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„Selectivity and bioactivity of genistein on isolated guinea pig organs“

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Acknowledgment

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In memory to my beloved grandmother, I would like to express my profound gratitude to her. Thanks to my mother and my brother for always being by my side throughout the good and the bad times. All my accomplishments would have not been the same without their support.

Abstract

The main focus of this diploma thesis was to get an insight into the activity profile of genistein on different organ systems and to highlight its potency. Therefore, data concerning the heart rate, the ionotropy and the relaxation of smooth muscle tissue were collected using ex vivo experiments. These testings included five organ types excised from a guinea pig, which were suspended in an organ bath and exposed to the test substance in different concentrations. To approach possible vasodilating and spasmolytic effects, isometric measurements of the contraction force on the Aorta descendens, the Truncus pulmonalis and the terminal ileum were performed. Furthermore, the Atrium cordis dextrum was used to assess the chronotropic effects of genistein, while test series on the papillary muscle elucidated the effects on the cardiac contraction force.

It became apparent that the test substance possesses negative chronotropic and positive ionotropic properties. On the smooth muscle tissue genistein exerted vasodilating and spasmolytic effects.

Since the lowest EC_{50} value has been described for the terminal ileum, additional experiments explaining a possible mechanism of action were conducted on that system. For this kind of approach, the nitric synthase was chosen to be blocked by the antagonist N^{ω}-nitro-L-arginine prior to the addition of the test substance. The results pointed out that genistein does not cause relaxation by activating the nitric synthase, but by other, here not specified, means.

In summary, it can be said that genistein is having undoubtedly the distinction of being not that selective, especially because it executed effects on all five organ specimens. This matter of fact makes it difficult to suggest this promiscuous substance for a specific therapeutic field. However, due to its pleiotropic properties and its potential beneficial effects, more in vivo studies are needed to consider a subtle judgment of genistein.

Kurzfassung

Gegenstand dieser Diplomarbeit war es, das pharmakologische Wirkprofil von Genistein zu eruieren und dessen Wirkstärke auf bestimmte Organe zu erfassen. Dafür wurden Parameter wie Chronotropie, Ionotropie sowie Daten zum Ausmaß einer Vasodilatation mittels ex vivo Experimenten erhoben. Die Versuchsreihen wurden an Meerschweinchenorganen aus der Linie TRIK vorgenommen und umfassen den rechten Vorhof, den Papillarmuskel, die Aorta, die Pulmonararterie und das terminale Ileum. Genistein wurde dann in verschiedenen Konzentrationen in das entsprechende Organbad injiziert und etwaige Veränderungen in der Kontraktionskraft bzw. Schlagfrequenz wurden registriert. Die Versuche am rechten Vorhof gaben Aufschluss über den chronotropen Effekt, während die vom Papillarmuskel die Inotropie der Testsubstanz untersuchten.

Die Schlussfolgerung der Ergebnisse ergab eine negative Chronotropie und eine positive Ionotropie, die man Genistein zuschreiben konnte. Außerdem übte das Phytoestrogen eine vasodilatierende und spasmolytische Wirkung auf die glatte Muskulatur aus.

Nachdem der niedrigste EC_{50}-Wert für den Darm beschrieben wurde und Genistein dort seine stärkste Wirkung entfalten konnte, wurden weitere isometrische Messungen, die auf einen möglichen Wirkungsmechanismus abzielen, an diesem Organ durchgeführt. Hierfür wurde die Stickstoffmonoxid-Synthase durch den Antagonisten N^{ω}-Nitro-L-Arginin inhibiert, um dann erst die Testsubstanz zu applizieren. Die Resultate legten nahe, dass das nicht der zugrunde liegende Wirkmechanismus von Genistein ist, sondern andere, hier nicht näher beschriebene, Mechanismen.

Zusammenfassend kann man sagen, dass Genistein eine geringe Selektivität aufweist, da es an allen fünf Organtypen eine merkliche Wirkung zeigt. Aus diesem Grund fällt es zurzeit schwer, Genistein als zielgerichtetes Therapeutikum für eine bestimmte Erkrankung zu empfehlen. Nichtsdestotrotz sind weitere Studien am Menschen notwendig, um eine endgültige und vernünftige Einschätzung dieser Substanz vornehmen zu können.

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List of abbreviations

EC_{50}	Half maximal effective concentration
CoA	Coenzyme A
SLGT-1	Sodium/glucose cotransporter 1
FDA U.S.	Food and Drug Administration
HCN	Hyperpolarization-activated cyclic nucleotide-gated cation channel
NO	Nitric oxide
cGMP	Cyclic guanosine monophosphate
cAMP	Cyclic adenosine monophosphate
DMSO	Dimethyl sulfoxide
NaCl	Sodium chloride
KCl	Potassium chloride
$NaHCO_3$	Sodium hydrogen carbonate
KH_2PO_4	Potassium dihydrogenphosphate
$CaCl_2$	Calcium chloride
$MgSO_4$	Magensium sulphate
HCl	Hydrogen chloride
NO	Nitric oxide
N	Newton
mN	Millinewton
MLCK	Myosin light chain kinase
HCN	Hyperpolarization-activated cyclic nucleotide-gated cation channel
IK	Potassium current
IKr	Rapid component of IK
IKs	Slow component of IK
IP_3	Inositol trisphopshate
SEM	Standard error of the mean
mV	Millivolt

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1. Introduction

Dietary habits are different around the world and deserve a great deal of attention as foods can prevent or either cause diseases. Based on this consecution, scientists eye-catched not so long ago the potential beneficial effects and the medicinal value of soy-isoflavones on our general health condition and especially on mammalian cancer.

Evidence in favour of this theory was shown in a recent meta-analysis demonstrating that a high intake of soy products is one of the reasons for a lower risk of breast cancer in pre- and postmenopausal women in Asian countries. These cancer preventive properties are attributed to the substance genistein, most commonly found in soy beans (Chen et al., 2014).

This is also correspondent with the average dietary isoflavone intake of an Asian, which adds up to 25-50 mg/d, while in comparison to our latitude, people have an intake of 2 mg/d (Messina, Nagata, & Wu, 2006; van Erp-Baart et al., 2003).

Such promising findings and the fact that isoflavones are part of people´s nutrition caused especially genistein to be content of many trials and preclinical testings.

1.1 Genistein

One of the well-established therapeutic fields of genistein are menopausal complaints. Nevertheless, it is assumed that genistein can favour a number of benevolent effects on different kinds of diseases due to its polypharmacological profile.

1.1.1 Structure and physico-chemical properties of genistein

Genistein belongs as a 3-phenlychromanderivative to the isoflavonoids, also referred as phytoestrogens, and is by definition a bioactive secondary metabolite most commonly found in the legume family Fabaceae (Teuscher, Melzig, & Lindequist, 2012).

Furthermore, it is best soluble in dimethyl sulfoxide, acetone and ethanol. The solubility in water is not near as good (Spagnuolo et al., 2015).

The two hydroxyl groups located on each end of the molecule (figure 1) are one of the special structural characteristics of this substance, which allow the development of hydrogen bonds inside the binding pocket of the estrogen receptor and thus inducing a conformational change (Steinhilber, Schubert-Zsilavecz, & Roth, 2010).

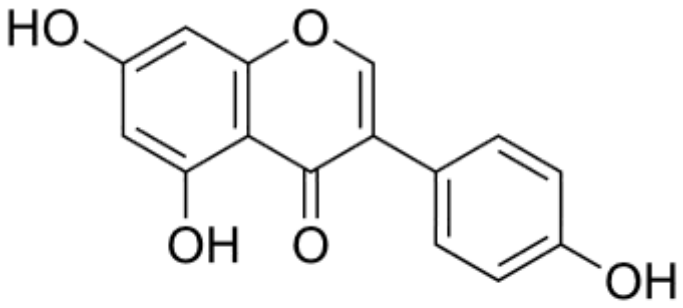


Figure 1 1: 5,7-Dihydroxy-3-(4-hydroxyphenyl)chromen-4-one (Wikipedia, 15 July 2018)

1.1.2 Biosynthesis

Malonyl CoA, a polyketide, and 4-cumaroyl CoA, a phenylpropane, combine through condensation to an intermediate product. Subsequent reactions executed by the chalcone synthase and the chalcone-flavanone isomerase lead to the structure of the flavanone called naringenin. Oxidation and reduction as well as structural rearrangements result in the different flavonoid groups. The shift of the phenolic-group from position 2 to 3 on the back bone is important to create the isoflavones (Dingermann, Hiller, Schneider, & Zündorf, 2004).

Furthermore, scientists managed to synthesize genistein by the means of biotechnology in Saccharomyces cerevisiae and in the leaves of Nicotiana tabacum L. (Katsuyama, Miyahisa, Funa, & Horinouchi, 2007; Yu et al., 2000).

1.1.3 Occurrence

One of the most common sources high in isoflavones are soy-based foods. This would include soy cheese, soy milk, soy meat and many more. The content of genistein inside these natural products differs depending on the quality and on the preparation methods that are used (Spagnuolo et al., 2015).

For example, fermentation of soy bean products can increase the content of genistein (Lee et al., 2014).

Furthermore, established herbal preparations containing genistein are often used as an alternative approach for postmenopausal complaints.

1.1.4 Pharmacokinetics of genistein

In natural products isoflavones are present as aglycones and in a certain amount as glycoside (Spagnuolo et al., 2015). Hence, the absorption of genistein could vary.

The aglycone is directly absorbed by passive diffusion in the small bowel and in the colon, whereas the glycoside, genistin, must be split from its sugar part first in order to gain permeability. This happens with the aid of glucosidases located in the brush-border membrane of the enterocytes. However, in addition to that, a sodium/glucose cotransporter 1 (SGLT-1) located in the small intestine is capable of transporting the glycoside form without previous enzymatic cleavage into the cell (Adzersen & Strowitzki, 2003; Hahn, Ströhle, & Wolters, 2006; Setchell, 1998).

In some extent the ingested isoflavone is also metabolized by the gut flora before entering the systematic circulation of the human body (Biesalski, Köhrle, & Schümann, 2002; Hahn et al., 2006; Setchell, 1998).

The oral bioavailability of genistein is considered not to be that high due to its poor solubility in water (Motlekar, Khan, & Youan, 2006). Genistin however, is reaching higher concentrations, because of its polar properties attained from the attached sugar component (Kwon et al., 2007).

Moreover, defined information concerning the distribution and in what extent this substance is stored in the human body is missing. Bolca et al. reported that isoflavones are most commonly distributed to the fatty tissue as well as to the glandular tissue of the breast in females (Bolca et al., 2010).

Additional studies conducted in rat models also confirmed a favouring cumulation in the breast, ovaries and uterus in the female animals. In the male ones, the prostate was the preferred spot for accumulation (Hahn et al., 2006; Kulling & Watzl, 06.2003).

Referring to the metabolism, Genistein-7-O-glucoronide is the metabolite primary excreted by the kidney. The remaining amount is eliminated biliary and underlies the enterohepatic circulation (Bolca et al., 2010; Hahn et al., 2006; Kulling & Watzl, 06.2003).

1.1.5 Effects of genistein

1.1.5.1 Estrogenic and antiestrogenic effects of genistein

Randomized, controlled studies have shown that the substitution of estrogen and progesteron is correlated with certain health risks, e.g. cardiovascular events and an increased incidence of mammalian carcinoma (Hulley et al., 1998; Rossouw, 2002).

Therefore, there are calls for new treatments. Nowadays different food supplements including dry extracts of soy bean and red clover are offered in community pharmacies and are implemented as an alternative option to the hormone replacement therapy in postmenopausal women.

As endocrine disruptors phytoestrogens intervene in our hormone system. A certain structural analogy to the native hormone estrogen is responsible for the binding to the corresponding receptors. There are two subtypes of nuclear estrogen receptors, namely ERalpha and ERbeta, both distributed in different tissues of the body and therefore executing diverse effects. Estrogen is a steroidal hormone consisting of a lipophilic middle part, carrying two hydroxyl groups in-between a distance of 1,245 nm. In comparison, genistein also features two hydroxyl groups in that same distance. This is known to be the tie-breaking point on the phytoestrogens´ ability to bind to these

specific receptors and conduct estrogen-like effects, although not as severe as the native ligand (Steinhilber et al., 2010).

However, depending on endogenous plasma concentrations of estradiol, genistein serves as partial-agonist or antagonist. At low levels, usually seen in postmenopausal women, phytoestrogens can reinforce the estrogenic effects. Vice versa, at high estrogen levels, isoflavones act antagonistic. That is why they are also referred as Phyto-Selective Estrogen Receptor Modulators (Teuscher et al., 2012).

In conclusion, the current state in the literature is quite equivocal and the heterogeneity of the performed studies is making it very difficult to pick a side regarding the effectiveness and safety of genistein on that matter. A review by Krebs et al., 2004, which included 25 published comparative clinical trials from between 1966 and 2004, came even to the conclusion that extracts from the red clover and soy bean show no significant reduction in hot flushes (Krebs, Ensrud, MacDonald, & Wilt, 2004).

Moreover, based on the poor data, the North American Menopause Society and the Drug Commission of the German Medical Association discourage the use of isoflavone extracts as an alternative to estrogen therapy. People should consume such products in moderation as there is a lack of evidence on the long-term effects as well.

1.1.5.2 Antitumor and pro-carcinogenic effects of genistein

There is plenty of evidence which highlights the chemopreventive effects of genistein on different kinds of tumors. The fundamental mechanisms of action are quite complex and encompass different pathways as well as interventions into the cell cycle and the gene expression of tumor cells (Spagnuolo et al., 2015).

Experiments conducted on rats show promising data that an early exposure on genistein-containing soy can lower the susceptibility to breast cancer and thus probably explaining why Asian women suffer less from this type of malignant disease (Lamartiniere, Zhang, & Cotroneo, 1998).This is also in correspondence with several case-control studies emphasizing that a nutrition high in isoflavones from soy is related to a lower breast cancer risk (Dai et al., 2001; A. H. Wu, Yu, Tseng, Twaddle, & Doerge, 2004; Yamamoto, Sobue, Kobayashi, Sasaki, & Tsugane, 2003).

Moreover, genistein shows synergistic effects on breast cancer cells when administered together with chemotherapeutic agents like docetaxel or tamoxifen (Mai, Blackburn, & Zhou, 2007; Satoh et al., 2003).

In contrast to that, it is noteworthy that there are also some animal studies which describe pro-carcinogenic effects. Mice exposed at a young age to phytoestrogens like genistein are prone to suffer from malignant diseases of the uterus later in life (Hänsel & Sticher, 2007).

These controversial findings suggest that there is still a necessity to gather more specific data under which circumstances genistein might be beneficial and when it is causing the exact opposite.

1.1.5.3 Osteoprotective and osteoanabolic effects of genistein

Statements about genistein and his favourable effects on attenuating or even increasing the bone mineral density are quite equivocal.

Via the estrogen receptors genistein is inhibiting the osteoclasts. However, the effects are considered not to be sufficient enough so that genistein could be used as a good preventive measurement or as an effective therapy option against osteoporosis (Blaschek, 2016; Kulling & Watzl, 06.2003; Kurzer, 2003; Watzl & Leitzmann, 1999).

1.1.5.4 Antioxidant effects of genistein

As an isoflavone genistein averts lipid peroxidation. Hence a high intake of this antioxidant protects the body from reactive oxygen species (Vitalstofflexikon).

1.1.5.5 Lipid-lowering effects of genistein

According to the FDA soy has a prophylactic impact on cardiovascular diseases, cholesterol and triglyceride levels (Hänsel & Sticher, 2007).

1.1.5.6 Effects on the glucose balance

An assessment of the European Medicines Agency from the year 2018 presented an increased insulin secretion in rats fed with a diet high in soy-isoflavones (Agency, 2018).

To what extent this evidence could be applied to humans, remains open and needs further investigation.

1.1.5.7 Immunological effects of genistein

A refined analysis of the Department of Food Science and Human Nutrition, USA, from the year 1999, demonstrated that a reinforced activation of natural killer cells can be expected by a normal intake of genistein (Zhang, Song, Cunnick, Murphy, & Hendrich, 1999).

However, concentrations above 10 µmol/l appearing in the blood contribute to an inhibition of the immune function**,** suggesting that a high intake of soy-isoflavones can be detrimental (Wang, Higuchi, & Zhang, 1997)**.**

1.1.5.8 Antithrombotic effects of genistein

Genistein carries out favourable effects on the cardiovascular system. A variety of studies support the fact that genistein prevents the activation as well as the aggregation of thrombocytes and thus prohibits a dangerous thrombus formation (Vitalstofflexikon).

1.1.5.9 Effects of genistein on the thyroid function

There are evident case reports that genistein might induce hypothyroidism via inhibition of the thyroid peroxidase (Hänsel & Sticher, 2007).

1.1.5.10 Cardiac and vascular effects of genistein

In agreement with the values found in the literature genistein already appears to be a promising antiarrhythmic agent as it can directly block the HCN channel in the sinoatrial node. Hence, less electrical impulses are generated resulting in a lower heart rate (Bois, Guinamard, Chemaly, Faivre, & Bescond, 2007).

Further electrophysiological experiments also suggest that during an action potential genistein conducts inhibitory effects on Ca^{2+} and K^{+} currents (Liew, Stagg, Chan, Collins, & MacLeod, 2004).

Moreover, valuable data confirming a stimulatory effect on myocardial contractility by genistein was presented by Li et al. in 2008 and shed new light on the mechanism of action, which includes an inhibition of the tyrosine kinase, eventually resulting in a Ca^{2+} mobilization within the myocytes (H. Li et al., 2008).

Concerning its impact on the vasculature, genistein proved to ameliorate blood flow due to vasodilatation in a double-blind, placebo controlled, randomized trial performed on healthy postmenopausal women (Squadrito et al., 2002).

It has been proposed that the underlying mechanism on the relaxation of smooth muscle tissue by genistein is an inhibition of the calcium influx, but not an enhanced release of NO or vasodilator prostanoids from the endothelium. Neither does the content of cGMP resume a pivotal role in terms of the relaxation via this soy-isoflavone (H.-F. Li, Wang, & Qu, 2004).

2. Aim

The main purpose of this thesis was to assess the selectivity and bioactivity of genistein by using isometric measurements performed on isolated guinea pig organs. These findings should help to draw conclusions if the test substance can be proposed for further development or rather can be used in a specific therapeutic field other than its common medical indication. Depending on the type of tissue, parameters including ionotropy, chronotropy and the magnitude of relaxation were evaluated.

The ionotropy and the chronotropy of the test substance were determined on the musculature of the heart. This included the Atrium cordis dextrum and the Musculus papillaris.Testings on the Aorta descendens, the Truncus pulmonalis and the terminal ileum gave an insight about the vasodilating as well as the spasmolytic action of genistein on the smooth muscle tissue.

Furthermore, the EC_{50} value would be determined as soon as genistein showed a significant effect on one of the specific organ specimens. Some final experiments aiming to describe a possible mechanism of action were also included in the evaluation.

3. Material and Methods

3.1 Test substance

Genistein was analysed in several test series on isolated guinea pig organs.

3.2 Preparation of the test solution

The test solution was always prepared in a vial just before testing started to ensure reproductivity of the whole experiment. DMSO was used as an appropriate solvent for genistein. A certain amount of the test substance (Table 1) was weighed and dissolved in the corresponding amount of organic solvent, guaranteeing a concentration of 100 µmol/l at the end of every experiment in the respective organ bath.

Table 1 Amount of test substance

Substance	Molar mass [g/mol]	Volume of the organ bath [ml]	Quantity of sample [mg]
Genistein	270.24	8	0.21
		25	0.67

3.3 Nutrient solution

Organs which are excised from the test animal must be put as fast as possible in a nutrient solution. This oxygen saturated and electrolyte-rich solution guarantees the organs functioning outside its organism for several hours and hence enabling a smooth execution of the ex vivo experiments. Fumigation with the gas mixture called Oxymix

(95% oxygen;5% carbon dioxide) provided sufficient oxygen supply for the tissues and maintained a physiological ph value of 7.2-7.4. Energy for the organs was provided by the containing glucose.

The instructions for the preparation are based on Reiter (Reiter, 1967) and represent a modified Krebs-Henseleit solution. However, throughout this thesis it will be referred to as Tyrode (Table 2).

Table 2 Components of the Tyrode

Substance	**Molar mass [g/mol]**	**Stock solution [g/l]**	**ml Stock solution / l Tyrode**	**Concentration [mmol/l]**
NaCl	58.44	200.05	33.60	115.02
KCl	74.55	10.07	35.00	4.73
NaHCO3	84.01	25.00	83.70	24.91
MgSO4	120.37	29.40	1.18	0.29
KH2PO4	136.09	248.00	1.18	2.15
CaCl2	110.98	136.00	3.20	3.92
Substance	**Molar mass [g/mol]**	**Stock solution [g/l]**	**g / l Tyrode**	**Concentration [mmol/l]**
Glucose	180.16	Pure substance	1.98 g	10.99

Table 3 Components of 2l Tyrode

Stock solution/ Solid	Volume/Mass
NaCl	67.2 ml
KCl	70 ml
NaHCO3	167.4 ml
MgSO4	2.36 ml
KH2PO4	2.36 ml
CaCl2	6.4 ml
Glucose	3.96 g

At the beginning of each day an amount of 2l of solution, separated in two 1l volumetric flasks, was prepared (Table 3). There was put an emphasis on a distinct order of adding the single components to the solution. This was important as the solution must be clear and not showing any forms of precipitation. Otherwise it could not be used for the experiments.

NaCl, KCl, $NaHCO_3$, $MgSO_4$ and KH_2PO_4 were mixed together, while the glucose was first fully dissolved in a separate beaker and then added to the mixture. The next step included filling the flask up to two thirds with distilled water and fumigate it with “Oxymix” for at least twenty minutes. After doing that, $CaCl_2$ would be added drop by drop to avoid precipitation. The final step included filling the flask up to the mark with distilled water and proceed fumigation.

3.4 Potassium chloride solution

A potassium chloride solution was prepared each day in two different concentrations depending on which organ it would be used on. Therefore, an appropriate amount of solid KCl was dissolved in 100ml Tyrode (Table 4). Adding this kind of solution into the organ bath of the terminal ileum, Aorta descendens and Truncus pulmonalis would cause a contraction of maximum capacity. Thus, a possible spasmolytic and vasodilating effect of the test substance could be examined.

Table 4 Components of the potassium chloride solutions

Organ	mass(KCl) [g]	Volume (Tyrode) [ml]	Concentration [mM]
Terminal Ileum	0.45	100	60
Aorta descendens	0.67	100	90
Truncus pulmonalis	0.67	100	90

3.5 Experimental animals

There is an existing histological similarity of guinea pig organs from the TRIK line to those of human tissue. Above all, they also feature quite similar ion channels and receptors compared to humans, making them the perfect fit for this kind of thesis.

Only organs from female animals were used with an average age of 10 weeks.

3.6 Removal of organs and preparation techniques

3.6.1 Removal of organs

Manual cervical dislocation, a common method of animal euthanasia, was used to secure a fast and a painless death of the animal. The heart, the ileum, the pulmonary artery and the aorta were immersed into beakers full of nutrient solution for further preparation immediately after they got removed from the body as keeping the organs alive was a priority (see figure 2).

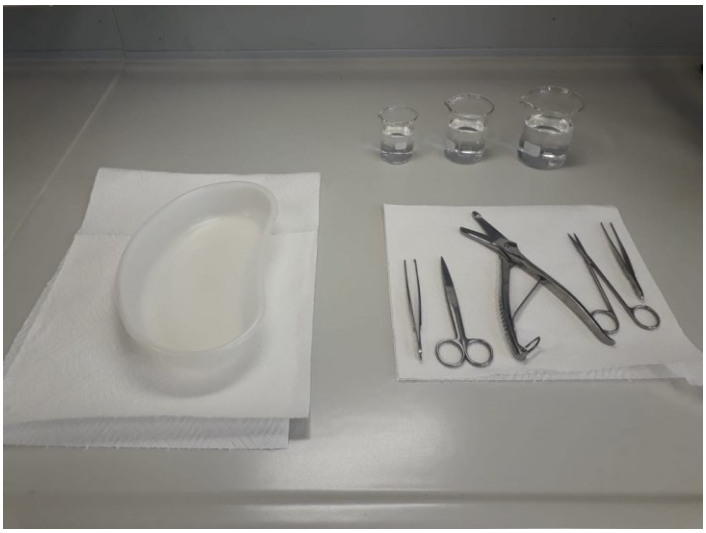


Figure 2 Tools for removal and beakers filled with nutrient solution for the organs

3.6.2 Preparation of the organs

Before the organs could be attached to the corresponding apparatus, further preparation steps needed to be carried out. This included excess fatty tissue removal, washing procedures with a pipette, removal of coagulated blood and in some cases attaching “S”-shaped silver hooks to the organs. To create an appropriate surface for this kind of procedures, a cork was fixed with a ruber tube in a Petri dish filled with nutrient solution (see figure 3). By the means of two small needles the organs were fixed on the cork, assuring thereby a stable position of the tissue.

Figure 3 Preparation tools and reflected-light microscope

3.6.2.1 Preparation of the right atrium (Atrium cordis dextrum)

The sinoatrial node is located in the right atrium. This group of cells is capable of producing a spontaneously electric impulse, that travels through the heart via the electrical conduction system causing the heart to contract ("Der Brock Haus: In Fünfzehn Bänden - Dreizehnter Band," 1999). For this reason, it is utterly important not to apply any injury or distension to the tissue. Otherwise damage would occur and lead inevitably to the uselessness of the organ.

Following the removal from the guinea pig, the heart was fixed onto a cork in a Petri dish. Throughout preparation the nutrient solution was changed several times in order to wash out all the blood and ensure a proper vision. During the second step the right atrium was isolated from the remaining tissue and deposited onto another cork flooring of a second Petri dish, where two silver hooks were attached by sewing threads. Not only a precise and proper handling was required, but also a quick performance of the several actions was demanded. Unless, there would be a risk that the right atrium could not resume pumping when it is coupled to the apparatus later on.

3.6.2.2 Preparation of the pulmonary artery (Truncus pulmonalis)

The Truncus pulmonalis was cut into small rings, as soon as it was freed from surrounding, excess fat and muscle tissue. The obtained ring-shaped preparations did not need any silver hooks and could be clamped directly to the experimental apparatus.

3.6.2.3 Preparation of the papillary muscle (Musculus papillaris)

The preparation of the papillary muscles demanded more sophisticated steps. Initially, the heart was butterflied and unfolded like a book. The papillary muscles were then preferably obtained from the left ventricle. Moreover, the removal of the Purkinje fibres was of high importance as these kind of conduction fibres induce cardiac action potentials by themselves and would distort the values. Finally, a single silver hook was

attached to each muscle by using sewer threads (see figure 4). This kind of measurement facilitates the coupling to the corresponding apparatus.

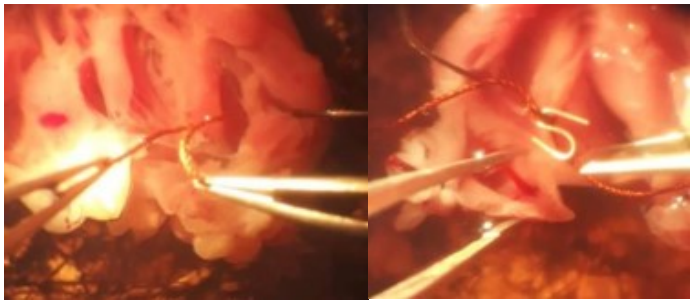


Figure 4 Attachment of the silver hooks to the papillary muscles

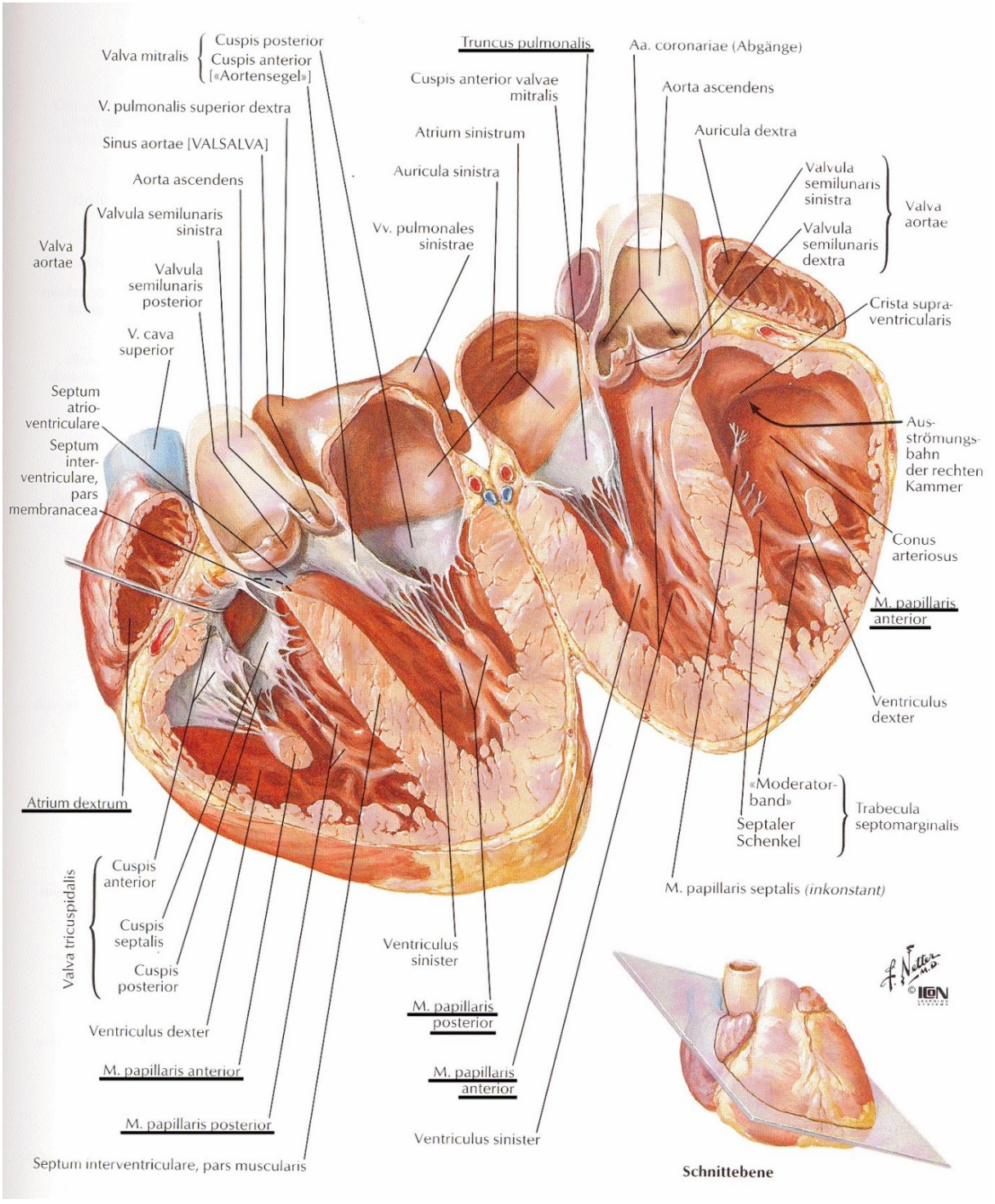


Figure 5 Cross section and anatomical structure of the heart (Netter, 2003)

3.6.2.4 Preparation of the small intestine (terminal ileum)

For the following test series, a thirty-centimetres-long part of the terminal ileum was removed from the abdomen of the guinea pig. The upper end was marked with a sewing thread and served as an orientation. Furthermore, a better handling could be ensured through this kind of measurement. The terminal ileum was subsequently cut into small pieces of about one centimetre. At this point of action, it should be mentioned that each end of the ileum was cut diagonally, making it easier to attach the silver hooks on both sides with the aid of sewing threads (see figure 6). Besides, this kind of cut also enabled an optimal flow of the nutrient solution and the test substance through the lumen.

Finally, the lumen of the intestine was flushed out using a pipette. This step is necessary to get rid of possible residues, before attaching it to the apparatus.

Figure 6 Piece of the terminal ileum attached with hooks on both sides

3.6.2.4 Preparation of the aorta (Aorta descendens)

This organ specimen was removed under the assistance of a second person. While one unfolded the thorax, the other person would have a better access to the aorta, which is attached alongside the spinal column. The aorta was then abstracted and it was also important not to stretch it out to much or otherwise damage could occur. Afterwards the aorta was positioned on the cork in the Petri dish for further preparation. In order to adopt the size for the apparatus, the vessel was cut into small rings of about two millimetres, which could easily be suspended into the organ bath (see figure 7).

Figure 7 Preparation steps of the aorta

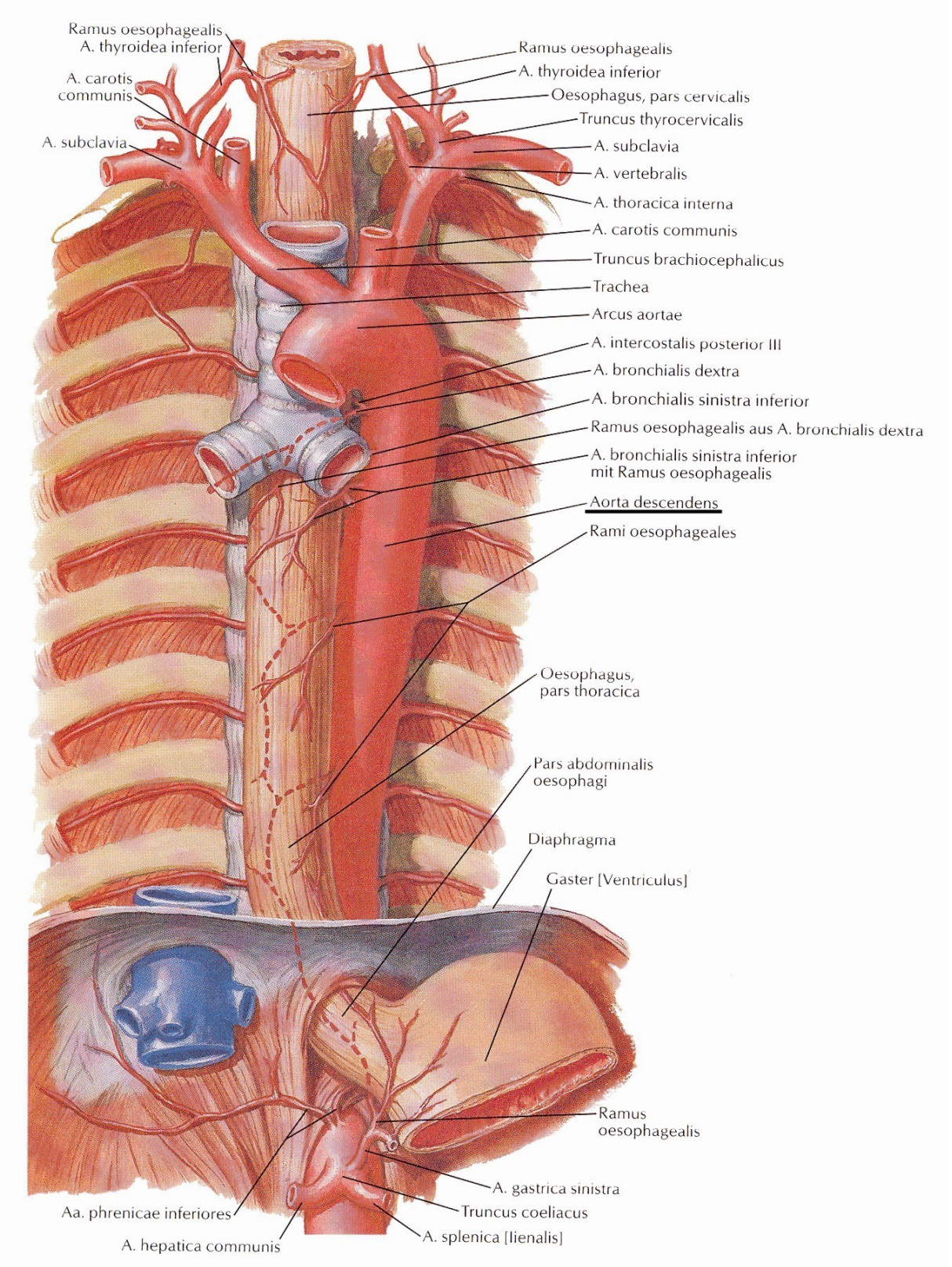


Figure 8 Anatomical structure of the aorta (Netter, 2003)

Converting a mechanical motion into an electrical signal was the underlying mechanism used to measure the organs´ contraction force under the influence of the test substance. Therefore, two different experimental set-ups were used, which included a force transducer to perform such measurements. Apparatus I was used for the right atrium, the aorta, the pulmonary artery and the terminal ileum, whereas apparatus II was constructed only for the papillary muscle. A silver wire was connecting the force transducer with the organ placed in the organ bath.

During the experiment the tested organ would then pull on that silver wire as it is contracting and the force transducer would recognize each of these contraction processes by using a strain gauge. Thus, the mechanical motion was converted into an electrical signal. Subsequently the signal was amplified by an amplifier and either recorded on graph paper by a plotter or digitally with a software on a Laptop.

Meeting standards and enabling a good reproducibility of the conducted experiments were a priority. That applies to the maintenance of a constant temperature as well as pH-level and guaranteeing an optimal oxygen supply of the organs for the entire test duration.

3.7 Experimental set-up

3.7.1 Apparatus Ia

The set-up of apparatus Ia (see figure 9) was used to perform experiments on all the above-mentioned organ specimens, except the papillary muscle. It consists of a metal framework holding the organ chamber. The organ chamber is a double-walled glass vessel. This kind of construction allows tempered water to flow in-between these two walls and hence securing a constant temperature of 37.5 ± 0.5°C. Gas supply was managed through a hose system at the bottom of the vessel and regulated by a hose clamp. A glass frit prevented the formation of very big bubbles, which may have caused potential disturbances to the test system. However, the fine bubbles formed via the glass frit achieved an evenly distribution of the test substance inside the organ bath.

The capacity of the vessel amounts to 25ml. The organs were placed in the middle of the chamber inside the nutrient solution. While the aorta and the pulmonary artery were directly attached to a metal rod, the right atrium and the terminal ileum could be fastened to the apparatus using the previously affixed tiny silver hooks. Subsequently the upper end of the organ was then connected to a silver wire, which was coupled to the force transducer. Finally, before recording could be started, a specific pretension was applied to the organs using the fine adjustment knob at the top. Thereby a level of reproducibility could be achieved.

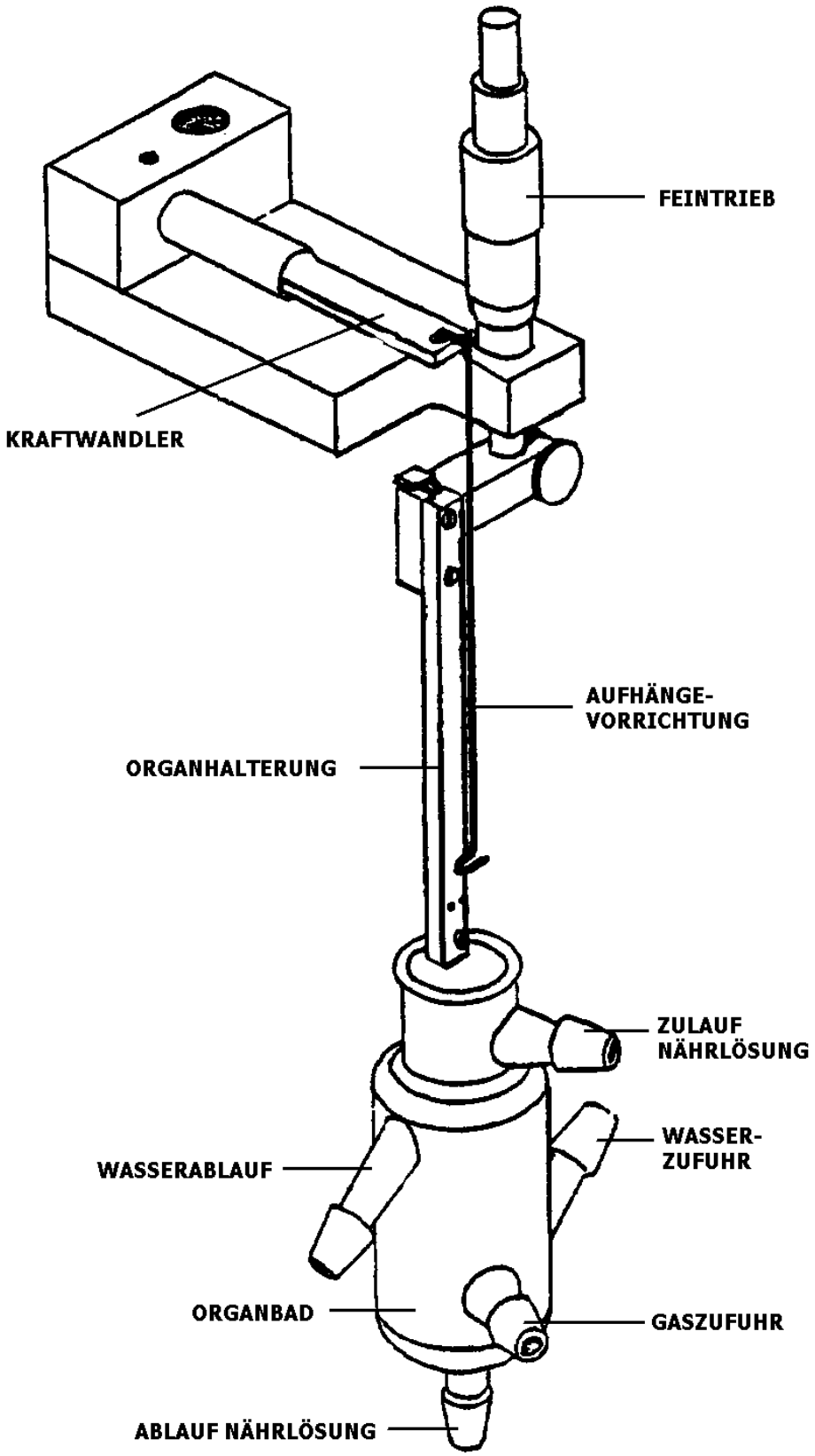


Figure 9 Schematic drawing of apparatus Ia

3.7.2 Apparatus Ib

Some of the experiments with the pulmonary artery and the aorta were conducted on apparatus Ib. (Figure 10) It features a more convenient and modern equipment in comparison to apparatus Ia. Furthermore, it has some slight modification concerning the set-up, which proved advantageous for this specific organ specimens.

Other than the attachment on apparatus Ia, two triangular clamps were carefully put through the vascular lumen. These clamps were connected to a sewing thread. Hence, the small pieces of the blood vessels could be mounted on a Teflon rod at the bottom and linked with the force transducer on the top.

The method of recording represents another distinct difference between apparatus Ia and Ib. This time, the signals were not recorded on a graph plotter as with apparatus Ia, but digitally on a notebook. The software used for this purpose was LabScribe 2.

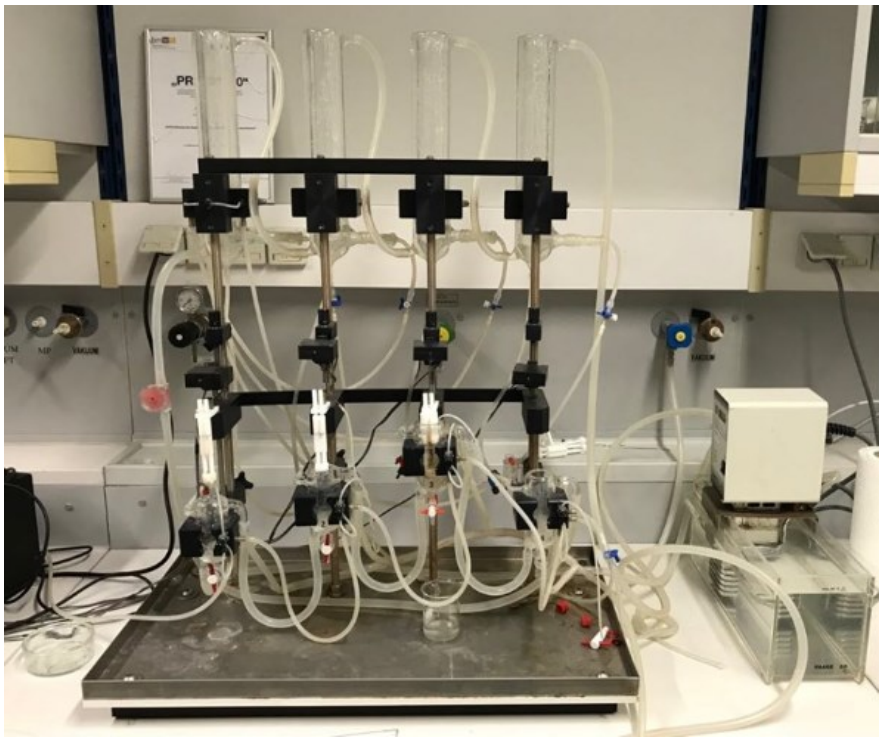


Figure 10 Photo of apparatus Ib

3.7.3 Apparatus II

The set-up of apparatus II was adequate for performing experiments on the Musculus papillaris (figure 11). A small vessel made of plexiglass served here as a chamber with a volume of 25ml. This was immersed directly into a water bath. To enable a good physiological environment, the adjusted temperature of the water bath was 35.5 ± 0.5°C throughout the whole experiment. As with apparatus Ia oxygen supply was arranged via a hose system at the bottom of the vessel.

The papillary muscle was hooked on a silver wire, while half of the tissue was gently squeezed between a platinum electrode and a small piece of plexiglass (see figure 14). The silver wire was connected to the force transducer, sending the signal to an amplifier and as a result registered contractions ended up recorded on a graph paper by a plotter.

Moreover, electrical stimulation was necessary to induce contraction in the papillary muscle. Therefore, an electrical pulse generator connected with the platinum electrode generated electrical pulses in a certain frequency. The coarse adjustment knob on the metal stand helped then to put the metal rod with the organ in the right position inside the organ bath. Before carrying out further steps, a certain pretension was applied by the fine adjustment knob to guarantee a good reproducibility.

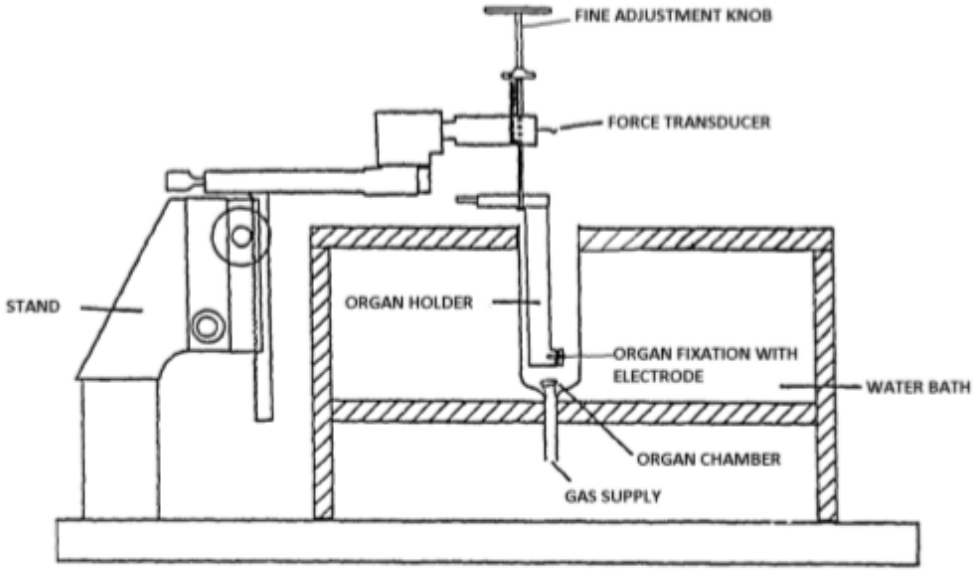


Figure 11 Schematic drawing of apparatus II

3.7.4 The force transducer

The conversion of a mechanical signal into an electrical signal is governed by a force transducer. The signal is then amplified by an amplifier and recorded on a graph paper by a plotter or digitally (figure12).

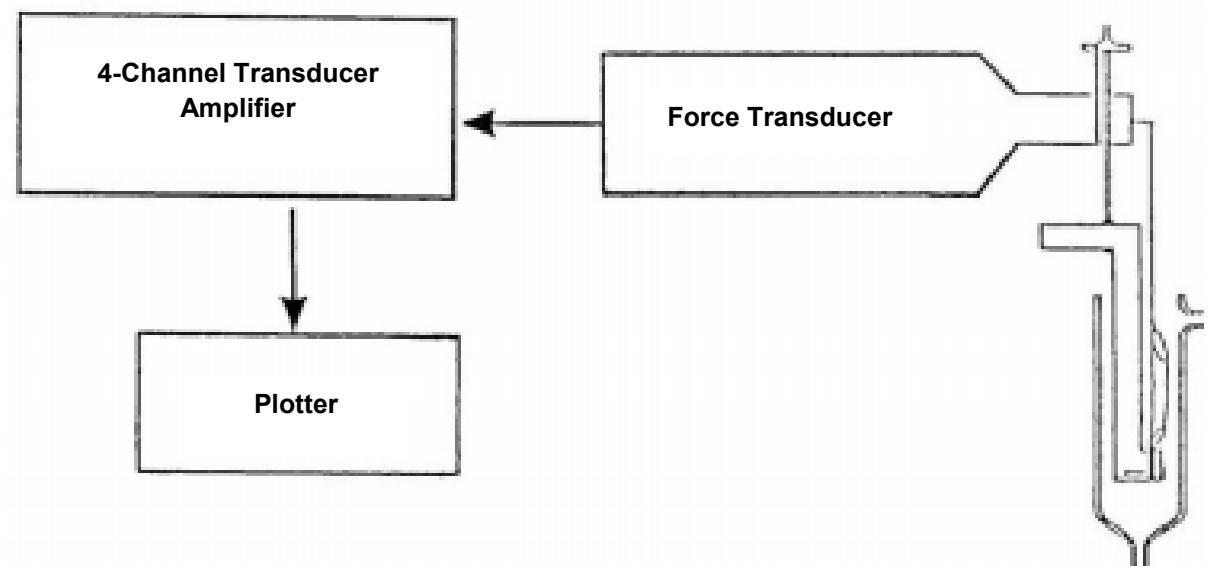


Figure 12 Schematic drawing of the signal processing

3.7.5 Gas supply by Oxymix

A good oxygen supply was of high importance to guarantee a proper functioning of the organs during all steps of preparation and the experiments. “Oxymix” is composed of 95% oxygen and 5% carbon dioxide. This ratio allowed to keep the pH value in the organ bath at balance (7.2 – 7.4). Furthermore, it contributed to an even distribution of the test substance inside the organ bath.

3.8 Experimental procedure

All five organ specimens underwent basically the same experimental procedures.

At the beginning it had to be made sure that the apparatus is working properly and that there were no residues left from previous experiments in the organ bath. For that reason, the organ bath was rinsed two times with HCl and two times with distilled water. After accomplishing these precautions, the vessel was filled with 25ml of nutrient solution and got fumigated. It would then take a couple of minutes until the nutrient solution adjusted the right temperature. In the meantime, the organs were attached to

the apparatus. Although a careful handling of the organs was of importance, a certain pretension had to be applied to them due to reproducibility. The extent of this pretension regulated by a fine knob differed from one organ to other and was determined empirically in preceding experiments. As soon as the organ was suspended in the organ bath a control period of at least 45 minutes was initiated. Afterwards the test substance was solved in a vial and added to the organ bath in a distinct pattern depicted in table 5. End of the experiment marked the passing of 45 minutes after the last addition of the test substance and thus simulating steady state at the highest substance concentration.

Table 5 Scheme of the test substance addition

Time [min]	Test solution [µl]	Concentration in the organ bath [µmol/l]
45/Steady state	3	3
90	7	10
135	20	30
180	70	100

3.8.1 Experimental procedure – Atrium cordis dextrum

Due to the liability of the right atrium to the conditions of the outer environment it was important to keep the time for preparation short.

When the nutrient solution got the right temperature, the organ specimen was clamped by his two tiny hooks onto the apparatus and got submersed into the organ bath (figure 13). The potential on the plotter was adjusted to 5mV and the recording speed was set to 5mm/sec. In case the deflection got smaller during the experiment, the potential was readjusted to 2mV or 1mV. Otherwise one could not be able to count the recorded beats.

Subsequently a pretension of 10.4 mN was applied by revolving the fine adjustment knob until a deflection of 10.4 cm appeared on the plotter. By switching on the amplifier and using the setting dial, the pen of the plotter could be manoeuvred back to position zero. From this point on, the organ was left to adjust to the ex vivo conditions in the bath for about 20 – 30 minutes and get the chance to resume pumping. The experiment would then start with a control phase of at least 45 minutes. A measurement of the pulse rate was carried out every five minutes for 12 seconds. This would correspond to 6 mm on the graph paper. Often the control phase continued beyond these 45 minutes. However, reaching a constant frequency prior to the addition of the test substance was of necessity. A constant frequency after 45 minutes was considered when the frequency did not change with a maximum deviation of one beat over three consecutive measurements.

After the control phase the test substance was carefully added in the pattern depicted in table 5 by an Eppendorf-pipette and all the following measurements were conducted in the same manner as mentioned. After 180 minutes the experiment was terminated.

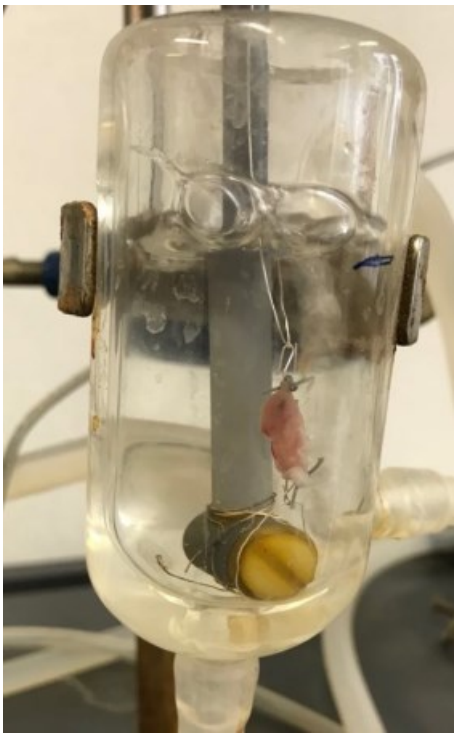


Figure 13 Right atrium suspended in the organ bath containing nutrient solution

3.8.2 Experimental procedure – Musculus papillaris

In contrast to the right atrium the papillary muscle does not possess any spontaneous activity and therefore it must be stimulated by electrical pulses to induce contraction. As in chapter 3.6.2.3 already mentioned the Musculus papillaris was sandwiched between a platin electrode and a small piece of plexiglass (see figure 14). Through this electrode a square wave pulse of 10ms in a frequency of 60 Hz was applied using the A310 Accupulser.

It was utterly important not to empty the catecholamine storage vesicles, otherwise this would result in a loss of function of the papillary muscle. For this purpose, the electrical pulses were set to be 10% higher than the stimulus threshold. (Furchgott, de Gubareff, & Grossman, 1959).

However, it happened that the muscle showed still some activity without any stimulation due to not previously removed Purkinje cells. In this case, the muscle would be exchanged by another piece to avoid any affectations of the test procedure. Sometimes the spontaneous activity would even fade in the progress of the adaptation phase.

Applying a pretension of 3.92 mN required a setting of 5mV on the plotter. After finishing the equilibration period of 20-30 minutes, the control phase could be initiated. This time, six amplitudes were recorded every five minutes for at least 45 minutes. Sometimes the control phase would take longer.

The amplitudes were then measured out by a ruler and had to be at least 2cm long and the deviation of three consecutive measurements should not be bigger than 1mm in order to proceed with the substance addition. The pipetting scheme is illustrated in table 5 and the following procedures remained the same as in the control phase.

Furthermore, it must be emphasised that changing the applied voltage on the organ to 2mV or 1mV was only possible before the test began. These alterations depended on the individual strength of the organ specimen and were necessary in some cases.

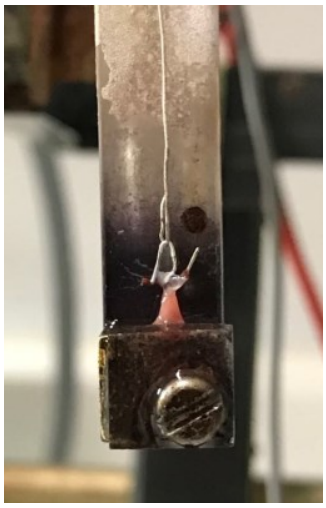


Figure 14 Musculus papillaris sandwiched between a platin electrode and a plexiglass

3.8.3 Experimental procedure – terminal ileum

A small piece of the terminal ileum was attached with two little hooks to the apparatus Ia (see figure 15). The precise preparation has been already explained in chapter 3.6.2.4.

The recorder was set to 5mV and the fine adjustment knob was turned until a deflection of 5cm was reached. Hence, a pretension of 4.9 mN was achieved on the organ specimen. Moreover, the pace of the recorder was adjusted to 1mm/sec.

During the adaptation phase of 20-30 minutes the peristaltic movements of the organ could be observed as wavy motions on the graph paper. In the meantime, 25ml of 60mM potassium chloride solution (see chapter 3.4) were prepared to be at hand. When the 20-30 minutes elapsed, the recorder was set back to zero and the nutrient solution was quickly exchanged by the potassium chloride solution to prompt a maximum contraction of the organ. Before the test substance could be added one had to wait until a plateau phase was reached, which should be at least 5 cm in height. The following steps are indicated in table 5 and do not differentiate from the previous procedures performed on other organs.

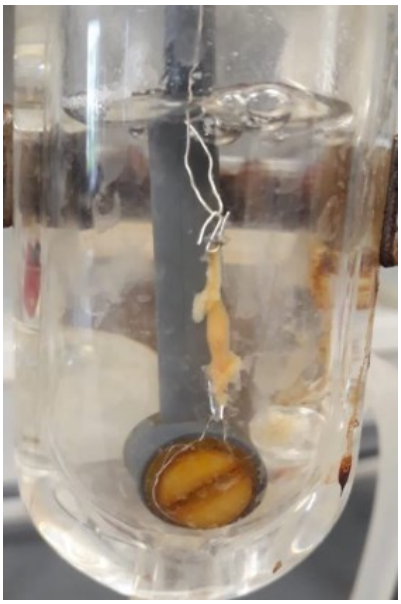


Figure 15 Terminal ileum suspended in the organ bath containing nutrient solution

3.8.4 Experimental procedure – Aorta descendens

The ring-shaped structure allowed to set up a direct connection to the silver wires on the metal rod (see figure 16). For the adaption phase the voltage of the recorder was regulated up to 10 mV. With the fine adjustment knob a pretension of 19.6 mN was applied, marking a deflection of 10cm on the graph paper.

As soon as the adaption phase ended, the voltage was reduced to 5 mV and the recorder was readjusted to zero again.

The nutrient solution was then replaced with 25ml of a 90 mM potassium chloride solution to foster a maximum contraction. A plateau phase reaching at least 5 cm above the base line on the graph paper was required to be able to continue the experiment. Table 5 indicates further procedures regarding the addition of the test substance.

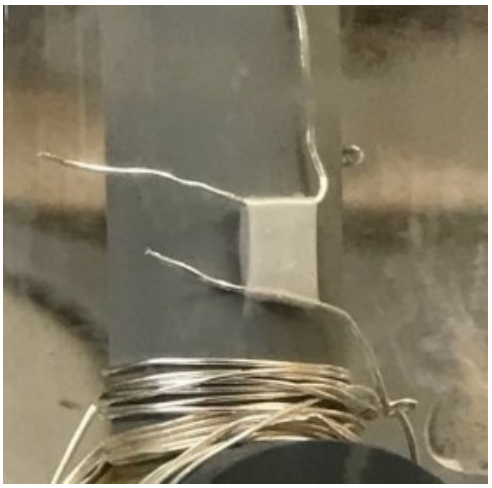


Figure 16 Aortic ring suspended in the organ bath containing nutrient solution

3.8.5 Experimental procedure – Truncus pulmonalis

The experimental procedures on the specimen of the Truncus pulmonalis were similar to those of the aorta described in 3.8.4. However, this time 5 mV were applied and a deflection of 10cm on the recorder lead to the required pretension of 9.8 mN (figure 17).

Figure 17 Pulmonary ring suspended in the organ bath containing nutrient solution

3.8.6 Experimental procedure to determine a mechanism of action of the test substance

Final experiments evaluating a possible mechanism of action were conducted on the terminal ileum as genistein showed the highest activity on that specimen. For this kind of approach, the nitric synthase was chosen to be blocked by the antagonist N^{ω}-nitro-L-arginine prior to the addition of the test substance. The set up and the preparation of the organ was the same as already described in the chapters 3.6.2.4 and 3.8.

However, as soon as the plateau phase was reached, N^{ω}-nitro-L-arginine was added to the organ bath causing a slight increase in contraction force. After 45 minutes genistein was applied in a dose correspondent to its EC_{50} value. Finally, after another 45 minutes the experiment was terminated.

3.8.6.1 N^{ω}-nitro-L-arginine

N^{ω}-nitro-L-arginine is a commonly used compound approaching the biological effects of nitric oxide. It carries out an irreversibly block on the nitric oxide synthase (Bansinath, Arbabha, Turndorf, & Garg, 1993).

3.8.6.2 Nitric oxide synthase

The nitric oxide synthase is an enzyme which catalyses the production of nitric oxide by using the amino acid L-arginine. This unique apolar gas is also known as the endothelium derived relaxing factor, as it leads to a relaxation of the smooth muscle tissue via the activation of the guanylate cyclase (Huppelsberg & Walter, 2005).

3.9 Evaluation methods

The effects of the test substance on each organ were either recorded on graph paper or digitally. This was up to the recording device that was used.

The obtained graphs on paper were measured with a ruler to evaluate the change in contraction force or in case of the right atrium the number of contractions. Programs like Microsoft Excel and SigmaPlot 9 would then be used to help converting the collected data and depicting the values in a rational manner for a better analysis.

Data obtained by the apparatus Ib was automatically processed.

3.9.1 Atrium cordis dextrum

Influencing specific ion channel currents or activating receptors located in the heart could result in a change of chronotropy. Such actions of cardiotropic substances are considered as chronotropic effects (Burger & Wachter, 1986).

Only measurements recorded at each steady-state status of the applied concentration were taken into consideration. Because the recording was conducted in a time of 12 seconds every five minutes, the rate had to be multiplied by five to get the beats per minute. The test substance was then specified having either a neutral, negative or positive chronotropic effect.

3.9.2 Musculus papillaris

The series of experiments performed on the papillary muscle had the purpose to reveal a possible ionotropic effect of the test substance. Inotropic effects are those that have an impact on the force of the muscular contraction (Burger & Wachter, 1986).

Again, only the last three measurements of each concentration were considered. But this time the height of the recorded amplitude was of importance. With a ruler the

recorded amplitudes were measured out in centimetres and afterwards converted into millinewton in relation to the voltage that was applied during the experiment.

3.9.3 Smooth muscle tissue (Aorta descendens, Truncus pulmonalis, terminal ileum)

In these kinds of experiments a maximum contraction of the organ specimens was achieved by a potassium solution before adding the test substance. After reaching the plateau, a mark with a pencil was applied on the graph at each addition of the test substance and at the end of the experiment. From these markings the distance between the curve and the zero line was measured out in centimetres by a ruler. Finally, this data was implemented into a Microsoft Excel chart and converted into millinewton in correspondence to the applied voltage during the experiment.

A vasodilating effect was observed if the contraction decreased to a higher extent under the influence of the substance. A rather steady decline of the curve would not be classified as an effect of the substance.

For evaluation of the computer-assisted experiments, the exact time was written down when the test substance was added. These marked checkpoints were alike the marks on the curve of a plotter recording. Hence, a shift in contraction force, specified in millinewton, could be detected after the addition of genistein.

3.9.4 Statistics

Dimethyl sulfoxide was used to create the test solution. This is worth mentioning as this kind of solvent has vasodilating properties on its own and therefore could distort the measurements performed on the smooth muscle tissue. To avoid that, the activity of DMSO was tested beforehand and its effect was deducted from the results by multiplying them with the factor 0,98. This new calculated data was then used for further analysis.

With the help of the software Sigma Plot 9.0. dose-response curves were created and the statistical evaluation was executed. The mean, the standard error of the mean (SEM) and the standard deviation were calculated. On the plotted curves the EC_{50} value could be ascertained in µmol/l.

To determine statistical significance (p) of the results, the paired-t-test was performed. Results having a significance level of $p < 0.05$ were significant. Furthermore, a significance level of $p < 0.01$ was considered highly significant, while $p < 0.001$ indicated very high significance.

4 Results

In the following section the results of each organ will be presented separately. This includes a table of data, a dose-response curve and a representative example of an original recording.

The table depicts the mean of the contraction force (fc) **[mN]** or the mean of the heart rate indicated by Hertz **[Hz]**, its relative percentage change (fc) **[%]**, the number of experiments (n) and the significance level (p).

The dose-effect relationship is illustrated by a curve in which the ordinate represents the change in contraction force **[%]** and the abscissa the concentration of the substance in the organ bath **[µmol/l]**. At a decrease of contraction force by 50% two lines were drawn characterizing the EC_{50} value on the curve.

In addition, arrows on the original recordings are marking the points of the addition of genistein.

Apart from that, the selected recordings of the Atrium cordis dextrum and the Musculus papillaris illustrate the effects at a specific concentration of the test substance after an elapsed time of 45 minutes. Furthermore, time **[min or s]** is represented on the abscissa and the contraction force **[mN]** on the ordinate.

4.1 Results of genistein on the right atrium

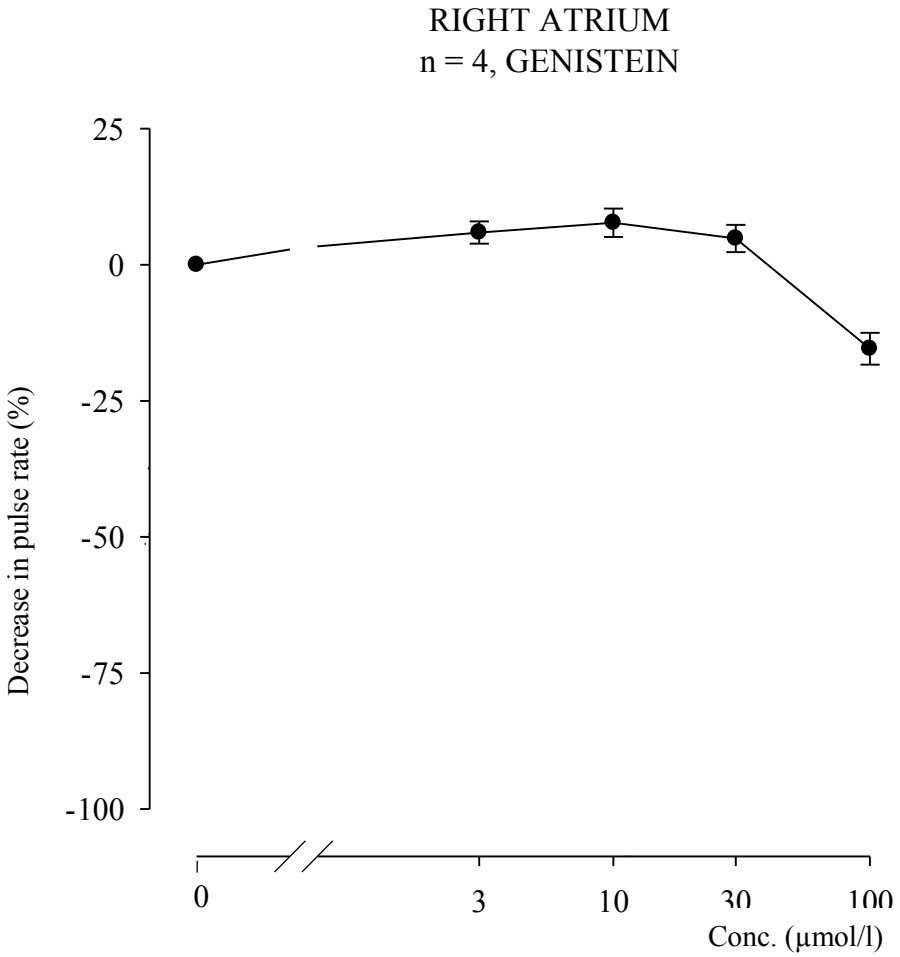
On the right atrium experiments inquiring a possible chronotropic effect of genistein were conducted. Before the test substance could be added, the organ had to be attached into the organ bath and run through an adaption phase due to the new environment. The exact procedure of the experiment can be read in chapter 3.8.1.

Table 6 Effect of genistein on the specimens of the right atrium

Concentration [µmol/l]	f±SEM [Hz]	f±SEM [%]	Number of experiments (n)	Probability (P)
0 (Control)	216.25±7.18	0.00±0.00	4	-
3	228.75±4.73	5.94±2.04	4	n.s.
10	232.50±4.33	7.73±2.62	4	n.s.
30	226.25±4.27	4.83±2.51	4	n.s.
100	182.50±5.20	-15.42±2.94	4	0.05

The results shown in table 6 reveal that a positive chronotropic effect occurs up to a concentration of 10 µmol/l, whereas higher concentrations lead to the opposite effect resulting in a negative chronotropy.

Figure 18 Dose-response curve of genistein on the right atrium



As can be seen in figure 18 on the abscissa the logarithmic concentration of genistein [µmol/l] is plotted. The ordinate instead represents the relative change in heart rate in percent [%]. Additionally, the four big black spots are illustrating the means obtained at different concentrations of the test substance (0 µmol/l, 3 µmol/l,10 µmol/l, 30 µmol/l, 100 µmol/l). The magnitude of the Standard error of the mean is indicated by lines crossing the black dots.

Genistein tended to induce a positive chronotropic effect up to a concentration of 10 µmol/l before the curve shows a drop-off at higher concentrations of the test substance. A half maximal effective concentration could not be observed.

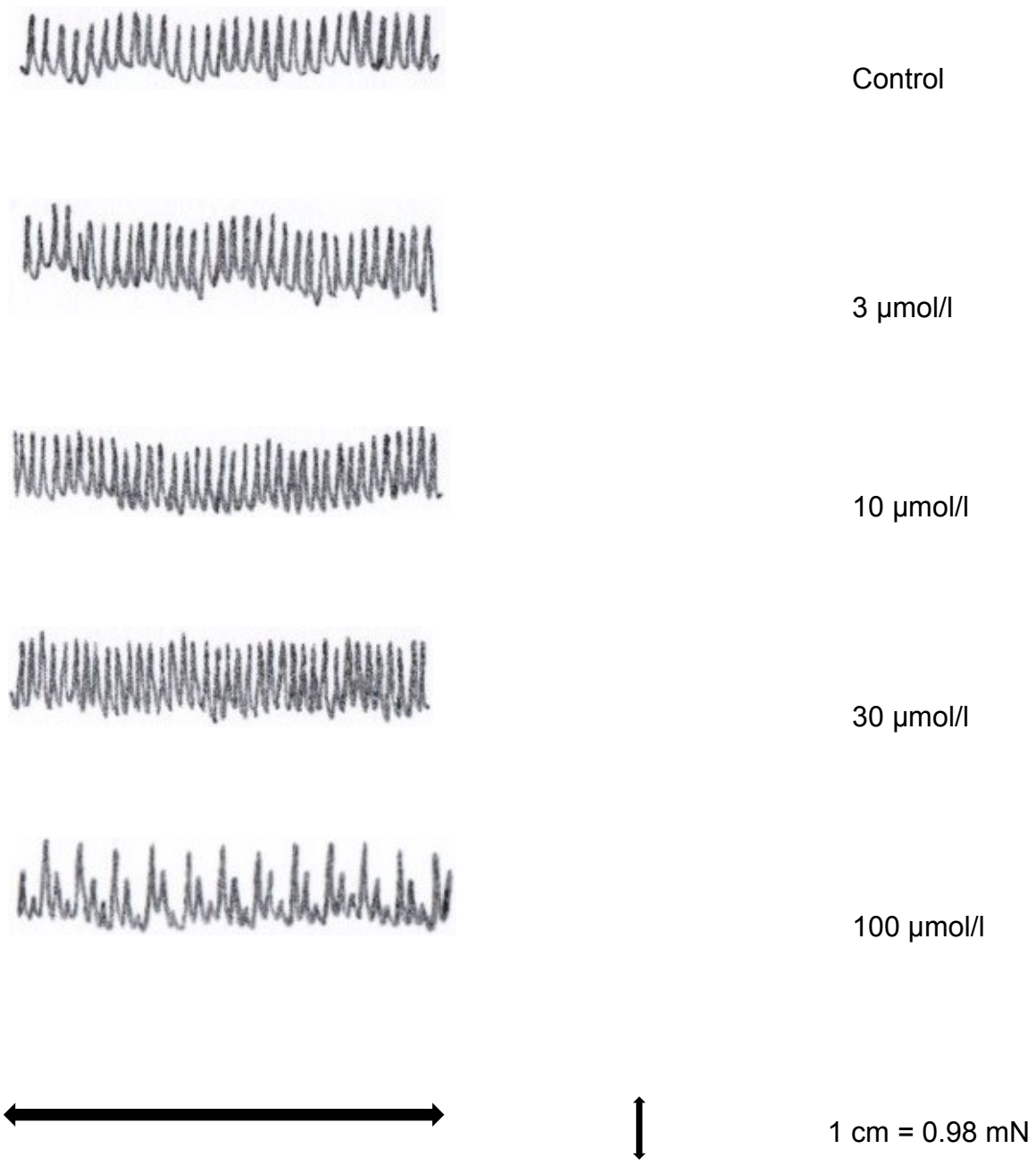


Figure 19 Original recordings of the right atrium

4.2 Results of genistein on the papillary muscle

Experiments on the papillary muscle delivered data on a potential ionotropic effect of genistein. The exact procedure is described in chapter 3.8.2.

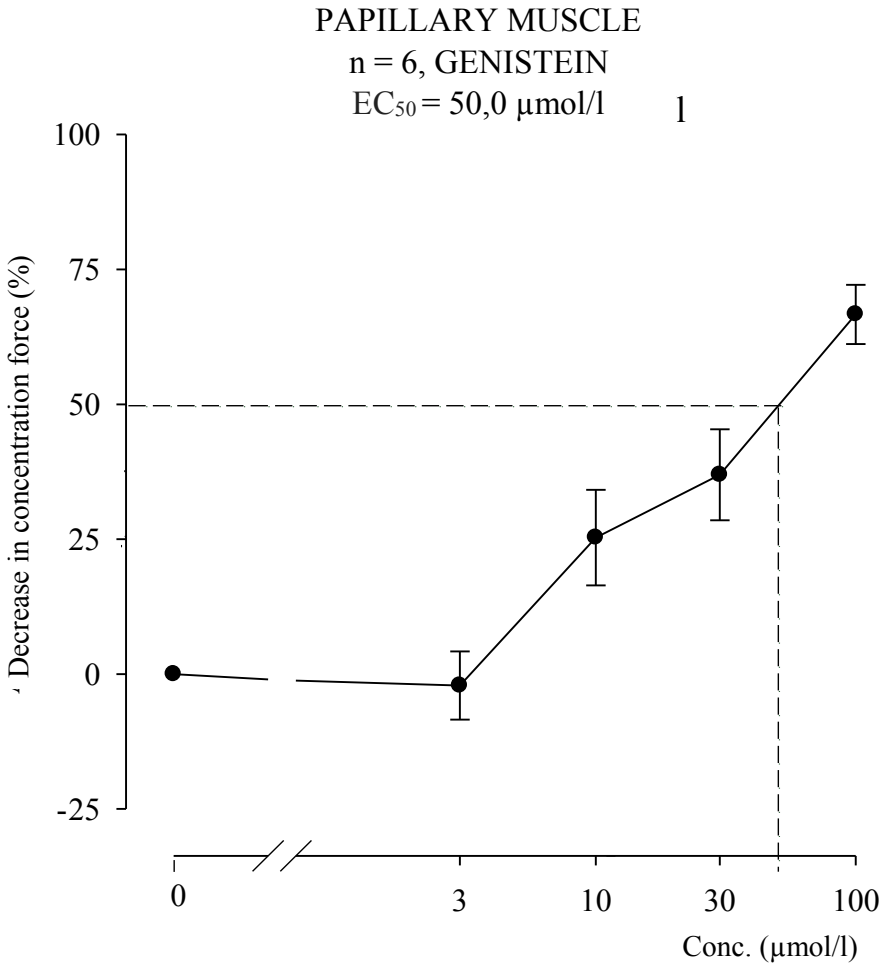
In reference to table 7 the papillary muscle lost 2.11% of its contraction force at concentrations of 3 µmol/l, respectively. However, concentrations starting at 10 µmol/l and above lead to an obvious positive ionotropy. Corresponding to this, a remarkable rise of 66.66% in contraction force was recorded before termination.

The evaluated EC_{50} value amounts to 50.0 µmol/l.

Table 7 Effect of genistein on the specimens of the papillary muscle

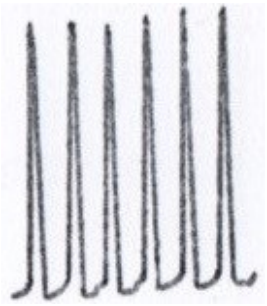
Concentration [µmol/l]	fc±SEM [mN]	fc±SEM [%]	Number of experiments (n)	Probability (P)
0 (Control)	1.13±0.11	0.00±0.00	6	-
3	1.11±0.13	-2.11±6.32	6	n.s.
10	1.40±0.14	25.30±8.85	6	0.05
30	1.53±0.13	36.93±8.43	6	0.05
100	1.88±0.19	66.66±5.50	6	0.01

Figure 20 Dos e-response curve of genistein on the papillary muscle

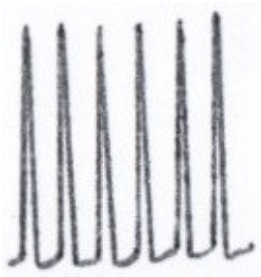


As can be seen in figure 20 on the abscissa the logarithmic concentration of genistein [µmol/l] is plotted. The ordinate instead represents the relative change of contraction force in percent [%]. Additionally, the four big black spots are illustrating the means obtained at different concentrations of the test substance (0 µmol/l, 3 µmol/l,10 µmol/l, 30 µmol/l, 100 µmol/l). The magnitude of the Standard error of the mean is indicated by lines crossing the black dots.

Towards concentrations above 3 µmol/l the curve slopes upward. Two dashed lines are pointing out the EC_{50} value of 50.0 µmol/l on the graph. Genistein exerts a significant ionotropic effect when applied in higher concentrations. For that reason, the EC_{50} value is found to be that high.

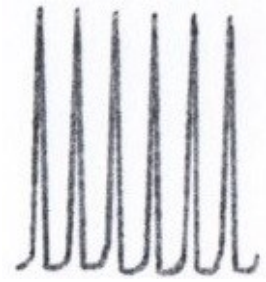


Control



3 µmol/l

10 µmol/l



30 µmol/l

100 µmol/l

1 cm = 0.98 mN

Figure 21 Original recordings of the papillary muscle

4.3 Results of genistein on the aorta

The evaluation of the vasodilating effects on the aorta included five experiments. The procedure has already been mentioned in chapter 3.8.4.

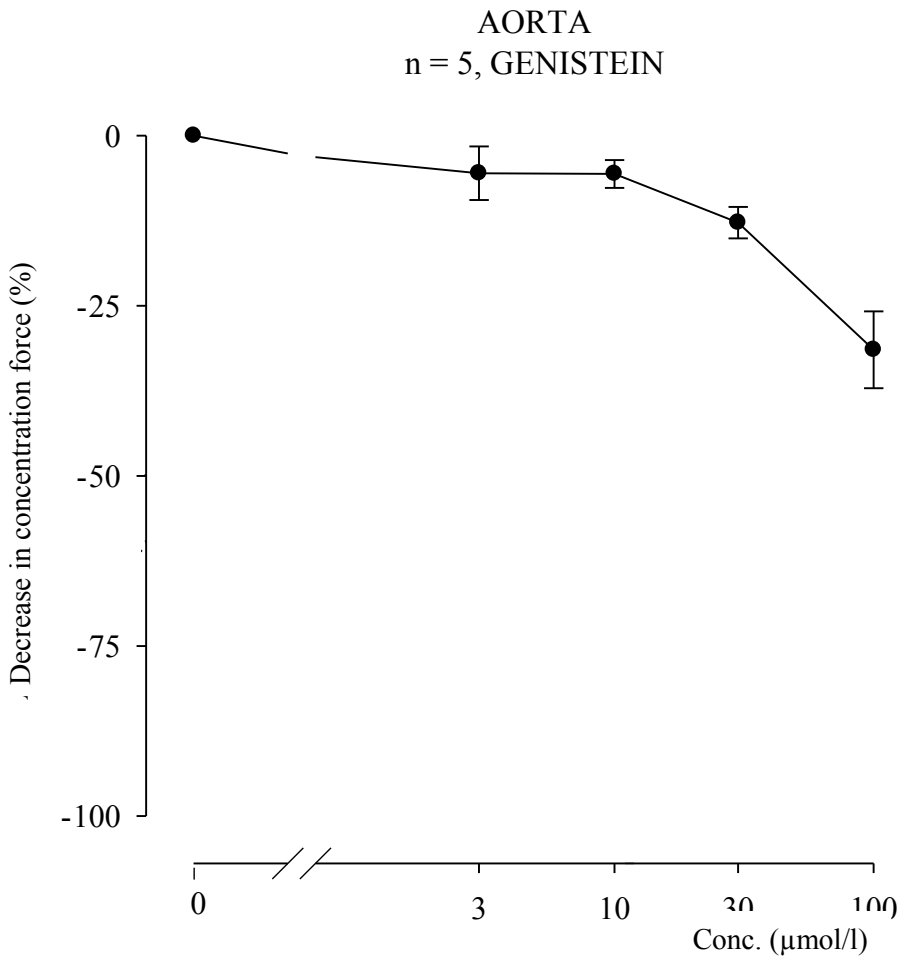
Initially, a minor but noticeable vasodilatation induced due to a low dose-exposure to genistein could be observed (table 8). Between the concentrations of 3 and 10 µmol/l almost no difference of contraction force was registered. Concentrations of 30 and 100 µmol/l delivered a vasodilating effect of a higher extent and produced a reduction in contraction force of 31.45%, respectively.

An EC_{50} value could not be evaluated. In order to do so, concentrations above 100 µmol/l are needed.

Table 8 Effect of genistein on the specimens of the aorta

Concentration [µmol/l]	fc±SEM [mN]	fc±SEM [%]	Number of experiments (n)	Probability (P)
0 (Control)	20.23±4.59	0.00±0.00	5	-
3	18.87±4.25	-5.51±3.94	5	n.s.
10	18.99±4.22	-5.61±2.05	5	n.s.
30	17.55±3.93	-12.77±2.32	5	0.05
100	14.19±3.80	-31.45±5.64	5	0.05

Figure 22 Dose-response curve of genistein on the aorta



As can be seen in figure 22 on the abscissa the logarithmic concentration of genistein [µmol/l] is plotted. The ordinate instead represents the relative change of contraction force in percent [%]. Additionally, the four big black spots are illustrating the means obtained at different concentrations of the test substance (0 µmol/l, 3 µmol/l,10 µmol/l, 30 µmol/l, 100 µmol/l). The magnitude of the Standard error of the mean is indicated by lines crossing the black dots.

The course of the curve is rather flat and is characterized by big drop-off after administering the last 70 µmol/l of genistein into the organ bath.

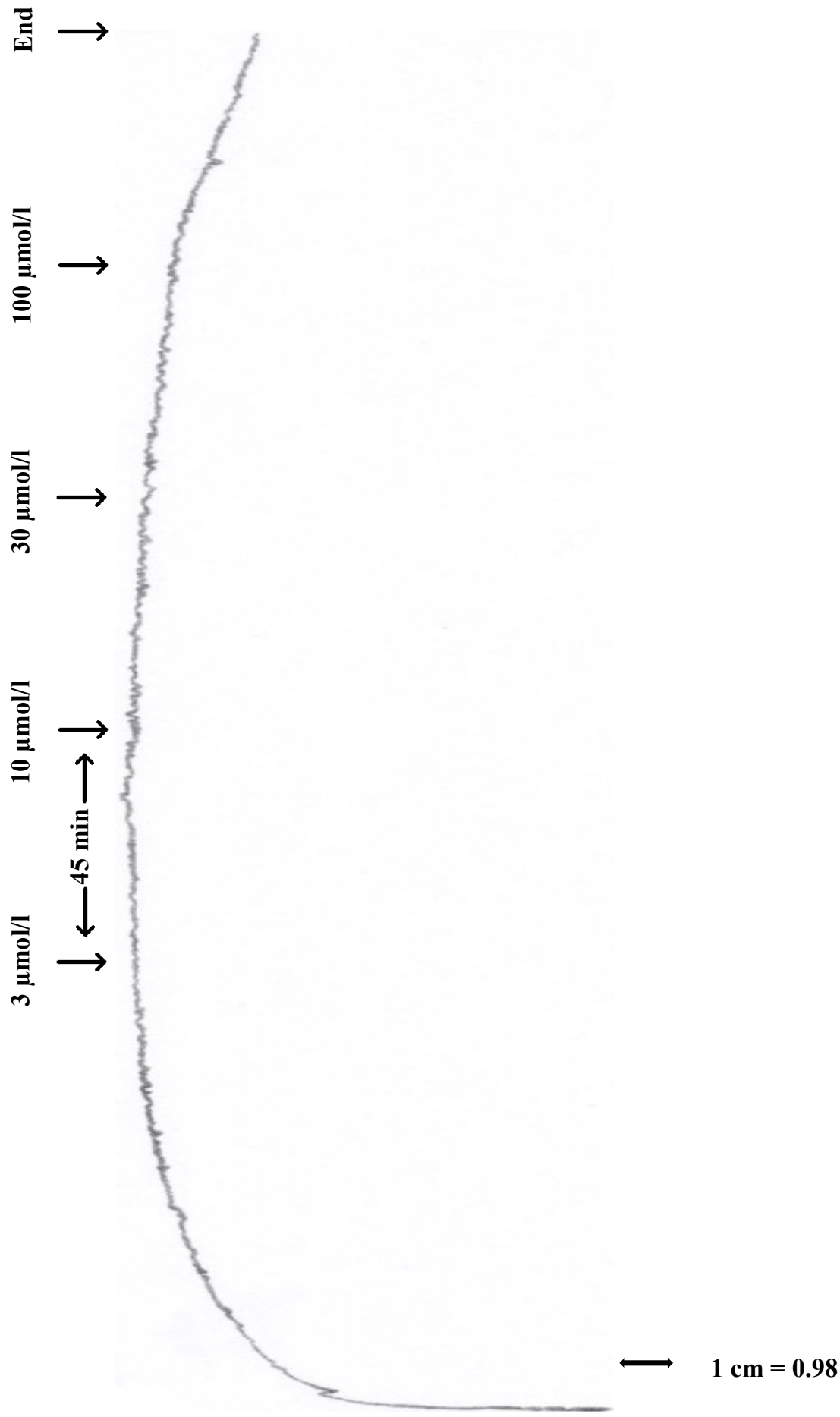


Figure 23 Original recording of the aorta

4.4 Results of genistein on the pulmonary artery

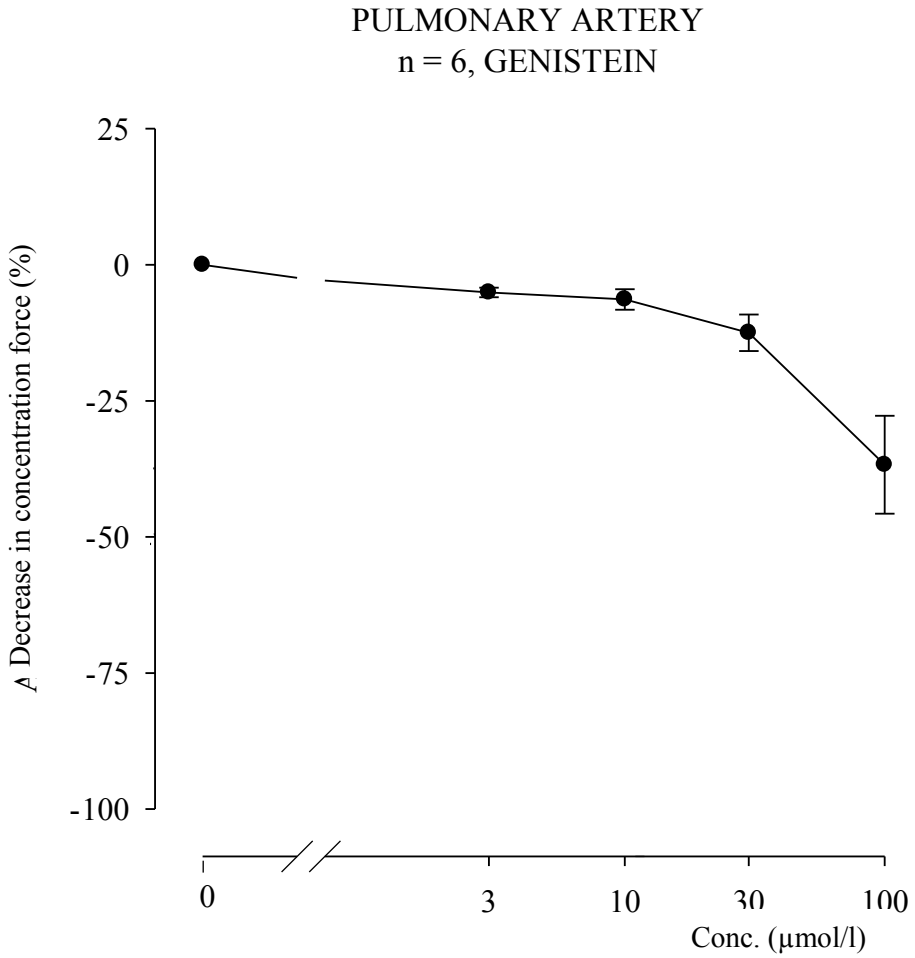
In comparison to the results shown for the aorta (table 8), quite similar outcomes on the pulmonary artery could be observed under the influence of the test substance (table 9). While the vasodilatation of the blood vessel was moderate at concentrations of 3 and 10 µmol/l, the relaxation got more distinct at 100 µmol/l of genistein.

However, the effect was not strong enough to evaluate a possible EC_{50} value on that organ specimen.

Table 9 Effect of genistein on the specimens of the pulmonary artery

Concentration [µmol/l]	fc±SEM [mN]	fc±SEM [%]	Number of experiments (n)	Ptobability (P)
0 (Control)	18.37±2.61	0.00±0.00	6	-
3	17.50±2.56	-5.08±0.87	6	n.s.
10	17.38±2.68	-6.35±1.89	6	n.s.
30	16.45±2.75	-12.48±3.36	6	0.05
100	12.31±2.93	-36.72±9.00	6	0.05

Figure 24 Dose-response curve of genistein on the pulmonary artery



As can be seen in figure 24 on the abscissa the logarithmic concentration of genistein [µmol/l] is plotted. The ordinate instead represents the relative change of contraction force in percent [%]. Additionally, the four big black spots are illustrating the means obtained at different concentrations of the test substance (0 µmol/l, 3 µmol/l,10 µmol/l, 30 µmol/l, 100 µmol/l). The magnitude of the Standard error of the mean is indicated by lines crossing the black dots.

The course of the curve is rather flat and is characterized by big drop-off after administering the last 70 µmol/l of genistein into the organ bath.

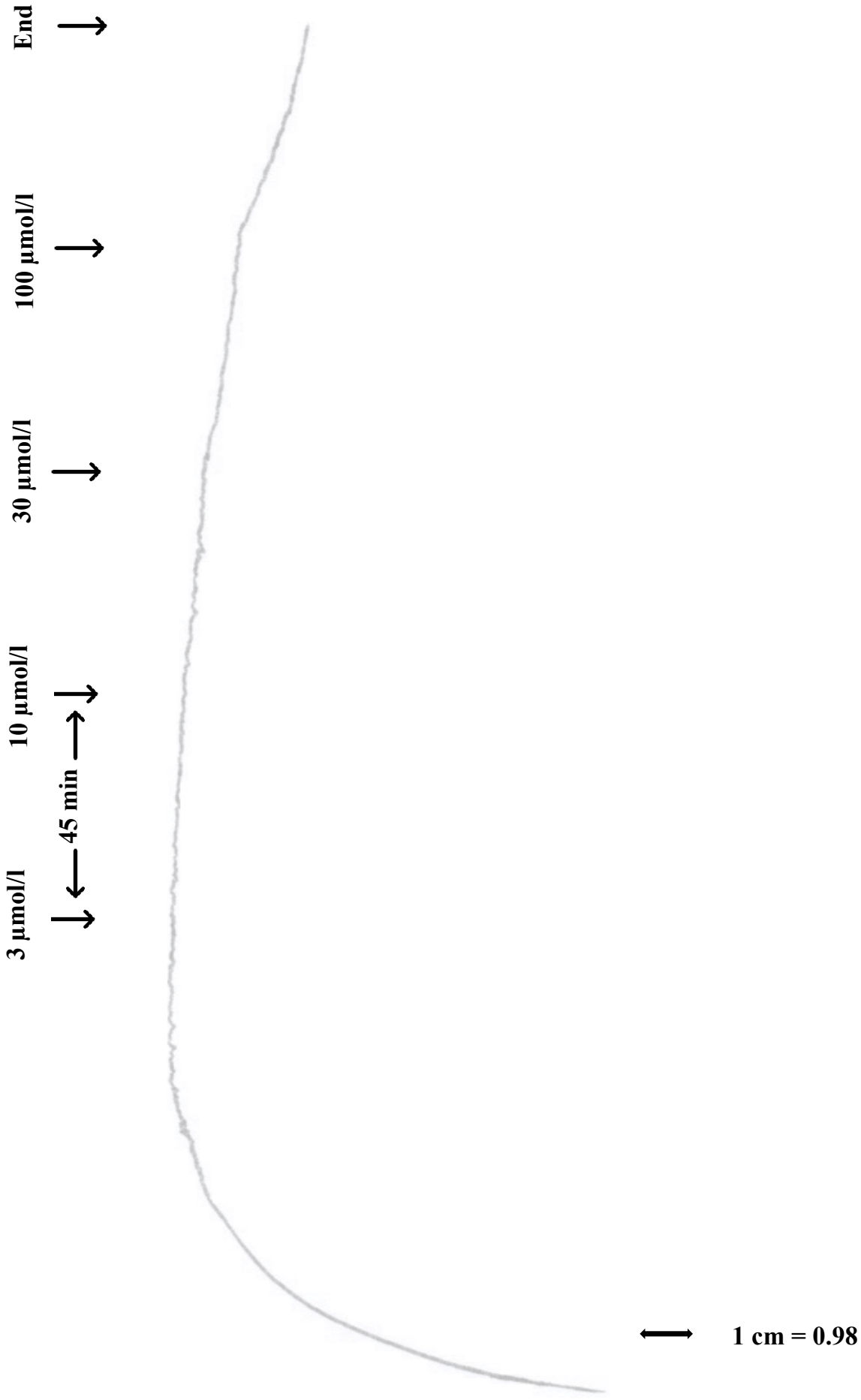


Figure 25 Original recording of the pulmonary artery

4.5 Results of genistein on the terminal ileum

The main purpose of this test series was to examine a possible spasmolytic effect of genistein. Preparation methods of the terminal ileum and the exact procedure of these experiments are thoroughly explained in the chapters 3.6.2.4 and 3.8.3. Table 10 comprises all the collected data.

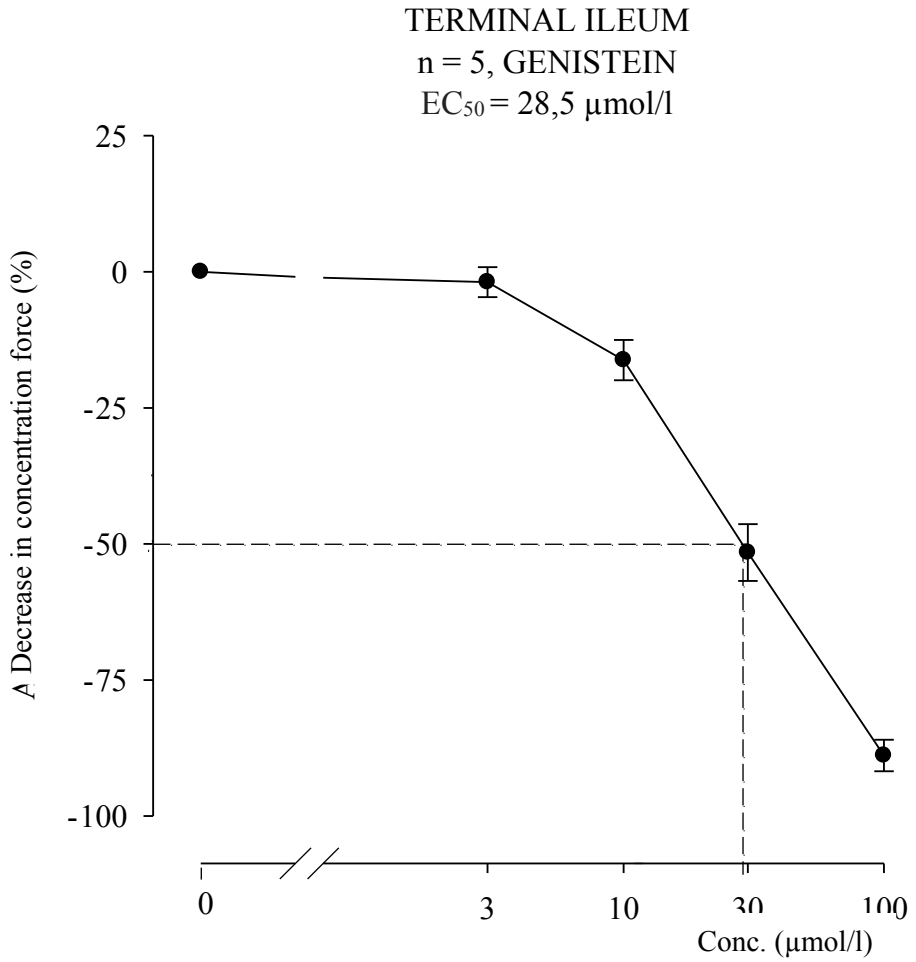
Genistein exerted by far the biggest effect on this organ specimen. An initial dose of 3 µmol/l seemed not to affect the smooth muscle tissue of the terminal ileum significantly, but there was a progressive decline in contraction force as soon as the concentration in the organ bath rose (10 µmol/l, 30 µmol/l, 100 µmol/l). By the end of the experiment a vasodilatation up to 88.86% was detected, respectively.

Furthermore, 28.5 µmol/l represent the half maximal effective concentration of genistein for this organ.

Table 10 Effect of genistein on the specimens of the terminal ileum

Concentration [µmol/l]	fc±SEM [mN]	fc±SEM [%]	Number of experiments (n)	Probability (P)
0 (Control)	9.60±1.38	0.00±0.00	5	-
3	9.46±1.44	-1.91±2.76	5	n.s.
10	8.08±1.24	-16.23±3.70	5	0.05
30	4.76±0.93	-51.58±5.23	5	0.01
100	1.03±0.23	-88.86±2.89	5	0.01

Figure 26 Dose-response curve of genistein on the terminal ileum



As can be seen in figure 24 on the abscissa the logarithmic concentration of genistein [µmol/l] is plotted. The ordinate instead represents the relative change of contraction force in percent [%]. Additionally, the four big black spots are illustrating the means obtained at different concentrations of the test substance (0 µmol/l, 3 µmol/l,10 µmol/l, 30 µmol/l, 100 µmol/l). The magnitude of the Standard error of the mean is indicated by lines crossing the black dots.

Two dashed lines are pointing out the EC_{50} value of 28.5 µmol/l on the graph. The spasmolytic effect is rather high and therefore the test substance induces a corresponding half-maximum response on the terminal ileum at such a relatively low concentration compared to the EC_{50} value of the papillary muscle.

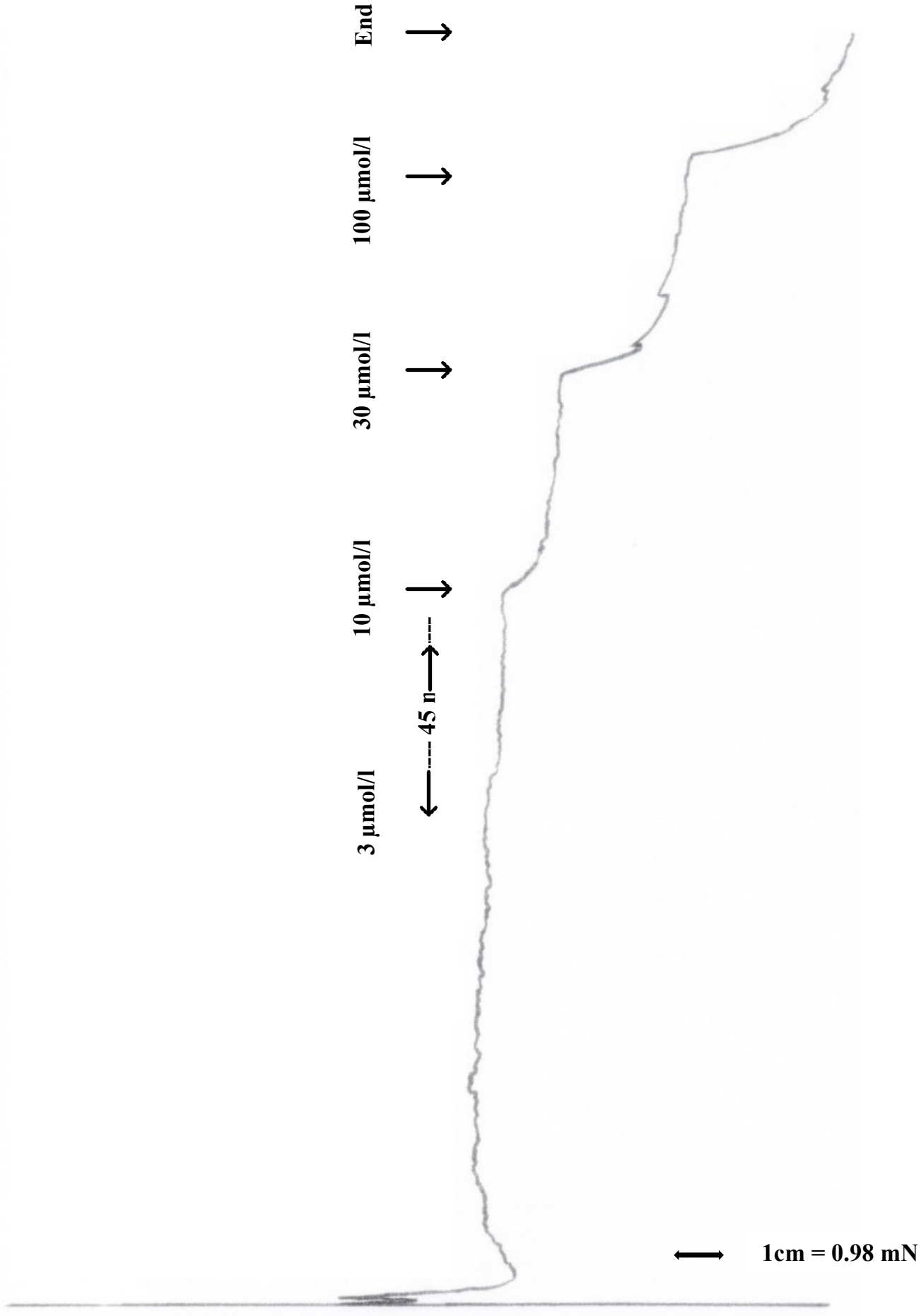


Figure 27 Original recording of the terminal ileum

4.6 Mechanism of action of genistein on the terminal ileum

N^{ω}-nitro-L-arginine was chosen to block the nitric synthase, an enzyme which is responsible for producing the vasodilating substance nitric oxide (Huppelsberg & Walter, 2005).

The objective of this kind of approach is to determine, whether this pathway could be a possible mechanism of action used by genistein (see chapter 3.8.6).

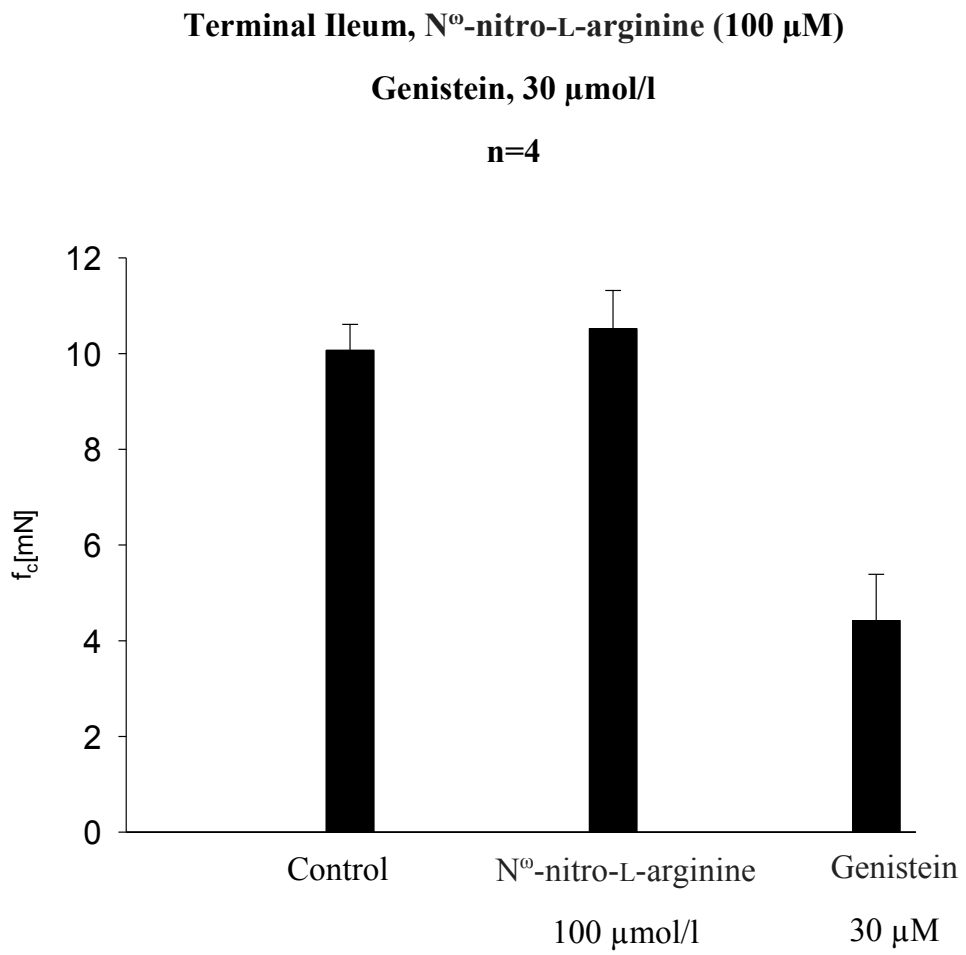
After a control phase N^{ω}-nitro-L-arginine was added into the organ bath causing the terminal ileum to contract slightly stronger (+4,06%) due to its antagonism. 45 minutes later, genistein was applied in a concentration representing the EC_{50} value stated in chapter 4.5. Instead of using the exact 28.5 µmol/l it was rounded up to 30 µmol/l.

The summarized data is outlined in table 11 and figure 28 clearly indicates a similar decrease of contraction force as without the antagonist.

Table 11 Results for the mechanism of action with 100 µmol/l Nω-nitro-L-arginine

Concentration [µmol/l]	fc±SEM [mN]	fc±SEM [%]	Number of experiments (n)	Probability (P)
Control	10.07±0.54	0.00±0.00	4	-
N^{ω}-nitro-L-arginine 100 µmol/l	10.52±0.80	4.06±2.36	4	n.s.
Genistein 30 µmol/l	4.42±0.97	-56.98±8.24	4	0.05

Figure 28 Bar chart of a possible mechanism of action with 100 µmol/l nitroarginine



Each bar represents the mean values. The lines sticking out at the top of the bars are illustrating the standard error of the mean. Moreover, the abscissa indicates the concentration and the ordinate displays the contraction force.

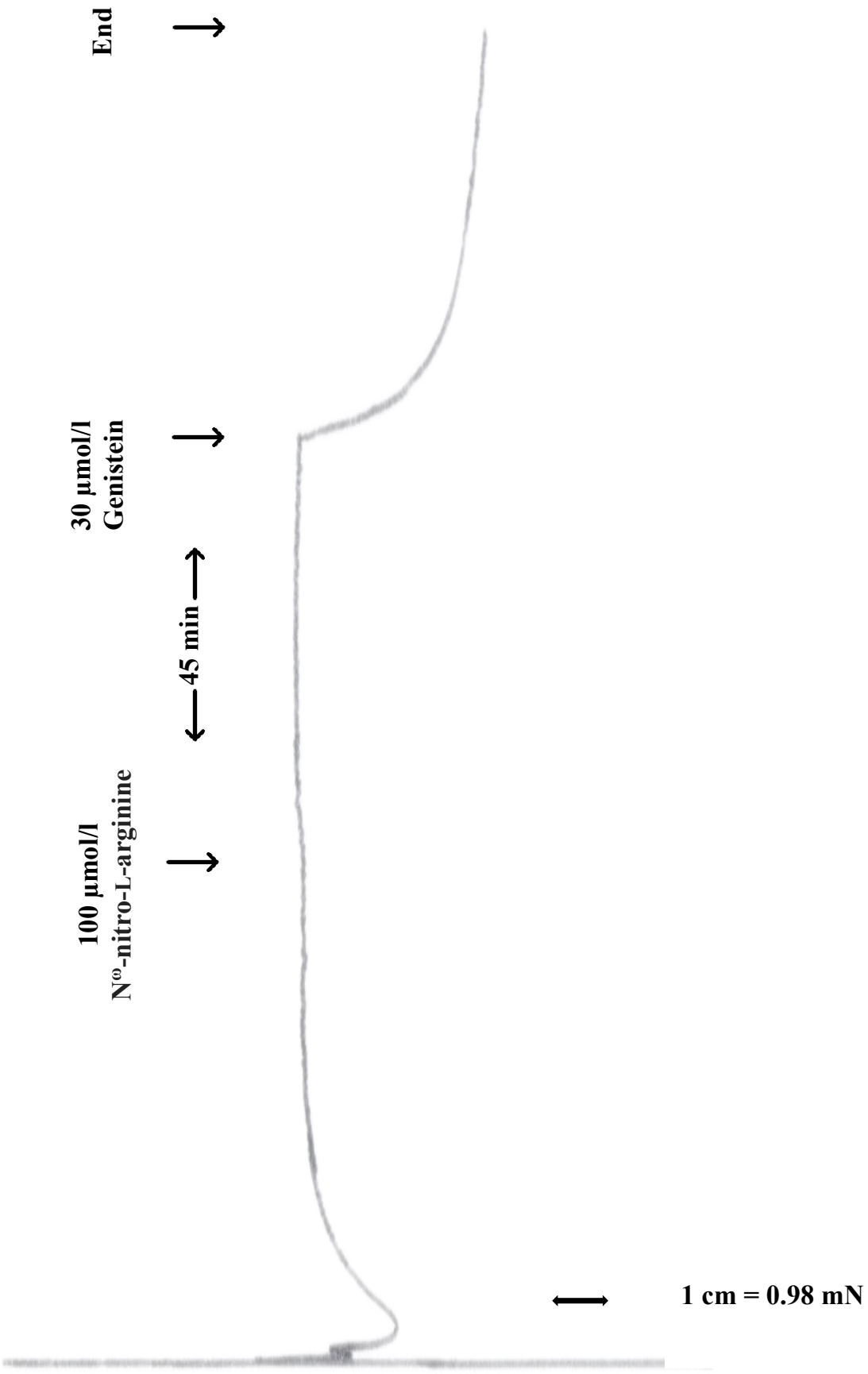


Figure 29 Original recording of the experiment with 100 µmol/l Nω-nitro-L-arginine

5. Discussion

In the context of this diploma thesis it has been shown that the phytoestrogen genistein triggers an effect on all five organ specimens. For more detailed discussion, the previous mentioned results are put to comparison in this section and are class-divided in smooth muscle tissue and in striated muscle tissue.

The group representing the smooth muscle tissue encompasses the Aorta descendens, the Truncus pulmonalis and the terminal ileum. These experiments delivered data on vasodilating and spasmolytic effects of the test substance.

On the other hand, the Atrium cordis dextrum and the Musculus papillaris are combined to a separate paragraph for further interpretation of the chronotropic and ionotropic effects on the striated muscle tissue. However, it must be kept in mind that the musculature of the heart has a construct, which embodies structure elements of both, the smooth muscle tissue and the striated muscle tissue.

Moreover, a possible mechanism of action is elucidated.

5.1 Effect of genistein on striated muscle tissue

Table 12 addresses the chronotropic and ionotropic effects on the striated muscle tissue. Only an EC_{50} value for the papillary muscle could be determined.

Table 12 Comparison of the effect of genistein on striated muscle tissue

Organ	fc±SEM [%] at 10 µmol/l	fc±SEM [%] at 100 µmol/l	EC_{50} [µmol/l]
Atrium cordis dextrum	7.73±2.62	-15.42±2.94	-
Musculus papillaris	25.30±8.85	66.66±5.50	50.0 µmol/l

5.1.1 Effect of genistein on the Atrium cordis dextrum

All four experiments conducted on the right atrium revealed a dose-depended change in the heart rate

It became apparent that genistein induced a humble positive chronotropic effect up to concentrations of 10 µmol/l (7.73±2.62%), whereas higher concentrations (100 µmol/l) disembogued in a negative chronotropy with a reduction of -15.42±2.94% in beats per minute, respectively (table 12). These values agree well with those reported in the literature.

There is evidence that genistein can reduce the maximal conductance in the native hyperpolarization-activated cyclic nucleotide-gated cation channel (HCN) in rabbits through an inhibition of the tyrosine kinase pathway (J.-Y. Wu & Cohen, 1997).

It also has been suggested that genistein directly blocks the HCN channel and thereby reducing the heart rate via a reduction of the slope of the diastolic depolarization (Altomare, Tognati, Bescond, Ferroni, & Baruscotti, 2006; Bois et al., 2007; Shibata, Ono, & Iijima, 1999).

Furthermore, the binding cannot be attributed to a certain gating or functional state of the channel, neither was the block voltage or current dependent (Altomare et al., 2006).

Besides, Washizuka et al., 1998, further investigated, whether the IK currents in a guinea pig heart are modified by genistein. The findings in this study supported the notion that genistein preferentially suppresses the slow component of IK (IKs), while the rapid component (IKr) is not affected at all. Thus, producing a prolongation of the refractory period, which results in a decline of contraction rate (Washizuka, Horie, Obayashi, & Sasayama, 1998).

The IK current is mainly responsible for the repolarisation during an action potential. Due to its slow activation it is also referred as the delayed rectifier and is segmented in a rapid component (IKr) and a slow component (IKs) (Sanguinetti & Jurkiewicz, 1990).

Considering these summarized evaluations from the literature, it is not surprising that genistein exerted a negative chronotropic action on the right atrium. Moreover, an EC_{50}

value could not be calculated as the test substance was not potent enough to carry out a half maximal effect on the right atrium (see figure 18).

However, based on the required data in this thesis, it can be surely concluded that genistein might serve as a template for some new antiarrhythmic drugs.

5.1.2 Effect of genistein on the Musculus papillaris

In contrast to the testings on the right atrium, genistein proved to have a stronger influence on the papillary muscle. It was noticeable that a concentration of 3 µmol/l consequently lead to a slight decrease of contraction force (-2.11±6.32%) (see table7).

However, as soon as the test substance accumulated in the organ bath to higher concentrations (above 10 µmol/l), a rapid rise in the amplitude could be recognised. A concentration of 100 µmol/l resulted in a significant increase of 66.66±5.50% in contraction force, respectively (table 12).

The experiments also demonstrated a rather high EC_{50} (50µmol/l) value for genistein (see figure 20).

These findings are in agreement with the previous work done by Li et al., 2008, that genistein have an excitatory effect on the isolated guinea pig left ventricular papillary muscles. The results of this study indicated that genistein (1–100 µM) was able to increase contraction in a concentration-related fashion in male and female animals. (H. Li et al., 2008).

Moreover, it has been reported that genistein inhibits the tyrosine kinase (Akiyama et al., 1987). As a consequence, an enhancement in cardiac excitation-contraction coupling through a modulation in cyclic adenosine monophosphate (cAMP) levels could be observed (Kerfant, Rose, Sun, & Backx, 2006).

Some studies also demonstrated that genistein stimulates cAMP accumulation in a variety of cell systems and tissues. As a signalling molecule, cAMP is involved in the regulation of Ca^{2+} mobilization into the sarcoplasmic reticulum and further affects the Ca^{2+} sensitivity of the myofilament in cardiac myocytes (H. Li et al., 2008).

In addition to that, Liew et al. ,2004, reported that genistein increases the Ca^{2+} content of the sarcoplasmic reticulum in guinea pig ventricular myocytes from both sexes (Liew et al., 2004).

To conclude, what is well explained by the proposals of Li et al., 2008, genistein facilitates Ca^{2+} mobilization via an inhibition of tyrosine kinase, which eventually lead to an increased cAMP level within the cell (H. Li et al., 2008). For that reason, genistein might prove beneficial in people who suffer from heart insufficiency.

5.2 Effect of genistein on smooth muscle tissue

The performed experiments on the three organs listed in table 13 helped to gain data on possible interactions of genistein with the smooth muscle tissue. Therefore, the Aorta descendens and the Truncus pulmonalis were used to examine a potential vasodilating effect of the test substance.

Besides the isometric measurements on the blood vessels, test series on the terminal ileum focused on exhibiting an eventual spasmolytic effect of the phytoestrogen.

Only an EC_{50} value for the terminal ileum could be determined.

Table 13 Comparison of the effect of genistein on smooth muscle tissue

Organ	fc±SEM [%] at 10 µmol/l	fc±SEM [%] at 100 µmol/l	EC50 [µmol/l]
Aorta descendens	-5.61±2.05	-31.45±5.64	-
Truncus pulmonalis	-6.35±1.89	-36.72±9.00	-
Terminal ileum	-16.23±3.70	-88.86±2.89	28.5 µmol/l

Additionally, it is more than clear that genistein executed a higher response on the smooth muscle tissue than on the striated muscle tissue (table12; table 13). Especially on the terminal ileum genistein lead almost to a full spasmolysis with a decrease of -88.86±2.89% in contraction force.

5.2.1 Effect of genistein on the Aorta descendens

The results summarized in table 13 show that the two blood vessel samples were dilatating under the influence of genistein, although not to a great extent.

Referring to the Aorta descendens, a -5.61±2.05% loss in contraction force was recorded at a concentration of 10 µmol/l. This trend continued up to the maximum cumulative substance concentration in the organ bath by the end of the experiment (100 µmol/l), counting a loss of -31.45±5.64 % in contraction force. No EC_{50} value could be determined due to the moderate effect on that organ system.

Similar isometric tension measurements where presented in an equivalent study for rabbit aortic arteries. This refined analysis showed that NO and cGMP do not play any role in terms of relaxation induced by genistein, neither does an endothelium-derived release of vasodilator prostanoids (H.-F. Li et al., 2004).

Moreover, there is evidence that under a pre-treatment with noradrenaline, genistein manages to decrease muscle tension of the vasculature in vitro. It has been proposed that this is due to an inhibition of calcium influx. However, these findings regarding the Ca^{2+} antagonistic property of genistein do not allow conclusions to be made whether this block results via receptor-operated calcium channels or if genistein simply counteracts the Ca^{2+}- release through IP_3 from the endoplasmic reticulum (H.-F. Li et al., 2004).

The above-mentioned approaches regarding the action mechanism are also supported by the findings of Figtree et al., 2000. The authors of this study could underline and confirm the calcium antagonistic properties of genistein also on isolated coronary rabbit arteries. However, these acute effects are suggested not to be due to an activation of ERalpha by the isoflavone (Figtree et al., 2000).

5.2.2 Effect of genistein on the Truncus pulmonalis

A similar outcome compared to the Aorta descendens was observed with the test series ran with the Truncus pulmonalis. These findings correspond with the fact that the two organs are quite similar in their general structure. At the final concentration of 100 µmol/l the reduction in contraction force increased up to -36.72±9.00%. Just like the Aorta descendes, the effect of genistein was not strong enough to specify an EC_{50} value.

To conclude, these results suggest that genistein acts protective on the cardiovascular system. Further optimization of the test substance, in order to increase bioavailability and selectivity, might help to introduce this isoflavone or derivatives to the common treatment of hypertension, angina pectoris and pulmonary hypertension.

5.2.3 Effect of genistein on the terminal ileum

By evaluating the results of the experiments on the terminal ileum, it became evident that genistein possess a great selectivity for this organ (table 13). A slight spasmolytic effect was already observed at 3 µmol/l (see table 10), which promised to intensify further. Nevertheless, it was not possible to achieve a full spasmolysis by the end of the experiment, but the contraction force dropped by a percentage of -88.86±2.89% (100 µmol/l). The EC_{50} value amounts to 28.5 µmol/l, being the lowest EC_{50} value to be calculated in this thesis. However, it is considered too high for a potential usage.

It is well-known that flavonoids could induce spasmolysis on a precontracted intestinal smooth muscle tissue (Santos-Fagundes et al., 2015).

For that reason, it was not surprising to find the obtained results on the inhibition of the guinea pig intestinal peristalsis consistent with those of Gharzouli et al., 2003, which also reported a loss of peristaltic motor activity by genistein administered at concentrations of 100 µmol/l (Gharzouli & Holzer, 2004).

But still, the pharmacological analysis on possible mechanisms of action remains complicated as there are a multiplicity of sites and pathways conducting such

antiperistaltic effects (Gharzouli & Holzer, 2004; Holzer, Lippe, Tabrizi, Lenard, & Bartho, 1997).

5.2.4 Evaluation of the potential mechanism of action of genistein

Additional testings on the terminal ileum approached a possible mechanism of action of genistein. In this context it should be kept in mind that there are many other possible pathways and signalling cascades, which lead to a relaxation of a muscle that were not included in this analysis.

Details regarding the exact procedure and how the nitric synthase was blocked are further explained in chapter 3.8.6. As a consequence to the inhibition of this enzyme, it has been expected that a subsequent addition of genistein would not show any decrease in contraction force. Unless, this is not the key mechanism of action.

The results in table 11 point out that there is a similar reduction in contraction force (-56.98±8.24%) after the addition of 30 µmol/l of genistein like without the antagonist (see table 10). Hence, it is suggested that genistein does not cause relaxation via an activation of the nitric synthase.

Concerning the current status of the literature, there is some evidence, addressing possible mechanisms, but none of them support the NO-synthase blocking mechanism as a plausible one for the antispasmodic activity on the terminal ileum.

Similar to the discussion of the right atrium, studies have suggested that the relaxation occurs due to interference with downstream signalling mechanisms by genistein, including protein kinases and a change of Ca^{2+} availability within the cells (Abdel-Latif, 2001; Duarte et al., 1993; Vuorela, Vuorela, Törnquist, & Alaranta, 1997).

Moreover, a paper already published back in the year of 1992, showed that it is very improbable that genistein inhibits the release of Ach from the ileal myenteric plexus and thus cause relaxation. However, it is due to an increase of cAMP levels in the ileum that makes a block of the phosphodiesterase more than likely to be one of the major mechanisms conducted by genistein (Herrera, Marhuenda, & Gibson, 1992).

Santos et al., 2015, observed interactions of genistein on Ca^{2+} and K^{+} channels on rabbit duodenum, which lead to the conclusion that these kinds of interactions may

attribute to the inhibitory action of genistein on the gastrointestinal peristalsis (Santos-Fagundes et al., 2015).

To conclude, it seems that genistein promotes relaxation most possibly due to intervention of downstream signalling pathways including a change in Ca^{2+} and cAMP disposability. Nonetheless, there is a necessity of further research on that topic to shed more light on the complex action machinery of genistein.

5.4 Potential usage of genistein

In summary, it can be said that genistein is having undoubtedly the distinction of being not that selective, especially when considering that genistein executed effects on all five organ specimens. This matter of fact makes it difficult to endorse this promiscuous substance for a specific therapeutic field. However, genistein may play a part in designing novel vasodilating, antiarrhythmic and cardiotonic compounds as the data reveals to be promising.

Furthermore, it also should be added that the performed experiments do not provide defined information about bioavailability, distribution, metabolism, and cumulation in specific compartments. Parameters such as toxicity are also missing.

To conclude, isoflavonoids are part of the nutrition chain as well and therefore makes it important to continue testing genistein and its polypharmacological effects so that a subtle judgment of the test substance can be made.

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