Ovarian blood flow responses to electroacupuncture stimulation depend on estrous cycle and on site and frequency of stimulation in anesthetized rats

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Stener-Victorin, Elisabet, Shigeko Fujisawa, and Mieko Kurosawa. Ovarian blood flow responses to electroacupuncture stimulation depend on estrous cycle and on site and frequency of stimulation in anesthetized rats. J Appl Physiol 101: 84–91, 2006. First published March 2, 2006; doi:10.1152/japplphysiol.01593.2005.—Electroacupuncture (EA) stimulation was applied to the abdomen and hindlimb muscles at three different frequencies (2, 10, and 80 Hz) during the estrus or diestrus phase. Involvement of sympathetic nerve to EA stimulation was investigated by spinal cord transection. Abdominal EA stimulation at 10 Hz increased the OBF response, whereas hindlimb EA stimulation at 10 Hz and abdominal and hindlimb stimulation at 80 Hz decreased the OBF response; 2-Hz EA caused no OBF response. The OBF response to abdominal EA was more pronounced in the estrus than the diestrus phase. The OBF response to abdominal and hindlimb EA stimulation at both 10 and 80 Hz was almost abolished, both after severance of the sympathetic nerves and after spinal cord transection. In conclusion, the OBF response to both abdominal and hindlimb EA stimulation was mediated as a reflex response via the ovarian sympathetic nerves, and the response was controlled via supraspinal pathways. Furthermore, the OBF response to segmental abdominal EA stimulation was frequency dependent and amplified in the estrous phase.

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CLINICAL AND BASIC RESEARCH studies have demonstrated that acupuncture regulates the blood flow of different organ systems such as the heart (9, 27), the skin, and the muscles (14, 17) in both animals and humans. Furthermore, our laboratory has shown that electrical stimulation of acupuncture needles, electroacupuncture (EA) treatment, reduces a high blood flow impedance, i.e., a high pulsatility index measured by transvaginal ultrasonography, in the uterine arteries to normal levels in women (21) and induces ovulation and normalized hormonal disturbances in anovulatory women with polycystic ovary syndrome (22).

Recently, our group investigated the ovarian blood flow (OBF) response to somatic afferent stimulation with EA at different frequencies and intensities in anesthetized rats (19, 20). EA stimulation was then applied bilaterally to needles inserted in the abdominal (ABD) and hindlimb (HL) muscles with the primary aim to elucidate whether OBF would change in response to EA stimulation. We found that low-frequency, 2-Hz burst pulses increased OBF as a reflex response via the ovarian sympathetic nerves, whereas high-frequency, 80-Hz pulses decreased OBF as a passive response after systemic circulatory changes.

The rat ovary receives sympathetic innervation from the ovarian plexus nerve (OPN) and the superior ovarian nerve (SON) (8) and parasympathetic innervation from the vagal nerve (5, 6). It has been reported that the ovarian sympathetic nerves, not the vagal nerves, are responsible for regulating blood flow in the ovary (24).

It has been reported that sympathetic supraspinal organization occurs when stimulus input enters cervical or lumbar enlargements (18). On the other hand, spinal organization has been reported to exist when stimulus input enters the spinal segments where respective autonomic nerves emerge (18). Sympathetic innervation of the rat ovary emerges from the spinal cord at the segments T9–T10 (7); thus ABD muscle EA stimulation may modulate sympathetic output via segmental reflexes. EA stimulation of the HL muscles produces afferent input to the lumbar enlargement (23) and might therefore modulate sympathetic outflow via supraspinal reflexes. In our laboratory’s previous study evaluating the effect of EA on OBF responses, both the ABD and HL muscles were stimulated together bilaterally (19, 20). Thus it would be of interest to further evaluate the effect of different EA frequencies at individual sites of stimulation, for example, the ABD or the HL alone.

Another variable that might influence OBF is the estrous cycle, because the concentration of estradiol varies throughout the cycle (1). Increasing evidence supports the role of estradiol as a modulator of autonomic tone (16). In female subjects, circulating estrogen has been shown to decrease sympathetic tone by increasing density and enhancing the function of α2-adrenoceptors, which lowers basal plasma levels of norepinephrine (2, 10). Therefore, it can be hypothesized that the OBF response pattern to EA stimulation may vary during the estrous cycle.

The present study aimed to further elucidate the role of different site and different frequencies of short-term EA stimulation and to study these parameters in different phases of the estrous cycle. We investigated the OBF response to 2-, 10-, and 80-Hz EA stimulation of needles placed in the ABD muscles (segmental stimulation) and in the HL muscles (extrasegmental stimulation) during two phases of the estrous cycle: estrus and...
diestrus. Furthermore, we studied the involvement of spinal and supraspinal reflexes in OBF responses to EA stimulation by spinal cord transection.

MATERIALS AND METHODS

The experimental protocol was reviewed and approved by the Animal Ethics Committee at the International University of Health and Welfare. The experiments conformed to the American Physiology Society’s Guiding Principles for Research Involving Animals and Human Beings. The experiment was performed on 23 virgin adult cycling Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 211–300 g. Rats were housed two to each cage at a controlled temperature of 22°C with a 12:12-h light-dark cycle and with rat chow and water provided ad libitum. The estrous cycle of each animal was monitored by a vaginal smear obtained between 8:00 AM and 10:00 AM for 10 consecutive days before each experiment. The different stages of the estrous cycle were determined according to the predominant cell type present in the vaginal smear. Fourteen rats were investigated in the estrus phase and 13 in the diestrous phase.

Surgical Procedures

Anesthesia was induced with an intraperitoneal injection of urethane (1.1 g/kg, Wako, Tokyo, Japan). The trachea was intubated, and respiration was monitored with a ventilator (model SN-480-7 ventilator, Shinano, Tokyo, Japan). The right jugular vein was cannulated for administration of anesthesia and lactated Ringer solution. The trachea was intubated, and respiration was monitored with a ventilator (model SN-480-7 ventilator, Shinano, Tokyo, Japan). The right jugular vein was cannulated for administration of anesthesia and lactated Ringer solution. The right carotid artery was cannulated, and both mean arterial blood pressure (MAP) and heart rate were measured continuously. Systolic pressure was maintained above 90 mmHg by administration of 4% Ficoll 70 (Pharmacia Fine Chemicals, Uppsala, Sweden). Body temperature, which was monitored in the rectum, was maintained at ~37 ± 0.1°C by a heating pad and an infrared lamp (model ATB-1100, Nihon-Kohden, Tokyo, Japan). During the experiment, additional urethane (10% of the initial dose) was given intravenously as necessary to maintain an adequate depth of anesthesia.

OBF Recordings

The ABD wall was opened, and the left ovary was identified and gently placed on a small plate. To avoid movements in response to EA stimulation, several rods were used to fix the lateral and distal part of the ABD wall surrounding the ovary. Blood flow was measured by a laser-Doppler flowmeter (model ALF21, Advance, Tokyo, Japan). The laser-Doppler flowmeter probe (type H, 1.0-mm diameter) was gently placed on the section of the surface of the ovary that was devoid of any visible large vessels. OBF and MAP were continuously recorded in a computer, and data were calculated between 1 min before and 6 min after onset of EA stimulation. The magnitude of the OBF and MAP response is expressed as a percentage of the prestimulus control value (1 s before onset of stimulation). A stable blood flow signal was recorded at least 2 min before EA stimulation.

Severance of Sympathetic Efferent Nerves

The SON was denervated by severing it and the bundle of the suspensory ligament of the ovary. The left OPN was separated from the vein and artery of the ovary and cut ~2 cm from the ovary. In most cases, denervation was performed in the same animals whose OBF responses were recorded under the nerve-intact condition. In addition, in some animals OBF responses were recorded only under the denervated condition.

Transection of the Spinal Cord

The spinal cord was transected at the first thoracic (T1) level in anesthetized rats. The operation took ~30 min. OBF recording was started to ~1 h after spinal transection. The systolic blood pressure was kept above 90 mmHg by injection of 4% Ficoll 70 (Amersham Biosciences, Uppsala, Sweden) after spinal transection.

Experimental Protocol and EA Stimulation

Acupuncture needles of stainless steel (Xeno Hegu, Svenska, Landsbro, Sweden) and a diameter of 0.3 mm were used in all experiments. EA was performed by connecting two acupuncture needles to an electrical stimulator (model SEN-3301, Nihon-Kohden) and stimulating with square-wave pulses of 0.5-ms duration at different frequencies (see below) with a fixed intensity of 10 mA during 30 s. The intensity of 10 mA was chosen because it has previously been found to excite all afferents, including unmyelinated group III and IV fibers (13–15).

Site of stimulation. EA stimulation was applied to two different sites: 1) For ABD stimulation, two needles were inserted obliquely 7 mm into the oblique externa and interna muscles on the left side, ~2 cm from the midline at the level of the 12th rib with a distance of 1.2 cm between the needles, in the same segment from which the ovarian sympathetic nerves emerge, T9–T10 (7), i.e., segmental stimulation. 2) For HL stimulation, two needles were inserted perpendicularly 7 mm into the left gastrocnemius muscle, the proximal needle at the level of the knee joint and the distal needle 1.2 cm below, in the segment far from where ovarian sympathetic nerves emerge, L1–L5 (23), i.e., extrasegmental stimulation.

Estrous cycle. To determine the effect of the estrous cycle on the OBF response to EA stimulation, experiments were performed in two different phases: 1) estrus, ~12 h after ovulation when levels of estradiol, progesterone, and luteinizing hormone are low in the rat (1); and 2) diestrus, 2 days after estrus when levels of estradiol and progesterone are higher, and levels of luteinizing hormone are still low, compared with the estrus phase (1).

EA frequency. To determine whether the OBF response is dependent on the frequency of EA stimulation in the two estrus phases, EA stimulation was applied at three different frequencies to each site and in each cycle, two low frequencies (2 Hz and 10 Hz) and one high (80 Hz).

Statistics

Data were analyzed using the software package StatView for Windows, SAS Institute, version 5.01. (Cary, NC). Group comparisons were made using repeated measures with two-way ANOVAs followed by Dunnett’s multiple post hoc comparison tests when appropriate. Changes over time within each group were analyzed by repeated measures with two-way ANOVAs followed by Dunnett’s multiple range tests for post hoc comparisons. All results are reported as means ± SE. A P value <0.05 was considered significant.

RESULTS

Effects of HL Stimulation on the OBF Response in Different Cycles and at Different Frequencies

Figure 1, A–F, shows sample recordings made during the estrus phase of the OBF and MAP responses at each frequency of HL stimulation. Figure 2, A–F, shows summarized data from both the estrus and diestrus phases of OBF and MAP responses at each frequency of HL stimulation.

In six rats in the estrus phase, OBF and MAP under the resting condition before HL stimulation were 495.9 ± 15.7 mV and 95.1 ± 3.0 mmHg, respectively, whereas in six rats in the diestrus phase, OBF and MAP under the resting condition before HL stimulation were 488.5 ± 43.0 mV and 94.1 ± 4.9 mmHg, respectively. There were no statistical differences between the values of OBF and MAP in estrus and diestrus phase. HL stimulation at 2 Hz had no significant effect on OBF in either the estrus or diestrus phase, whereas MAP increased
significantly during stimulation in rats in the estrus phase but not in rats in the diestrus phase (Fig. 2, A and B). Despite lack of effect over time, there was a significant difference ($P < 0.05$) between the estrus and diestrus phases in the OBF response, mimicking a small increase of OBF in the estrus phase and a small decrease in the diestrus phase (Fig. 2A).

HL stimulation at 10 Hz significantly decreased OBF immediately after the cessation of stimulation, in both the estrus and diestrus phases; OBF returned to prestimulus control levels within 2–3 min after the onset of stimulation (Fig. 2C). MAP was significantly increased during the first 20 s of stimulation in the estrus phase and returned to prestimulus control levels immediately thereafter (Fig. 2D). The responses of MAP in rats in the diestrus phase were variable. There were no differences between estrus and diestrus in OBF responses at 10-Hz EA (Fig. 2E).

HL stimulation at 80 Hz significantly decreased the OBF response immediately after the cessation of stimulation in both the estrus and diestrus phases; with 2–3 min, OBF had returned to prestimulus control levels (Fig. 2E). MAP showed no significant response to 80-Hz EA stimulation in either the estrus or the diestrus phases (Fig. 2F). There were no differences between estrus and diestrus in OBF responses at 80-Hz EA (Fig. 2E).

**Severance of Ovarian Sympathetic Nerves and Effects of HL Stimulation on the OBF Response in Different Cycles and at Different Frequencies**

The ovarian sympathetic nerves SON and OPN were severed to investigate their involvement in OBF regulation during and after HL stimulation at 10 and 80 Hz.

When the SON and OPN were severed, the decrease in OBF response seen in rats with intact sympathetic nerves after the cessation of HL stimulation at both 10 and 80 Hz failed to
occur in either the estrus or the diestrus phase (Fig. 3, A and C). The MAP response after severance of the SON and OPN was similar to that in rats with intact sympathetic nerves in both the estrus and diestrus phases at both 10- and 80-Hz EA stimulation (Fig. 3, B and D).

Effects of ABD Stimulation on the OBF Response in Different Estrous Cycles and at Different Frequencies

Figure 4, A–F, shows sample recordings made during the estrus phase of the OBF and MAP responses at each frequency of ABD stimulation. Figure 5, A–F, shows summarized data from both the estrus and diestrus phases of the OBF and MAP responses at each frequency of ABD stimulation.

In seven rats in the estrus phase, OBF and MAP under the resting condition before ABD stimulation were 474.5 ± 29.7 mV and 97.1 ± 2.7 mmHg, respectively, whereas in six rats in the diestrus phase, OBF and MAP under the resting condition before ABD stimulation were 430.3 ± 33.3 mV and 99.8 ± 2.4 mmHg, respectively. There were no statistical differences between the values of OBF and MAP in estrus and diestrus phase.

ABD stimulation at 2 Hz had no significant effect on either OBF or MAP in either the estrus or the diestrus phase (Fig. 5, A and B).

ABD stimulation at a frequency of 10 Hz produced a significant decrease in OBF 10–20 s after the cessation of stimulation in the estrus phase (Fig. 5C). This decrease evolved into a significant increase in OBF between 3 and 5 min after onset of 10-Hz ABD stimulation (Fig. 5C). The OBF response to ABD stimulation at 10 Hz in the diestrus phase resulted in a brief decrease after the onset of stimulation without significant decreases at 10–20 s after the cessation of stimulation. A significant increase in OBF was also observed in the diestrus phase between 4 and 5 min after onset of 10-Hz ABD stimulation (Fig. 5C). The MAP response to ABD stimulation at 10 Hz resulted in a significant transient decrease after the onset of stimulation in both the estrus and diestrus phases, but it was more pronounced in the estrus phase (Fig. 5D). There was a significant difference between the estrus and diestrus phases in the OBF response to ABD stimulation at 10-Hz EA but not in the MAP response (Fig. 5, C and D).

ABD stimulation at 80 Hz resulted in a profound decrease in OBF in the estrus phase (Fig. 5E). MAP also underwent a pronounced transient decrease in response to 80 Hz (Fig. 5F). The OBF and MAP responses during the diestrus phase followed the same patterns as those in the estrus phase but were significantly less pronounced (Fig. 5, E and F).
Severance of Ovarian Sympathetic Nerves and Effects of ABD Stimulation on the OBF Response in Different Estrous Cycles and at Different Frequencies

After severance of the SON and OPN, the initial decrease and later increase in the OBF response to ABD stimulation at 10 Hz was abolished. In rats in the diestrus phase, the OBF underwent a transient decrease during 80-Hz ABD stimulation after severance of the ovarian sympathetic nerves, coincident with a decrease in MAP (Fig. 6, A and C). The MAP response after severance of the SON and OPN was similar to that in rats with intact sympathetic nerves in both the estrus and diestrus phases at both 10- and 80-Hz EA stimulation (Fig. 6, B and D).

Fig. 6. Responses of OBF and MAP to EA stimulation of the ABD at 2, 10, and 80 Hz. Summarized responses of OBF (A, C, and E) and MAP (B, D, and F) are shown. Values are means ± SE. See Fig. 2 for details.

Transcetion of the Spinal Cord

The spinal cord was transected in three animals in the estrus phase to elucidate the involvement of supraspinal reflex pathways in OBF regulation during and after EA stimulation at 10 and 80 Hz applied to ABD and HL. The estrus phase was chosen since the pattern of the OBF response was more pronounced in this cycle.

Figure 7, A–H, shows sample recordings of OBF and MAP responses to HL (A–D) EA stimulation and to ABD (E–H) EA stimulation at 10 and 80 Hz after spinal cord transection. The OBF response was completely abolished at both frequencies and during both HL and ABD EA stimulation. Similar results were obtained in other two rats.

Fig. 7. Responses of OBF and MAP to EA stimulation of the ABD at 2, 10, and 80 Hz. Summarized responses of OBF (A) and MAP (B) are shown. Values are means ± SE. See Fig. 2 for details.

Fig. 5. Responses of OBF and MAP to EA stimulation of the ABD at 2, 10, and 80 Hz. Summarized responses of OBF (A, C, and E) and MAP (B, D, and F) are shown. Values are means ± SE. See Fig. 2 for details.


DISCUSSION

The present study aimed to extend our knowledge on the OBF response to short-term EA stimulation, and it clearly demonstrated that the response differs depending on the site of stimulation, the frequency of EA stimulation, and the estrous cycle. The novel findings of the present study were that somatic afferent stimulation with EA causes changes in OBF via reflex responses via the ovarian sympathetic nerves via supraspinal pathways. We also demonstrated a significant difference in the OBF response to EA stimulation between the estrus and diestrus phases.

OBF Response to Sites and Frequency of EA Stimulation

In the present study, we were able to distinguish between the effects of different sites and frequencies of stimulation. Interestingly, ABD EA stimulation at 10 Hz resulted in a transient decrease directly after stimulation began followed by a long-lasting increase in the OBF response, whereas HL stimulation at the same frequency lowered the OBF response. OBF changes during both ABD and HL 10-Hz EA were independent of changes in MAP.

The response pattern to 2-Hz EA stimulation of a large area that included both the ABD and the HL in our laboratory’s previous study (19) was similar to that of ABD 80-Hz and HL 80-Hz EA stimulation.

Uchida et al. (25) demonstrated that the OBF response to pinching (noxious stimulation) was greatly affected by changes in MAP. In our study, OBF and MAP responded more or less independently to EA stimulation. One plausible explanation for the discrepancy in MAP involvement in the OBF response between EA and pinching (noxious) stimulation of the skin might be that pinching of the skin activates a larger number of type IV afferent fibers compared with muscles stimulation and therefore has a stronger influence on systemic circulatory...
variables such as MAP than on OBF. In line with this observation, OBF responses to 80-Hz EA stimulation of a large area that includes the ABD and HL was greatly influenced by changes in MAP (19).

The present study clearly demonstrates that the OBF response to ABD EA stimulation was frequency dependent, which was illustrated by a transient decrease, followed by, after cessation of 10-Hz EA, a long-lasting increase, and, after cessation of 80-Hz EA, by a pronounced decrease in OBF response. It can be speculated that excitatory and inhibitory responses compete with each other, but we do not know the mechanisms. Interestingly, it has been shown that the vascular supply to the ovary is under adrenergic vasomotor control (4), that sympathetic efferents regulate OBF via $\alpha_1$-adrenoceptors (26), and that all $\alpha_1$-adrenoceptors subtypes are expressed in the ovaries of rats (11). However, the involvement of adrenoceptors in the control of the OBF response to EA stimulation remains to be elucidated. Also, the release of neuropeptides may be involved in OBF regulation, and neuropeptide Y (NPY; a potent vasoconstrictor) and vasointestinal peptide-containing nerve fibers have been found in relation to ovarian blood vessels (4). Furthermore, it has been suggested that norepinephrine released from ovarian sympathetic nerves is subjected to a dual modulatory influence by norepinephrine and NPY and that this regulatory effect is exerted via prejunctional $\alpha_2$-adrenoceptors (3). Interestingly, NPY has been found to be mostly released during high-frequency stimulation which supports the involvement of NPY in situations and dysfunctions associated with enhanced sympathetic (3). Also, relaxing and contracting factors such as nitric oxide derived from the endothelium seem to be important for the maintenance and increase of OBF during the preovulatory period (12).

OBF Response After Severance of Ovarian Sympathetic Nerves and After Spinalization

The involvement of sympathetic efferents to the ovary on OBF response to EA stimulation was elucidated by severance of the SON and OPN nerves. The OBF response after both ABD and HL EA stimulation at 10 and 80 Hz was almost abolished after severance of the ovarian sympathetic nerves. These findings indicate that the OBF response to both ABD and HL EA stimulation at 10 and 80 Hz was mainly mediated as a reflex response via the ovarian sympathetic nerves.

To further extend our knowledge of the control of the OBF response to EA stimulation, we performed additional experiments with spinal cord transection to elucidate whether the reflex centers for the OBF responses to EA stimulation are located in the spinal cord or in supraspinal structures. It has been reported that sympathetic supraspinal organization occurs when stimulus input enters cervical or lumbar enlargements, whereas spinal organization exists when stimulus input enters the spinal segments where respective autonomic nerves emerge (18). This leads assumption that EA to the ABD, but not EA to the HL, produces OBF response in spinalized animals; however, interestingly, the OBF response to both ABD and HL stimulation with 10- and 80-Hz EA was abolished after spinalization. This finding clearly demonstrates that OBF responses to EA stimulation are primarily controlled via supraspinal reflex pathways (see summarizing Fig. 8).

Contrary to our findings, Uchida et al. (25) found that pinching the skin of the hind paw was strictly related to supraspinal reflex pathways, whereas ABD pinching was regulated via both spinal and supraspinal reflex pathways. Again, the type of stimulation is different and might explain the divergence in the OBF responses.

Involvement of Estrous Cyclicity in the OBF Response to EA Stimulation

Another novel finding in the present study was that rats in the estrus phase exhibited a more pronounced OBF response compared with those in the diestrous phase to ABD EA stimulation at both 10 Hz and 80 Hz.

In our laboratory’s previous study, however, we were unable to distinguish responses of OBF between the different cycles (20). Similar responses in rats with any estrus cycles were also observed in the study by Uchida et al. (25). One reason might be that the stimulation in the present study was less intensive compared with the two previous studies (19, 25).

The reasons for the discrepancy in the OBF response to EA stimulation during the estrous cycle might be that the sympathetic efferents are affected by the release of ovarian estradiol, as suggested by Saleh et al. (16). As mentioned before, circulating estradiol seems to decrease sympathetic tone by increasing the density and enhancing the function of presynaptic $\alpha_2$-adrenoceptors (10). Furthermore, Saleh et al. showed that 17β-estradiol increases baroreflex sensitivity (16), which might further explain the difference in OBF responses during the estrous cycle. It is also possible that estrogen increased the sensitivity of the somatovisceral reflexes.

In conclusion, the present study demonstrates that the OBF response to both ABD and HL EA stimulation was mediated as a reflex response via the ovarian sympathetic nerves and that they were controlled via supraspinal pathways. Furthermore, the OBF response to segmental ABD EA stimulation was frequency dependent and amplified in the estrus phase.

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