Gut region-specific alterations of the endogenous heme oxygenase system and pro-inflammatory cytokines in the enteric neurons of streptozotocin-induced diabetic rat model

Summary of Ph.D. Thesis

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1. Introduction

The enteric nervous system (ENS) is the intrinsic nervous system of the gastrointestinal (GI) tract which is structurally organized into two major ganglionated plexuses: myenteric (Auerbach’s) and submucous (Meissner's) plexus. Enteric neurons are responsible for the regulation of GI functions such as mucosal secretion, motility, blood flow, immune and inflammatory processes. Therefore, any dysregulation in the ENS can lead to GI complications, like gastroparesis, motility disorders or delayed gastric emptying.

It is reported that type 1 diabetes mellitus (T1DM) patients often suffer from GI motility abnormalities like nausea, vomiting, constipation and diarrhea. The main underlying cause of these GI complications is the altered expression of nitric oxide synthase (NOS) in the gut.

Nitric oxide (NO) plays a significant role as a non-adrenergic non-cholinergic neurotransmitter in regulating blood flow, sphincters, and motility of the gut. We have recently demonstrated that nitrergic myenteric neurons located in different intestinal
segments display different susceptibilities to diabetic damage. They also exhibit different levels of responsiveness to insulin treatment, which indicates the importance of the molecular difference in the neuronal microenvironment in the pathogenesis of diabetic nitrergic neuropathy. We have also demonstrated that the mesenterial capillaries supplying the myenteric ganglia and the faecal-associated microbiota are also main targets of diabetic injuries.

Growing evidence indicates that long-lasting hyperglycaemia induces oxidative stress, decreases the effectiveness of the endogenous antioxidant protection and therefore plays a major role in the initiation of diabetes-related neuronal damage. Heme oxygenase (HO) is a rate limiting enzyme in the heme catabolism which produces biologically active carbon monoxide, iron and bilirubin. Three main HO isoforms, encoded by different genes, have been reported. These isoforms have different expression patterns mainly in tissues and cells. HO2, a constitutive enzyme, is highly expressed in neuronal tissues and acts as a critical mediator of cellular homeostasis, whereas HO1, a stress-inducible protein, expressed in relatively low level in most tissues. The induction of HO1 have been reported to play a protective role against oxidative stress, ischaemia-reperfusion, hypoxia, reactive oxygen species and inflammation. In addition, in rat ileum the antioxidant HO2 protects those NOS-containing neurons in which it is co-localized from oxidative stress. Due to the beneficial effects of the HO system, these endogenous antioxidants can be very important players in the prevention of oxidative injury and diabetic GI complications.

Besides alterations of endogenous antioxidant system, the pro-inflammatory cytokines are also considered to be important cellular mediators in ENS pathology. It has been reported that the expression of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin 1 beta (IL1β) and interleukin 6 (IL6) are strongly upregulated in inflammatory as well as in neurological diseases. Other than the deleterious effect to neurons, it has been reported that pro-inflammatory cytokines also act as neuroprotective agents in several disease conditions. However, to date only few studies have reported the importance of HO system and pro-inflammatory cytokines in the enteric neurons in type 1 diabetic rats.

2. Aims

**HO system in the myenteric plexus**

- Is there any gut segment-specific difference in the number of myenteric HO1- and HO2-IR neurons?
- Is there any effect of diabetes on the HO-nNOS colocalization in the myenteric plexus of the different intestinal segments?
- Is there any effect of the diabetes on the serum and tissue level of HO1 and HO2?

**HO system in the submucous plexus**
- Is there any segment-specific effect of diabetes and immediate insulin treatment on the proportion of the nNOS-, HO1- and HO2-IR neurons?

**Pro-inflammatory cytokines**
- Is there any gut segment-specific difference in the expression of TNFα and IL6 in the myenteric ganglia and surrounding capillary endothelium of the control, diabetic and insulin-treated rats?
- Is there any effect of the diabetes and insulin-treatment on the level of TNFα, IL1β and IL6 in tissue homogenates containing the myenteric or submucous plexus?

### 3. Materials and methods

In this study, we established a streptozotocin (STZ)-induced diabetic rat model which is suitable for investigating the effects of chronic hyperglycaemia and immediate insulin replacement in the ENS and its microenvironment along the GI tract.

**Animal model**

For the 10-week chronic hyperglycaemic study the rats were divided randomly into two groups: STZ-induced diabetics (n=6) and sex- and age-matched controls (n=10). Besides these two groups, for the investigation of the HO system in the submucous plexus and the expression of the pro-inflammatory cytokines, a third group, an insulin-treated diabetic group was also used (n=4).³

**Blood collection and tissue handling**

Ten weeks after the onset of hyperglycaemia, the blood samples were collected for glycated haemoglobin (HbA1c) assay and enzyme-linked immunosorbent assay (ELISA). After the blood collection, the gut segments of the control, STZ-induced diabetic and insulin-treated diabetic rats were dissected and rinsed in 0.05 M phosphate buffer (PB, pH 7.4). A 10 cm-long intestinal segments were taken from the duodenum (1 cm distal to the pylorus), the ileum (1 cm proximal to the ileo-caecal junction) and the proximal colon and were processed for immunohistochemical studies and ELISA.

**HbA1c assay**

Serum level of HbA1c was analysed by standard clinical chemistry assay on an Automated Chemistry Analyzer (BiOLis 24i, Tokyo Boeki Machinery Ltd, Tokyo, Japan).¹¹
Measurement of serum protein levels by ELISA

Serum levels of HO1 and HO2 were measured by means of quantitative ELISA according to the manufacturer’s instructions (SunRed Biotechnology, Shanghai, China). Optical density was measured at 450nm (Benchmark Microplate Reader; Bio-Rad, Budapest, Hungary). The serum HO1 and HO2 concentrations were expressed as ng/ml.

Double-labelling immunohistochemistry

After fixation of the different gut segments in 4% paraformaldehyde solution, whole-mounts from myenteric and submucous plexus were prepared and processed for the double-labelling immunohistochemistry. The samples were incubated overnight with primary antibodies (nNOS, HO1, HO2, HuC/D, peripherin) in various combinations. We used secondary antibodies (Alexa Fluor 488, Cy3) to evaluate the myenteric ganglia containing HO-immunoreactive (IR) and nNOS-HO-IR neurons and the submucous ganglia containing nNOS-IR, HO1-IR, and HO2-IR neurons under different experimental conditions.

Postembedding immunohistochemistry

Small pieces of the gut segments were fixed, embedded and processed for quantitative postembedding immunohistochemistry. Ultrathin sections from each block were preincubated in 1% bovine serum albumin, then in primary antisera (TNFα and IL6) overnight, followed by the species-specific secondary antibodies (anti-rabbit or anti-mouse IgG conjugated to 18 nm colloidal gold), with extensive washing in-between. Counting was performed on digital photographs of 5 myenteric ganglia and 5 mesenteric capillaries per intestinal segment in each experimental group at a magnification of 20000×. The total number of gold particles per unit area was counted in the entire profile of the myenteric ganglia and the endothelium of capillaries in the vicinity of the myenteric plexus with the ImageJ software (Wayne Rasband; National Institutes of Health, USA).

Measurement of tissue HO1, HO2, TNFα, IL1β and IL6 concentrations

Tissue homogenates from different intestinal segments containing the myenteric or submucous plexus were centrifuged at 5000 rpm for 20 min at 4 °C and used to determine the total protein content. Then the tissue total protein content was measured using the Bradford protein micromethod. Tissue homogenates obtained from the different intestinal segments were used to measure levels of HO1, HO2, TNFα, IL1β and IL6 by means of quantitative ELISA according to the manufacturer’s instructions (SunRed Biotechnology, Shanghai, China). Optical density was measured at 450 nm (Benchmark Microplate Reader; Bio-Rad, Budapest, Hungary). Tissue concentrations of HO1, HO2 were expressed as ng/mg protein, while TNFα, IL1β and IL6 were expressed as pg/mg protein.
**Statistical Analysis**

Statistical analysis was performed with one-way ANOVA and the Newman–Keuls test. All analysis was carried out with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). A probability of P<0.05 was set as the level of significance. All data were expressed as mean ± SEM.

**4. Results**

**Evaluation of HO-IR and nNOS-HO-IR neurons in myenteric ganglia**

In the controls, the number of HO-IR and nNOS-HO-IR myenteric neurons was the lowest in the ileal and the highest in the colonic segment. In diabetics, it increased the most extensively in the ileum and was also elevated in the colon. Although the total number of nitrergic neurons decreased in all the segments, the proportion of nNOS-IR neurons co-localizing with HOIs was enhanced robustly in the ileum and colon of diabetics.

**Evaluation of nNOS-IR, HO1-IR, and HO2-IR neurons in submucous ganglia**

The total number of submucous neurons and the proportion of nNOS-, HO1- and HO2-IR subpopulations were not affected in the duodenal ganglia of control, diabetic and insulin-treated rats. While the total neuronal number did not change in either the ileum or the colon, the density of nitrergic neurons exhibited a 2- and 3-fold increase in the diabetic ileum and colon, respectively, which was further enhanced after insulin replacement.

The proportion of HO1- and HO2-IR submucous neurons to the total number of submucous neurons was high in the colon of controls (38.4-50.8%), whereas it was significantly lower in the small intestinal segment (0.0-4.2%, P<0.0001). Under pathophysiological conditions the only alteration detected was an increase of the proportion of HO-IR neurons in the ileum and a decrease in the colon of insulin-treated diabetic rats.

**Intracellular distribution of TNFα and IL6 in the myenteric ganglia and capillary endothelium**

The number of TNFα- and IL6-labelling gold particles was strictly region-dependent in control animals with the numbers increasing to the distal direction. In diabetics, the number of TNFα-labelling gold particles was significantly increased in the duodenal, decreased in colonic myenteric ganglia while it did not show any significant difference in ileal ganglia. However, the number of IL6-labelling gold particles was not affected by diabetes in different gut regions.

Regarding the capillary endothelium, the TNFα gold labelling was slightly altered in duodenal and colonic segments, however, significant increase was only observed in the
ileum of diabetic rats. In contrast, the density of IL6-labelling particles were not significant changed in capillary endothelium even under different experimental conditions. The diabetes-related alterations of TNFα- and IL6 expression were not protected by the immediate insulin replacement in any of the investigated intestinal segments.

**Tissue levels of pro-inflammatory cytokines in the smooth muscle layers and the myenteric plexus**

In tissue homogenates of control rats, the expression of TNFα, IL1β, and IL6 was strictly region-dependent. In the diabetic group, tissue levels of TNFα and IL1β were significantly increased only in the duodenum, whereas in the ileum and colon, levels were unaffected when compared to controls. The concentration of IL6 was decreased in the ileal segment, whereas it was unaltered in the duodenum and colon compared to controls. The immediate insulin treatment induced massive changes in TNFα and IL1β concentrations only in the duodenal segment, while IL6 concentrations were slightly elevated in the duodenum and decreased significantly in the ileum.

**Tissue levels of pro-inflammatory cytokines in the mucosa-submucous plexus**

In tissue homogenates of mucosa and submucosa including the submucous plexus of control rats, the expression of TNFα were strictly region-dependent. In diabetics, TNFα levels were significantly decreased in the ileum and colon, while in the duodenum it slightly, but not significantly increased when compared to controls. Interestingly, IL1β and IL6 were undetectable in the mucosa-submucosa of both control and diabetic groups.

5. Discussion

**HO system in the myenteric plexus**

In support of our earlier finding that the susceptibility of nitrergic myenteric neurons to experimentally induced diabetes is strictly regional\(^3\), the present study provides evidence of gut segment-specific diabetes-related induction of the endogenous HO system and also intestinal region-dependent enhanced co-localization of HO1 and HO2 with nNOS in myenteric neurons. The occurrence of HO1- or HO2-containing myenteric ganglia, and the presence of those ganglia which include nNOS-HO1 or nNOS-HO2 co-localized neurons, was most pronounced in the colon and less represented in the ileum of control rats. Similarly, the number of HO1- or HO2- and nNOS-HO1- or nNOS-HO2-IR neurons was the highest in the colon and the lowest in the ileum under control conditions. Interestingly, in the control duodenum, although the number of HO1- or HO2- and nNOS-HO1- or nNOS-HO2-IR neurons was less than in the colon, still the presence of either HO-IR or
nNOS-HO-IR neurons representing myenteric ganglia was nearly as explicit as in the colon.

In a previous study\textsuperscript{12} we demonstrated evidence that region-specific accumulation of reactive oxygen species leads to regionally distinct activation of antioxidant and apoptotic marker molecules in proximal and distal part of the gut in STZ-induced diabetic rats. This data also suggests that the distal part of the gut is more vulnerable to oxidative stress than the proximal. Therefore, we propose that in the colon, where the baseline oxidative environment is far from optimal, the basal expression of HOs as essential players of the endogenous defence mechanisms is the most pronounced. We suggest that in the ileum, the level of the HO proteins is extremely low in controls, which may result in much lower protection against different pathological stimuli. We also assume that as a result of the adequate oxidative environment and definite baseline expression of HOs and their co-localization with nNOS in the myenteric ganglia in the proximal small intestine, the nitrergic neurons get greater protection and can tolerate hyperglycaemia-related oxidative stress better. Moreover, the highest levels of HO1 and HO2 expression in control duodenum tissue homogenates (including the smooth muscle layers of the gut wall and the myenteric plexus between them) also highlights an intensified protective environment in this particular gut segment and emphasizes the importance of the neuronal microenvironment\textsuperscript{3,13}.

In diabetics, the number of HO1- or HO2-representing myenteric ganglia, as well as the number of those ganglia which contain nNOS-HO1 or nNOS-HO2 co-localized neurons were markedly elevated in the ileum. Likewise, the number of HO1- or HO2-IR and nNOS-HO1 or nNOS-HO2-IR neurons were altered in a region-dependent manner in diabetic rats. On the basis of our previous data, we suggest that the diabetes-related massive changes in the composition of the ileal gut microbiota\textsuperscript{5} may contribute to the enhanced mucosal immune response and the greatest induction of endogenous HO system in this particular gut segment. Accordingly, more nitrergic neurons started to produce HO enzymes. Moreover, this also means that those nNOS-positive neurons which are not expressing HOs will likely degenerate. Similar alterations were seen in the colon, from which we assume that HO-containing nitrergic neurons enjoy higher protection, whereas the others are heavily affected by diabetic damage. In the diabetic duodenum, besides a decreasing number of nNOS neurons, the number of co-localized myenteric neurons did not alter significantly, leading to the same conclusion. In summary, we propose that the reduced number of nNOS neurons means cell loss in the diabetic ileum and colon but a change in neurochemical character in the duodenum\textsuperscript{3,5}.
**HO system in the submucous plexus**

In the present study we demonstrate that the total submucous neuronal number is similar in the duodenum, ileum and colon in the controls, and remains unchanged in diabetes as well as after insulin replacement. This is in agreement with the observation of da Silva et al\(^{14}\), however their investigations are limited to the ileum. Our data reflect that STZ-induced diabetes does not induce degenerative changes in the total population of submucous neurons, unlike our previous data about the myenteric plexus\(^3\). We suggest, in accordance with the opinion of Lopes et al\(^{15}\), that submucous neurons may have greater resistance to reactive oxygen and nitrogen species under diabetic conditions. Our results demonstrate that not only total neuronal density but also the nitrergic subpopulation of the two plexuses are affected differently by the diabetic state. An important finding of this study is that HO1- and HO2-IR neurons are more abundant in the colon than in the small intestinal segments in the controls and STZ treatment did not result in any significant changes in the investigated regions. It is possible that under worsening oxidative circumstances the pro-oxidant basal state in the colon acts as a preconditioning factor inducing the higher physiological activity of both isoforms of the antioxidant HO enzymes.

We found no significant changes in the total number of submucous neurons of the three intestinal regions of insulin-treated diabetic rats. The density of nitrergic neurons showed similar patterns in the two plexuses after insulin replacement, an increase was found from proximal to distal direction, which means significant changes in the ileum and the colon as compared to untreated diabetic rats. As in the case of the nitrergic subpopulation, not the duodenal, but the ileal and colonic HO1- and HO2- IR submucous neurons responded to insulin treatment, but in an opposite way. However, there were no differences between the control and diabetic groups in these two regions and we found an increased density in the ileum, but a decreased abundance in the colon of insulin-treated diabetic rats.

Our results suggest that the three investigated gut segments have different levels of responsiveness to immediate insulin replacement in diabetes regarding the nitrergic and HO-IR subpopulation of submucous neurons. This is confirmed by our earlier findings in the myenteric neurons and in their microenvironment including the capillary endothelium adjacent to the myenteric ganglia\(^4\). The environmental changes of myenteric ganglia were described, such as the alteration of the faeces-associated microbiota in diabetes and insulin-treatment\(^5\). On the other hand, our results are in accordance with the previous contradictory data about the beneficial or harmful effects of insulin\(^3\).
Distribution of TNFα- and IL6-labelling gold particles in the myenteric ganglia

In this study, we quantified TNFα- and IL6-labelling gold particles in the myenteric ganglia and capillaries in their vicinity in control, diabetic and insulin-treated diabetic rats. We provide evidence that the distribution of these pro-inflammatory cytokines is strictly gut region-specific. The density of TNFα- and IL6-labelling gold particles was increased from proximal to the distal part of the GI tract of controls. Interestingly, the density of TNFα-labelling gold particles was significantly higher in the ganglia of diabetic and insulin-treated diabetic duodenum compared to the controls. This could explain that myenteric neurons express this cytokine at high levels in order to protect them from invading pathogenic microbes, thereby preventing tissue injury in this particular gut segments. Our result is correlated with Gonçalves et al.\textsuperscript{16} whose study reported that TNFα plays a significant role in controlling pathogenic bacterial populations and preventing tissue injury in the intestine of TNFRp55/\textsuperscript{-/-} mice. It has also been reported that expression of TNFα plays a dual role in T1DM, this study indicates that islet-specific TNFα expression abrogates the ongoing autoimmune process when induced late, but not in the early phase of T1DM pathogenesis\textsuperscript{17}.

The tissue levels of TNFα and IL1β (including the smooth muscle layers of the gut wall and the myenteric plexus between them) were increased significantly in tissue homogenates of the duodenum of diabetic and insulin-treated diabetic animals, while their levels did not change in the ileum and the colon.

In the ileum, the density of TNFα-labelling gold particles was slightly increased, while in the colon it was significantly decreased in diabetics compared to the control group. It is in correlation with our previous results, we suggested that the colon was the only segment where the anti-apoptotic bcl-2 expression was increased and severe necrosis was also confirmed by electron microscopy in diabetic rats\textsuperscript{12}. The density of TNFα-labelling gold particles was significantly decreased in the insulin-treated diabetics compared to the control group which indicates that immediate insulin treatment did not prevent the expressional changes of TNFα in the duodenum and colon. Interestingly, tissue levels of TNFα and IL1β were unaltered in the ileal and colonic myenteric plexus of different experimental groups, while the level of TNFα in submucous samples decreased significantly in the ileum and colon when compared to controls. Surprisingly, the density of IL6-labelling gold particles was unaffected by diabetes in the myenteric ganglia of duodenum, ileum, and colon. Our finding is in correlation with the results published by Hundhausen et al.\textsuperscript{18}: IL6 expression was declined in the serum of patients with long-standing T1DM, however, its expression was higher during the early stage of the disease.
The density of IL6 gold particles was significantly increased in the duodenum of insulin-treated diabetics compared to diabetics and the control group. This change is in line with our previous results, where immediate-insulin treatment prevented the diabetes-related alteration of the microcirculation in the duodenum, but was not effective in the distal gut segments.

In the ileum, the density of IL6-labelling gold particles in myenteric neurons remained at the same level in the diabetic and the insulin-treated diabetic group. In our previous study, we reported that the nitrergic subpopulation was reduced in the ileum of diabetic rats. Taken together, we suggest that IL6 did not play any potential role in the myenteric neurons of the ileal segment. Similarly, in the colon, the density of IL6-labelling gold particles in the myenteric neurons slightly changed in diabetics when compared to control group. In contrast, in the insulin-treated diabetic group, IL6 expression was downregulated in the myenteric ganglia of the colon.

**Distribution of TNFα- and IL6-labelling gold particles in the capillary endothelium**

Similarly to our results in myenteric ganglia, the density of TNFα- and IL6-labelling gold particles in the endothelium showed segment-specific differences along the GI tract. The density of TNFα-labelling gold particles was slightly increased in the capillary endothelium of diabetic duodenum when compared to the controls. Several studies have reported that TNFα is a vascular permeability-increasing agent which activates number of cell adhesion molecules and chemoattractants. However, in contrast to this notion, our previous results reported that the capillary basement membrane and vascular permeability were unaffected in the duodenum of diabetic rats and the present findings support that TNFα did not mediate capillary inflammation in the duodenum of diabetic rats.

In the ileum, the number of TNFα gold particles was significantly increased in diabetics when compared to controls. This observation correlates well with our previous study, where structural, functional, and molecular alterations were observed in the capillary endothelium of ileal diabetic rats, although the immediate-insulin treatment did not result in the change of the number of TNFα gold particles. In the colon, the number of TNFα gold particles remained at the same level in the capillary endothelium of the diabetics when compared to the control group. However, the immediate-insulin treatment decreased the density of TNFα gold particles in the colon, but did not reach the control level. In contrast, in our previous results, we demonstrated that capillary adjacent to the myenteric plexus was highly damaged in the colon of the diabetic rats.

Taken together, the number of IL6-labelling gold particles was slightly increased in the capillary endothelium of duodenum, ileum, and colon of the diabetic rats. Interestingly, in
the insulin-treated diabetic group, IL6 expression was upregulated in the endothelium of duodenum.

6. Conclusions

The present study demonstrates evidence for intestinal region-specific diabetes-related alterations in the expression of endogenous HO enzymes and pro-inflammatory cytokines in enteric neurons and their microenvironment in experimental diabetes. We summarize our most important findings as follows:

- We provide evidence for gut segment-specific diabetes-related induction of the endogenous HO system and also for intestinal region-dependent enhanced co-localization of HO1 and HO2 with nNOS in myenteric neurons. We presume that those myenteric nitrergic neurons which do not co-localize with HOs are the most seriously affected by diabetic damage.
- We demonstrate that the neurochemical character of nitrergic submucous neurons exhibit gut region-dependent changes in diabetic and insulin-treated diabetic rats. We show that HO1-IR and HO2-IR submucous neurons are present in small amounts in the small intestine, but in high abundance in the colon of control and diabetic rats, and they have segment-specific responsiveness to immediate insulin replacement.
- The present study reveals a strictly gut-region dependent expression of TNFα and IL6 in the myenteric ganglia and supplying capillary endothelium of control, diabetic and insulin-treated diabetic rats. The density of TNFα and IL6-labelling gold particles is gradually decreasing from duodenum to colon in myenteric neurons of diabetic rats. The density of TNFα-labelling gold particles in the duodenal endothelium did not show significant differences in the different animal groups, while the density of IL6 was slightly elevated in diabetics, and significantly higher in the insulin-treated rats compared to controls.

7. References

LIST OF PUBLICATION (MTMT number: 10053168)
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Publications related to thesis

Full papers (IF: 7.958)


Oral/Poster presentations


Publications not related to thesis

Full papers


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Supervisor’s/Coauthor’s declaration

I, undersigned Mária Bagyánszki, the coauthor and supervisor of Lalitha Chandrakumar’s PhD work hereby certify that I am familiar with the PhD thesis of the applicant Lalitha Chandrakumar entitled ‘Gut region-specific alterations of the endogenous heme oxygenase system and pro-inflammatory cytokines in the enteric neurons of streptozotocin-induced diabetic rat model’.

The applicant’s contribution was prominent in obtaining the followings: establishment of the diabetic rat model, quantitative fluorescent and post embedding immunohistochemistry, statistical analyses.

I did not and will not use these results in getting academic research degree. There is no other PhD student who can use these results in a doctoral process.

Mária Bagyánszki

Coauthor’s declaration

We, undersigned Nikolett Bódi, Zita Szalai and Diána Mezei the coauthors of Lalitha Chandrakumar’s papers hereby certify that we are familiar with the PhD thesis of the applicant Lalitha Chandrakumar entitled ‘Gut region-specific alterations of the endogenous heme oxygenase system and pro-inflammatory cytokines in the enteric neurons of streptozotocin-induced diabetic rat model’.

We did not and will not use these results in getting academic research degree.

Nikolett Bódi          Zita Szalai          Diána Mezei