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MEINEN ELTERN
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### ABBREVIATIONS

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>amplitude</td>
</tr>
<tr>
<td>AMPK</td>
<td>adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>CRI</td>
<td>constant-rate infusion</td>
</tr>
<tr>
<td>CSM</td>
<td>circular smooth muscle</td>
</tr>
<tr>
<td>DRM</td>
<td>detergent-resistant membrane</td>
</tr>
<tr>
<td>ENS</td>
<td>enteric nervous system</td>
</tr>
<tr>
<td>F</td>
<td>frequency</td>
</tr>
<tr>
<td>G protein</td>
<td>guanosine nucleotide-binding protein</td>
</tr>
<tr>
<td>GX</td>
<td>glycine xylidide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>ICC</td>
<td>interstitial cells of Cajal</td>
</tr>
<tr>
<td>IR</td>
<td>ischaemia and reperfusion</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>potassium ion</td>
</tr>
<tr>
<td>LSM</td>
<td>longitudinal smooth muscle</td>
</tr>
<tr>
<td>MAF</td>
<td>mean active force</td>
</tr>
<tr>
<td>MEGX</td>
<td>monoethylglycinexylidide</td>
</tr>
<tr>
<td>MLC</td>
<td>myosin light chain</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>sodium ion</td>
</tr>
<tr>
<td>Na&lt;sub&gt;v&lt;/sub&gt;</td>
<td>voltage-gated sodium channel</td>
</tr>
<tr>
<td>pH</td>
<td>negative decadic logarithm of the hydrogen ion activity</td>
</tr>
<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt;</td>
<td>negative decadic logarithm of the acid dissociation constant</td>
</tr>
<tr>
<td>POI</td>
<td>postoperative ileus</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family member A</td>
</tr>
<tr>
<td>RM</td>
<td>repeated measures</td>
</tr>
<tr>
<td>SM</td>
<td>smooth muscle</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
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SUMMARY

Karen Beatrix Tappenbeck - Lidocaine effects in equine jejunal circular and longitudinal smooth muscle in vitro

In horses which undergo gastrointestinal surgery, postoperative ileus (POI) frequently occurs as a severe complication especially following small intestinal lesions (ROUSSEL et al. 2001). For POI, incidence rates of 10-19% were reported (FREEMAN et al. 2000; ROUSSEL et al. 2001; COHEN et al. 2004) combined with death rates of up to 86% (HUNT et al. 1986). POI is characterised by a reduced propulsive motility of the intestine, clinically accompanied by abdominal pain, missing of gastrointestinal sounds, a deficiency in defaecation and urination, accumulation of fluid in the stomach and small intestine resulting in nasogastric reflux, changes in the mucous membrane colour, prolonged capillary refill time and elevated pulse rates (HUNT et al. 1986). For treating POI in horses, lidocaine, a local anaesthetic (LÖSCHER 2006) and class IB antiarrhythmic agent (UNGEMACH 2006), currently is the most frequently applied drug (VAN HOOGMOED et al. 2004), although its prokinetic mechanism of action has not been finally elucidated. Previous studies demonstrated that lidocaine concentration-dependently increased the intestinal smooth muscle (SM) contractility in vitro (GUSCHLBAUER et al. 2010a). Therefore aim of the present PhD project was to further examine the efficacy of lidocaine for increasing the contractility of equine intestinal SM in vitro and thereby to assess possible mechanisms underlying the observed effects. For this purpose, equine jejunal SM strips were prepared and treated with lidocaine in vitro.

It was hypothesised that the contractility-enhancing effects of lidocaine resulted from its insertion into cell membranes of SM cells and consecutive alterations of cell membrane properties, modulated by its molecular structure and lipophilicity. The impact of lidocaine on cell membrane properties was suggested to include improved cell membrane integrity, assessed by a reduced release of creatine kinase (CK) from SM cells (GUSCHLBAUER et al. 2010a). In order to determine the influence of lidocaine’s molecular structure and lipophilicity on SM contractility and CK release, in vitro effects of lidocaine were compared to the effects of four other, structurally related substances, namely mexiletine, bupivacaine, tetracaine and procaine. It was
shown that all substances concentration-dependently increased the SM contractility, followed by a decrease in contractility at high concentrations. However, the course of contractility-enhancing effects differed between the different substances. Only mexiletine, which is structurally closely related to lidocaine, provided contractility-enhancing effects similar to lidocaine and like lidocaine reduced the CK release from SM. For this reason it was concluded that lidocaine and mexiletine may interact with SM cell membranes with comparable effects presumably resulting from their structural and lipophilic similarity.

In order to further examine the impact of lidocaine on cell membrane properties, a second study was performed regarding effects of lidocaine on detergent-resistant membrane microdomains (DRMs) in equine intestinal SM. It was hypothesised that lidocaine disrupts DRMs in SM cells and/or interstitial cells of Cajal (ICC). Lidocaine was found to cause redistribution of the DRM marker protein flotillin-2 in fractions of sucrose density-gradients, indicating DRM disruption. As DRM disruption was shown to alter activity of membrane ion channels as summarised by PRISTERA and OKUSE (2012), it was suggested that the lidocaine-induced increase in SM contractility might be based on DRM disruption and subsequent changes in cellular ion fluxes.

So far, studies regarding contractility-enhancing effects of lidocaine focussed on the circular SM (CSM) layer (GUSCHLBAUER et al. 2010a, 2011; Study 1). Therefore, the third study conducted during the present PhD project aimed to compare lidocaine effects on the contractility of CSM and longitudinal SM (LSM) of equine small intestine as both SM layers contribute to the physiological intestinal motility. As a result, lidocaine concentration-dependently increased the contractility in both CSM and LSM, but higher lidocaine concentrations were required for provoking significant effects in LSM. High lidocaine concentrations caused a decrease in CSM contractility in accordance with previous studies (GUSCHLBAUER et al. 2010a; Study 1) but did not induce a decline in LSM contractility. Data in literature suggested differences in characteristics of CSM and LSM cells (MURTHY et al. 1991; LIU and HUIZINGA 1993; KUEMMERLE et al. 1994; MCKIRDY et al. 2004). Consequently, it was suggested that differences in lidocaine effects on CSM and LSM might result from these differences in molecular characteristics.
Although the enteric nervous system (ENS) takes major part in controlling intestinal motility, so far recent in vitro studies were performed in intestinal SM in which the ENS was deactivated using tetrodotoxin (TTX) in order to determine lidocaine effects on intrinsic SM contractility (GUSCHLBAUER et al. 2010a, 2011; Studies 1, 3). Therefore, a fourth study was conducted comparing contractility-enhancing effects of lidocaine in SM in the presence and absence of TTX. Obtained results illustrated that the increase in CSM and LSM contractility at low to medium-ranged lidocaine concentrations was not altered by TTX application, as well as the decrease in CSM contractility at high lidocaine concentrations. The activity of the ENS was reduced at high lidocaine concentrations in both CSM and LSM. In conclusion, lidocaine seemed to increase the SM contractility by directly affecting SM cells and/or ICC independent from ENS activity. In vivo, high lidocaine concentrations might induce adverse effects on intestinal motility due to decreased CSM contractility and ENS activity.

Despite the fact that the contractility-enhancing effects of lidocaine were reliably reproducible in vitro, the applicability of the observed results to in vivo situations remains limited due to missing drug metabolism and lack of systemic effects under in vitro conditions. Furthermore, lidocaine concentrations which increased the SM contractility in vitro significantly exceeded therapeutic blood concentrations following in vivo infusion of lidocaine (MALONE et al. 2006). As the obtained results suggested a mechanism of action based on the insertion of lidocaine into cell membranes of SM cells and/or ICC, it was hypothesised that this insertion might be associated with lidocaine accumulation within the intestinal wall. Hence it was speculated that lidocaine accumulation might result in higher tissue compared to plasma concentrations, corresponding to effective lidocaine concentrations in vitro - a hypothesis which needs to be evaluated in further pharmacological studies.
ZUSAMMENFASSUNG

Karen Beatrix Tappenbeck - Wirkungen von Lidocain an der glatten Zirkulär- und Longitudinalmuskulatur des equinen Jejunums in vitro


Es wurde die Hypothese aufgestellt, dass die kontraktilitätssteigernde Wirkung des Lidocains aus seiner Einlagerung in Zellmembranen glatter Muskelzellen mit nachfolgenden Änderungen der Zellmembraneigenschaften resultiert, moduliert durch seine molekulare Struktur und Lipophilie. Es wurde vorgeschlagen, dass der Einfluss des Lidocains auf die Zellmembraneigenschaften eine Verbesserung der Zellmembranintegrität beinhaltet, erfassbar durch eine reduzierte Freisetzung von VI

Um den Einfluss von Lidocain auf die Eigenschaften von Zellmembranen näher zu untersuchen, wurde eine zweite Studie durchgeführt, die sich mit der Wirkung von Lidocain auf Detergenzien-resistente Mikrodome in Zellmembranen (DRMs) der equinen Darmmuskulatur befasste. Es wurde die Hypothese aufgestellt, dass Lidocain DRMs in glatten Muskelzellen und/oder interstitiellen Zellen nach Cajal (ICC) zerstört. Es wurde gezeigt, dass Lidocain eine Umverteilung des DRM-Markerproteins Flotillin-2 in Fraktionen von Saccharosedichtegradienten verursachte, was auf eine Zerstörung von DRMs hindeutete. Da gezeigt wurde, dass die Zerstörung von DRMs die Aktivität von membranständigen Ionenkanälen veränderte, wie von PRISTERA und OKUSE (2012) zusammengefasst, wurde vorgeschlagen, dass der Lidocain-induzierte Anstieg in der Kontraktilität der glatten Muskulatur auf Zerstörung von DRMs und nachfolgenden Änderungen in zellulären Ionenströmen basieren könne.

Bislang haben sich Studien zur kontraktilitätsteigernden Wirkung von Lidocain auf die glatte Ringmuskulatur konzentriert (GUSCHLBAUER et al. 2010a, 2011; Studie 1). Aus diesem Grund wurde im Zuge des vorliegenden PhD-Projektes eine dritte Studie durchgeführt mit dem Ziel, die Wirkungen von Lidocain auf die glatte Ring-


VIII
1 INTRODUCTION

1.1 Intrinsic motility of small intestine

Physiologically, the motility of the intestinal tract fulfils various tasks including ingesta transport, mixing of luminal contents and clearance from noxious substances. In the intestine, different patterns of contractions are known, depending on food intake and the gastrointestinal segment as summarised by FURNESS (2012). The musculature of the intestine consists of two components – the inner circular (CSM) and the outer longitudinal smooth muscle (LSM) layer (CAREY 1921) (Figure 1).

Contractions of the CSM and LSM layer are precisely coordinated, with a lower spontaneous rate of contractions in the LSM (MELVILLE et al. 1975; SARNA 1993; MCKIRDY et al. 2004). Thereby contractions of the small intestinal CSM were accompanied by relaxation of the LSM (SARNA 1993). Whereas the LSM layer is thought to contribute to mixing of luminal contents, nutrient absorption and

Figure 1: Haematoxylin-eosin-stained slice of equine distal jejunum. M = mucosa; S = submucosa; CSM = circular smooth muscle; LSM = longitudinal smooth muscle
transmission of electrical activity (MELVILLE et al. 1975; SARNA 1993; SHAFI IK et al. 2002), ingesta transport seems to be mainly mediated by the CSM layer (SARNA 1993; SHAFI IK et al. 2002). Activity of both smooth muscle (SM) layers appears to be regulated differentially as suggested by results of MURTHY et al. (1991), LIU and HUIZINGA (1993), KUEMMERLE et al. (1994) and MCKIRDY et al. (2004).

For coordinating intestinal SM contractile activity, interstitial cells of Cajal (ICC) play an important role. Information on the impact of ICC activity on intestinal functions was summarised by HAGGER et al. (1997). Like SM cells, ICC originate from mesenchymal precursor cells. They generate rhythmic alterations in the membrane potential, known as slow waves, which are spread among the ICC and SM cells via gap junctions. SM contractions are initiated when additional stimuli are superimposed on slow waves. Thus impaired ICC functions may contribute to various gastrointestinal motility disorders (HENNIG et al. 2010). In equine small intestine, ICC were mainly found between the CSM and LSM layer in the area of the myenteric plexus, forming networks with some short extensions into the LSM layer, and in smaller numbers as stellate and bipolar cells within the CSM layer (HUDSON et al. 1999). In horses, ICC density was highest in the ileum, the pelvic flexure and the body of the caecum (HUDSON et al. 1999).

In addition to ICC, the enteric nervous system (ENS) takes major part in controlling intestinal motility. The following paragraph provides a short summary on ENS structure and functions, based on reviews by HOL ZER (2002), HANSEN (2003a, b) and FURNESS (2012). Being part of the autonomous nervous system, the ENS is located within the wall of the whole gastrointestinal tract. It consists of a dense, interactive neuronal network and is divided into the myenteric plexus, located between the CSM and LSM layer, and the submucosal plexus positioned in the submucosal layer. In humans, the ENS consists of about 400-600 million neurons which is comparable to the number of neurons in the spinal cord (FURNESS 2006). Enteric neurons themselves are classified as sensory, inter- and motor neurons which are connected via synapses and form circuits of reflexes. Sensory neurons include intrinsic and extrinsic afferent neurons and comprise mechano-, chemo- and thermoreceptors. Via interconnecting neurons, they transfer obtained information to motor neurons which in turn can be divided into different types: Muscle motor neurons mediate SM contraction, secretomotor and vasomotor neurons regulate
secretion and blood flow and some neurons innervate endocrine cells and in this way take part in controlling intestinal functions. In addition to motility, the ENS influences and coordinates secretory processes, microcirculation, inflammation and immune responses in the gastrointestinal tract. Although it may act autonomously, activity of the ENS can be modulated by the central nervous system via sympathetic and parasympathetic signals.

1.2 Excitation-contraction coupling in intestinal smooth muscle

In general, initial SM contraction is promoted by the entry of calcium ions (Ca\(^{2+}\)) into the cytosol from the extracellular space and, to a lower extent, from intracellular stores. Ca\(^{2+}\) forms a complex with the protein calmodulin and this Ca\(^{2+}\)-calmodulin-complex causes phosphorylation of caldesmon via a protein kinase. Caldesmon blocks the binding site of myosin at the actin filaments. Upon phosphorylation, caldesmon binds to the Ca\(^{2+}\)-calmodulin-complex and thus dissociates from the myosin binding site. Additionally, the Ca\(^{2+}\)-calmodulin-complex activates the myosin light chain (MLC) kinase which subsequently phosphorylates MLC and thus permits interaction of myosin with actin filaments. In the following, cross-bridge cycling occurs, moving actin and myosin filaments against each other which results in SM contraction (Figure 2). Following the initial Ca\(^{2+}\) influx, Ca\(^{2+}\) is rapidly removed from the cytosol via Ca\(^{2+}\)-pumps or by exchange of sodium ions (Na\(^{+}\)) versus Ca\(^{2+}\) by Na\(^{+}/Ca\(^{2+}\) exchangers, mediating SM relaxation.

Sustained contraction of SM occurs due to inhibition of MLC phosphatase, an enzyme mediating dephosphorylation of MLC, and activity of Ca\(^{2+}\)-independent MLC kinases. Both inhibition of MLC phosphatase and activity of Ca\(^{2+}\)-independent MLC kinases are partly mediated by the small guanosine nucleotide-binding protein (G protein) Ras homolog gene family member A (RhoA). RhoA is activated by specific guanine nucleotide exchange factors via exchange of guanosine diphosphate for guanosine triphosphate, and in turn activates Rho kinase. In addition to Rho kinase activation, RhoA reduces MLC phosphatase activity by a pathway including phospholipase D and protein kinase C (Figure 2). More detailed information on mechanisms involved in SM contraction is given in reviews by WEBB (2003), MURTHY (2006) and RATTAN et al. (2010).
Introduction

Figure 2: Mechanisms involved in smooth muscle contraction as suggested by WEBB (2003), MURTHY (2006) and RATTAN et al. (2010). Initial smooth muscle contraction is triggered by influx of calcium ions (Ca$^{2+}$) into the cytosol, while sustained contraction is mediated by activation of the small guanosine nucleotide-binding protein Ras homolog gene family member A (RhoA). MLC = myosin light chain; MLC-P = phosphorylated MLC; RhoGEFs = guanine nucleotide exchange factors; GDP = guanosine diphosphate; GTP = guanosine triphosphate; PLD = phospholipase D; PKC = protein kinase C

1.3 Gastrointestinal motility disorders in horses - postoperative ileus

Disturbances of gastrointestinal motility are widely spread among horses and represent a major cause for euthanasia in this species. For example, in the years 1994-1998, more than half of all horses (57.7%) admitted to the Faculty of Veterinary Medicine at the University of Leipzig suffered from gastrointestinal disease, 12% of which showed strangulating lesions of the small intestine (GROSCHE 2000). A comparison of data in literature by GROSCHE (2000) revealed colic incidences of about 3-10% in the horse population with death rates between 5 and more than 40%. Although colic surgery is performed in order to restore physiological intestinal functions, recovery of the patients is frequently complicated by reduced propulsive motility of the intestine, referred to as postoperative ileus (POI).
For POI, incidences between 10-19% (FREEMAN et al. 2000; ROUSSEL et al. 2001; COHEN et al. 2004) were reported, with death rates of up to 86% in affected horses (HUNT et al. 1986). Symptoms and clinical findings related to POI were abdominal pain, missing of gastrointestinal sounds, deficiency in defaecation and urination, accumulation of fluid in the stomach and small intestine resulting in nasogastric reflux, changes in mucous membrane colour, prolonged capillary refill time and elevated pulse rates (HUNT et al. 1986). Increased risk for developing POI was associated with lesion sites located in the small intestine, prolonged duration of anaesthesia and surgery, resection and anastomosis (especially jejunocaecostomy), increased haematocrit and high serum concentrations of total protein and albumin (FREEMAN et al. 2000; ROUSSEL et al. 2001; COHEN et al. 2004). So far, the pathogenesis of POI has not been finally elucidated. A multifactorial origin is discussed, based on tissue inflammation (KALFF et al. 1999; LITTLE et al. 2005), sympathetic stimulation (DUBOIS et al. 1973) and endotoxemia (KING and GERRING 1991). These pathological processes were thought to be caused by luminal distension and subsequent decompression of the intestinal tube (DABAREINER et al. 2001), the operative manipulation (DUBOIS et al. 1973; ROUSSEL et al. 2001) and ischaemia and reperfusion (IR) of intestinal tissues (DABAREINER et al. 2001; LITTLE et al. 2005).

1.4 Lidocaine for therapy of postoperative ileus

1.4.1 Lidocaine characteristics

For treating POI in horses, lidocaine was shown to be the most frequently applied prokinetic substance, usually administered as bolus infusion of 1.3 mg/kg body weight (BW) followed by constant rate infusion (CRI) of 0.05 mg/kg BW/min (VAN HOOGMOED et al. 2004). Originally, lidocaine is well-known as amino-amide local anaesthetic and class IB antiarrhythmic agent and was first synthesised by Nils Löfgren and Bengt Lundquist in 1943. In veterinary medicine, lidocaine is widely used for infiltration and nerve block anaesthesia and at higher concentrations as well for surface anaesthesia (LÖSCHER 2006). Additionally, lidocaine is applied intravenously or intramuscular for treating ventricular extrasystoles, cardiac
tachyarrhythmias, ventricular fibrillation and intoxications with cardiac glycosides (UNGEMACH 2006).

Structurally, lidocaine (Figure 3) consists of a lipophilic aromatic ring, a hydrophilic amino group and an intermediate chain formed by an amide linkage (COURTNEY and STRICHARTZ 1987). Its local anaesthetic mechanism of action is thought to be based on direct binding to and blockade of voltage-gated sodium channels (Na\textsubscript{v}) in nerve cell membranes and hence impaired initiation and propagation of action potentials. Beside a direct action on Na\textsubscript{v}, indirect effects of lidocaine on Na\textsubscript{v} via the cell membrane have been discussed as well (STRICHARTZ and RITCHIE 1987).

![Figure 3: Molecular structure of lidocaine.](image)

The molecular weight of lidocaine is 234.34 g/mol. This parameter influences its diffusion rate in free solution and, in combination with its molecular structure, provides information on the space required by the molecule (COURTNEY and STRICHARTZ 1987).

Like all local anaesthetics, lidocaine is a weak base with a pK\textsubscript{a} (negative decadic logarithm of the acid dissociation constant) of 7.77 ± 0.04 (STRICHARTZ et al. 1990). At certain pH (negative decadic logarithm of the hydrogen ion activity), pK\textsubscript{a} values determine the balance between the charged, protonated form of local anaesthetics and their uncharged base with increased pH resulting in elevated relation of base to cation. Whereas the uncharged base is assumed to facilitate membrane permeation of the local anaesthetic and thus speed onset of local anaesthetic effects (reviewed by HEAVNER 2007), the protonated form is thought to be pharmacologically more active (STRICHARTZ and RITCHIE 1987).
The potency of local anaesthetics is influenced by their lipophilicity, as this parameter strongly determines their insertion into cell membranes (COURTNEY and STRICHARTZ 1987; STRICHARTZ and RITCHIE 1987). Nonetheless, the predictive power of lipophilic properties concerning membrane concentration of local anaesthetics is thought to be limited (COURTNEY and STRICHARTZ 1987). Compared to other local anaesthetics, lidocaine features medium-ranged lipophilicity with an octanol:buffer partition coefficient of 366 ± 31 at 36°C and pH 7.4 (STRICHARTZ et al. 1990).

In plasma, lidocaine binds preferentially to α1-acid glycoprotein (ARTHUR 1987). In equine plasma after in vitro supplementation of 2 µg/mL, about 53.06 ± 10.28% of lidocaine were protein-bound (MILLIGAN et al. 2006). Thereby it has to be noted that increasing plasma concentrations decrease protein binding of local anaesthetics (TUCKER et al. 1987) and therefore increase drug toxicity (CATCHLOVE 1972).

Like all amino-amide local anaesthetics except prilocaine, lidocaine is metabolised in the liver (ARTHUR 1987). Therefore, clearance of lidocaine from plasma is thought to depend on hepatic blood flow which in turn is influenced by cardiac function. Thus, reduced cardiac function in form of diminished heart rate and mean arterial blood pressure presumably contributed to higher serum lidocaine concentrations in anaesthetised compared to awake horses (FEARY et al. 2005). In horses with colic, the terminal half-life of lidocaine was calculated as 65 ± 33 min (mean ± SD) following bolus infusion of 1.3 mg/kg BW for 15 min and subsequent CRI of 0.05 mg/kg BW/min for 60-90 min (FEARY et al. 2006). Its major metabolites monoethylglycinexylidide (MEGX) and glycinexylidide (GX) are pharmacologically active and like lidocaine potentially feature a toxic risk (ARTHUR 1987).

In general, undesired side effects associated with lidocaine application comprise allergic, neuro- and myotoxic reactions and symptoms caused by central nervous and cardiovascular disturbances as summarised by HEAVNER (2007). In horses, undesired side effects in context with lidocaine administration included muscle fasciculations, serious ataxia proceeding to collapse and reduced quality of recovery from general anaesthesia. Additionally, behavioural changes indicated by anxiety, mild sedation and visual disturbances were reported, while the cardiovascular system appeared to be less sensitive to lidocaine overdose compared to other species (MEYER et al. 2001; VALVERDE et al. 2005; MALONE et al. 2006). Discontinuation
of drug administration lead to rapid recovery of the patients (MEYER et al. 2001) as lidocaine is cleared from plasma within minutes (RODEN 1996).

1.4.2 Lidocaine effects in vivo

Regarding the impact of lidocaine administration on intestinal functions in vivo, it is important to distinguish between effects in clinically healthy horses and in horses suffering from gastrointestinal disease. In healthy horses, no reinforcing effects of lidocaine treatment could be detected with regard to intestinal electrical activity (MILLIGAN et al. 2007; OKAMURA et al. 2009). On the contrary, long-term lidocaine infusion even reduced the total faecal output during the first day of infusion and slowed transit time of faeces without alterations in moisture content (RUSIECKI et al. 2008).

On the other hand, in horses suffering from gastrointestinal disease lidocaine application was associated with shorter hospitalisation time and decreased duration of reflux (MALONE et al. 2006). Response to lidocaine treatment correlated with an earlier time point of first defaecation (MALONE et al. 2006). Similarly, in human patients undergoing colorectal surgery, perioperative administration of lidocaine induced earlier return of gastrointestinal motility, shortened duration of the hospital stay and reduced inflammatory response to surgery (HERROEDER et al. 2007). Prophylactic lidocaine treatment reduced the risk for developing POI and improved short-term survival rates in horses (TORFS et al. 2009).

After colic surgery, horses are often treated with flunixin meglumine, a non-steroidal anti-inflammatory drug, in order to reduce pain and to prevent endotoxemia (HUNT et al. 1986). On the other hand, flunixin meglumine was shown to decrease barrier function in IR-injured equine mucosa. These deleterious effects of flunixin meglumine were antagonised by administration of lidocaine (COOK et al. 2008). As disturbed integrity of the mucosal barrier was identified as one factor responsible for IR-induced local and systemic damage as reviewed by KONG et al. (1998), protective effects of lidocaine on the intestinal mucosa may contribute to its positive effects on intestinal functions.
1.4.3 Lidocaine effects in vitro

In vitro, lidocaine dose-dependently increased amplitudes of contractions in non-injured SM from proximal duodenum, but not from the pyloric antrum or middle portion of the jejunum of horses (NIETO et al. 2000). Direct comparison of equine jejunum subjected to artificial short-term IR injury and non-injured control samples revealed that lidocaine effects on SM contractility were more pronounced in IR-injured samples (GUSCHLBAUER et al. 2010a). In these experiments, lidocaine induced a concentration-dependent increase in contractility, followed by a plateau phase and a decrease in contractility at high concentrations. Additionally, lidocaine reduced the release of creatine kinase (CK) from SM. Both enhanced SM contractility and reduced CK release were suggested to be based on restored cell membrane integrity (GUSCHLBAUER et al. 2010a). This suggestion was affirmed as in vivo lidocaine infusion diminished IR-induced looseness of submucosal and muscular tissue in equine jejunum. Reduced looseness of tissues denoted impaired oedema formation, possibly due to reduced permeability of endothelial cell membranes (GUSCHLBAUER et al. 2010b). Additionally, in vivo lidocaine infusion during short-term ischaemia increased contractility of equine jejunum in vitro, although it did not diminish CK release (GUSCHLBAUER et al. 2011). In studies on cultured cells, lidocaine reduced the secretion of pro-inflammatory cytokines, stimulated the release of anti-inflammatory molecules and alleviated cell injury provoked by pro-inflammatory cytokines (LAHAV et al. 2002; DE KLAVER et al. 2003).

1.4.4 Proposed mechanisms of action

The in vitro effects of lidocaine as pointed out above imply a positive impact on SM contractility, which might contribute to its prokinetic effects in vivo. Thereby contractility-enhancing effects of lidocaine may be mediated by direct stimulating effects on SM cells and ICC (GUSCHLBAUER et al. 2010a), possibly caused by improved cell membrane integrity and subsequent preservation of the cellular energy metabolism (TAKEO et al. 1989). Lidocaine effects on cell membrane integrity might be brought about by insertion of lidocaine into cell membranes of SM cells. One possible mode of interaction of lidocaine with cell membranes was suggested by KAMATA et al. (2008) who showed that lidocaine affected distinct microdomains in
erythrocyte membranes, referred to as lipid rafts or detergent-resistant membranes (DRMs). Thus lidocaine effects on DRMs might contribute to its contractility-enhancing effects. As inflammatory processes are thought to be involved in the development of POI (KALFF et al. 1999; LITTLE et al. 2005), anti-inflammatory properties of lidocaine may add to its therapeutic effects in vivo, as well as pain relief and blockade of inhibitory sympathetic reflexes as suggested by RIMBÄCK et al. (1990) and KUO et al. (2006). In addition, reduced release of non-adrenergic-non-cholinergic neurotransmitters (MALONE et al. 2006), decreased intracellular Ca$^{2+}$ accumulation detaining formation of reactive oxygen species (reviewed by CASSUTTO and GFELLER 2003; COOK and BLIKSLAGER 2008) and diminished amounts of circulating catecholamines (RIMBÄCK et al. 1990) have been discussed in this context, pointing to a wide range of positive influence of lidocaine in horses affected by intestinal motility disorders.
2 PHD PROJECT – AIMS AND HYPOTHESES

2.1 Aim of the PhD project

Despite the increasing body of evidence suggesting positive impact of lidocaine on impaired gastrointestinal motility, its prokinetic mechanism of action still needs to be clarified. Gastrointestinal motility is partly mediated by intrinsic activity of SM cells and ICC. Direct stimulatory effects of lidocaine on these cells were thought to contribute to prokinetic effects of lidocaine in vivo. Hence, aim of the present PhD project was to further examine the efficacy of lidocaine treatment in equine intestinal SM in vitro and thereby to assess possible mechanisms underlying the observed effects. For this purpose, four different studies were conducted which are presented in Manuscripts 1-4.

2.2 Studies conducted during the present PhD project

Study 1 - Manuscript title: Lidocaine and structure-related mexiletine induce similar contractility-enhancing effects in ischaemia–reperfusion injured equine intestinal smooth muscle in vitro

Aim: The first study performed during the present PhD project compared effects of lidocaine to those of four structurally related local anaesthetics and aimed to determine the influence of the molecular structure and lipophilicity of lidocaine on its contractility-enhancing effects in intestinal SM in vitro.

Hypothesis: It was hypothesised that lidocaine effects on SM contractility are based on its insertion into membranes of SM cells, depending on its structural and lipophilic characteristics.

Study 2 - Manuscript title: Lidocaine affects detergent-resistant membranes of equine jejunal smooth muscle in vitro

Aim: In previous in vitro studies (GUSCHLBAUER et al. 2010a; Study 1), lidocaine reduced CK release from intestinal SM implying an interaction of lidocaine with SM cell membranes. Therefore aim of the second study was to examine the impact of
lidocaine administration on characteristics of specific cell membrane microdomains called DRMs.

*Hypothesis:* It was hypothesised that lidocaine disrupts DRMs in equine jejunal SM.

**Study 3 - Manuscript title: In vitro effects of lidocaine on contractility of circular and longitudinal equine intestinal smooth muscle**

*Aim:* Prior to the present PhD project, lidocaine was shown to increase contractility in CSM (GUSCHLBAUER et al. 2010a, 2011; Study 1) but effects on LSM had not been examined, yet. Therefore aim of the third study was to compare lidocaine effects on contractility of equine jejunal CSM and LSM *in vitro.*

*Hypothesis:* It was hypothesised that lidocaine enhances contractility in both SM layers.

**Study 4 - Manuscript title: Impact of tetrodotoxin application and lidocaine supplementation on equine jejunal smooth muscle contractility and activity of the enteric nervous system *in vitro***

*Aim:* So far, previous *in vitro* studies (GUSCHLBAUER et al. 2010a, 2011; Studies 1, 3) focussed on lidocaine effects in SM in the presence of tetrodotoxin (TTX), in order to deactivate the ENS and to exclusively examine direct lidocaine effects on SM cells and ICC. The fourth study of this PhD project aimed to assess the impact of TTX application on spontaneous intestinal SM contractility and contractility-enhancing lidocaine effects *in vitro,* and to investigate the influence of lidocaine administration on ENS activity.

*Hypothesis:* It was hypothesised that neither spontaneous SM contractility nor contractility-enhancing effects of lidocaine are altered by TTX application, but lidocaine administration reduces ENS activity.
3 METHODOLOGICAL BACKGROUND INFORMATION

3.1 Horses included in the present PhD project

Samples collected during the different studies were obtained from 23 horses of various breeds (Table 1) who were assigned to euthanasia for reasons other than gastrointestinal disorders.

Table 1: Individual data of all horses included in the present PhD project and their integration into the different studies.

<table>
<thead>
<tr>
<th>Horse</th>
<th>Breed</th>
<th>Gender</th>
<th>Age (years)</th>
<th>BW (kg)</th>
<th>Study</th>
<th>Unpublished data</th>
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</thead>
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<td>545</td>
<td>1</td>
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<tr>
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<td>15</td>
<td>526</td>
<td>1</td>
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<tr>
<td>3</td>
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<td>mare</td>
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<td>1</td>
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<tr>
<td>4</td>
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<td>630</td>
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</tr>
<tr>
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<td>mare</td>
<td>16</td>
<td>460</td>
<td>1</td>
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</tr>
<tr>
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<td>15</td>
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<tr>
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<tr>
<td>22</td>
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<td>mare</td>
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<td>3,4</td>
<td>2,3</td>
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<tr>
<td>23</td>
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<td>gelding</td>
<td>16</td>
<td>591</td>
<td>2,3,4</td>
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</table>

\(^a\text{BW = body weight; }^b\text{Aims of Studies 1-4 are pointed out in Chapter 2.2 and results are presented in Manuscripts 1-4; }^c\text{Unpublished data: }^d\text{Smooth muscle contractility after prolonged duration of ischaemia (Chapter 5.1), }^e\text{Concentration-dependent lidocaine effects on creatine kinase release (Chapter 5.2), }^f\text{Tissue lidocaine concentrations after in vitro incubation (Chapter 5.3)}
The horses featured different gender (20 mares; 2 stallions; 1 gelding) and a huge variation in age (3-27 years; mean ± SEM 16 ± 1 years) and body weight (350-675 kg; mean ± SEM 534 ± 16 kg). The most frequently reported reasons for euthanasia were lameness (6 horses) and old age (5 horses). Reduction of the population and unsuitability for breeding were indicated in 3 respectively 2 cases, whereas arthrosis, headshaking and ataxia were each registered in one horse. In some horses two of the above reasons were combined, and in 6 horses reasons for euthanasia were unknown.

### 3.2 Ischaemia-reperfusion injury

During colic surgery, apparently damaged intestine is resected whereas adjacent segments which appear to be morphologically intact are spared and adjusted in order to restore continuity of the intestinal tube. Nonetheless, also these parts of the intestine schedule loss of contractile function compared to completely unaffected tissues and thus may contribute to the development of POI (MALONE and KANNAN 2001).

Although studies conducted during the present PhD project solely comprised experimental *in vitro* approaches, the study design aimed to imitate *in vivo* situations following colic surgery as faithfully as possible. For this reason, prior to sample collection a segment of equine jejunum was harmed by a standardised, artificial IR injury which was intended to simulate mild muscular damage such as may occur in non-resected equine intestine. Whereas in Study 1 only IR-injured tissues were used, Studies 2 and 3 compared IR-injured to non-injured control tissues. Study 4 was only conducted in non-injured tissues.

### 3.3 Deactivation of the enteric nervous system

In previous scientific research, TTX at concentrations of 1 µmol/L has been frequently used for deactivating the ENS in order to study intrinsic activity of SM cells and ICC (BODDY et al. 2004; GUSCHLBAUER et al. 2010a, 2011). TTX is a highly potent neurotoxin which was first isolated from the puffer fish family Tetraodontidae and since then was found in a variety of other species. TTX specifically binds to the
outer pore of Nav, blocks the influx of Na$^+$ into nerve and muscle cells and thus impairs the propagation of action potentials. More detailed information on TTX is provided by reviews of LEE and RUBEN (2008), HANIFIN (2010) and NIETO et al. (2012). Whereas in Studies 1 and 3 of this PhD project ENS activity was blocked using TTX, Study 4 compared spontaneous SM contractility and contractility-enhancing effects of lidocaine in SM in the absence and presence of TTX.
4 MANUSCRIPTS

4.1 Manuscript 1

Lidocaine and structure-related mexiletine induce similar contractility-enhancing effects in ischaemia–reperfusion injured equine intestinal smooth muscle in vitro

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4.2 Manuscript 2

Lidocaine affects detergent-resistant membranes of equine jejunal smooth muscle \textit{in vitro}

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Short Communication

Lidocaine affects detergent-resistant membranes of equine jejunal smooth muscle in vitro

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Abstract

Lidocaine is the most commonly chosen prokinetic for treating postoperative ileus in horses, a motility disorder associated with ischaemia-reperfusion injury of intestinal tissues. Despite the frequent use of lidocaine, the mechanism underlying its prokinetic effects is still unclear. Previous studies suggested that lidocaine altered cell membrane characteristics of smooth muscle cells. Therefore the present study aimed to elucidate effects of lidocaine administration on characteristics of detergent-resistant membranes in equine jejunal smooth muscle. Lidocaine administration caused significant redistribution of flotillin-2, a protein marker of detergent-resistant membranes, in fractions of sucrose-density-gradients obtained from ischaemia-reperfusion injured smooth muscle solubilised with Triton X-100. It was concluded that lidocaine induced disruption of detergent-resistant membranes which might affect ion channel activity and therefore enhance smooth muscle contractility.

Keywords: Contractility; Detergent; Flotillin; Lidocaine; Lipid rafts
Lidocaine is frequently applied for treating postoperative ileus (POI) in horses (Van Hoogmoed et al., 2004). POI occurs as a fatal complication especially after small intestinal surgery (Cohen et al., 2004) and is characterised by reduced propulsive motility of the intestine. Concerning its pathogenesis, POI is thought to be associated with ischaemia and reperfusion (IR) of intestinal tissues (Little et al., 2005).

It was suggested that lidocaine ameliorated smooth muscle (SM) contractile performance in vitro by changing SM cell membrane characteristics (Guschlbauer et al., 2010). One possible mode of interaction of lidocaine with the cell membrane was identified by Kamata et al. (2008) who showed that lidocaine reversibly disrupted membrane lipid rafts. Lipid rafts are defined as distinct membrane microdomains enriched in cholesterol and sphingolipids which are relatively insoluble in cold, non-ionic detergents (review: Pristera and Okuse, 2012). For this reason they are also referred to as detergent-resistant membranes (DRMs). It was proposed that lidocaine may interact with DRMs of intestinal SM cells or interstitial cells of Cajal, possibly provoking changes in ion channel activity and thus modulating SM contractility. The present study examined consequences of lidocaine treatment on DRMs in equine jejunal SM. It was hypothesised that administration of 50 mg/L lidocaine disrupts DRMs, assessed by redistribution of the DRM marker protein flotilllin-2 in fractions of sucrose-density-gradients.

Non-injured control and IR-injured intestinal samples were collected from healthy horses following a protocol described previously (Tappenbeck et al., 2013). SM strips of equal size (2x2 cm) were prepared by removing mucosa and submucosa and were incubated in modified Krebs-Henseleit Buffer (in mmol/L: 117.0 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 1.2 NaH$_2$PO$_4$, 11.0 Glucose, 25.0 NaHCO$_3$; 38 °C; pH 7.4; continuously aerated with 95% O$_2$ and 5% CO$_2$) at 38 °C for 60 min. In lidocaine-treated SM, the buffer solution
was supplemented with 50 mg/L lidocaine. Following incubation, tissues were homogenised, suspended in 3 mL PBS with 150 μL protease inhibitor mix and treated with either 0.5% Triton X-100 (Sigma-Aldrich) or 1% Lubrol WX (MP Biomedicals). After 2 hours solubilisation and 15 min centrifugation at 4 °C, the supernatant was collected and normalised for protein content. For sucrose-density-gradient centrifugation, 1 mL 80% sucrose was overlaid with 500 μL lysate mixed with 500 μL 80% sucrose, 7 mL 30% and 1 mL 5% sucrose. After centrifugation (SW 40 Ti rotor, Optima™ XL Series, Beckman Coulter, 33,000 rpm, 4 °C, 18 hours), 9 mL of the gradient were collected from top to bottom as 1 mL aliquots and protein precipitation was performed in each aliquot (96% ethanol, -20 °C, overnight). After subsequent centrifugation (20 min, 4 °C) the pellets were collected, dissolved in 40 μL Lämmli SDS-PAGE loading buffer (150 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerine, 0.02% bromophenol blue) and heated for 5 min at 95 °C. Western Blot analysis was performed with one gradient fraction per lane (SDS-PAGE: 12% gels; Roti-PVDF membranes, pore size 0.45 μm, Carl Roth; primary antibody: flotillin-2 (B-6), mouse monoclonal IgG1, molecular weight 45 kDa, Santa Cruz Biotechnology; secondary antibody: Immunopure Goat Anti-Mouse IgG (H+L), Thermo Scientific). Bands were visualised by chemiluminescence (SuperSignal West Femto, Maximum Sensitivity Substrate, Thermo Scientific) and were analysed semi-quantitatively (Quantity One, version 4.6.7, Bio-Rad Laboratories). One-Way repeated measures analysis of variance with Tukey’s post hoc tests (GraphPad Prism 4.0, GraphPad Software) were used to compare differences in lanes 1, 2, 3, 4-7, 8 and 9 between treatment groups. All values were expressed as means ± SE of N=5 horses. Levels of significance were set at P<0.05, P<0.01 and P<0.001.

In control SM treated with either detergent, flotillin-2 was mainly found in fractions 1-3 of the sucrose-density-gradients (Fig. 1A and B, upper panel). IR injury did not affect distribution of the DRM marker protein (Fig. 1A and B, middle panel, Fig. 2), but by
administration of 50 mg/L lidocaine, flotillin-2 was distributed throughout the whole gradient in Triton X-100-DRMs (Figure 1A, lower panel). This shifting of flotillin-2 in Triton X-100-DRMs was statistically significant (Fig. 2A). In DRMs extracted with Lubrol WX (Figure 1B) no shifting of flotillin-2 was detected (Fig. 2B).

As expected, DRMs of control SM accumulated in the low-density, floating fractions of the sucrose-density-gradients (Brown and Rose, 1992), which were fractions 1-3 in this experimental setting. Administration of 50 mg/L lidocaine caused redistribution of the DRM marker protein flotillin-2 in DRMs isolated by Triton X-100 but not by Lubrol WX, indicating disruption of these DRMs. Thus our data suggest an impact of lidocaine on DRMs which are exposed at the outer leaflet of the membrane, as Lubrol WX-DRMs contain predominantly components of the inner membrane leaflet (Delaunay et al., 2008). It is tempting to speculate that lidocaine could exchange cholesterol in the Triton X-100-DRMs by virtue of its partial similarity to cholesterol. Disruption of DRMs altered activity of ion channels which are involved in modulating membrane potentials as well as action potentials (review: Pristera and Okuse, 2012). Hence it was proposed that changes in ion fluxes due to lidocaine-induced DRM-disruption might enhance SM contractility.

IR injury did not significantly influence distribution of flotillin-2, suggesting that IR injury did not profoundly affect DRM characteristics. However, Bessho et al. (2005) demonstrated that IR injury reduced the amount of NMDA receptor subunits in DRMs but did not alter the content of flotillin-1. Therefore missing redistribution of flotillin-2 after IR injury as observed in this study does not necessarily exclude functional changes in the affected tissues.
In conclusion, alterations in DRM characteristics might constitute a future mechanistic approach for further elucidating the mechanism underlying prokinetic effects of lidocaine in intestinal SM. Further investigations are needed to determine lidocaine-induced concentration-dependent functional changes in ion channels located in DRMs.

Acknowledgement

The authors would like to thank Susanne Hoppe and Kathrin Hansen for their excellent assistance during experimental procedures.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper. The presented data will be published as part of a PhD thesis at the University of Veterinary Medicine, Hannover, Germany.

References


Figure legends

Fig. 1. Representative Western Blot results concerning the distribution of the detergent-resistant membrane (DRM) marker protein flotillin-2 (45 kDa) in equine jejunal smooth muscle after sucrose-density-gradient centrifugation and Western Blot analysis with one gradient fraction per lane. DRMs were isolated using Triton X-100 (Fig. 1A) or Lubrol WX (Fig. 1B). Distribution of flotillin-2 was examined in non-injured control tissues without lidocaine supplementation (control –lidocaine) and in tissues subjected to a defined, artificial ischaemia-reperfusion (IR) injury without (IR-injured –lidocaine) and with lidocaine treatment (IR-injured +lidocaine). Density gradient fraction 1 featured the lowest and fraction 9 the highest sucrose density.

Fig. 2. Statistical analysis of Western Blot results concerning the distribution of the detergent-resistant membrane (DRM) marker protein flotillin-2 in fractions of sucrose-density-gradients. Numbers at the X-axis refer to the sucrose-density-gradient fractions received from each treatment group (non-injured control SM without lidocaine supplementation: control –lidocaine; ischaemia-reperfusion- (IR-) injured SM without lidocaine supplementation: IR-injured –lidocaine; IR-injured SM treated with lidocaine: IR-injured +lidocaine). Density gradient fraction 1 featured the lowest and fraction 9 the highest sucrose density. DRMs were isolated using Triton X-100 (Fig. 2A) or Lubrol WX (Fig. 2B). Density in each fraction was expressed as % of total density of all nine fractions. One-Way repeated measures analysis of variance with Tukey’s post hoc test was used to compare differences in density of lanes 1, 2, 3, 4-7, 8 and 9 between the different treatment groups. All values were expressed as means ± SE of N=5 horses. Levels of significance were set at *p<0.05, **p<0.01 and ***p<0.001.
**Figure 1**

A Fraction 1 2 3 4 5 6 7 8 9

45 kDa

Control -lidocaine

IR-injured -lidocaine

IR-injured +lidocaine

B Fraction 1 2 3 4 5 6 7 8 9

45 kDa

Control -lidocaine

IR-injured -lidocaine

IR-injured +lidocaine
Figure 2

A

B

% of total density

Control - lidocaine
IR-injured - lidocaine
IR-injured + lidocaine

% of total density

Control - lidocaine
IR-injured - lidocaine
IR-injured + lidocaine
4.3 Manuscript 3

*In vitro* effects of lidocaine on contractility of circular and longitudinal equine intestinal smooth muscle

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4.4 Manuscript 4

Impact of tetrodotoxin application and lidocaine supplementation on equine jejunal smooth muscle contractility and activity of the enteric nervous system in vitro

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Original Article

Impact of tetrodotoxin application and lidocaine supplementation on equine jejunal smooth muscle contractility and activity of the enteric nervous system in vitro

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Abstract

Despite poor understanding of its prokinetic mechanism of action, lidocaine is the most frequently used agent for improving intestinal motility in horses suffering from postoperative ileus (POI). *In vivo*, intestinal motility is controlled by the enteric nervous system (ENS). Nonetheless, recent *in vitro* studies were performed in smooth muscle (SM) in which the ENS was deactivated using tetrodotoxin (TTX), in order to determine lidocaine effects on intrinsic SM contractility. Aim of the present study was to elucidate the impact of TTX application on intestinal SM contractility and contractility-enhancing lidocaine effects *in vitro*, and to investigate the influence of lidocaine administration on ENS activity. Hypothetically, lidocaine effects on SM contractility are not altered by TTX application, but lidocaine administration reduces ENS activity. SM strips were treated with lidocaine in the absence or presence of TTX. Alterations in SM contractility were assessed by isometric force development, and ENS activity was evaluated by calculating the contractile response of SM to electric field stimulation. The lidocaine-induced increase in SM contractility at low to medium-ranged concentrations was not altered by TTX application. High lidocaine concentrations reduced both circular SM contractility and ENS activity. In conclusion, lidocaine seemed to increase SM contractility by directly affecting SM cells and interstitial cells of Cajal without contribution of the ENS. Reduced circular SM contractility and ENS activity at high lidocaine concentrations might impair intestinal motility *in vivo*. Improved understanding of underlying mechanisms is thought to be relevant for therapeutic use of lidocaine in cases of POI.

*Keywords:* Enteric nervous system; Horse; Lidocaine; Motility; Small intestine
Introduction

Physiologically, coordinated small intestinal motility depends on rhythmic intrinsic spontaneous smooth muscle (SM) contractility based on activity of interstitial cells of Cajal (Hennig et al., 2010). Superordinate, intestinal motility is controlled by the enteric nervous system (ENS), which acts via circuits of reflexes and communicates with the central nervous system as summarised by Furness (2012). The ENS can be deactivated using tetrodotoxin (TTX), a potent neurotoxin which binds to voltage-gated sodium channels, inhibiting the initiation and propagation of action potentials as reviewed by Lee and Ruben (2008).

Lidocaine, a local anaesthetic and class IB anti-arrhythmic agent, is a widely used prokinetic for stimulating intestinal motility in horses suffering from postoperative ileus (POI) (Van Hoogmoed et al., 2004). POI occurs as severe complication especially after small intestinal surgery (Cohen et al., 2004) and is characterised by reduced propulsive motility of the intestine. Lidocaine has been shown to ameliorate intestinal motility dysfunctions in vivo (Cohen et al., 2004; Malone et al., 2006; Torfs et al., 2009) and SM contractility in vitro (Guschlbauer et al., 2010, 2011; Tappenbeck et al., 2013a, b). Despite its frequent application in patients, cellular targets and mechanisms involved in its prokinetic effects are still unclear.

Several studies demonstrated contractility-enhancing effects of lidocaine on intestinal SM in which the ENS was deactivated by TTX, suggesting a mechanism of action on the level of SM cells and of interstitial cells of Cajal (ICC) (Guschlbauer et al., 2010, 2011; Tappenbeck et al., 2013a, b). However, no study has examined the extent to which application of TTX might alter spontaneous SM contractility and contractility-enhancing effects of lidocaine in vitro. Therefore on the one hand, purpose of the present study was to compare spontaneous SM contractility and contractility-enhancing effects of lidocaine in equine small
intestine *in vitro* in the absence and presence of TTX. As lidocaine exerted local anaesthetic
effects by blocking signal transduction in nerve cell membranes (Strichartz and Ritchie,
1987), increasing lidocaine concentrations were supposed to inhibit enteric nervous functions.
Thus on the other hand, the present study aimed to examine the impact of increasing lidocaine
concentrations on ENS activity, assessed by contractile response of SM to electric field
stimulation (EFS) (Boddy et al., 2004; Guschlbauer et al., 2010, 2011; Tappenbeck et al.,
2013a, b). Both approaches are targeted on identification of mechanisms and cellular
structures which could be influenced by lidocaine administration, thereby explaining its
prokinetic effect. It was hypothesised that spontaneous SM contractility and contractility-
enhancing lidocaine effects are not affected by application of TTX, while lidocaine
administration reduces ENS activity.

**Materials and methods**

*Sample collection*

Samples were collected from nine mares and one gelding aged 3-27 years (mean ± SE
18.3 ± 2.6 years). Horses were kept in individual stalls with free access to water. They were
fed hay and concentrated feed three times a day. All horses were dewormed and did not show
any clinical signs of gastrointestinal disorders. After premedication with 0.8-1.1 mg/kg
bodyweight (BW) xylazine intravenously (IV) and induction of anaesthesia (0.05 mg/kg BW
diazepam IV and 2.2 mg/kg BW ketamine IV), balanced anaesthesia was maintained using
isoflurane in 100% oxygen combined with continuous rate infusion of xylazine (0.7 mg/kg
BW/hour). The mean arterial blood pressure was kept above 60 mmHg by infusing
dobutamine, lactated Ringer’s solution and hydroxylethylstarch as needed. During a standard
median laparotomy, a 25 cm segment of equine jejunum located 1.5 m orally to the ileocecal
fold was resected. Following resection, the horses were euthanised (35 mg/kg BW
pentobarbital IV) during general anaesthesia. All procedures were approved by the State Office for Consumer Protection and Food Safety in accordance with the German Animal Welfare Law (application number: 33.12-42502-04-11/0572).

Tissue preparation

After resection, the intestinal segment was transferred into modified Krebs-Henseleit Buffer (KHB; in mmol/L: 117.0 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 11.0 Glucose, 25.0 NaHCO₃; 38 °C; pH 7.4; continuously aerated with 95% O₂ and 5% CO₂) and prepared as described previously (Guschlbauer et al., 2010). In short, mucosa and submucosa were removed and SM strips of equal size and weight (0.5x1.0 cm; 0.0194 ± 0.0004 g wet tissue) were prepared by cutting full thickness SM strips in direction of the circular or longitudinal SM fibres, referred to as circular (CSM) and longitudinal smooth muscle (LSM) strips. SM strips were placed into individual organ bath filled with 12 mL KHB and connected to isometric force transducers (HBM). In order to achieve maximum isometric force development, SM strips were stretched with 2 g of initial tension in accordance with a study of Nieto et al. (2000).

Electric field stimulation and lidocaine administration

All SM strips were stimulated using EFS (10 s, 10 Hz, 30 V) at regular intervals of 15 min, starting after 35 min equilibration time. Five min after the first EFS, 1 μmol/L TTX (BIOTREND Chemicals) was administered to half of the SM strips in order to block responses of the ENS (Boddy et al., 2004). Every 15 min lidocaine (lidocaine hydrochloride, Sigma-Aldrich), dissolved in distilled water, was added cumulatively to half of the organ baths starting 30 min after administration of TTX (details are given in figure legends). As lidocaine was applied to the organ bath at regular intervals, each lidocaine concentration
resembled a distinct time point during experimental procedures. Therefore time-dependent
effects on SM contractility in tissues without lidocaine supplementation could be compared to
lidocaine-induced changes in lidocaine-treated tissues. The experimental setting pointing out
the use of SM strips obtained from each horse is outlined in Fig. 1. Isometric force
development of the SM strips was continuously registered throughout the trial and analysed
during 3 min evaluation periods directly before EFS.

Contractility

Isometric force development of the SM strips, described by frequency (F; Peaks/min),
amplitude (A; mN) and mean active force of contractions (MAF; mN), was defined as
contractility. For calculating F, all peaks during an evaluation period were registered. Thereby
peaks were defined as contractions with amplitudes ≥ 0.2 g (= 1.96 mN). A described the
mean amplitude of all peaks during the evaluation period. In contrast to A, MAF was specified
as mean of all values registered during the evaluation period, corrected by baseline values.
Changes in F, A and MAF throughout the trial were calculated by subtracting basal values
before TTX application from data received from each further evaluation period and were
defined as Delta F, Delta A and Delta MAF.

Activity of the enteric nervous system

The contractile response of SM strips to EFS, assessed by the difference between MAF
values before and after EFS, was defined as impulse. For calculating impulses, the beginning,
end and duration of each impulse were identified. Thereby the first value exceeding baseline
values following EFS was taken as beginning of the impulse. Starting from this time point,
MAF of consecutive 20 s-intervals were calculated. The impulse ended when the MAF of a 20
s-interval, corrected by MAF values before beginning of the impulse, was ≤ 0. For quantifying
the impulse (mN x s), MAF (mN) during the whole impulse was multiplied with the duration
of the impulse (s).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software) for
Microsoft. Values for Delta F, Delta A, Delta MAF and impulses were normally distributed
and were presented as means ± SE of N=10 horses with each mean consisting of n=2
values/horse. Two-Way repeated measures (RM) ANOVA were performed regarding the
impact of TTX application (factors ‘lidocaine concentration and ‘TTX application’) and
lidocaine treatment (factors ‘lidocaine concentration’ and ‘lidocaine treatment’) on Delta F,
Delta A, Delta MAF and impulses. Paired Student’s t tests were used to compare Delta F,
Delta A, Delta MAF and impulses at each time point in tissues with or without TTX
application and in tissues with or without lidocaine supplementation. Additionally, in
lidocaine-treated tissues concentration-dependent effects on Delta F, Delta A and Delta MAF
were calculated within the CSM and LSM layer by Paired Student’s t test. P values <0.05
were regarded as statistically significant.

Results

Data concerning the influence of TTX application and lidocaine administration on SM
contractility and on ENS activity were presented in separate paragraphs, respectively. For
assessing the impact of TTX application, SM strips with and without TTX application (+TTX;
-TTX) were compared within the CSM or LSM layer (Fig. 1, a). Effects of lidocaine
administration were examined by comparison of lidocaine-treated and untreated SM strips
(+L; -L) within each SM layer (Fig. 1, b).
Smooth muscle contractility - Influence of tetrodotoxin application

Application of TTX did not affect Delta F, Delta A and Delta MAF as assessed by Two-Way RM ANOVA (factors ‘lidocaine concentration’ and ‘TTX application’, results not shown). Paired Student’s t tests only revealed one significant difference in Delta F of lidocaine-treated LSM (Fig. 2).

Smooth muscle contractility - Influence of lidocaine administration

Lidocaine administration affected Delta F, Delta A and Delta MAF, but its effects differed in CSM and LSM (Two-Way RM ANOVA, factors ‘lidocaine concentration’ and ‘lidocaine treatment’; Table 1). Paired Student’s t tests comparing SM with or without lidocaine supplementation indicated that increasing lidocaine concentrations caused an increase in contractility of CSM and LSM with or without TTX application (data not shown).

Comparison of Delta F, Delta A and Delta MAF within lidocaine-treated SM revealed a decrease in Delta A and Delta MAF of CSM with or without TTX application at lidocaine concentrations of 200 mg/L compared to 100 mg/L (Fig. 3A and 4A; A: CSM without TTX: P<0.001, CSM with TTX: P<0.001; MAF: CSM without TTX: P<0.05). In LSM, no lidocaine-induced decrease in contractility was observed.

Activity of the enteric nervous system - Influence of tetrodotoxin application

TTX application eliminated impulses in both CSM and LSM (Two-Way RM ANOVA, interaction: P<0.001, factor ‘lidocaine concentration’: P<0.001, factor ‘TTX application’: at least P<0.05). Elimination of impulses persisted throughout the whole experimental period in TTX-treated SM strips (Fig. 5).

Activity of the enteric nervous system - Influence of lidocaine administration
Lidocaine administration concentration-dependently altered impulses of CSM strips which were not pre-treated with TTX (Two-Way RM ANOVA, factors ‘lidocaine concentration’ and ‘lidocaine treatment’: interaction $P<0.001$). A decrease in impulses of CSM was detected at lidocaine concentrations of 100 mg/L ($P<0.05$) and 200 mg/L lidocaine ($P<0.001$) compared to tissues without lidocaine supplementation. In LSM strips without TTX, a decrease in impulses was only detected at lidocaine concentrations of 200 mg/L lidocaine ($P<0.05$). At lower lidocaine concentrations, no effect on impulses was observed in CSM and LSM.

**Discussion**

*Impact of tetrodotoxin application on smooth muscle contractility and activity of the enteric nervous system*

Spontaneous contractility of small intestinal SM was not altered by application of TTX. Although both TTX-sensitive and TTX-resistant sodium channels were found in intestinal SM (Smirnov et al., 1992; Holm et al., 2002), TTX-resistant sodium channels seemed to predominate (reviewed by Beyder and Farrugia, 2012). Thus on the one hand, missing influence of TTX on spontaneous SM contractility might result from the presence of mainly TTX-resistant sodium channels in equine intestinal SM. On the other hand, sodium fluxes appeared to be less important for intestinal SM contraction compared to calcium fluxes (Snape and Tan, 1985), thus impaired sodium channel activity might not affect SM contractility.

Enteric neuronal cells express a variety of TTX-sensitive sodium channels (Bartoo et al., 2005) which are essential for action potential propagation. Accordingly, administration of 1 μmol/L TTX abolished impulses immediately and permanently, indicating effective
deactivation of the ENS throughout the whole trial. The consistent SM contractility despite effective blockade of the ENS implied that spontaneous SM contractility originated solely from SM cells and ICC.

*Impact of lidocaine supplementation on smooth muscle contractility*

Increasing lidocaine concentrations induced an increase in contractility of CSM and LSM without alterations due to TTX application, followed by a decrease in CSM contractility at high lidocaine concentrations. Differences in reactions of CSM and LSM to lidocaine administration confirmed results of Tappenbeck et al. (2013b). Several previous studies already documented reinforcing effects of lidocaine on SM contractility in the presence of TTX and thus suggested a mechanism of lidocaine action on the level of SM cells and/or ICC (Guschlbauer et al., 2010, 2011; Tappenbeck et al., 2013a, b). Although sodium fluxes are thought to play a minor role in SM contraction (Snape and Tan, 1985) as pointed out above, they seemed to contribute to the generation of slow waves in ICC (Strege et al., 2003). Hence, to the best of our knowledge this is the first study demonstrating that contractility-enhancing effects of lidocaine are not modulated by blockade of TTX-sensitive sodium channels in intestinal SM.

Beside sodium channels, local anaesthetics were shown to act on potassium and calcium channels (reviewed by Scholz, 2002) which exert major influence on SM contraction (reviewed by Farrugia, 1999), possibly contributing to contractility-enhancing effects of lidocaine in intestinal SM. Furthermore, lidocaine effects on ion permeability in renal brush border membranes were shown to be concentration-dependent, causing a decrease in ion permeability at low concentrations and an increase at higher concentrations (Schell and
Wright, 1987). Additional studies are needed in order to further investigate concentration-dependent influence of lidocaine administration on ion channel activities in intestinal SM.

Alternative mechanisms which have been proposed in context with prokinetic lidocaine effects include anti-inflammation (Lahav et al., 2002; Kuo et al., 2006; Herroeder et al., 2007), pain reduction (Kuo et al., 2006) and blockade of sympathetic inhibitory reflexes (Rimbäck et al., 1990). Of these, pain reduction and blockade of sympathetic inhibitory reflexes can be excluded as mechanisms underlying contractility-enhancing effects of lidocaine under in vitro conditions. Nonetheless they may contribute to curative effects of lidocaine in vivo. Furthermore, improved membrane integrity resulting in preservation of high-energy phosphates and ATP metabolites has been discussed as a potential reason for improved muscle contractility (Takeo et al., 1989).

Impact of lidocaine supplementation on activity of the enteric nervous system

High lidocaine concentrations reduced impulses of both CSM and LSM. Thereby in CSM, contractility and impulses decreased simultaneously. Since impulses were assessed by measuring the contractile response of SM to EFS, it was impossible to differentiate if the reduction in impulses of CSM occurred due to diminished ENS activity or the lack of ability of CSM to contract under these high lidocaine concentrations. However, LSM still exhibited a rise in contractility at these high lidocaine concentrations but also featured declining impulses. Hence in LSM, the observed decrease in impulses at high lidocaine concentrations could not be charged to reduced SM contractile function but presumably appeared due to reduced ENS activity. In general, lidocaine is thought to block sodium channels in nerve cell membranes for exerting its local anaesthetic effects (Strichartz and Ritchie, 1987). Hence, it
was suggested that the reduction in ENS activity might occur due to lidocaine-induced reduction in sodium channel activity.

To summarise, two major findings were obtained from the present study: First, lidocaine effects on SM contractility were independent of TTX-sensitive sodium channel activity. Secondly, ENS activity appeared to be reduced by high lidocaine concentrations.

Applicability of the observed in vitro results to in vivo situations

Although enhanced SM contractility is thought to add to improved intestinal motility in vivo, applicability of the observed results to in vivo situations is limited as lidocaine concentrations applied to the organ bath exceeded therapeutic plasma concentrations in vivo (Malone et al., 2006). Concerning these discrepancies, it was suggested that an interaction of lipophilic lidocaine with cell membranes of SM cells and/or ICC might result in drug accumulation in these cells, producing higher tissue concentrations compared to plasma concentrations which might correspond to effective lidocaine concentration in vitro. So far, in vivo tissue lidocaine concentrations were only measured after rather short infusion periods under general anaesthesia (Guschlbauer et al., 2011; Teepe, 2012). Thus further studies are needed in order to determine in vivo tissue concentrations of lidocaine after long-term infusion.

Conclusions

Overall, the present study represented an in vitro approach destined to further elucidate possible mechanisms and to identify structural targets underlying contractility-enhancing effects of lidocaine in equine intestinal SM. At low to medium-ranged concentrations, lidocaine seemed to increase intrinsic SM contractility by directly affecting
SM cells and/or ICC without participation of the ENS. However, \textit{in vivo} intestinal motility does not only depend on intrinsic SM contractility but as well on ENS activity. As high lidocaine concentrations caused a decrease in both CSM contractility and ENS activity, the question arises whether these depressive effects might impair intestinal motility \textit{in vivo}. As lidocaine may accumulate in SM cells and ICC, further studies are needed to investigate \textit{in vivo} tissue lidocaine concentrations after long-term infusion for evaluating associated risks.

\textbf{Conflict of interest statement}

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

The presented data will be published as part of a PhD thesis at the University of Veterinary Medicine, Hannover, Germany.

\textbf{Acknowledgements}

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\textbf{References}


Manuscripts

Table 1

Effects of lidocaine treatment on frequency (ΔF), amplitude (ΔA) and mean active force (ΔMAF) of contractions.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>TTX application</th>
<th>Parameter</th>
<th>Conc.</th>
<th>Treatment</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSM</td>
<td>- TTX</td>
<td>ΔF</td>
<td>&lt;0.01</td>
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<td></td>
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<td>ΔA</td>
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<td>ΔMAF</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
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<td></td>
<td>+ TTX</td>
<td>ΔF</td>
<td>&lt;0.01</td>
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<td>ΔA</td>
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<td></td>
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<td>ΔMAF</td>
<td>&lt;0.001</td>
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<tr>
<td>LSM</td>
<td>- TTX</td>
<td>ΔF</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
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<td></td>
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<td>ΔA</td>
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<td>ΔMAF</td>
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<td></td>
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<td>ΔMAF</td>
<td>&lt;0.001</td>
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</tr>
</tbody>
</table>

 Differences in ΔF, ΔA and ΔMAF were assessed by Two-Way repeated measures ANOVA (factors ‘lidocaine concentration’ and ‘lidocaine treatment’) in circular (CSM) and longitudinal smooth muscle (LSM) in the absence (-TTX) or presence (+TTX) of tetrodotoxin (TTX).

 Factor ‘lidocaine concentration’ (conc.) regarded the influence of increasing lidocaine concentrations on ΔF, ΔA and ΔMAF.

 Factor ‘lidocaine treatment’ (treatment) examined differences in lidocaine-treated compared to untreated tissues.

 Interactions between factors ‘lidocaine concentration’ and ‘lidocaine treatment’ indicated that the influence of increasing lidocaine concentrations in lidocaine-treated tissues, respectively the influence of time in untreated tissues, differed in lidocaine-treated and untreated smooth muscle.
\[ \text{bd} \] \text{P values <0.05, <0.01 and <0.001 indicated significant influence of factors 'lidocaine concentration' and 'lidocaine treatment' on Delta F, Delta A and Delta MAF, as well as an interaction between both factors; ns = not significant} \]
Figure legends

Fig. 1. Experimental setting pointing out the different treatment groups and the number of smooth muscle (SM) strips within each treatment group (in parentheses) obtained from each horse. Full thickness SM strips were cut in direction of the circular or longitudinal SM layer, referred to as circular (CSM) or longitudinal (LSM) SM strips. Half of the SM strips were incubated with/without tetrodotoxin (+TTX/-TTX). Lidocaine was added to half of the SM strips (+L), whereas the other half remained untreated (-L). For assessing the impact of TTX application, SM strips with and without TTX application were compared within the CSM or LSM layer (a). Effects of lidocaine administration were examined by comparison of lidocaine-treated and untreated SM strips within each SM layer (b).

Fig. 2. Frequency (Delta F) of contractions in circular smooth muscle (CSM; Fig. 2A) and longitudinal smooth muscle (LSM; Fig. 2B) with or without application of tetrodotoxin (+TTX; -TTX) in the presence (+L) or absence (-L) of increasing lidocaine concentrations. As lidocaine was applied to the organ bath at regular intervals, each lidocaine concentration resembled a distinct time point during experimental procedures, with concentrations of 5, 25, 50, 100 and 200 mg/L lidocaine at 80, 95, 110, 125 and 140 min after start of the experiment. Data were given as means ± SE of N=10 horses. Paired Student’s t tests comparing Delta F in the absence and presence of TTX were performed for each time point, with *P<0.05, **P<0.01 and ***P<0.001 indicating significant differences.

Fig. 3. Amplitude (Delta A) of contractions in circular smooth muscle (CSM; Fig. 3A) and longitudinal smooth muscle (LSM; Fig. 3B) with or without application of tetrodotoxin (+TTX; -TTX) in the presence (+L) or absence (-L) of increasing lidocaine concentrations. As lidocaine was applied to the organ bath at regular intervals, each lidocaine concentration...
resembled a distinct time point during experimental procedures, with concentrations of 5, 25, 50, 100 and 200 mg/L lidocaine at 80, 95, 110, 125 and 140 min after start of the experiment. Data were given as means ± SE of N=10 horses. Paired Student’s t tests comparing Delta Δ in the absence and presence of TTX were performed for each time point, with *P<0.05, **P<0.01 and ***P<0.001 indicating significant differences.

Fig. 4. Mean active force (Delta MAF) of contractions in circular smooth muscle (CSM; Fig. 4A) and longitudinal smooth muscle (LSM; Fig. 4B) with or without application of tetrodotoxin (+TTX; -TTX) in the presence (+L) or absence (-L) of increasing lidocaine concentrations. As lidocaine was applied to the organ bath at regular intervals, each lidocaine concentration resembled a distinct time point during experimental procedures, with concentrations of 5, 25, 50, 100 and 200 mg/L lidocaine at 80, 95, 110, 125 and 140 min after start of the experiment. Data were given as means ± SE of N=10 horses. Paired Student’s t tests comparing Delta MAF in the absence and presence of TTX were performed for each time point, with *P<0.05, **P<0.01 and ***P<0.001 indicating significant differences.

Fig. 5. Activity of the enteric nervous system, assessed as impulses, in the presence or absence of tetrodotoxin (+TTX; -TTX) in tissues with or without supplementation of increasing lidocaine concentrations (+L; -L). Impulses were measured in circular smooth muscle (CSM; Fig. 5A) and longitudinal smooth muscle (LSM; Fig. 5B). As lidocaine was applied to the organ bath at regular intervals, each lidocaine concentration resembled a distinct time point during experimental procedures, with concentrations of 5, 25, 50, 100 and 200 mg/L lidocaine at 80, 95, 110, 125 and 140 min after start of the experiment. Data were given as means ± SE of N=10 horses. Paired Student’s t tests comparing impulses in the
absence and presence of TTX were performed for each time point, with \( *P<0.05, **P<0.01 \) and \( ***P<0.001 \) indicating significant differences.
Figure 1

Equine jejunal SM

CSM (8)

+TTX (4)

-L (2)

+L (2)

-TTX (4)

-L (2)

LSM (8)

+TTX (4)

-L (2)

+L (2)

-L (2)

a

b

a

b

a

b
Figure 2

A: CSM

B: LSM
Figure 3

![Graph A: CSM](image)

![Graph B: LSM](image)

- CSM - TTX - L
- CSM + TTX - L
- CSM - TTX + L
- CSM + TTX + L

- LSM - TTX - L
- LSM + TTX - L
- LSM - TTX + L
- LSM + TTX + L
Figure 4

A: CSM

B: LSM

[Graph showing data for CSM and LSM over time]

[Graph showing data for CSM and LSM over time]
Figure 5

- **A: CSM**
  - Graph showing time (min) on the x-axis and Impedance (mS x V) on the y-axis.
  - Different lines represent different conditions:
    - CSM - TTX - L
    - CSM + TTX - L
    - CSM - TTX + L
    - CSM + TTX + L
  - Significant differences marked by asterisks: *** indicates p < 0.001, ** indicates p < 0.01, * indicates p < 0.05.

- **A: LSM**
  - Similar graph structure to CSM.
  - Lines represent:
    - LSM - TTX - L
    - LSM + TTX - L
    - LSM - TTX + L
    - LSM + TTX + L
  - Significant differences also marked by asterisks: *** indicates p < 0.001, ** indicates p < 0.01, * indicates p < 0.05.
5 UNPUBLISHED DATA

From the horses used in this PhD project (Table 1) some additional data were collected which were not included in Manuscripts 1-4 and therefore are presented in the following chapters.

5.1 Smooth muscle contractility after prolonged duration of ischaemia

5.1.1 Background

A previous in vitro study by GUSCHLBAUER et al. (2010a) demonstrated a marked decrease in SM contractility due to 15 min of standardised, artificial ischaemia followed by 15 min of reperfusion. In the same study, lidocaine was shown to be more effective in increasing contractility of IR-injured compared to non-injured SM. Therefore it would be of interest to determine consequences of prolonged duration of ischaemia on spontaneous SM contractility and to examine the ability of lidocaine to improve contractile functions in SM subjected to varying duration of ischaemia. We hypothesised that increased duration of ischaemia further reduces spontaneous SM contractility, but these effects are counterbalanced by lidocaine administration. In three preliminary experiments, SM contractility after 30 min of ischaemia was compared to contractility of non-injured control SM and SM subjected to 15 min of ischaemia.

5.1.2 Materials and methods

Samples were collected from two stallions and one mare which were 7, 25 and 3 years old (Table 1). Surgical procedures were performed as described in Manuscript 3 except that in addition to an intestinal segment subjected to 15 min of ischaemia, a second intestinal segment was simultaneously subjected to 30 min of ischaemia. Both intestinal segments were allowed to reperfuse for 15 min. Preparation of CSM and LSM strips (size: 0.5 x 1.0 cm; weight: mean ± SEM 0.024 ± 0.001 g wet tissue) and measurement of isometric contractile activity were conducted as pointed out in Manuscripts 3 and 4.
After testing ENS and SM viability, the ENS was deactivated using 1 µmol/L TTX (Manuscript 3). Spontaneous contractile activity of CSM and LSM strips was assessed by determining frequency ($F$; Peaks/min), amplitude ($A$; mN) and mean active force ($MAF$; mN) of contractions and was defined as CSM and LSM contractility. $F$, $A$ and $MAF$ were calculated for 3 min evaluation periods at regular intervals of 15 min with definitions of $F$, $A$ and $MAF$ according to the description in Manuscripts 1, 3 and 4.

Values are presented as means ± SEM of N=3 horses with n=1 value/horse. Normal distribution of data could not be tested due to the small number of animals included in this experimental approach. Differences in SM contractility following prolonged duration of ischaemia were analysed by Two-Way repeated measures (RM) analysis of variance (ANOVA) (factors ‘time’ and ‘duration of ischaemia’) and One-Way RM ANOVA with Tukey’s post hoc test for all treatment groups at each time point. Paired Student’s t-tests for each time point were used to compare contractility of SM subjected to 15 or 30 min of ischaemia to contractility of control SM and to assess differences in contractility of SM following 15 or 30 min of ischaemia. Levels of significance were set at $P<0.05$, $P<0.01$ and $P<0.001$.

5.1.3 Results

According to results of Two-Way RM ANOVA and One-Way RM ANOVA with Tukey’s post hoc test, the duration of ischaemia did not significantly influence $F$, $A$ and $MAF$ of intestinal SM. Results of Paired Student’s t-tests revealed an increase in $A$ and $MAF$ of CSM after 30 min of ischaemia compared to non-injured control CSM (Figure 4).
Figure 4: Frequency, amplitude and mean active force of contractions of circular and longitudinal equine jejunal smooth muscle. Smooth muscle was non-injured (control) or subjected to 15 or 30 min of ischaemia followed by 15 min of reperfusion. Paired Student’s t-tests for each time point revealed significant differences in contractility of circular smooth muscle subjected to 30 min of ischaemia compared to control circular smooth muscle. Data are presented as means ± SEM of N=3 horses with *P<0.05 indicating significant differences.

5.1.4 Evaluation of obtained results

The present preliminary experiments aimed to compare spontaneous SM contractility at different degrees of tissue damage, induced by prolonged duration of ischaemia. The underlying hypothesis was based on previous findings showing that 15 min of ischaemia markedly reduced contractility of equine jejunal SM (GUSCHLBAUER et al. 2010a). It was presumed, that doubling the duration of ischaemia would amplify these effects as for example ASKENASY et al. (2001) found that membrane permeability, which was suggested to be associated with decreased
SM contractile performance (GUSCHLBAUER et al. 2010a), correlated with the duration of ischaemia in rat heart muscle. Unexpectedly, 30 min of ischaemia did not reduce but enhanced contractility in equine intestinal CSM. In contrast to CSM, contractility of LSM was not altered due to IR injury.

In general, IR injury is thought to diminish SM contractile activity due to formation of reactive oxygen metabolites (reviewed by KONG et al. 1998) and degenerative changes in the SM layer (PONTELL et al. 2011). Changes in ion channel activity altering fluxes of potassium ions (K⁺) or Ca²⁺ have also been discussed in context with hypoxia (reviewed by TAGGART and WRAY 1998). Nonetheless, evidence is provided concerning mechanisms which might increase SM contractility following IR injury. These include stimulatory effects of prostaglandins which might be released due to inflammatory processes following IR of the intestinal wall. For example, VAN HOOGMOED et al. (2000) showed that different prostaglandins increased LSM contractility in equine large intestine, depending on the concentration of prostaglandins and the localisation within the large intestine from which SM samples were collected. These results were as well observed in the human gastrointestinal tract where a sensitivity gradient to prostaglandin administration was detected, declining from the stomach to the sigmoidal colon (BENNETT et al. 1981). These findings may be based on a prostaglandin-induced increase in ICC activity (PARK et al. 2011).

Thus, concerning results of the present experiments, the observed increase in CSM contractility following 30 min of ischaemia might be attributed to IR-induced release of prostaglandins. After 15 min of ischaemia, prostaglandin release might not yet be strong enough for inducing a significant rise in contractility. However, BENNETT et al. (1981) and VAN HOOGMOED et al. (2000) documented more pronounced stimulating effects of prostaglandins on LSM. Therefore, if prostaglandin release was responsible for the increase in CSM contractility, an even greater increase in contractility would be expected in LSM. On the contrary, results of the present experiments showed that LSM contractility was not altered by IR injury. BENNETT et al. (1981) and VAN HOOGMOED et al. (2000) used tissue samples from non-injured intestine for testing the effects of different prostaglandins. Thus, discrepancies in reactions of CSM and LSM to prostaglandins in these previous studies and the present experiments might result from different sensibility of both SM
layers to IR injury (PONTELL et al. 2011). Molecular differences between CSM and LSM cells (Chapter 1.1; Manuscript 3) might contribute to the varying response of CSM and LSM to ischaemic damage. Alternatively, prostaglandin effects in equine small intestine, which was used in the present experiments, might be converse to those effects detected in equine large intestine (VAN HOOGMOED et al. 2000).

Overall, it has to be noted that the consequences of prolonged duration of ischaemia as presented in Figure 4 only referred to data obtained from three horses with one SM strip per horse. Due to this small number of animals and high inter-individual variations, the obtained results have to be interpreted with caution. Additional studies on a greater number of animals are needed in order to confirm the observed results and to determine the efficacy of lidocaine for increasing SM contractility after prolonged duration of ischaemia.

5.2 Concentration-dependent lidocaine effects on creatine kinase release

5.2.1 Background

CK is widely accepted as marker for cellular damage and cell membrane permeability in muscle and was suggested to pass through the muscle cell membrane via small clefts, formatted by mechanical stress (OZAWA et al. 1999). In rat heart muscle, prolonged duration of ischaemia correlated with increased membrane permeability, which was indicated by elevated CK release, augmented distribution of the extracellular marker cobalticyanide in the intracellular space and reduced hypo-osmotic swelling of muscle cells (ASKENASY et al. 2001). TAKEO et al. (1989) demonstrated that lidocaine treatment exerted positive effects on contractile functions of rabbit heart muscle following hypoxia and reoxygenation. They suggested that administration of lidocaine preserved the membrane integrity of cardiac muscle cells and consecutively lead to retention of high energy phosphates and adenosine triphosphate metabolites which in turn maintained the cellular energy metabolism. A similar mechanism of lidocaine might be involved in improving contractility of SM, as previous in vitro studies (GUSCHLBAUER et al. 2010a; Manuscript 1) indicated a positive impact of lidocaine on the cell membrane permeability of equine jejunal SM cells, assessed by reduced CK release.
So far, contractility-enhancing effects of lidocaine were shown to be concentration-dependent (GUSCHLBAUER et al. 2010a). Thereby increasing lidocaine concentrations induced an increase in contractility, followed by a decrease in contractility at high concentrations. If the observed increase in SM contractility depended on improved cell membrane integrity, it would be paralleled by a concentration-dependent decrease in CK release. Therefore, aim of the present study was to determine CK release from non-injured control and IR-injured equine jejunal SM following incubation with increasing lidocaine concentrations in vitro. It was hypothesised that increasing lidocaine concentrations reduce CK release in a concentration-dependent manner, whereas high lidocaine concentrations augment CK release.

5.2.2 Materials and methods

Data were collected from N=8 horses (7 mares and 1 gelding) aged between 3 and 27 years (Table 1). Surgical procedures were conducted as described in Manuscript 3 and SM strips (size: 2 x 2 cm; weight: mean ± SEM 0.26 ± 0.01 g) were prepared as described in Manuscript 1 by removing mucosa and submucosa.

The SM strips were incubated for 60 min at 38°C in organ baths containing 5 mL modified Krebs-Henseleit Buffer (Manuscripts 1-4) supplemented with lidocaine. Control SM strips received 50 mg/L lidocaine, whereas IR-injured SM strips were incubated with 2.5, 5, 25, 50 or 100 mg/L lidocaine. Basal values were obtained from control and IR-injured SM strips incubated without lidocaine supplementation. Following incubation, CK activity in the incubation buffer was measured as indicator for CK release using an automated analyser as described in Manuscript 1.

Data obtained from this experimental approach were normally distributed. Influence of IR injury and administration of 50 mg/L lidocaine on CK activity were assessed by Two-Way RM ANOVA (factors ‘lidocaine administration’ and ‘IR injury’). Additionally, Paired Student’s t-tests were used to compare CK release from control and IR-injured SM with or without administration of 50 mg/L lidocaine. Effects of increasing lidocaine concentrations on CK release from IR-injured SM were calculated by One-Way RM ANOVA with Tukey’s post hoc test. In the control group treated with 50 mg/L lidocaine, the value obtained from one horse was below the limit
of detection. Therefore this horse was excluded from the calculation and results are presented as means ± SEM of N=7 horses. \( P \) values <0.05, <0.01 and <0.001 were regarded as statistically significant. \( P \) values <0.1 were defined as a trend.

5.2.3 Results

Compared to control SM, IR injury increased CK release from equine intestinal SM without and with lidocaine supplementation (Paired Student’s t-test; Figure 5). Administration of 50 mg/L lidocaine alleviated CK release from intestinal SM (Two-Way RM ANOVA, factor ‘lidocaine administration’: \( P<0.05 \), factor ‘IR injury’: not significant). Thereby lidocaine did not alter CK release from control SM, but tended to reduce CK release from IR-injured SM. Compared to untreated control SM, CK release from lidocaine-treated IR-injured SM tended to remain elevated (Paired Student’s t-tests; Figure 5).

Administration of increasing lidocaine concentrations significantly altered CK release from IR-injured SM (One-Way RM ANOVA: \( P<0.001 \)). In contrast to Paired Student’s t-test (Figure 5), One-Way RM ANOVA did not detect any differences between untreated IR-injured SM and IR-injured SM treated with 50 mg/L lidocaine (Figure 6). In comparison to administration of 2.5, 5 or 25 mg/L lidocaine, CK release was reduced following application of 50 mg/L lidocaine. Treatment with 100 mg/L lidocaine did not provide any significant effects (Figure 6).
5.2.4 Evaluation of obtained results

Results of the present study contradicted previous findings stating that 25 mg/L lidocaine significantly reduced CK release from equine jejunal SM which was subjected to a comparable IR injury and incubated under similar experimental conditions (GUSCHLBAUER et al. 2010a). The present results also did not support the hypothesis that lidocaine altered CK release from SM cells concentration-dependently in accordance with changes in SM contractility. Nonetheless, CK release tended to be reduced at lidocaine concentrations of 50 mg/L compared to untreated IR-injured SM (Figure 5), consistent with the concentration range between 25 and 100 mg/L in which the lidocaine-stimulated SM contractility reached its plateau (GUSCHLBAUER et al. 2010a). Thus, relevance of reduced CK release in context with improved SM contractility due to lidocaine administration remains questionable. Differences between data in literature and the observed results cannot be explained, yet.
5.3 Tissue lidocaine concentrations after \textit{in vitro} incubation

5.3.1 Background

Regarding results of previous \textit{in vitro} studies (GUSCHLBAUER et al. 2010a; Manuscripts 1, 3), lidocaine most effectively increased the contractility of IR-injured equine jejunal CSM in the concentration range between 20-100 mg/L. Accordingly, these \textit{in vitro} concentrations were at least ten times higher than effective blood concentrations \textit{in vivo} (1-2 mg/L; MALONE et al. 2006) and clearly exceeded serum concentrations associated with lidocaine intoxication in healthy horses (mean ± SD 3.24 ± 0.74 mg/L; MEYER et al. 2001). As lidocaine was suggested to exert contractility-enhancing effects on SM via interactions with SM cell membranes (Manuscript 1), it was hypothesised that during infusion, lidocaine accumulates in intestinal SM resulting in elevated tissue compared to plasma concentrations. Thereby tissue concentrations were suggested to correspond to effective lidocaine concentrations \textit{in vitro}.

So far, studies in literature comparing tissue and serum (respectively plasma) concentrations of lidocaine in anaesthetised horses failed to detect a clear association between both parameters. GUSCHLBAUER et al. (2011) found that after short-term \textit{in vivo} infusion (10 min bolus infusion with 1.3 mg/kg BW, followed by 5 min CRI with 0.05 mg/kg BW/min) lidocaine concentrations in serum and jejunal SM of horses were highly variable and did not show any significant differences. Likewise, six hours of lidocaine infusion (15 min bolus infusion with 1.3 mg/kg BW, followed by CRI with 0.05 mg/kg BW/min up to 360 min) did not result in any significant discrepancies between mean plasma and tissue lidocaine concentrations (TEEPE 2012). However, after 60 min infusion time lidocaine concentrations reached a steady state in equine plasma, whereas in the extracellular fluid a continuous slight increase in lidocaine concentrations was observed throughout the trial (TEEPE 2012). This result indicated that despite steady plasma lidocaine concentrations, lidocaine concentrations might still increase in the intestinal wall.

During \textit{in vivo} lidocaine infusion, inter-individual differences between horses such as different drug metabolism or cardiovascular parameters contribute to high variability of the obtained results. For this reason, aim of the present study was to
measure the lidocaine content in intestinal SM following incubation with increasing lidocaine concentrations under well standardised *in vitro* conditions.

### 5.3.2 Materials and methods

Samples were received from N=8 horses (7 mares and 1 gelding) aged between 3 and 27 years (Table 1). During a routine median laparotomy, a segment of distal jejunum was subjected to a defined, artificial IR injury as described in Manuscripts 1 and 3, consisting of 15 min of ischaemia followed by 15 min of reperfusion. Following resection, SM strips (size: 2 × 2 cm; weight: mean ± SEM 0.26 ± 0.01 g) were prepared as pointed out in Manuscript 1 by removing mucosa and submucosa.

The SM strips were incubated for 60 min at 38°C in 5 mL modified Krebs-Henseleit Buffer (Manuscripts 1-4) containing 2.5, 5, 25 or 100 mg/L lidocaine. Following incubation, the SM strips were shock frozen in liquid nitrogen and stored at -80°C. Lidocaine was extracted from SM by a method modified according to BLIGH and DYER (1959) and established by TEEPE (2012). Briefly, about 50 mg of tissue samples were homogenised using a potter (B. Braun Melsungen AG, Melsungen, Germany) after adding 10 µg bupivacaine (bupivacaine hydrochloride, Fluka/Sigma-Aldrich Chemie GmbH, Munich, Germany, dissolved in distilled water) as internal standard for determination of recovery rates and 200 µL of distilled water. Extraction was performed using methanol (2 mL) and chloroform (1 mL), the latter of which was added after samples had been incubated for 2 min with methanol. After further homogenisation of samples using a potter (B. Braun) and 30 min of mixing by rotation (overhead shaker Reax 2, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), samples were centrifuged for 5 min with 3000 rpm at 7°C (Heraeus Megafuge 16R, rotor TX-200, bucket 3659, Thermo Fisher Scientific Inc., Waltham, MA, USA). The supernatant was mixed with 1 mL chloroform and 1 mL distilled water for 5 min using a shaker (VXR basic Vibrax, IKA-Werke GmbH & Co. KG, Staufen, Germany) and again centrifuged with 3000 rpm at 7°C for 10 min (Heraeus Megafuge 16R, Thermo Fisher Scientific). The upper phase was discarded. Centrifugation (5 min with 3000 rpm at 7°C, Heraeus Megafuge 16R, Thermo Fisher Scientific) and discarding was repeated before the lower phase was dried by evaporation under air pressure at 40°C (TCS metal block thermostat, vapotherm I,
Barkey GmbH & Co. KG, Leopoldshöhe, Germany). The remaining pellet was dissolved in 1 mL of an eluent (100 mL acetonitril, 400 mL phosphate buffer (0.340 g KH$_2$PO$_4$ and 0.445 g Na$_2$HPO$_4$ in 500 mL distilled water at pH 5.5), 5 mL acetoacetate (100%) and 5 mL triethylamine), filtered and stored at -80°C.

Lidocaine concentrations in samples were measured by high performance liquid chromatography (HPLC) at constant flux rates of 1.5 mL/min in combination with UV/VIS detection at 280 nm using a system consisting of autosampler (model 507e, Beckman Coulter GmbH, Fullerton, CA, USA), pump (model 126, Beckmann), detector (model 168, Beckmann), software (24 Karat 5.0, Beckmann), column (LiChroCART 25-4 (C18, 5µm), Merck KGaA, Darmstadt, Germany), pre-column (LiChroCART 4-A (5 µm), Merck) and heater (SPH 99, 40°C, Spark Holland B.V., Emmen, The Netherlands). Limits of detection were 47 ng/mL for lidocaine and 238 ng/mL for bupivacaine, whereas limits of quantification were 93 ng/mL for lidocaine and 477 ng/mL for bupivacaine. For quantification, drug concentrations in samples were compared to standard concentrations of lidocaine and bupivacaine (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 2.5, 5.0, 7.5 and 10.0 µg/mL lidocaine and bupivacaine hydrochloride dissolved in distilled water).

HPLC analysis was kindly conducted by Jessica Stahl, Department of Pharmacology, Toxicology and Pharmacy, University of Veterinary Medicine, Hannover, Germany.

Data concerning lidocaine concentrations in equine intestinal SM were normally distributed. Influence of increasing lidocaine concentrations in the incubation buffer on lidocaine concentrations in equine intestinal SM was assessed by One-Way RM ANOVA with Tukey’s post hoc test. Correlations between buffer and tissue lidocaine concentrations were examined by linear and non-linear regression analyses. Data were given as means ± SEM of N=8 horses with levels of significance at $P<0.05$, $P<0.01$ and $P<0.001$.

5.3.3 Results

The mean recovery rate for bupivacaine was 73%. Increasing lidocaine concentrations in the incubation buffer increased the tissue lidocaine content (One-Way RM ANOVA, $P<0.001$). Following incubation with 100 mg/L, lidocaine...
concentrations were significantly higher than after incubation with 2.5, 5 or 25 mg/L (Figure 7). Both linear and non-linear regression analysis detected a correlation between lidocaine concentrations in the incubation buffer and in intestinal SM (Figure 8).

5.3.4 Evaluation of obtained results

If occurring, insertion of lidocaine into intestinal SM was expected to become apparent soon after starting the incubation, as lidocaine was shown to be incorporated into human erythrocytes within 30 seconds (NISHIGUCHI et al. 1993). Thus, if lidocaine accumulated in intestinal SM, an exponential increase in tissue lidocaine content should readily appear after 60 min of incubation.

As expected, rising lidocaine concentrations in the incubation buffer resulted in increased tissue lidocaine content. Nonetheless, results obtained from the present experiments were ambiguous. Theoretically, a linear correlation would point at free diffusion of lidocaine between buffer solution and SM tissue, whereas an exponential increase in SM lidocaine concentrations would indicate directed transport and lidocaine accumulation. In the present study, linear and non-linear regression analyses featured similar results, presumably due to the limited number of different

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**Figure 7:** Lidocaine concentrations in ischaemia-reperfusion-injured equine jejunal smooth muscle after 60 min in vitro incubation with different lidocaine concentrations. Results were obtained from N=8 horses and are presented as means ± SEM. Results of One-Way repeated measures analysis of variance with Tukey’s post hoc test revealed significant differences between treatment groups with ***P<0.001.

**Figure 8:** Linear and non-linear regression analyses regarding tissue lidocaine concentrations after 60 min of incubation with different lidocaine concentrations in the incubation buffer. Results were obtained from N=8 horses and are presented as means ± SEM. Slope of linear regression line deviated from zero with P<0.001.
lidocaine concentrations. In consequence, the question whether lidocaine accumulates in intestinal SM in vivo resulting in higher tissue compared to plasma concentrations cannot be answered, yet.

Further experiments comprising a greater range of different lidocaine concentrations might be able to determine whether lidocaine accumulates in intestinal SM. Alternatively, tissue lidocaine content could be measured at different time points during long-term incubation, with constant buffer lidocaine concentrations reflecting therapeutic plasma lidocaine concentrations in vivo.
6 DISCUSSION

6.1 Efficacy of lidocaine treatment

6.1.1 Efficacy of lidocaine treatment in vitro

In the present PhD project, samples were received from horses of different breeds, gender, age and body weight which were assigned to euthanasia for reasons other than gastrointestinal disorders. Despite this heterogeneous study population, lidocaine was consistently effective in increasing intestinal SM contractility in vitro. This confirmed results of previous studies, in which lidocaine effects were as well discovered in horses featuring different characteristics (GUSCHLBAUER et al. 2010a, 2011). Although horses used in this PhD project were not representative of the overall population of horses, it can be concluded that the observed results apply for a wide variety of horses.

Lidocaine stimulated contractility in both CSM and LSM of equine jejunum, although effective concentrations differed between the SM layers. Thus overall, lidocaine exhibited high efficacy for increasing SM contractile activity under the given in vitro conditions.

6.1.2 Optimal concentration range for increasing smooth muscle contractility

Regarding results of previous studies (GUSCHLBAUER et al. 2010a) and studies performed during this PhD project (Manuscripts 1, 3), lidocaine consistently enhanced contractility of intestinal SM at concentrations ranging from 20 to 100 mg/L. In accordance with these findings, lidocaine disrupted DRMs in membranes of intestinal SM at concentrations of 50 mg/L. DRM disruption was suggested to increase activity of cellular ion channels (Manuscript 2), resulting in improved SM contractility. However, DRM disruption was not associated with deteriorated cell membrane integrity, indicated by the finding that the release of CK from IR-injured SM cells tended to be reduced at lidocaine concentrations of 50 mg/L (Manuscript 1; Chapter 5.2). Reduced CK release was interpreted as indicator for improved cell membrane integrity, which was suggested to result in preserved energy metabolism and subsequently in increased SM contractile activity in accordance with a study by
TAKEO et al. (1989). However, a concentration-dependent decrease in CK release due to administration of increasing lidocaine concentrations could not be demonstrated (Chapter 5.2). On the contrary, at lidocaine concentrations of 2.5-25 mg/L, CK release from SM was elevated compared to concentrations of 50 mg/L. Thus an involvement of improved cell membrane integrity in contractility-enhancing lidocaine effects remains questionable.

In contrast to lidocaine concentrations between 20-100 mg/L, lidocaine concentrations higher than 100 mg/L caused a decrease in CSM contractility (GUSCHLBAUER et al. 2010a; Manuscript 3). These adverse effects on CSM contractility might be explained by concentration-dependent effects of lidocaine on ion channel activity. Thereby low to medium-ranged concentrations may increase activity of ion channels, whereas high concentrations may reduce ion channel activity. Such converse effects of lidocaine on ion transport were indicated by SCHELL and WRIGHT (1987), who found that low or high lidocaine concentrations decreased or increased ion permeability of renal brush border membranes, respectively. Nonetheless, the mechanism underlying reduced SM contractility at high lidocaine concentrations remains unclear and further studies are needed in order to clarify this aspect. Despite diminished SM contractility at high lidocaine concentrations, lidocaine did not seem to provide cell toxic effects as contractility of SM was shown to recover after removing lidocaine (Manuscript 1).

When discussing an optimal concentration range for the lidocaine-induced increase in SM contractility, differences in lidocaine effects on CSM and LSM have to be taken into account, as well. In contrast to CSM, LSM still exhibited an increase in contractility at high lidocaine concentrations as used in this PhD project (Manuscript 3). However, LSM contractile activity seemed to have minor impact on the directed transport of chyme through the intestinal tract compared to CSM, as pointed out more detailed in Chapter 1.1 and Manuscript 3. For this reason, lidocaine concentrations which are able to increase contractility in CSM are considered to be more relevant with respect to overall stimulating effects of lidocaine on gastrointestinal motility in vivo.

When regarding the above results, lidocaine concentrations between 20-100 mg/L appeared to be most effective in increasing SM contractility, while they simultaneously modulated cellular structures which might be involved in its
contractility-enhancing effects. However, concentrations of 20-100 mg/L widely exceeded therapeutic blood lidocaine concentrations \textit{in vivo} (MALONE et al. 2006). As lidocaine was suggested to directly act on SM cells and ICC (GUSCHLBAUER et al. 2010a; Manuscript 4) the question arises whether lidocaine administration causes tissue concentrations far higher than plasma concentrations (Chapter 5.3), corresponding to the suggested optimal concentration range for improving SM contractility. In this context, tissue lidocaine concentrations exceeding this optimal concentration range would abolish previous positive effects on SM contractility, whereas lower concentrations would fail to exert best efficacy. In the present PhD project, increasing lidocaine concentrations in the organ bath resulted in elevated lidocaine content in intestinal SM \textit{in vitro} (Chapter 5.3). Nonetheless, accumulation of lidocaine in SM samples, resulting in higher tissue compared to buffer lidocaine concentrations, could not be clearly demonstrated.

According to the above explanations, the optimal concentration range for increasing SM contractility \textit{in vitro} comprised lidocaine concentrations corresponding to the plateau phase of its contractility-enhancing effects (GUSCHLBAUER et al. 2010a). However, lidocaine already exerted contractility-enhancing effects on intestinal SM at lower concentrations, although the observed effects were not yet statistically significant (GUSCHLBAUER et al. 2010a; Manuscripts 1, 3). It remains to be clarified, if \textit{in vivo} maximum activation of SM contractility was actually desirable. On the contrary, maximum lidocaine effects on SM contractility might be associated with hypermotility rather than restored physiological motility of the intestine.

Subsequent studies on tissue lidocaine concentrations following \textit{in vitro} incubation with or \textit{in vivo} infusion of lidocaine, possibly combined with measurement of \textit{in vivo} intestinal motility, might provide further evidence for an optimal concentration range of lidocaine in equine intestinal SM.

\textbf{6.1.3 Applicability of the obtained \textit{in vitro} results to \textit{in vivo} situations}

Despite the fact that contractility-enhancing effects of lidocaine were reliably reproducible, the observed \textit{in vitro} results cannot easily be extrapolated to \textit{in vivo} situations. As pointed out above (Chapters 5.3.1, 6.1.2), lidocaine concentrations applied to the organ bath \textit{in vitro} exceeded \textit{in vivo} blood target concentrations
Discussion

(MALONE et al. 2006) about 10-100 times and presumably would be associated with undesired side effects if occurring in equine serum \textit{in vivo} (MEYER et al. 2001). \textit{In vitro}, these concentrations are needed for inducing statistically significant effects on SM contractility. Concerning these discrepancies between effective lidocaine concentrations \textit{in vivo} and \textit{in vitro}, it was hypothesised that lipophilic lidocaine accumulated in the intestinal wall resulting in tissue concentrations far higher than plasma concentrations (Chapters 5.3.1, 6.1.2). However, until this hypothesis is confirmed, data obtained from the described \textit{in vitro} approaches have to be interpreted with caution.

In contrast to \textit{in vivo} situations, lidocaine was not metabolised under the prevailing \textit{in vitro} conditions as lidocaine metabolism takes place in the liver (ARTHUR 1987). In consequence, effects of the major lidocaine metabolites MEGX and GX which are pharmacologically active could not be determined in experiments of this PhD project.

Additionally, in horses suffering from POI, effects such as pain relief, anti-inflammation and suppression of inhibitory sympathetic reflexes might contribute to the therapeutic impact of lidocaine as suggested by RIMBÄCK et al. (1990) and KUO et al. (2006). In the current experiments, an influence of these effects was excluded by examining isolated SM strips in an \textit{in vitro} setting. Thus, the observed results only described direct pharmacological effects of lidocaine on the SM layer.

\textit{In vivo}, POI may occur due to tissue damage at varying locations throughout the gastrointestinal tract. Nonetheless, the present \textit{in vitro} studies only examined effects of lidocaine on a very small segment of equine intestine, located at a defined position in the distal jejunum. An \textit{in vitro} study by NIETO et al. (2000) reported beneficial influence of lidocaine on SM contractility in the proximal part of the duodenum of horses, but not in the jejunum. Efficacy of lidocaine in the large intestine was indicated by studies stating that \textit{in vivo} lidocaine administration exerted beneficial effects on bowel functions in human patients who underwent colorectal and colonic surgery (KUO et al. 2006; HERROEDER et al. 2007). In horses, lidocaine was frequently chosen as prokinetic agent both after small and large intestinal lesions (VAN HOOGMOED et al. 2004). However, to the best of our knowledge no study systematically compared lidocaine effects on SM contractility throughout the whole equine gastrointestinal tract. Thus, differences in lidocaine activity along the intestinal
axis cannot be excluded and need to be examined in further studies, in order to prove the validity of the results obtained during this PhD project.

Concerning the severity of tissue damage, the present experiments were conducted under well standardised conditions including an artificial IR injury. Compared to strangulating obstructions of equine intestine *in vivo*, duration of the applied *in vitro* IR injury was rather short in order to simulate mild muscular damage such as may occur in non-resected equine intestine. Nevertheless, tissue injury following ischaemic bowel obstructions and subsequent colic surgery *in vivo* might differ from the given *in vitro* approach. For this reason further *in vitro* studies are needed to determine lidocaine effects on contractility of SM along the intestinal axis at varying degrees of tissue injury.

6.2 Possible mechanisms of contractility-enhancing lidocaine effects

6.2.1 Ion channels and ion transport systems

*In vitro*, lidocaine appeared to enhance SM contractility without alterations in lidocaine effects due to TTX application (Manuscript 4). These results indicated that lidocaine exerted beneficial effects on SM contractility by solely affecting SM cells and/or ICC. Reduced cell membrane permeability (GUSCHLBAUER et al. 2010a; Manuscript 1) and disruption of DRMs in SM (Manuscript 2) due to lidocaine application suggested that lidocaine interacted with cell membranes of its target cells.

In SM cells and ICC, a variety of membrane ion channels takes part in modulating the membrane potential and in initiating contractions as summarised by FARRUGIA (1999). These include $K^+$, $Ca^{2+}$ and $Na^+$ channels which were shown to respond to local anaesthetic treatment (reviewed by SCHOLZ 2002). Therefore it was proposed that changes in ion fluxes might be involved in lidocaine-induced effects on SM contractility. Alterations in activity of other cellular structures like the $Na^+\text{-}K^+$-pump, $Ca^{2+}$-pumps or the $Na^+/Ca^{2+}$-exchanger might as well mediate changes in cell excitability and contractions as these structures contribute to restoring and maintaining the original intra- and extracellular ion distribution (reviewed by FLEMING 1980; WEBB 2003). When considering possible lidocaine effects on ion fluxes in SM, concentration-dependent actions on ion channel activity have to be taken into account. Even converse reactions due to increased or decreased drug
concentrations are imaginable as shown for ion permeability in rabbit renal brush border membranes (SCHELL and WRIGHT 1987). Therefore in the following, consequences of both increased and decreased ion channel activity are briefly discussed.

In excitable cells, Na\(^+\) ions normally constitute the major ion influx at membrane depolarisation, inducing the development and propagation of action potentials. In gastrointestinal SM, Na\(^+\) influx appears to play a minor role for membrane depolarisation and SM contraction compared to Ca\(^{2+}\) influx (SNAPE and TAN 1985), but it is thought to modulate the slow waves generated by ICC (STREGE et al. 2003) and might affect enteric nervous functions. Therefore, blockade of Na\(^+\) channels could contribute to impaired SM contractility at high lidocaine concentrations \textit{in vitro} and adverse lidocaine effects \textit{in vivo}.

In contrast to Na\(^+\) influx, outward-directed K\(^+\) fluxes are responsible for membrane repolarisation and take major part in setting the resting membrane potential (reviewed by FARRUGIA 1999). Blockade of K\(^+\) channels could increase excitability of cells due to delayed repolarisation or could slightly move the resting membrane potential towards more positive values and thus permit easier triggering of action potentials. In turn, increased outward-directed K\(^+\) fluxes could enforce repolarisation and thereby reduce cell excitability.

Ca\(^{2+}\) influx is an essential prerequisite for SM contractions (Chapter 1.2). Hence, facilitated Ca\(^{2+}\) influx was proposed to enhance SM contractions whereas inhibited Ca\(^{2+}\) fluxes were considered to reduce contractility. On the other hand, intracellular Ca\(^{2+}\) overload in consequence of IR was thought to be involved in formation of reactive oxygen metabolites and in activation of different inflammatory enzymes, especially in the heart and brain as summarised by CASSUTTO and GFELLER (2003) and COOK and BLIKSLAGER (2008). Blocking of Ca\(^{2+}\) channels reduced IR induced tissue damage (reviewed by KONG et al. 1998). As IR-induced inflammation was suggested to be involved in impaired intestinal motility (LITTLE et al. 2005), blockade of Ca\(^{2+}\) channels by lidocaine might as well ameliorate contractile function in IR-injured intestinal SM. Although IR injury did not significantly affect SM contractility in the present PhD project, the described mechanisms may nonetheless account for prokinetic lidocaine effects \textit{in vivo}.
Mechanistically, changes in ion conductance might be induced either by direct interaction of lidocaine with ion channels or indirectly via modulation of cell membrane structure (STRICHARTZ and RITCHIE 1987). In case of the latter, lidocaine effects on DRMs in intestinal SM have to be taken into account (Manuscript 2).

Despite the above explanations, so far no data is available confirming effects of lidocaine on ion channel activity in SM in context with changes in contractility. Therefore annotations on these effects so far remain hypothetic.

6.2.2 Alternative mechanisms

Beside actions on membrane ion channels, alternative mechanisms have to be taken into account when discussing prokinetic effects of lidocaine. As pointed out in the introduction (Chapter 1.2), Ca\(^{2+}\) influx into the cytosol is an essential prerequisite for SM contraction. Subsequent cellular processes associated with SM contraction include phosphorylation of MLC. Accordingly, blocking of mechanisms which promote the removal of Ca\(^{2+}\) from the cytosol might increase SM contractility, as well as processes regulating MLC phosphorylation and thus may contribute to contractility-enhancing effects of lidocaine.

Enzymes involved in removing Ca\(^{2+}\) from the cytosol include Ca\(^{2+}\)-pumps and Na\(^{+}\)/Ca\(^{2+}\) exchangers. If lidocaine reduced activity of these enzymes, intracellular Ca\(^{2+}\) levels would remain elevated favouring SM contraction.

Dephosphorylation of MLC is mediated by MLC phosphatase, an enzyme which is partly controlled by the small G protein RhoA. In vascular SM, RhoA activation was inhibited by adenosine monophosphate-activated protein kinase (AMPK) (WANG et al. 2011), a key enzyme for regulating the cellular energy metabolism in skeletal muscle (MU et al. 2001) which was also shown to modulate SM contractile force in case of metabolic challenge (RUBIN et al. 2005). AMPK reduced Rho kinase activity and MLC phosphorylation while it increased MLC phosphatase activity and consecutively permitted SM relaxation (WANG et al. 2011). Furthermore, AMPK phosphorylated MLC kinase, reducing its affinity for Ca\(^{2+}\)/calmodulin (HORMAN et al. 2008). Hence mechanisms increasing RhoA activity or inhibiting AMPK may as well contribute to increased SM contractility.
When discussing a potential impact of lidocaine administration on G protein function, also inhibitory effects have to be considered. In a study by XIONG et al. (1999), lidocaine was shown to block thyrotropin-releasing hormone-induced $K^+$ and $Ca^{2+}$ currents in anterior pituitary cells possibly by inhibiting G protein signalling. Inhibitory effects of lidocaine on G proteins were as well found in studies on oocytes and erythrocytes (HOLLMANN et al. 2001; KAMATA et al. 2008). Furthermore, results of KAMATA et al. (2008) suggested that reduced G protein function was associated with lidocaine-induced DRM disruption. These findings implied that lidocaine might increase SM contractility by disrupting SM DRMs, resulting in reduced G protein function and subsequent modulations in $K^+$ and $Ca^{2+}$ fluxes.

6.3 Impact of ischaemia and reperfusion on smooth muscle contractility

During surgery, only apparently injured intestinal segments are resected whereas morphologically intact intestine is used for anastomosis. These spared intestinal segments may nonetheless be functionally affected and thus may contribute to the development of POI (MALONE and KANNAN 2001). In the present studies, effects of lidocaine administration were examined in IR-injured compared to non-injured control SM. For IR injury, a standardised, artificial IR model (GUSCHLBAUER et al. 2010a) was chosen in order to simulate slightly damaged tissue which might remain in the abdominal cavity after colic surgery.

Surprisingly and in contrast to previous studies (GUSCHLBAUER et al. 2010a), no significant differences were determined in spontaneous contractility of artificially IR-injured compared to non-injured SM (Manuscript 3). Effects of IR injury were only detectable in form of elevated CK release from IR-injured SM (Chapter 5.2). Concerning the efficacy of lidocaine, as well only marginal differences were observed between control and IR-injured SM. These included a tendency towards decreased CK release from IR-injured but not from control SM upon administration of 50 mg/L lidocaine (Chapter 5.2). Additionally, higher lidocaine concentrations were required for inducing a significant increase in contractility of IR-injured compared to control SM (Manuscript 3).

In general, IR injury was thought to impair SM contractility (GUSCHLBAUER et al. 2010a) and to be involved in the development of intestinal motility disorders.
(PONTELL et al. 2011). Therefore on the one hand, in the present PhD project IR injury might not have been severe enough for impairing SM contractility, considering that sufficient IR injury would reliably induce this effect. On the other hand, both the previous (GUSCHLBAUER et al. 2010a) and the present studies used the same experimental protocol for tissue injury and were conducted at the same institutions and by the same staff. Thus, varying impact of IR injury on CSM contractility in the present and previous studies is unlikely to be based on experimental settings and cannot be explained, yet.

Despite these discrepancies, lidocaine administration reliably improved contractility of both control and IR-injured equine intestinal SM. Thus it may at least be assumed that missing consequences of IR injury did not adversely affect the outcome of the studies performed during the present PhD project.
7 MAJOR FINDINGS

Despite the heterogeneity of the study population including horses of various age, breed, gender and body weight, lidocaine reliably increased contractility of small intestinal SM, confirming results of previous in vitro studies (GUSCHLBAUER et al. 2010a, 2011). Together with data revealing contractility-enhancing effects of lidocaine in both CSM and LSM of equine jejunum (Manuscript 3) these results suggested high efficacy of lidocaine treatment in equine small intestine. As lidocaine effects were not altered by TTX application (Manuscript 4), lidocaine seemed to act primarily on SM cells and/or ICC. DRM disruption (Manuscript 2) and reduced CK release (Manuscript 1, Chapter 5.2) suggested an influence of lidocaine on cell membrane characteristics in these cells, possibly influencing activity of ion channels or mechanisms involved in sustained SM contraction. A direct impact of improved cell membrane integrity of SM cells, indicated by reduced CK release, on SM contractility remains questionable (Chapter 5.2). Data in literature indicated that CSM and LSM differ with respect to cellular characteristics (Manuscript 3, Chapter 1.1). Thus, molecular differences between CSM and LSM cells might point to structures which may be affected by lidocaine application and hence may suggest prokinetic mechanisms of action of lidocaine. Notwithstanding these results, external validity of the obtained data was limited (Chapter 6.1.3).
8 PERSPECTIVES

Additional studies determined to further elucidate the impact of contractility-enhancing effects of lidocaine for its therapeutic use in vivo might comprise the following experimental approaches.

As pointed out in Chapter 6.1.3, effective lidocaine concentrations in vitro exceeded therapeutic blood concentrations in vivo. We hypothesised that lidocaine might accumulate in the intestinal wall resulting in tissue concentrations considerably higher than therapeutic plasma concentrations (Chapter 5.3.1). In literature, comparative determination of tissue and plasma or serum lidocaine concentrations following in vivo infusion was limited to relatively short infusion periods (GUSCHLBAUER et al. 2011; TEEPE 2012). Together with high inter-individual variances (GUSCHLBAUER et al. 2011) this might explain why in these studies no significant differences were detected between tissue and plasma (serum) lidocaine concentrations. Therefore, currently a study is performed in succession to the present PhD project, destined to determine plasma and tissue concentrations of lidocaine and its active metabolites MEGX and GX following long-term infusion of lidocaine at therapeutic dosages (15 min bolus infusion of 1.5 mg/kg BW and subsequent CRI of 0.05 mg/kg BW/min for 71 hours and 45 min).

Analysis of tissue lidocaine concentrations following in vitro incubation as performed during the present PhD project did not allow any conclusion on how lidocaine was inserted into intestinal SM (Chapter 5.3). Both free diffusion of drug molecules and directed transport combined with drug accumulation in tissues are imaginable. Additional in vitro experiments including incubation of equine intestinal SM with a broader range of lidocaine concentrations might clarify this aspect and add to information obtained from long-term infusion of lidocaine as pointed out above. Alternatively, intestinal SM samples could be incubated with constant lidocaine concentrations, with tissue lidocaine content being measured at different time points during incubation.

At concentrations of 50 mg/L, lidocaine caused significant redistribution of the DRM marker protein flotillin-2 in DRMs, suggesting disruption of DRMs. DRM disruption in turn was proposed to alter activity of ion channels located in these areas of the cell membrane, resulting in increased SM contractility (Manuscript 2).
Functionally, 50 mg/L of lidocaine represented a concentration which significantly increased contractility in both CSM and LSM (Manuscript 3). As the increase and decrease in SM contractility due to lidocaine administration appeared to be concentration-dependent (GUSCHLBAUER et al. 2010a; Manuscripts 1, 3), additional studies examining the impact of increasing lidocaine concentrations on DRM integrity and composition might be a promising future approach in order to confirm an involvement of DRM disruption in the mechanism of action of lidocaine.

Additionally, studies examining lidocaine effects on ion fluxes in SM cells and ICC could provide major evidence concerning this potential mechanism of action of lidocaine. Alternatively, lidocaine effects on RhoA and AMPK pathways might further elucidate molecular mechanisms of lidocaine.

As lidocaine was unequally effective in the CSM and LSM layer (Manuscript 3), studies regarding differences in characteristics of CSM and LSM cells could offer new information on cellular targets which might be affected by lidocaine treatment.

Finally, regarding the present experimental protocol, contractility-enhancing properties of lidocaine were only tested in a restricted area of the gastrointestinal tract. Studies including intestinal segments obtained from different localisations throughout both the small and large intestine might further elucidate the efficacy of lidocaine along the intestinal axis. These studies might include different degrees of tissue damage as the applied short-term IR injury might not reflect severity of tissue damage in horses suffering from strangulating obstructions.
9 FINAL EVALUATION

Overall, direct effects of lidocaine on SM cells and/or ICC represent one mechanism by which lidocaine may improve gastrointestinal motility in vivo. So far, the proportion to which contractility-enhancement in SM contributes to therapeutic effects of lidocaine in horses suffering from POI is unclear as further local and systemic effects of lidocaine are as well thought to play a role. Thus further research is required in order to confirm the relevance of contractility-enhancing lidocaine effects in therapeutic use of lidocaine and to determine possible interactions between the different favourable consequences of lidocaine administration in vivo.
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