Novel sequential stress model for functional dyspepsia: Efficacy of the herbal preparation STW5

Heba Abdel-Aziz a,b,*, Wala Wadie c, Hala F. Zaki c, Jürgen Müller a, Olaf Kelber a, Thomas Efferth b, Mohamed T. Khayyal c

a Scientific Department, Steigerwald Arzneimittelwerk GmbH, Darmstadt, Germany
b Department of Pharmaceutical Biology, Institute of Pharmacy and Biochemistry, Johannes Gutenberg University, Mainz, Germany
c Department of Pharmacology, Faculty of Pharmacy, Cairo University, Cairo, Egypt

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A B S T R A C T

Background: Many screening procedures for agents with potential usefulness in functional dyspepsia (FD) rely on animals exposed to stress early in life (neonatal maternal separation, NMS) or in adulthood (restraint stress, RS).

Purpose: Since many clinical cases of FD have been associated with stress in early life followed by stress in adulthood, a sequential model simulating the clinical situation is described. To explore the validity of the model, the efficacy of STW5, a multicomponent herbal preparation of proven usefulness in FD, was tested.

Study design/methods: A sequential stress model established where rats are exposed to NMS after birth followed later by RS in adulthood. Stress hormones and ghrelin were measured in plasma, while responsiveness of stomach fundus strips to smooth muscle stimulants and relaxants was assessed ex-vivo. The effectiveness of treatment with STW5 a few days before and during exposure to RS in preventing changes induced by the stress model is reported and compared to its efficacy when used in animals subjected to RS alone.

Results: Responses to both stimulants and relaxants were reduced to various extents in the studied models, but treatment with STW5 tended to normalize gastric responsiveness. Plasma levels of ghrelin, corticosterone releasing factor, and corticosterone were raised by RS as well as the sequential model. Treatment with STW5 tended to prevent the deranged parameters.

Conclusion: The sequential stress model has a place in drug screening for potential usefulness in FD as it simulates more the clinical setting. Furthermore, the findings shed more light on the mechanisms of action of STW5 in FD.

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Introduction

Functional dyspepsia (FD) is one of the most common gastrointestinal disorders, affecting up to 15–30% of the general population worldwide (Miwa 2012; Vanheel et al. 2013). Because of the chronic relapsing nature of the disease and the limited treatment options, especially after withdrawal of the indication for metoclopramide (EMA 2013), FD is associated with significantly impaired quality of life and considerable health care costs (Vanheel et al. 2014).

The pathogenesis of FD remains largely unclear. Various studies suggest the implication of one or more factors, involving gastric motility/compliance impairment, visceral hypersensitivity, low grade inflammation in the duodenal mucosa and psycho-social disturbances (Moayyedi 2012).

Clinical studies have shown that adverse physiological or psychological experiences in early life are associated with the development of FD symptoms (Van Oudenhove et al. 2011) as well as acute stressful conditions in adulthood (Kim et al. 2013). Based therefore on the pivotal role stress seems to play in functional GI-disorders, available animal models for FD rely on exposing animals to various types of stress either in the neonatal period (early life stress) or in adulthood.
Based on the assumption that functional dyspepsia in adulthood may have originated from stress disorders in childhood and become aggravated through exposure to stress later in life, the present study was designed to expose animals to stress early in life (neonatal maternal separation) followed by exposure to acute stress in adulthood (restraint stress), in the hope of developing an experimental model of FD with closer resemblance to the complex clinical situation.

To explore the validity of this sequential stress model as a screening tool for developing new drugs for FD, we compared the impact of the sequential model with NMS and RS on different relevant parameters of stress. Furthermore, we studied the activity of STW 5 (Iberogast®), a multicomponent herbal preparation of proven clinical efficacy in the treatment of FD (Holtmann et al. 2013) and recommended by the guidelines of the German Society of Gastroenterology (Madisch 2013) for this condition. Additionally, we wanted to further investigate the mechanisms of action of STW 5 in FD.

Materials and methods

Animals

For experiments involving RS, adult male Wistar rats weighing 250–300 g each, were obtained from the Modern Veterinary Office for Laboratory Animals (Giza, Egypt). The animals were left to acclimatize for one week before subjecting them to experimentation. They were provided with a standard pellet diet and given water ad libitum. The animals were kept at a temperature of 22 ± 3 °C and a 12-h light/dark cycle as well as a constant relative humidity throughout the experimental period.

For experiments on NMS, primiparous timed-pregnant Wistar female rats were obtained from the same source (above) on gestational day 13–14. Mothers were individually housed in cages lined with bedding material on a 12-h light/dark cycle and at a temperature of 22 ± 3 °C. They were provided with a standard pellet diet and given water ad libitum. The study was carried out according to The European Communities Council Directive of 1986 (86/609/EEC) and approved by the Research Ethical Committee at the Faculty of Pharmacy, Cairo University (PT306).

Drugs

STW 5 was provided in the form of its commercial preparation (Iberogast®, batch number 130472) by Steigerwald Arzneimittelwerk GmbH (Darmstadt, Germany). The preparation consists of the 50% (v/v) hydroethanolic fresh plant extract of Iberis amara L. (Brassicaceae, 15%) whole plants and the 30% hydroethanolic extracts of Melissa officinalis L. (Lamiaceae, 10% leaves), Matricaria chamomilla (Compositae, 20%) flowers, Carum carvi L. (Apiaceae, 10% fruits), Mentha piperita L. (Lamiaceae, 5%), Angelica archangelica L (Apiaceae, 10% roots), Silybum marianum (L.) Gaertn. (Compositae, 10% fruits), Chelidonium majus L. (Papaveraceae, 10%) and Glycyrrhiza glabra L. (Leguminosae, 10%). The preparation and its single components are well characterized according to validated analytical methods as well reported (Kroll and Cordes 2006).

Neonatal maternal separation

Neonatal maternal separation (NMS) was performed as described by other authors (Coutinho et al. 2002; Ren et al. 2007). Briefly, animal pups were removed from their mother cage and housed individually in a separate room for 3 h/day (9:00 am–12:00 pm) from postnatal day 2 till postnatal day 21. After separation, pups were returned to their home cage. On postnatal day 22, pups were weaned. Studies were performed on male animals when the rats had reached adulthood (8–12 weeks). Male rats raised concurrently but not subjected to maternal separation served as controls.

Restraint stress

Animals were restrained in cylindrical and well-ventilated tubes for 90 min/day for 1 week (Zhang et al., 2008). They were sacrificed 24 h after the last restraining session.

Experimental design

Male rats from the same litter were used for this study. One group (designated Group A) was subjected to NMS then allowed to reach adulthood, while the other group (designated Group B) was not subjected to NMS and reached adulthood normally. The experimental study was started when both groups had reached adulthood. At this point in time, the adult rats from either group were randomly allocated to several subgroups (n = 8) and treated as follows:

- Rats from Group A:
  - (a) NMS control group: rats given vehicle for STW 5 (31% alcohol; 5 ml/kg p.o.) for 2 weeks, then sacrificed 24 h later.
  - (b) NMS/RS controls: rats given vehicle (5 ml/kg p.o.) for 1 week, then subjected to RS for a further week while continuing vehicle treatment. Animals were sacrificed 24 h later.
  - (c) NMS/RS treated group: rats given STW 5 (5 ml/kg) orally daily for 1 week then subjected to RS for a further week while continuing drug treatment. Animals were sacrificed 24 h later.

- Rats from Group B were allocated as follows:
  - (a) Normal control group: normal adult rats were given 31% alcohol (vehicle of STW 5; 5 ml/kg p.o.) for 2 weeks.
  - (b) RS control group: rats were given vehicle (5 ml/kg p.o.) daily for 1 week and later subjected to RS for a further week while continuing vehicle treatment. Animals were sacrificed 24 h later.
  - (c) RS treated group: rats given STW 5 (5 ml/kg) orally daily for 1 week then subjected to RS for a further week while continuing treatment. Rats were sacrificed 24 h later.

Determination of plasma ghrelin, CRF and corticosterone

Just before sacrifice, two blood samples were withdrawn from the retro-orbital plexus of each animal between 8:00 and 10:00 am under light ether anesthesia. One blood sample was collected in a tube containing EDTA-2K as an anticoagulant and centrifuged at 3000 rpm for 15 min at 4 °C. The separated plasma was subsequently stored at −20 °C for determination of corticotrophin-releasing factor (CRF) levels using rat specific ELISA kit obtained from Assaypro (St. Charles, MO, USA). The second sample was collected in a tube containing EDTA-2K, aprotinin, as a protease inhibitor (50 µl of aprotinin solution/ml whole blood). Aprotinin solution was prepared by dissolving 500 Kallikrein Inhibitor Unit of aprotinin in 50 ml normal saline. The tubes were rocked gently and then centrifuged at 3000 rpm for 15 min at 4 °C. The obtained plasma was immediately treated with 1 mol/l HCl (10% of sample volume) and stored at −20 °C for the determination of active octanoylated acyl-ghrelin using rat specific ELISA kit obtained from Sceti K.K. (Tokyo, Japan).

Stomach motility

After blood withdrawal, the animals were sacrificed by cervical dislocation and the stomach was excised. Longitudinal smooth muscle strips, 2 cm in length and 2–3 mm in width, were prepared from the gastric fundus. The strips were suspended under a 1 g load in a...
Fig. 1. Effect of restraint stress (RS), neonatal maternal separation (NMS) or both (NMS/RS) on carbachol-induced contractions of longitudinal fundus smooth muscle preparations in rats pre-treated with STW 5 (5 ml/kg) or vehicle. Cumulative dose–response curves were constructed ex-vivo. Data are expressed as mean ± SEM of at least five preparations from five different animals. ∗p ≤ 0.05.

30 ml water-jacketed organ bath containing Krebs–Henseleit solution of the following composition (mmol/l): NaCl (119.0); KCl (4.7); CaCl$_2$ (2.5); MgSO$_4$ (1.2); KH$_2$PO$_4$ (1.2); NaHCO$_3$ (25.0) and glucose (5.5). The bath was maintained at 37 °C and aerated with carbogen (95% O$_2$ + 5% CO$_2$). The preparation was connected to an isotonic lever transducer, Model B40 (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany), and recordings were made on a multipen chart recorder (Rikadenki-R50 series, Tokyo, Japan) via a bridge-amplifier Type 336 (Hugo Sachs Elektronik–Harvard Apparatus GmbH, March – Hugstetten, Germany). Each preparation was allowed to equilibrate for 1 h before testing its sensitivity toward different agents in the following sequence: carbachol, potassium chloride (KCl) serotonin and adrenaline. Cumulative concentration–response curves were constructed for the smooth muscle stimulants carbachol, KCl and serotonin on the basal contractility of the longitudinal muscle followed by cumulative concentration–response curves for the smooth muscle relaxant, adrenaline, on the same muscle strips pre-contracted with a submaximal dose of carbachol (eliciting 60–70% of the response provoked by the maximal dose).

Statistical analysis

All results are presented as mean ± standard error (SEM). For the biochemical parameters, one way analysis of variance (ANOVA) was used to compare means of different study groups followed by Dunn’s as post hoc test. To statistically compare the dose–response curves, the R-package “drc” was used (Ritz and Streibig 2005). It is an add-on package for the statistical language and environment R which allows simultaneous fitting of several non-linear regression models with the focus on analysis of multiple dose–response curves. The curves were simultaneously fitted with the four-parameter log-logistic dose–response model and a lack-of-fit test was performed comparing the four-parameter logistic model to a two-way ANOVA model, implying that the four-parameter logistic model provides an acceptable fit of the data. The effective dosage (ED) is appropriate for the comparison of different curves as it is a function of the parameters. Therefore the estimated ratios of ED50 were calculated and the null hypothesis that the indices are equal to 1 was tested.

Results

Effects of neonatal maternal separation and restraint stress on stomach motility

Subjecting the animals to RS or to NMS reduced the responsiveness of the stomach fundus preparation to carbachol significantly (p < 0.001, Fig. 1). However, the effects of restraint stress alone or in combination with NMS were significantly more pronounced than NMS alone (p = 0.01). Pre-treatment with STW 5 significantly safeguarded against the reduction in responsiveness in both groups (RS and NMS/RS), but the effect was more marked in the RS group (Fig. 1).

Contractile responses to KCl presented a similar picture, with a significantly more pronounced impairment in RS and NMS/RS animals than in those subjected to NMS alone (p < 0.001; Fig. 2). However, STW 5 showed only a tendency for improvement, that did not reach statistical significance (p = 0.27 and p = 0.08 respectively). This might have been due to the strong variation seen in tissue response to KCl in the current experimental setting.
Fig. 2. Effect of restraint stress (RS), neonatal maternal separation (NMS) or both (NMS/RS) on KCl-induced contractions of longitudinal fundus smooth muscle preparations in rats pre-treated with STW 5 (5 ml/kg) or vehicle. Data are expressed as the mean ± SEM of at least five preparations from five different animals. ∗p ≤ 0.05.

Fig. 3. Effect of restraint stress (RS), neonatal maternal separation (NMS) or both (NMS/RS) on serotonin-induced contractions of longitudinal fundus smooth muscle preparations in rats pre-treated with STW 5 (5 ml/kg) or vehicle. Data are expressed as the mean ± SEM of at least five preparations from five different animals. ∗p ≤ 0.05.

All three stress regimens significantly reduced serotonin-induced fundus contractions to different extents. Here again NMS alone had the least effect on tissue responses followed by RS and NMS/RS. Especially for the lower serotonin concentrations, the combined stress model was most potent in impairing the fundus response (Fig. 3). STW 5 treatment largely prevented the motility impairment in both models (RS and NMS/RS), leading to complete normalization in strips from animals subjected to RS.

Adrenaline was found to cause a concentration dependent relaxation of the normal rat fundus preparation pre-contracted with carbachol. All stress regimens reduced the relaxant effect of adrenaline, but the effects of RS and NMS/RS were yet again more
Fig. 4. Effect of restraint stress (RS), neonatal maternal separation (NMS) or both (NMS/RS) on adrenaline-induced relaxation of longitudinal fundus smooth muscle preparations pre-contracted with carbachol in rats pre-treated with STW 5 (5 ml/kg) or vehicle. Data are expressed as the mean ± SEM of at least five preparations from five different animals. *p < 0.05.

marked (Fig. 4). STW 5 significantly improved the relaxant response to adrenaline in the NMS/RS animals, restoring it nearly to normal values. However, the response in rats subjected to RS alone was not significantly influenced by the herbal preparation.

Effects of neonatal maternal separation and restraint stress on biochemical parameters

RS was found to markedly increase the plasma levels of active ghrelin while NMS tended to reduce the plasma active ghrelin levels although the effect was not statistically significant (Fig. 5). Subjecting the rats to both RS and NMS tended to increase active ghrelin, however to a lower extent than in animals subjected to RS alone. Pre-treatment with STW 5 before subjecting rats to RS or NMS/RS guarded against the increase in the plasma levels of active ghrelin in both stress models.

The stress hormone, corticotrophin-releasing factor (CRF), was found to be raised threefold in both single stress models, albeit the effect of NMS was not statistically significant (p = 0.10), possibly due to variations in response in the individual animals within the group (Fig. 6).

In the sequential stress model, however, the CRF level was boosted even further to reach a five-fold increase over control values (Fig. 6). Pre-treatment with STW5 curbed effectively this rise.

The rise in CRF levels was reflected in a threefold rise in corticosterone in animals exposed to RS or to NMS/RS, but to a much lesser extent (amounting to only 83%) in animals subjected only to NMS (Fig. 7). Interestingly, pre-treatment with STW5 was more effective in normalizing corticosterone levels in animals subjected to the combined model than in those subjected to RS alone.

Discussion

The aim of the present work was not only to develop an animal model for FD that can parallel the clinical situation where FD has been attributed to exposure to stress early in life and possibly aggravated by stress in adulthood, but also to further explore the mechanisms of action of STW5 in FD. Since stress is a key predisposing factor in the pathogenesis of FD, animals were subjected to early life stress through neonatal maternal separation (NMS) followed by restraint stress (RS) in adulthood. The sequela of this model on various parameters related to FD were compared to those after NMS or RS alone. The effect of STW5 in the sequential model was evaluated and compared to its effect in animals exposed only to RS.

Stress models have been reported to affect different parameters influencing gastric functions. These parameters include the plasma levels of active ghrelin and some stress hormones. Active ghrelin plays an important role in mediating gastric motility (Taniguchi et al. 2008) and has been reported to be reduced in some FD patients (Shindo et al. 2009). Other authors, however, showed that the plasma active ghrelin levels are elevated in FD patients and are correlated with subjective symptoms of the condition (Shinomiya et al. 2005). The reason for this discrepancy remains uncertain, warranting further investigation (Akamizu et al. 2010). In the present study the plasma levels of active ghrelin tended to be slightly reduced in animals subjected to NMS but were raised in those exposed to RS or NMS/RS. In agreement with these findings earlier studies reported a reduction in active ghrelin in NMS exposed rats (Cheung et al. 2010) and an elevation of this hormone in animals subjected to RS (Zheng et al. 2009).

Ghrelin secretion from the stomach is regulated by both cholinergic and adrenergic pathways. Sympathetic nerve stimulation has been
Fig. 5. Active ghrelin levels in plasma of rats subjected either to restraint stress (RS) or neonatal maternal separation (NMS) or both (NMS/RS). Data are expressed as the mean ± SEM of at least five animals. *p ≤ 0.05 vs. normal control. #p ≤ 0.05 vs. RS.

Fig. 6. Plasma corticotrophin-releasing factor (CRF) levels of rats subjected either to restraint stress (RS) or to neonatal maternal separation (NMS) or both (NMS/RS). Data are expressed as the mean ± SEM of at least five animals. *p ≤ 0.05 vs. normal control. #p ≤ 0.05 vs. NMS/RS.

shown to increase gastric ghrelin secretion in rats (Mundinger et al. 2006) and since RS stimulates the sympathetic pathway (Nakade et al. 2005) it is plausible to suggest that stress acts to up-regulate ghrelin expression following activation of the sympathetic nerves (Zheng et al. 2009). Exposure of animals to NMS or to RS led to an increase in the plasma level of the stress hormones, CRF and corticosterone, an effect which was not statistically significant in rats exposed to NMS but particularly marked in those subjected to RS. The rise may have been partly due to enhancing the activity of the hypothalamic pituitary adrenal (HPA) axis by RS (Zhang et al. 2008) and partly to mast cell degranulation of the gastric mucosa induced by stress, thereby releasing CRF as one of the mast cell products (Mayer and Tillisch 2011). Exposure of the rats to NMS before RS in the sequential model greatly enhanced the rise in CRF to a two-fold extent as compared to RS alone, albeit without affecting the increase in corticosterone level. STW5 was effective in protecting against the rise in both stress hormones, suggesting an effect on mast cells or possibly a central effect as well. This issue is currently under investigation.

The evidence for the interrelationship between the gut and the brain is overwhelming (Camilleri et al. 1986; Mayer and Tillisch 2011). In FD, impaired gastric accommodation has been correlated with a decreased cardiovagal tone and a decreased vagal regulation of intestinal transit (Hausken et al. 1993) and may contribute to symptom generation in afflicted patients (Tack et al. 2006). Since the fundus plays an important role in gastric accommodation and hence in FD (Tack 2007), its reactivity toward autonomic responses to parasympathetic and sympathetic agonists has been studied. Contractile
responses to carbachol were reduced by the three models of stress, confirming the involvement of the parasympathetic nervous system. Treatment with STW5 tended to guard against this desensitization of the gastric muscarinic receptors, confirming its targeting. The sympathetic receptors were also affected by the stress models, relaxing responses to adrenaline on the fundus strips being markedly reduced. The contribution of the sympathetic nervous system to gastric motility in rats following restraint stress was reviewed by Yano et al. (Yano et al. 1977). They also reported the inhibitory effect of adrenaline on the enhanced gastric motility after restraint stress. STW5 markedly normalized the responses to adrenaline, confirming normalization of sympathetic receptor function. Earlier studies had suggested the involvement of serotonin in vagally mediated gastric effects in mice and rats (Bulbring and Gershon 1967) and in the guinea pig (Meulemans et al. 1993). Because of the increasing evidence that serotonergic receptors are also involved in gastric accommodation, the contractile responses to serotonin on fundus strips were also studied in the three stress models. The responses were depressed to various extents by the different models, but were normalized after treatment with STW5 in both RS and the sequential model. The differences in contractile responses for acetylcholine and serotonin have been reported earlier (Mukai and Kubota 1980). In addition to the agents known to affect accommodation by acting on specific receptors, the effect of KCI was also studied. The activation of K+ channels results in cellular hyperpolarization due to K+ efflux which in turn causes the inhibition of Ca2+ influx through L-type Ca2+ channels and Ca2+ release from intracellular stores through inositol trisphosphate. Contractile responses by KCl in the models tested, but such decreased responses tended to be normalized by STW5.

Earlier studies had shown that STW5 had a dose-dependent relaxing effect when added in vitro to stomach fundus preparations. Moreover, its overall effects on the stomach were region-specific, relaxing the gastric corpus and fundus while tonicizing the antrum (Schemann et al. 2006). The drug was also reported to increase the relaxing effect when added to stomach fundus preparations. Interestingly, stress models tested against acetylcholine or histamine induced contractions (Schemann et al. 2006).

In conclusion, considering the various changes in the measured parameters induced by the different stress models, the present findings show that, although the impact of the NMS/RS model on fundus reactivity is not very different from that of RS alone, the stress hormones are most dramatically affected by the sequential model. Since stress is clinically a crucial factor in the etiology of FD, it would seem that this model is well suited for testing potentially effective drugs in this condition. In addition, the model provides further pharmacological evidence that psychological stress could affect gastric smooth muscle function.

Although it is not always easy to extrapolate experimental findings to the clinical situation, impaired gastric smooth muscle reactivity may at least in part play a contributing role toward the gastric abnormalities seen in FD patients. By protecting against many of the changes that could possibly play a role in the human condition, the present findings show supportive evidence for the clinical usefulness of STW5 in FD, especially when the latter is triggered by psychological stress. Derangement in autonomic and serotonergic responses of the stomach induced by stress tended to be normalized by STW5 in the laboratory setting at hand. STW5 was also effective in protecting against changes in plasma levels of CRF, corticosterone and active ghrelin induced by the stress model.

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Conflict of interest

Heba Abdel-Aziz, Jürgen Müller and Olaf Kelber are employed by Steigerwald Arzneimittelwerk GmbH, Darmstadt, Germany. All other authors have no conflict of interest.

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