle probably reflected insufficient estradiol production by the remaining ovary, although it is difficult to imagine that the potential of one ovary in fertile animals is not enough to adequately compensate consequences of UOE (5 weeks after the surgery) via hyperactivity and hypertrophy. Most likely, the cause of insufficient activity of the remaining ovary is due to the disruption of its functional control by the hypothalamus and the pituitary as a consequence of post-operative stress [5]. If this assumption is true, then the succinate-based substrate composition can be viewed as a potential corrector of estrous cycle; this was shown by Dilman et al [9] in the experiments using succinate and glutamate, and later supported by our team in the study evaluating possibility of correcting estrous phase ratios in female rats of post-reproductive age using SBC [10].

Indeed, administration of SBC to the rats, which have undergone UOE, significantly improved the situation: the NRP/RP ratio decreased to the levels observed in the control animals (Figure 1). Therefore, a SBC course stimulated compensatory hyperactivity of the remaining ovary. Noteworthy is the absence of a positive result after administration of the vitamin composition without SBC; the combination of vitamin and SBC (SBCV) successfully corrected the NRP/RP ratio (see Figure 1).

Thus, our assumption that unrealized potential of the remaining ovary may be due to the dysfunction in the control system (hypothalamus-pituitary-ovarian axis) that occurs under stress, when oscillation rhythm is disrupted and formation and secretion into the blood stream of follicle stimulating and luteinizing hormones are suppressed, can be considered substantiated. According to Selye [5], such disruptions may be consequences of an intense increase in the synthesis of adrenocorticotrophic hormone that occurs under stress. This leads to the need to quantitatively evaluate degrees of developing stress caused by UOE and the effects of the substrate compositions on this process.

In order to detect the stress state in animals [5], instead of comparing decreases in the thymus mass and increases in adrenal mass separately, similarly to the estrous cycle phase analysis, we used a relative parameter: the ratio of thymus weight to adrenal weight (T/A). This allowed to somewhat cope with heterogeneity in the samples in the groups, and to at least discover trends in the changes of the animals’ functional state (see Figure 2 above herein).

In the control experiments (no UOE), after 2 weeks of the SBC administration, compared to the intact group, a clear trend of an increased T/A ratio (+ 60 %) was observed. Combined actions of SBC and vitamins (SBCV) resulted in a significantly higher T/A ratio: by 236 % compared to the T/A ratio in the intact animals (p < 0.01).

An increase in the thymus weight was the main contributor to the increase of the T/A ratio; this clearly points to the development of the physiological “activation” state according to Garkavi et al. [1]. The “activation” state is characterized by predominance of the adrenergic (sympathetic) regulation, a higher resistance to harmful external influences, mobilization of substrates, an increase in the glycogen deposition, and readiness of individuals to withstand stress, combined with the stimulation of the immune system [1].

On the contrary, a course of vitamins without SBC slightly decreased the T/A ratio (~ 27 %) in the intact animals. This trend toward a decrease of the T/A ratio can hardly be considered as a positive effect. Probably it should be attributed to vitamin overdose.

Five weeks after UOE, there was a trend toward a decrease in the T/A ratio (~ 40 %). We regarded the decrease as a remaining sign of post-operative stress, in accordance to Selye’s views [5]. It is possible that this parameter would have been more pronounced if measured closer to the time of the surgery. A 2-week course
of SBCV that has started 3 weeks after UOE resulted in a sharp increase in the T/A ratio – by 350 % (p < 0.002) compared to the animals, which have undergone UOE, but have not received a SBC course. The increase in the T/A was so high that the T/A ratio in the UOE+SBCV group reached the same levels as those observed in the control group during “activation” state after the SBCV course (see Figure 2).

In fact, the SBCV course produced a marked anti-stress effect that resulted in the recovery of the T/A ratio and normalization of the regulation along the hypothalamus-pituitary-ovarian axis. The latter caused, as mentioned above, a compensatory activation of the hormonal production in the remaining ovary. As a result, the NPR/PR ratio of the estrous cycle in the operated animals that underwent a course of the SBCV dropped to the levels observed in the first 4 control groups of the animals who did not receive the surgery (Figure 1).

Cytobiochemical analysis of the effects of partial ovariectomy and the succinate-based composition treatment course

Based on the CBC analysis, in the control animals, who received a SBC treatment course and who were not subjected to UOE, there was an increase in the intensity of NBT to formazan formation that occurred during the oxidation of added succinate (SUC, Figure 3). This is likely due to an increased activity of SDG [6].

The largest increase in the color intensity was observed with SUC probe after a course of SBCV (see Figure 3 above herein). A more intense succinate oxidation may reflect sympathetic effects of SBC [6]. At the same time, there was an increase in the color intensity of formazan with EC and MAL probes. This is usually due to an increase of the exogenous substrate pool in the cells, specifically, due to fatty acids mobilization. Unlike the decrease in substrate oxidation during the first half of the tricarboxylic acid cycle (isocitrate, α-ketoglutarate and succinate), oxidation of fatty acids is not blocked by malonate (inhibitor of SDG). However, without additional experiments we cannot determine a possible cause of non-responsive-ness of the NBT reaction to malonate. Along with the activation of endogenous fatty acids oxidation, an increase in the free-radical reactivity of NBT (as a result of SDG inhibition) may also take place.

The CBC analysis of the samples from partially ovariectomized animals did not reveal any significant differences between UOE, UOE+SBC, and UOE+VIT groups (Figure 4). In these three groups, the levels of NBT conversion (in the blood smears with immobilized lymphocytes) remained at the levels similar to those observed in...
the intact animals (compare Figures 3 and 4). After UOE, only the course of vitamins in combination with SBC (SBCV group) resulted in significantly increased rates if the NBT reaction that occurred when added succinate was oxidized (SUC, see Figure 4 below herein).

Therefore, based on the three measurements – ratio of non-reproductive and reproductive estrous cycle period lengths, ratio of thymus and adrenal weights, and the quantitation of the NBT reaction that occurs during succinate oxidation – we determined that the most effective way to correct functional state of female rats with and without UOE is to use a relatively short course of succinate based composition enriched with vitamins (SBCV). The effect of SBCV can be described as an anti-stress effect. Noteworthy is the fact that a course of SBCV provided a complete compensatory activation of the remaining ovary, which was evidenced by a decrease of the non-reproductive to reproductive phases length ratio of the estrous cycle. The discovery that a SBCV course supports activity of the hormonal system was also apparent in the CBC analysis; there was a significant increase in the SDG activity: an increase in the formazan color formation that occurs when adding succinate and endogenous substrates during the development of “activation” state after a course of the succinate-based composition with vitamins in the control and UOE groups.

2. Following unilateral ovariectomy, we observed changes in the estrous cycle phases: an increase in the length of the non-reproductive period and shortening of the reproductive period; this points to hormonal deficiency that typically occurs under stress.

3. A two-week course of the succinate-based composition with vitamins administered 3 weeks after partial ovariectomy resulted in the development of “activation” state and recovery of the estrous cycle.

4. The cytobiochemical analysis showed an increase in the nitro blue tetrazol reducing activity when probing with exogenous succinate and endogenous substrates during the development of “activation” state after a course of the succinate-based composition with vitamins in the control and UOE groups.

Conclusions

1. A two week course of the succinate-based composition with vitamins resulted in a significant increase in the thymus to adrenal weight ratios in female rats; this points to the development of “activation” state: increased resistance to harmful stimuli and stressors.

2. Following unilateral ovariectomy, we observed changes in the estrous cycle phases: an increase in the length of the non-reproductive period and shortening of the reproductive period; this points to hormonal deficiency that typically occurs under stress.

3. A two-week course of the succinate-based composition with vitamins administered 3 weeks after partial ovariectomy resulted in the development of “activation” state and recovery of the estrous cycle.

4. The cytobiochemical analysis showed an increase in the nitro blue tetrazol reducing activity when probing with exogenous succinate and endogenous substrates during the development of “activation” state after a course of the succinate-based composition with vitamins in the control and UOE groups.

Statement on ethical issues
Research involving people and/or animals is in full compliance with current national and international ethical standards.

Conflict of interest
None declared.

Author contributions
Study conception and design: Maevsky E.I. and Uchitel M.L. Acquisition of data: Vasilieva A.A., Simonova M.A., Grishina E.V. Organization and carrying out animal operations: Bairamov A.A. Analysis and interpretation of data, drafting of manuscript: Maevsky E.I. Critical revision: Uchitel M.L., Grishina E.V.

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