University of Szeged

Faculty of Pharmacy

Department of Pharmacodynamics and Biopharmacy

DEVELOPMENT OF SMOOTH MUSCLE ELECTROMYOGRAPHIC METHOD: DETECTION AND INTERPRETATION OF SLOW WAVE ACTIVITIES IN GASTROINTESTINAL TRACT AND PREGNANT UTERUS

Summary of Ph.D. Thesis

KÁLMÁN FERENC SZŰCS

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Head: Dr. István Zupkó Ph.D.

Department of Pharmacodynamics and Biopharmacy

SUPERVISOR:

DR. RÓBERT GÁSPÁR PHARM.D., PH.D.

Kálmán Ferenc Szűcs Pharm.D.

DEVELOPMENT OF SMOOTH MUSCLE ELECTROMYOGRAPHIC METHOD: DETECTION AND INTERPRETATION OF SLOW WAVE ACTIVITIES IN GASTROINTESTINAL TRACT AND PREGNANT UTERUS

Final Exam Committee:

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1. INTRODUCTION

The detection of motility problems of smooth muscle organs has not yet been solved. It is currently considered that these motility disorders are idiopathic in origin; their diagnosis, prognosis and treatment are therefore not satisfactory.

The gut-brain axis creates a two-way communication network created by the complex system of the enteric nervous system (ENS), cerebral nerves and the humoral system. As a part of the ENS the GI smooth muscle has its own pacemaker cells, called the interstitial cells of Cajal (ICCs), which play a key role in the generation and propagation of the electric signal in GI contractility. ICCs generate slow-wave electric impulses to induce action potentials for contractions and determine the frequency of the signals. Functional GI malfunctions can be the consequences of dysregulation in the gut–brain axis. The altered gut-brain interaction can be in the background of functional GI disorders and other motility diseases, such as gastroesophageal reflux disease and irritable bowel syndrome. The investigation of the gut-brain axis and the enteric plexus activity helps to promote the understanding of GI diseases and the therapies for GI disorders and stress-induced GI malfunctions .

The other major unsolved problem is premature delivery, which is defined as delivery occurring before 37 completed weeks of gestation also caused by smooth muscle overactivity. Premature labour is the major contributor to perinatal mortality and morbidity, with an average rate of 10-12% in developing and developed countries and accounts for 75%–85% of all neonatal deaths.

Myometrial contraction is regulated by sexual and stress hormones, the autonomic nervous system, ion channels and transmitters. Dysregulation of the myometrial contractility can lead to either preterm or slow-to-progress labour. ICCs have been immunohistochemically detected in a variety of smooth muscle tissues as well, including the myometrium. These myometrial cells could behave as sensors, controlling myometrial contractility, depending on sexual and also the stress hormone levels. It has been suspected that ICCs are associated with myometrial motility disorders, which may have a role in the regulation of labour in the pregnant uterus.

Besides, preterm delivery is related to stressful life events, anxiety, depression, lack of psychosocial support and physical abuse. Patients with anxiety and depressive disorders also have a higher incidence of functional GI disorders that are frequently unrecognized, these organs respond to psychosocial distress through smooth muscle contractions.

Identification of the signals from the various smooth muscle organs is an essential requirement for the development of non-invasive clinical methods for the diagnosis of motility disorders of given parts of the GI tract and for the prediction of premature birth.

Motion artifacts are major obstacles to the various electromyographic examinations in wakeful subjects. The skeletal muscle induced motion artifacts have higher amplitude compared to the basal activity, which may cause large distortion in the measurement. Therefore these disturbing signals have to be filtered out, however, in the case of overlapping frequencies, filtering can result in significant data loss.

2. AIMS

The first aim of our study was to identify the slow wave frequency parameter of the gastrointestinal tract, and characterization of the uterine electrical activity of the pregnant rat. To attain this goal, we have developed a method with which to follow up the changes in the myoelectric activity of gastrointestinal tract and pregnant uterus in parallel with the mechanical contraction in anaesthetized rats.

The second aim was to follow up the stress condition induced alteration in the GI tract and to measure the effects of central nervous depressants by smooth muscle electromyography in wakeful rats.

To achieve these goals we initiated in vivo experiments in male and female rats.

3. MATERIALS AND METHODS

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/198/2013 and IV/3796/2015).

Sprague-Dawley rats were involved in our studies. Each animal was fasted for two hours before the experiments.

3.1 Detection of gastrointestinal myoelectric activity

Male rats were anaesthetized, thereafter the total GI tract was resected with the exception of one segment (stomach, small intestine or large intestine) from the abdomen. A bipolar thread electrode pair was inserted into the the target organ, while a bipolar disk

electrode pair was placed subcutaneously above the specific segment of the GI tract. An implantable strain gauge was sutured onto the surface of the stomach, ileum or caecum in order to detect the mechanical contractions.

The electric signals were recorded and analysed by an on-line computer and amplifier system for 60 min. The recorded signals were analysed by fast Fourier transformation (FFT). The frequency of the electric activity was characterized in cpm, and the magnitude of the activity was described as power spectrum density (PsD). The mechanical contractions were evaluated by area under the curve analysis of the primary contractility curves.

In the case of anaesthetized, non-GI tract-resected rats, a bipolar disk electrode was placed under the abdominal skin and 3 strain gauges were sutured one by one onto the surface of the stomach, ileum and caecum.

A dose of neostigmine (20 μg/kg) was administered and after 30 min a dose of atropine (300 μg/kg) i.v. for the GI tract-resected and intact rats. Following the addition of each drug, the AUC and FFT of 30-min periods were evaluated. The effects were expressed as percentages of the spontaneous activity. The AUC, cpm and PsDmax values were determined and compared statistically.

3.2 **Detection of myoelectric activity**

Female, full term pregnant rats were anaesthetized, thereafter the total GI tract was resected. A bipolar thread electrode pair was inserted into the myometrium, while a bipolar disk electrode pair was placed subcutaneously above the uterus. An implantable strain gauge was sutured onto the surface of the left uterine horn.

The electric signals were recorded and analysed by the same on-line computer and amplifier system for 60 min. The recorded signals were analysed by fast FFT. The highest peak of PsD was considered during the evaluation. The mechanical contractions were evaluated by AUC analysis of the primary contractility curves.

In the case of anaesthetized, non-GI tract-resected rats, a bipolar disk electrode was placed under the abdominal skin and 2 strain gauges were sutured one by one onto the surface of the uterus and caecum.

3.2.1 Pharmacological investigations

Two doses of oxytocin (1 μg/kg) were administered after recording the basal activity, 15 min. apart. After 30 min, a dose of terbutaline (50 μg/kg) was injected i.v. both for GI-resected and non-GI-resected rats. One dose of neostigmine (20 μg/kg) and one dose of atropine (300 μg/kg) i.v. were administered 30 min apart for non-GI-resected rats on the 22^{nd} day of pregnancy. Following the administration of each drug, the AUC and FFT of 30-min periods were evaluated. The effects were expressed as percentages of the spontaneous activity. The AUC, cpm and PsD_{max} values were determined and compared statistically.

3.3 **Stress measurements**

Male rats were anaesthetized, then a bipolar disk electrode pair was fixed subcutaneously above the gastrointestinal tract. The connecting cable of the sensor to the swivel was led subcutaneously and the terminal was led out through the skin of the neck.

The animals were not restricted in their movements for 30 min while recording basal GI tract activity (control). Then the rats were anaesthetized and fixed onto a glass plate by strong sticky belts. The rats were laid on the abdomen and were not able to move or turn around. After full awakening, the GI activity was recorded again for 30 min under this stress condition. When diazepam (5 mg/kg) or haloperidol (1 mg/kg) was administered intraperitoneally for the given groups of rats, the treatments were done after recording the basal activity. Then 30-min recording was carried out for each drug to determine their actions before and during stress condition.

The myoelectric signals were recorded and analyzed by the same computer system. The FFT of 30-min periods were evaluated. During the evaluation, the EMG spectrum of basal activity was compared to the activities after drug treatment or during stress period. The stress-induced alterations were expressed as percentage of the spontaneous activity.

To remove the motion artifacts, a digital cutter was built into the software. The edge values of the limiter were set by the motion artifact-free sections of the records. Thereby, we were able to cut the artifact signals by their obviously high outlier amplitude.

At the end of each period of 30 min, samples of 0.5 ml blood were collected from the tail veins to separate plasma for hormone assay and HPLC analysis. The organ samples for haloperidol determination were collected after termination by CO_2 inhalation.

The plasma concentration of corticosterone was measured by enzyme-linked immunosorbent assay (ELISA) from the collected samples. A Mouse/Rat Corticosterone ELISA kit was used for the quantification of corticosterone.

The drug concentration of the plasma or tissue homogenate samples was determined by HPLC analysis. The chromatographic separations were performed on a Kromasil Eternity C18 analytical column at 35 ºC. The detection wavelength was 245 nm.

For the ex vivo contractility studies in isolated organ bath the animals were terminated by CO_2 inhalation. The stomach, ileum and cecum were dissected, rinsed with Tyrode solution. The prepared organ strips were incubated for 60 min, then the control contractions of smooth muscle were evoked by KCl (25 mM) and a cumulative dose-response curve of 10^{-4}–10^{-9} M diazepam or haloperidol was obtained. The activity of gastric strips, ileal and cecal rings was measured with a gauge transducer. The haloperidol or diazepam effects were characterized by the percentage of the AUC values of recorded contractions before and after adding them into the organ bath. The analyzed periods were 5 min both for the control and the drug-altered contractions.

4. RESULTS

4.1 Gastrointestinal smooth muscle myoelectric activity

The primary EMG curves and mechanical contractions from partially GI tract-resected rats revealed the different characteristics of the stomach, ileum and caecum. When the primary EMG curves were transformed by FFT, the resulting cpm values differed for each individual GI tract segment.

The characteristic cpm values for the stomach, ileum and caecum were 3-5, 20-25 and 1-3 cpm, respectively (**Fig. 1A**). The maximum intensity of the signals (PsD_{max}) was higher with the thread electrodes in the given cpm ranges (**Fig. 1B**).

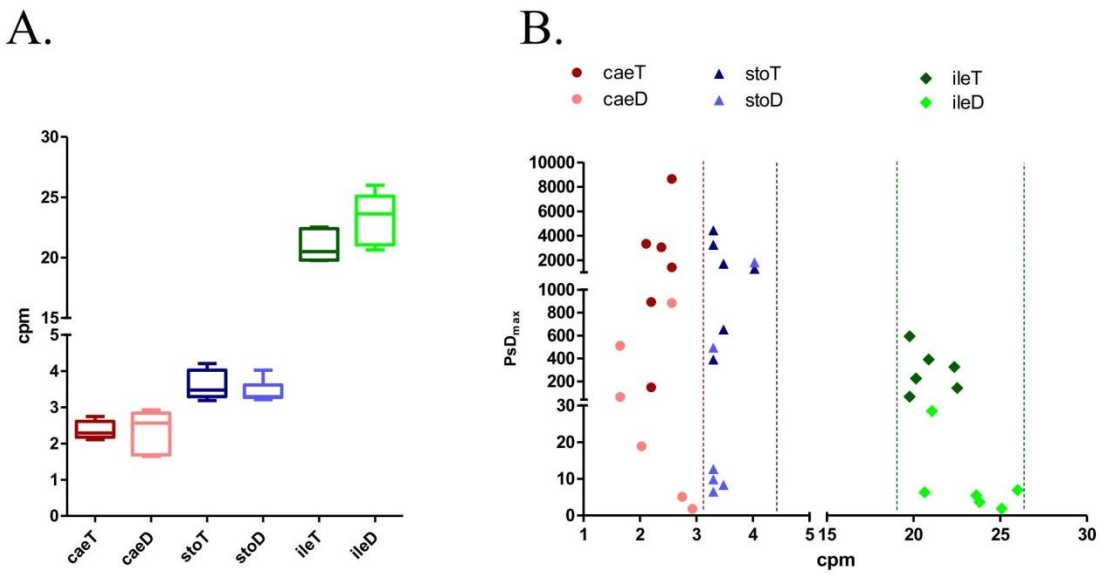

Figure 1. Cycles per minute (cpm) values of the spectra obtained by Fast Fourier transformation for the stomach (sto), ileum (ile) and caecum (cae), measured with thread (T) and disk (D) electrodes.

Neostigmine increased, while atropine decreased both the electrical and mechanical signals in the partially GI tract-resected rats. The FFT analysis revealed significant changes in the PsD_{max} values, while the AUC analysis demonstrated similar changes in the contractions in each investigated GI tract segment (**Fig. 2A-C**).

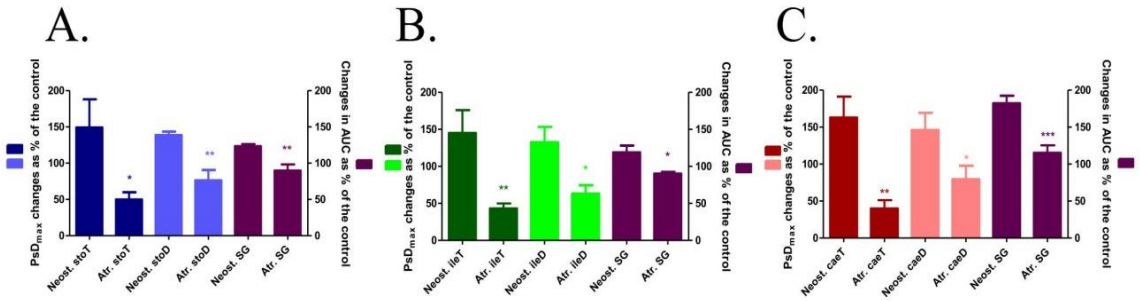

Figure 2. Changes in myoelectric and mechanical gastrointestinal (GI) activity after neostigmine (Neost.) and atropine (Atr.) treatments in partially GI tract-resected rats. The electric signals were detected with thread (T) and disk electrodes (D) and analysed by fast Fourier transformation with GI tract segment specific filtering. The contraction was measured with a strain gauge (SG). The intensity of electric signals was expressed as the maximum of the power spectrum density (PsD_{max}), while the mechanical contraction was evaluated by area under curve (AUC) analysis. In the stomach (sto) (**A**), ileum (ile) (**B**) and caecum (cae) (**C**), neostigmine increased, while atropine reduced the electric and mechanical activities of the smooth muscles relative to the basic activity (100%). ($p<0.05$: *; $p<0.01$: **; $p<0.001$: ***)

We also determined the pharmacological response in the non-GI tract-resected rats. Neostigmine increased, while atropine decreased the filtered electric and mechanical signals in all the GI tract segments. The extents of stimulation or inhibition were similar in the stomach, ileum and caecum (**Fig. 3A-C**).

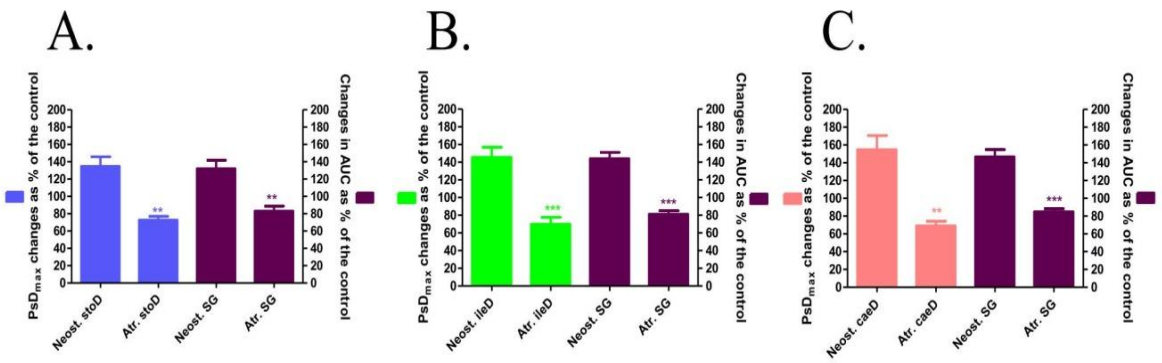

Figure 3. Changes in myoelectric and mechanical gastrointestinal (GI) activities after neostigmine (Neost.) and atropine (Atr.) treatments in non-GI tract-resected rats. The electric signals were detected with abdominal disk electrodes (D) and the contraction was measured by strain gauge (SG).

A very good correlation was found between the changes in the PsD_{max} and AUC values in all the GI tract segments (**Fig. 4A-C**).

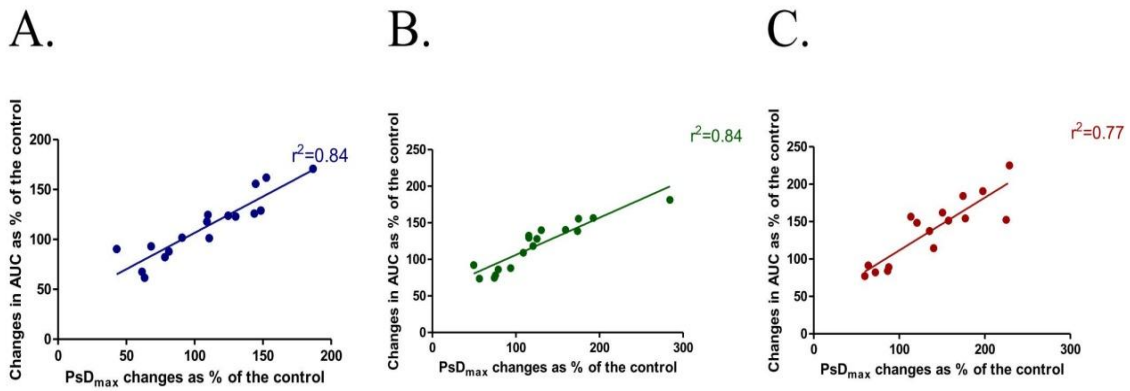

Figure 4. Correlations between myoelectric and mechanical responses induced by neostigmine and atropine treatments in non-gastrointestinal (GI) and GI tract-resected animals. Linear regression analysis revealed a very good correlation between the changes in PsD_{max} and AUC values in the stomach (**A**), ileum (**B**) and caecum (**C**).

4.2 **Pregnant uterus smooth muscle myoelectric activity**

Primary EMG curves from GI tract-resected rats represent characteristic myoelectric signals recorded by thread or disk electrodes. When the primary EMG curves were transformed by FFT, the resulting spectra had sharp and dominant peaks.

The characteristic cpm value for the uterus was found between 1-2.5 cpm measured by thread or disk electrodes (**Fig. 5A**). The PsD_{max} representing the maximum intensity of the signals was higher with the thread electrodes in the cpm range 1-2.5 (**Fig. 5B**).

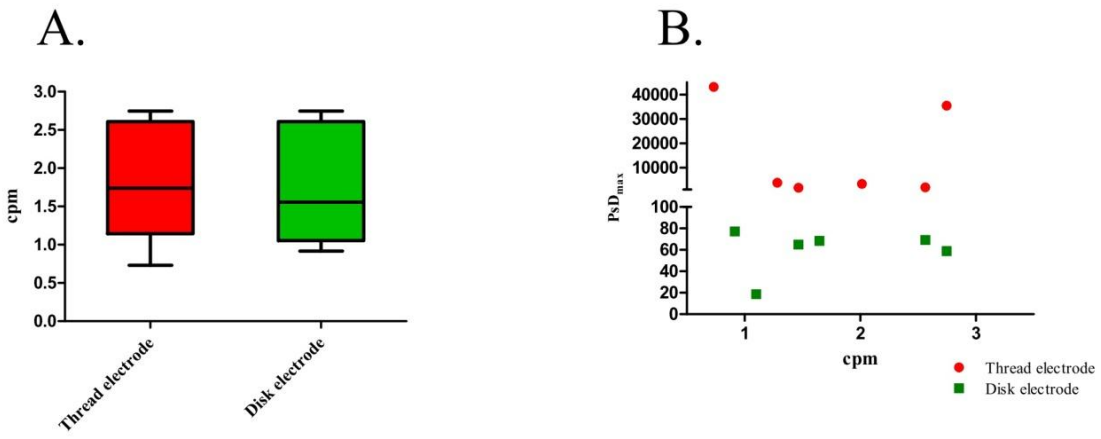

Figure 5. Cycles per minute (cpm) values of the spectra obtained by Fast Fourier transformation, measured with thread and disk electrodes. In the given cpm ranges, the PsD_{max} values detected with the thread electrodes were usually higher than those with the disk electrodes.

In pharmacological experiments oxytocin increased, while terbutaline decreased both the electrical and mechanical signals of the uterus in the GI tract-resected rats (**Fig. 6**).

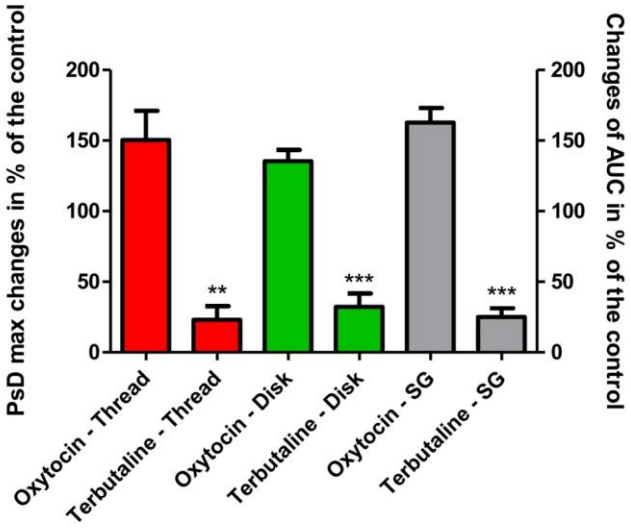

Figure 6. Changes in myoelectric and mechanical uterine activity after oxytocin and terbutaline treatments in GI tract-resected rats. Oxytocin increased, while terbutaline reduced the electric and mechanical activities of the smooth muscles relative to the basic activity (100%). ($p<0.05$: *; $p<0.01$: **; $p<0.001$: ***)

The FFT analysis revealed significant changes in the PsD_{max} values, while the AUC analysis demonstrated similar changes in the mechanical contractions. The extents of stimulation or inhibition were similar in both mechanical and electrical changes. A very good correlation was found between the changes in the PsD_{max} and AUC values measured by thread (**Fig. 7A**) or disk electrode (**Fig. 7B**) in comparison with SG signals.

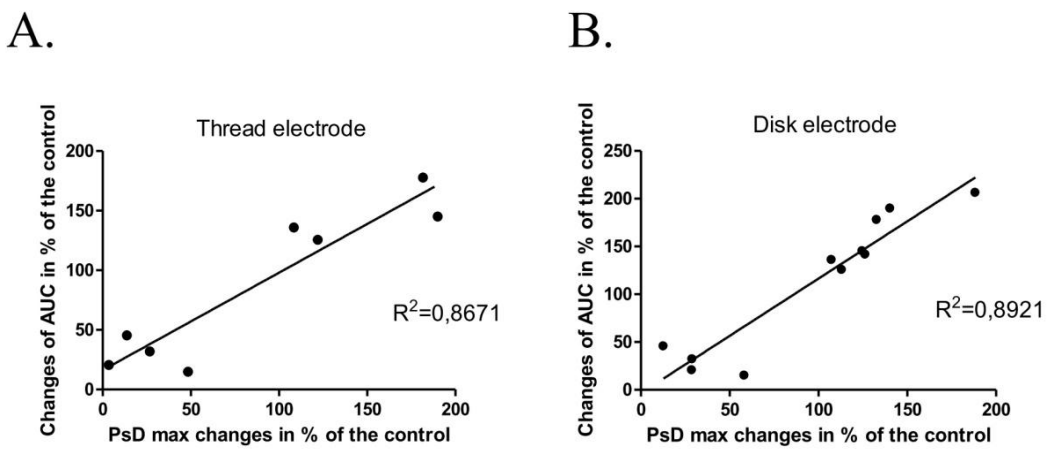

Figure 7. Correlations between myoelectric and mechanical responses induced by oxytocin and terbutaline treatments in GI tract-resected animals. Linear regression analysis revealed a very good correlation between the changes in PsD_{max} and AUC values in the uterine signals recorded by thread (**A**), or disk electrode (**B**).

The myoelectric and contractility responses of caecum and pregnant myometrium have been recorded for different drugs in parallel. We have administered neostigmine and atropine, which mainly act on the GI tract, and oxytocin and terbutaline, which have effects on the myometrium. Neostigmine and atropine treatment caused significant changes in the myoelectric signal of the caecum (**Fig. 8A**), while oxytocin and terbutaline had actions both the electrical and mechanical signals of the uterus only (**Fig. 8B**).

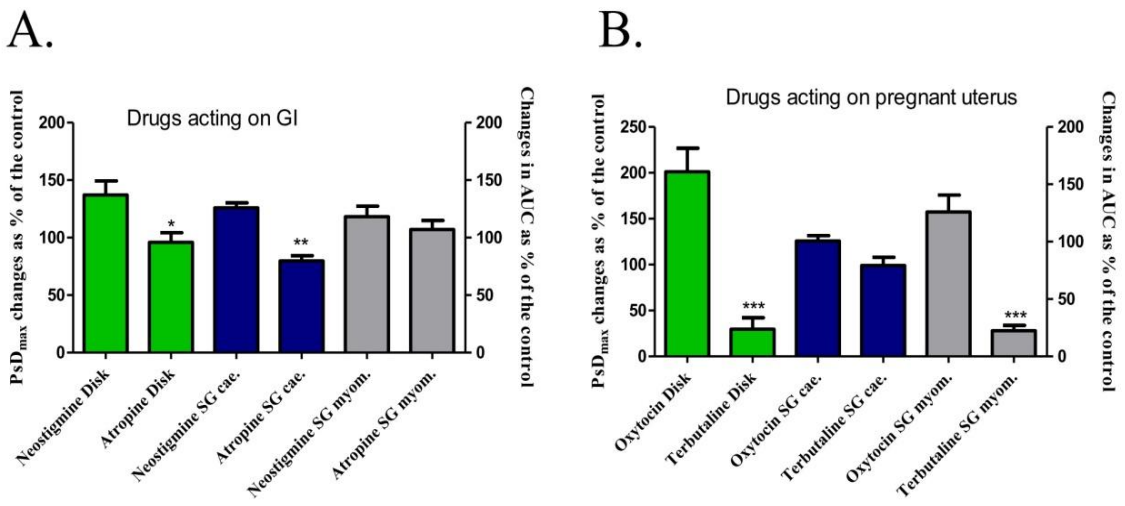

Figure 8. Changes in myoelectric and mechanical gastrointestinal (GI) and myometrial activities after neostigmine and atropine (**A**), or oxytocin and terbutaline (**B**) treatments in non-GI tract-resected rats. Significant changes were found in case of neostgimin-atropine treatment in cecal signals, while oxytocin-terbutaline treatment affected the uterine smooth muscle signals relative to the basic activity (100%). ($p<0.05$: *; $p<0.01$: **; $p<0.001$: ***).

4.3 **Detection of stress in wakeful rats**

The motion artifact was separated from the basal electric activity of the GI smooth muscle by the cutter built into the software. The digital cut reduced the motion artifact elevated PsD_{max} values to the range of values in anaesthetized rats, but did not further reduce the values detected in anesthetized rats.

Immobilization stress significantly increased the plasma level of corticosterone. In parallel, the PsD_{max} values in the whole GI tract were elevated 1.5-2-fold during immobilization. The greatest increase was found in the gastric values, although the increase in the gastric PsD_{max} values was not significantly higher than the increase in the small or large intestine values (**Fig. 9**).

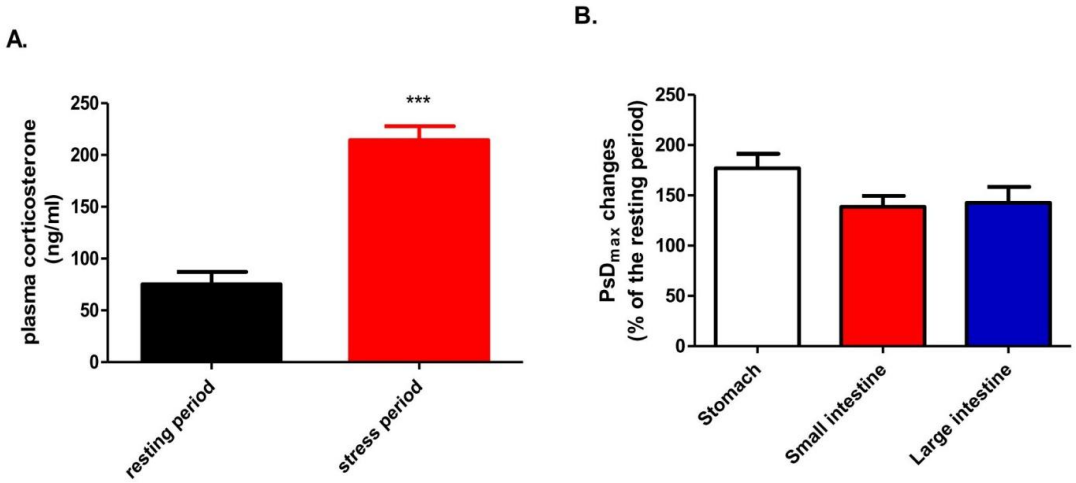

Figure 9. Immobilization stress induced a significant increase in the plasma corticosterone level of rat (***: $p<0.001$) (**A**). The stress condition also raised the power spectrum density (PsD_{max}) values of the FFT spectra from the stomach, small intestine and large intestine as compared with the resting period. (**B**).

Diazepam treatment reduced the resting EMG activity by 40-60%, while the stress-induced PsD_{max} values were set back to the resting level, reaching around 100% in all the three GI tract sections. Haloperidol treatment reduced the resting EMG activity by 40%, but immobilization stress was not able to induce significant elevation in the myoelectric activity of the whole GI tract **(Fig. 10)**.

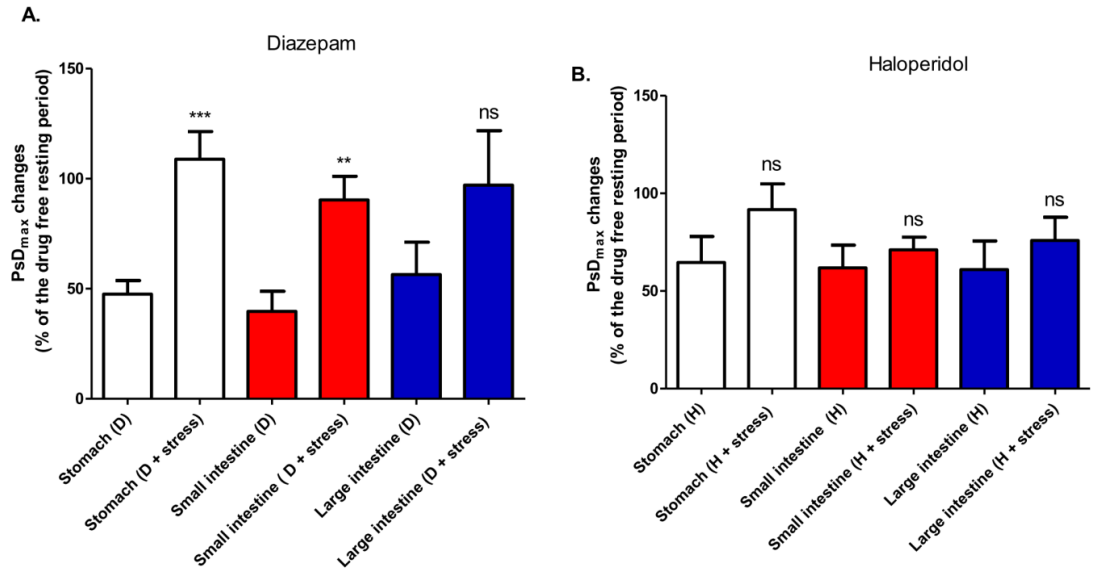

Figure 10. Changes in power spectrum density maximum (PsD_{max}) values after diazepam (**A**) or haloperidol (**B**) treatments in conscious rats under immobilization-induced stress. (ns: not significant; $p < 0.01$**; $p < 0.001$***)

Both diazepam and haloperidol treatments reduced the plasma corticosterone levels during the resting period by approximately 30 %, however, these alterations were not significant. Only haloperidol was able to blot out the stress hormone inducing effect of immobilization **(Fig. 11)**.

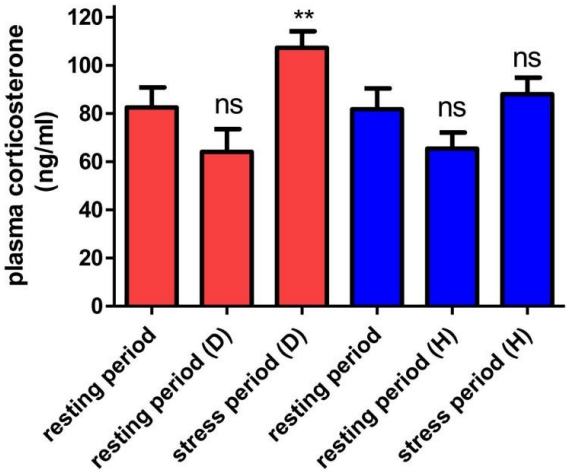

Figure 11. Alterations of plasma corticosterone levels by drug treatments (red columns: diazepam, blue columns: haloperidol) and immobilization stress in rats (D. diazepam; H: haloperidol; ns: not significant;*: $p<0.05$; **; $p<0.01$)

The plasma level of diazepam was 0.42 μg/ml at the end of the resting period, and its level was reduced to 0.13 μg/ml at the end of the stress period. Haloperidol was not detectable in the plasma even at the end of the resting period, but it was found in the brain, liver and lung. At the end of the stress period, the levels of haloperidol were still considerable in these organs.

Significant correlation was found between the drug induced change in the PsD_{max} values of the stomach, small intestine or large intestine and the change in corticosterone plasma levels. The levels of correlations were the same in all the three GI tract segments **(Fig. 12)**.

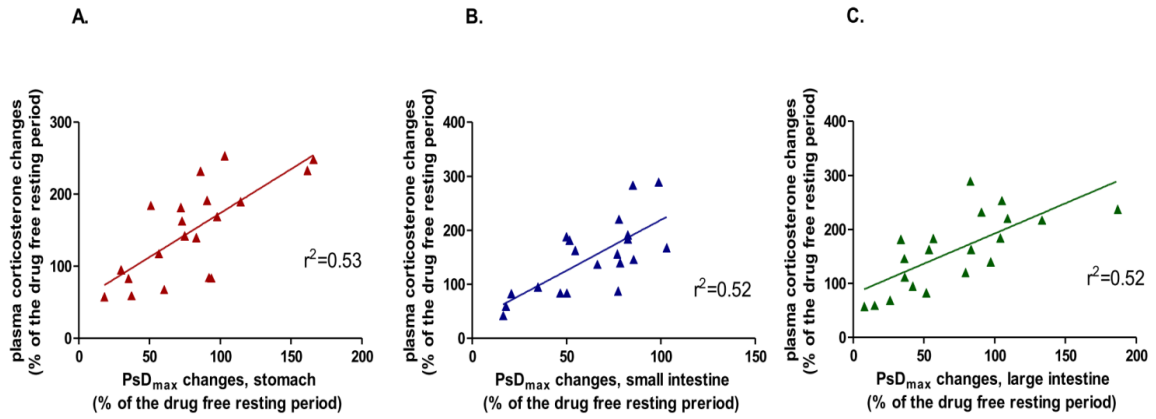

Figure 12. Correlation between the changes of the power spectrum density maximum (PsD_{max}) values in the given sections of the gastrointestinal system (**A**: stomach, **B**: small intestine, **C**: large intestine) and the changes of corticosterone plasma levels in rats. The linear regression analysis proved a significant correlation between PsD_{max} and corticosterone alteration.

Neither diazepam nor haloperidol elicited any significant relaxing effect on gastric strip, ileal or cecal rings in isolated organ studies.

5. DISCUSSION

Several attempts have been made to record the GI tract and uterine motilities with either implanted or surface electrodes *in vivo*, but the ideal method has still not been found. The focus during the development of our research model was to create a smooth muscle electromyographic tool for simple and reliable measurements on these organs with pharmacological and diagnostic aims. We applied simple electrode pairs, and we used built-in digital filters in the frequency range 0-30 cpm to separate the noise-free, slow-wave smooth muscle signals.

The slow waves of smooth muscle electric activity are usually characterized with cpm that is the 60-fold value of Hz. The FFT calculated range for the stomach (3-5 cpm) was in harmony with earlier findings in humans. That for the caecum (1-3 cpm) was lower, while that for the ileum (20-25 cpm) was higher than earlier findings in humans or rodents.

In order to determine the individual myoelectric signals of the pregnant uterus and the GI tract segments, we resected non-investigated parts. Two pairs of electrodes measured the myoelectric signals from the left segment: one was inserted into the organ, while the other was positioned under the skin of the abdomen. The most intensive signals were provided by the organ-inserted electrodes, and these signals were also measured from the abdomen to allow a comparison of the detectability of the activities between the two sites. We found that pregnant uterine smooth muscle frequency is between 1-3 cpm.

The experiments on the partially resected animals revealed that the stomach, ileum and caecum have different mechanical and myoelectric activities, which are well distinguishable from each other. However, the myoelectric signals of the pregnant uterus and caecum overlap.

The PsD_{max} values usually express the intensity of myoelectric signals. We observed a very good correlation between the changes in mechanical contractile response and the changes in electric PsD_{max} values. This means that the changes in PsD_{max} values recorded even on the abdominal wall perfectly reflect the real changes in the contractions of the GI tract and uterine smooth muscle.

The PsD_{max} values varied depending on the intensity of the contractions. We proved this correlation via the well-known GI tract motility increasing and decreasing effects of neostigmine and atropine, respectively. The oxytocin-induced increase and the terbutaline-induced decrease were clearly detectable via the observation of the changes in the PsD_{max} intensity of the myoelectric spectra from pregnant uterus.

The frequency range for the pregnant uterus and the colon myoelectric activity are almost equals therefore we launched a series of experiments with non-GI-resected pregnant animals to investigate how we can distinguish the myoelectric signals of these two types of smooth muscles. We found that drugs acting on myometrial activity altered the PsD_{max} with a good correlation to myometrial mechanical contractions, but they did not affect cecal activity. The drugs acting on the caecum elicited similar action on cecal activity without influencing the myometrial response. It means that we can follow the uterine contractions with the evaluation of PsD_{max} values when the uterine activity is predominant. During pregnancy the mass of the uterus is significantly increased, and its activity is predominant over the cecal contractions. Our method seems to be proper for the detection of the uterine activity in that late-pregnancy period.

On the other hand, the non-invasive and reliable measurement of stress condition by physiological parameters is still an unsolved clinical problem. Although the relationships between stress condition and organ systems like cardiovascular, dermal or GI systems are obvious, the level of stress is mostly appraised by psychological tests.

Acute stress response enhances the motility in the GI tract, which is controlled by peripheral neuropeptides and corticosterone-releasing hormone. We hypothesized that our EMG method can be applicable to awake rats and to measuring the correlation between GI tract motility and stress condition.

Our first task was to identify the EMG signals of motion artifacts and then develop a digital cutter to remove them from the record without significant loss of smooth muscle

data. Our results suggest that we have successfully kept the smooth muscle signals with the effective removal of motion artifact.

Immobilization can increase the stress factors within quite a short period of time, 30-60 min can be enough for the significant elevation of stress hormone levels in rodents. Both corticosterone and PsD_{max} values were increased during the immobilization, and the correlation found between the two parameters indicates that the severity of acute stress can be predicted by the detection of smooth muscle EMG in awake rats.

The plasma levels of both diazepam and haloperidol were checked at the end of resting period and at the end of stress period. The measurement of plasma levels was important to justify the presence of effective concentrations during our experiments.

While haloperidol blocked the rise of corticosterone during acute stress, diazepam was only able to reduce the increase in the stress hormone level. This difference between the effects of these two types of drug was clearly detectable in the smooth muscle EMG response of the GI tract. The isolated organ bath studies proved that neither diazepam nor haloperidol had a direct smooth muscle relaxing effect on the GI tract smooth muscles, therefore the responses found in awake rats originated from their central nervous system effects, possibly mediated through the brain-gut axis.

6. CONCLUSION

Based on our results, we believe that we have successfully developed a method for the *in vivo* detection of GI activity that serves a basis for non-invasive, reliable and specific GI tract segment measurements. We have clarified the different myoelectric activities of the stomach, ileum and caecum. Additionally, our preclinical method is able to investigate the pregnant myometrial activity, *in vivo*, and already suitable for pharmacological investigations for drugs acting on GI tract or uterine contractions.

It also can be concluded that our smooth muscle EMG instrument can measure the level of acute stress in awake rats, which shows correlation with the stress hormone plasma level. This finding is further evidence for the function of the brain-gut axis. Our technique is also applicable to the investigation of different drugs affecting the central nervous system through the GI tract myoelectric response.

We suppose that this method serves as a good basis for the development of a new clinical tool to investigate the pathophysiological processes in GI tract or pregnant uterus contractility in animals and assess the stress and anxiety levels of patients in different kinds of psychological disorders and during pharmacotherapy.

List of publications

This thesis is based on the following publications:

I. Szucs KF, Nagy A, Grosz G, Tiszai Z, Gaspar R: **Correlation between slow-wave myoelectric signals and mechanical contractions in the gastrointestinal tract: Advanced electromyographic method in rats**. *Journal of Pharmacological and Toxicological Methods* 2016, **82**:37-44.

[IF: 2.238]

II. Szucs KF, Grosz G, Sule M, Nagy A, Tiszai Z, Samavati R, Gaspar R: **Identification of myoelectric signals of pregnant rat uterus: new method to detect myometrial contraction**. *Croatian Medical Journal* 2017, **58**(2):141-148.

[IF: 1.619]

III. Szucs KF, Grosz G, Sule M, Sztojkov-Ivanov A, Ducza E, Marki A, Kothencz A, Balogh L, Gaspar R: **Detection of stress and the effects of central nervous system depressants by gastrointestinal smooth muscle electromyography in wakeful rats**. *Life Sciences* 2018, **205**:1-8.

[IF: 2.936]

Other publication unrelated to this thesis:

I. Berko S, Szucs KF, Balazs B, Csanyi E, Varju G, Sztojkov-Ivanov A, Budai-Szucs M, Bota J, Gaspar R: **Electroporation-delivered transdermal neostigmine in rats: equivalent action to intravenous administration**. *Drug Design, Development and Therapy* 2016, **10**:1695-1701.

[IF: 2.822]