Diploma Thesis

The Influence of *Zeng Ye Tang* on the Intestinal Epithelium

submitted by

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Graz, 30.06.2022

Declaration

I declare that I wrote the present work independently, that I have not used any sources other than those given and that I have explicitly marked all material which has been quoted either literally or by content from the sources used.

Graz, 30.06.2022 *Clara Luise Meentzen eh.*

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Abbreviations

ALI	Air-liquid interface
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
MEM	Minimum Essential Medium
MUC	Mucin
NEAA	Non-Essential Amino Acids
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
P/S	Penicillin/Streptomycin
qPCR	Real-time quantitative PCR
TCM	Traditional Chinese Medicine

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Abstract

Zeng Ye Tang is an important herbal preparation used in traditional Chinese medicine for the treatment of constipation for centuries. The present study examined the effect of *Zeng Ye Tang* on the intestinal mucosa and its mucus production.

To generate a colon epithelium, the colon carcinoma cell lines CaCo2 and HT29 were cultivated in a ratio of 3:1. In order to simulate physiological conditions, we used an air-liquid interface (ALI) culture, in which the cells are cultivated in cell culture inserts with a permeable polycarbonate membrane. The apical compartment is exposed to the air and the cells are supplied with cell culture media from the basolateral side. As a result, the cells differentiate in a maximum of three weeks and develop a basoapical polarity. This leads to a structured cell layer with mucus producing goblet cells.

After the cells had been cultivated, *Zeng Ye Tang* was added to the tissue in various concentrations and for different incubation times. The microscopic examination of the tissue and the computer-based evaluation of the microscopy images with the CellProfiler software showed versatile results. However, the intensity of the mucus was in some cases significantly reduced in the tissue samples treated with *Zeng Ye Tang*. This indicates a higher mucus production. In addition, in all samples the tissue increased in thickness with a higher concentration of *Zeng Ye Tang*.

Furthermore, the mRNA expression of various mucins occurring in the colon (MUC2, MUC5AC and MUC5B) was determined in the tissue samples using qPCR. The samples treated with *Zeng Ye Tang* showed a partially significant increase in the mucin mRNA expression.

Overall, the traditional Chinese formula *Zeng Ye Tang* seems to have a beneficial impact on the intestinal epithelium and the mucus production. However, in the future further in vitro and in vivo experiments will be necessary to demonstrate effects on the intestinal epithelium as well as to identify possible side effects.

Zusammenfassung

Zeng Ye Tang ist eine weit verbreitete pflanzliche Rezeptur aus der Traditionellen Chinesischen Medizin, welche schon seit Jahrhunderten therapeutisch bei Obstipationen eingesetzt wird. In dieser Studie wurde untersucht, wie sich *Zeng Ye Tang* auf die Darmschleimhaut und deren Schleimproduktion auswirkt.

Für die Etablierung eines Kolonepithels wurden die Kolonkarzinom-Zelllinien CaCo2 und HT29 in einem Verhältnis von 3:1 kultiviert. Um möglichst physiologische Bedingungen zu konzipieren, verwendeten wir eine Air-Liquid Interface (ALI) Kultur, bei der die Zellen in Zellkultureinsätzen mit einer permeablen Polycarbonatmembran kultiviert werden. Das apikale Kompartment wird dabei der Luft ausgesetzt und die Zellen ausschließlich basolateral mit Nährstoffen versorgt. Dies hat zur Folge, dass die Zellen sich nach spätestens drei Wochen differenzieren und eine basoapikale Polarität entwickeln. So entstand eine strukturierte Zellschicht mit schleimproduzierenden Becherzellen.

Nach der Kultivierung der Zellen, wurde dem Gewebe *Zeng Ye Tang* in verschiedenen Konzentrationen und bei unterschiedlicher Inkubationsdauer hinzugefügt. Die mikroskopische Betrachtung des Gewebes sowie die computerunterstützte Auswertung der mikroskopischen Bilder mit der Software CellProfiler ergaben gemischte Ergebnisse. Die Intensität des Mukus zeigte sich jedoch in den mit *Zeng Ye Tang* behandelten Gewebeproben teilweise deutlich verringert. Dies weist auf eine höhere Schleimproduktion hin. Zudem nahm das Gewebe bei allen Proben mit einer höheren Konzentration an *Zeng Ye Tang* an Dicke zu.

Zusätzlich wurde in den Gewebeproben die mRNA Expression verschiedener im Kolon vorkommender Mucine (MUC2, MUC5AC und MUC5B) mittels qPCR bestimmt. Hier zeigte sich bei den mit *Zeng Ye Tang* behandelten Proben eine teils signifikante Erhöhung der Mucin mRNA Expression.

Insgesamt scheint die chinesische Rezeptur *Zeng Ye Tang* einen positiven Effekt auf die intestinale Schleimhaut und deren Mukus-Produktion zu haben. Es sind jedoch in der Zukunft weitere in vitro und in vivo Experimente notwendig, um die positive Wirkung auf das Darmepithel nachzuweisen und auch mögliche Nebenwirkungen zu identifizieren.

1 Introduction

1.1 The Intestinal Epithelium

The main function of the intestine is to digest and absorb food as well as to mix and transport the intestinal contents. Nutrients, water, minerals and vitamins are absorbed in the small intestine. In the large intestine the absorption of water and ions takes place. It is colonized by a large number of bacteria, that, among other things, play a role in the development and differentiation of the immune system [1].

The intestinal wall consists of an epithelium and muscle layers and contains nerves and endocrine cells. The intestinal epithelium is folded multiple times. The presence of protuberances or invaginations of the mucosa as well as microvilli on the membrane of the mucosal cells increases the surface area of the small and large intestines by approximately 3000% [1].

The cells of the colon epithelium are constantly renewed starting from the crypt base, which contains stem cells that differentiate mainly into colonocytes, goblet cells or enteroendocrine cells [2]. They are connected to one another by tight junctions, desmosomes and adherens junctions [3]. The absorptive colonocytes are most abundant in the colon epithelium and the secretory goblet cells as well as enteroendocrine cells are located between them [4].

Goblet cells make up 4 to 16% of the epithelial cells, with an increasing proportion from the duodenum to the distal colon. They are specialized in producing and secreting mucus. One of its major components are the mucins, which give the mucus its gel-like properties. At present, more than 20 mucin types are known. They are broadly classified in secretory and membrane-associated mucins. The mucins produced by the goblet cells are the mucins MUC2, MUC5AC, MUC5B and MUC6, which all form large polymers. These mucins are secreted by the intestine, but also by the stomach and salivary glands. The transmembrane mucins (such as MUC3, MUC12 and MUC13) contribute to the structure of the glycocalyx, a filamentous layer of branched carbohydrates that form an important physical layer [3, 5, 6, 7].

Mucins are glycosylated proteins. The mucin composition can be different depending on the location in the intestine. The mucus is secreted constitutively but can be induced by various stimuli [2].

The MUC2 mucin is the major structural molecule of the intestinal mucus and therefore primarily secreted. It is a very large and complex glycoprotein and contains a protein core with two tandem repeat domains, which are rich in proline, serine and threonine molecules (therefore also called PTS domains), connected with O-linked glycans. Long, stiff bottle brush-like rods are created in which the glycans make up more than 80%. They give mucins their high water-binding capacity [3, 5, 8].

The secreted mucus forms a mucus layer, which is discontinuous in the small intestine, but continuous in the colon [3]. There are two different layers: an outer loose mucus layer and an inner dense layer, which is attached to the mucosa. The outer layer protects the epithelium from physical and chemical damage and contains bacteria, while the inner layer functions as a barrier between the intestinal epithelial cells and the microbiome. The inner layer near the epithelium is generally free of bacteria and contains Immunoglobulin A as well as antimicrobial peptides and enzymes [2]. A schematic structure of the colon epithelium is shown in figure 1.

A study showed that the epithelial cells of constipated rats contained less mucus than the epithelial cells of the control group. In addition, the mucus layer at the fecal surface was thinner. By lubricating, mucus normally facilitates the smooth movement and evacuation of non-digestible food solids in the intestine [9, 10].

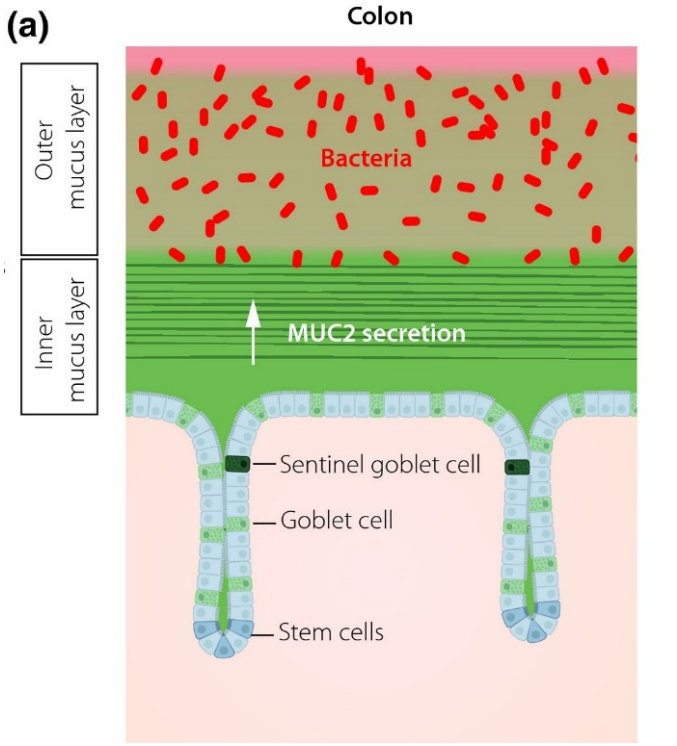

Figure 1: Structure of the Colon Epithelium [11]

a) The crypt base contains stem cells that differentiate into goblet cells, enterocytes, or others. The goblet cells secret the mucus, which forms two layers: an outer loose mucus layer, which contains bacteria and an inner dense mucus layer, which is generally free of bacteria and functions as a barrier between the epithelial cells and the microbiome.

1.2 The Air-Liquid Interface Culture

Cell culture is a well-established and fundamental technique in biology and medicine. A challenge with the conventional cell culture is the large discrepancy between the cell kinetics in vivo and in vitro. For the cells to behave as they would in their natural physiological environment not only the reproduction of the cells, but also the tissue architecture, cell-cell interactions and a specific physical microenvironment are important. Therefore, three-dimensional culture systems were developed, such as the air-liquid interface (ALI) culture [12].

This culture system is primarily used to mimic the intestinal tract, the respiratory tract, or the skin. Physical stress has a large impact on the proliferation and differentiation of various cell types. The ALI culture system mimics the intestinal tract or skin's microenvironment, as it is constantly exposed to air. It also maintains the homeostasis of the epidermis and dermis in the same manner observed in vivo [12].

In conventional methods, the cells are supplied with medium from the apical and basolateral side. In ALI cultures, medium is only present in the basal compartment, while the apical surface faces the air [13]. The culture system consists of an inner cell insert, an outer plastic dish and collagen gel scaffold [12].

The cells are seeded into the culture inserts with a porous membrane. To generate the air-liquid interface, the medium is removed from the apical compartment after proliferation and the cells are consequently exposed to the surrounding air. Nutrients can only be absorbed by the basolateral cell pole. That leads to differentiation after one to three weeks and the cells regain their full basoapical polarity. Some types of cells, such as goblet cells, produce in vivo-like mucus or fluid [14].

A schematic illustration of an ALI culture is shown in figure 2. Figure 3 shows the growth and differentiation of an epithelium grown at an ALI culture system over time.

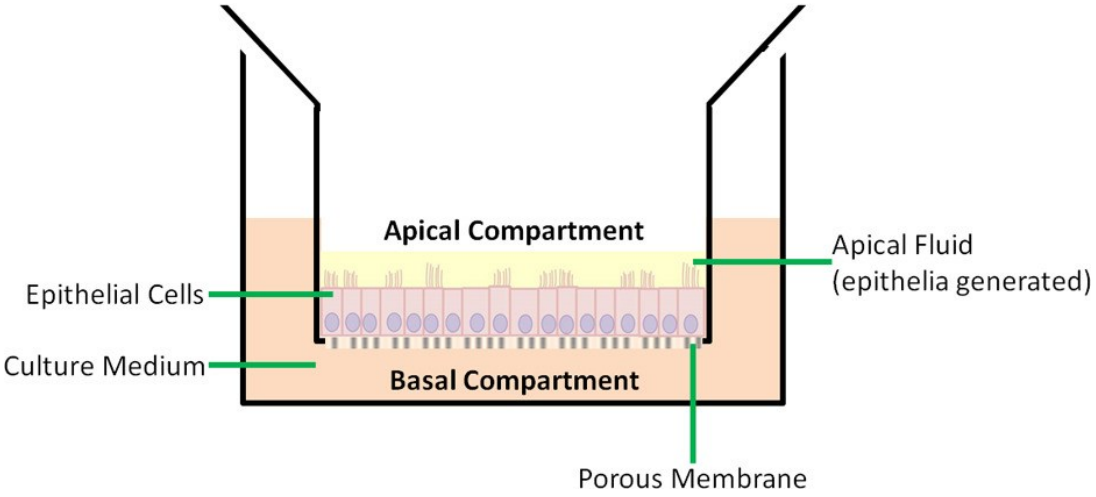

Figure 2: Schematic illustration of an air-liquid interface (ALI) culture with epithelial cells [14]
The ALI culture system consists of a basal compartment filled with culture medium and an apical compartment filled with mucus fluid secreted by epithelial cells like goblet cells. The compartments are separated by the epithelial cells and a porous membrane.

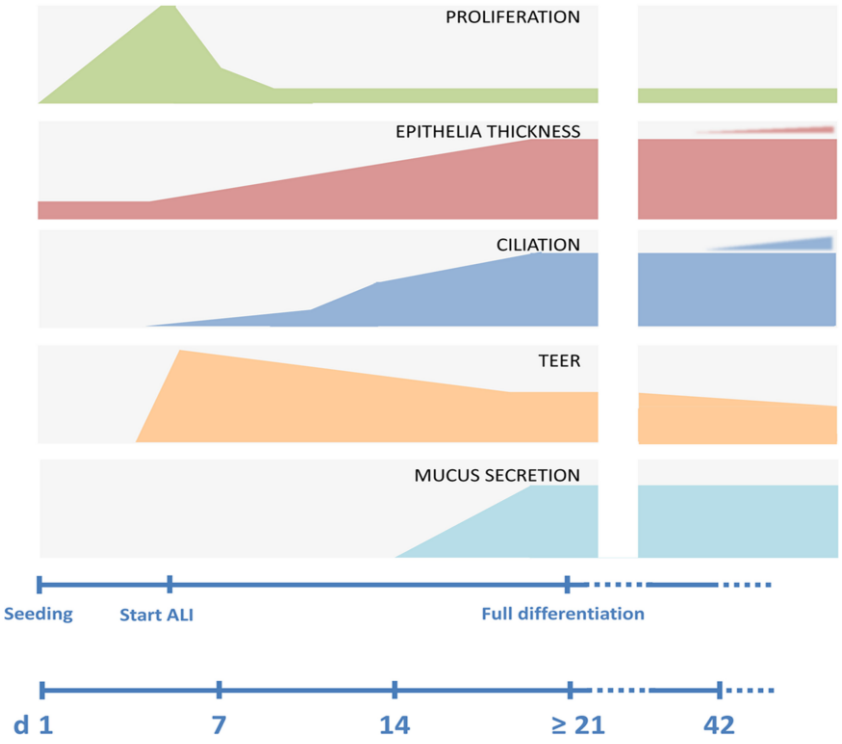

Figure 3: Epithelial growth and differentiation of an ALI culture over time [14]
The full differentiation of the epithelial cells in an ALI culture lasts about three weeks. Most of the proliferation occurs within the first week, while the mucus production begins after approximately two weeks.
TEER: transepithelial electrical resistance

1.2.1 Air-Liquid Interface Culture for the Colon Epithelium

The intestinal epithelium functions as a barrier between the lumen of the intestine and the internal microenvironment of the organism, and digests and transports nutrients from the intestinal lumen into the bloodstream. Maintaining intracellular connections and cellular polarity is important for these functions. In conventional cultures, the oxygen supply is difficult, as the cells are completely covered with medium. The intestinal epithelial cells are exposed to an alternating supply of oxygen via the bloodstream, which can regulate the absorption function of the epithelium [15].

According to a study by Constanze Nossol et al (2011), the ALI culture results in a greater number of cells and a greater thickness of the epithelial layer compared to a conventional culture. A comparison of the morphological parameters of the ALI culture with those of intestinal cells in vivo reveals great similarities. To cultivate an intestinal epithelium that is as close as possible to the in vivo epithelium, an adequate supply of oxygen is therefore necessary. This is achieved by ALI cultures, in which cultivated intestinal cells should form polarized and differentiated epithelial structures [15].

The invention of membrane culture systems, like the transwell system, which contains membranes with pores that mimic the basement membrane, also provides more physiological conditions [15].

For the ALI culture we used CaCo2 cells and HT29 cells in a ratio of 3:1, as this showed the best results in a previous study at the Department of Cell Biology, Histology and Embryology in 2018 [16]. Both cell lines were isolated from colon adenocarcinomas. In culture, the CaCo2 cell line differentiates into cells that resemble enterocytes in the intestinal epithelium. It forms polarized monolayers. HT29 cells are heterogeneous. The cell line contains a proportion of mucus-secreting cells and columnar absorptive cells [17].

1.3 Traditional Chinese Medicine

The traditional Chinese medicine (TCM) is one of the earliest models of medical treatment in the world, which is still practiced until today. Its knowledge and practice have been accumulated over 2000 years. The main therapeutic aspects of TCM include acupuncture, herbal preparations and other physical therapy (such as massage). Not only in China, but also in Korea, Japan and Taiwan, it is an important part of the medical system. TCM is also becoming increasingly popular in Europe and North America. Here it is considered part of the complementary or "alternative" form of medicine [18, 19, 20].

TCM takes a holistic approach. If the balance of the human body is disturbed by endogenous or exogenous factors, disease syndromes occur. The treatment is intended to restore the balance. Each patient is treated individually. In the diagnosis, a personal anamnesis including family history, characteristics of the pulse and physical signs, such as smell, facial complexion and the appearance of the tongue, play a role [20].

In TCM, the human body consists of five organs, six hollow organs, five senses, five body parts, four limbs, nine external openings and a meridian system that connects everything [20]. *Qi*, the life energy (or life force) flows through the meridians. To explain the human body, TCM also uses the theory of the five elements, which correspond to certain organs: Fire, earth, metal, water and wood. A central element of TCM is the concept of *yin* and *yang*, two opposing but complementary forces [21].

An important part of TCM is the use of medicinal herbs. Many herbs or preparations are mainly unexplored. However, in the last decades they have been increasingly researched and significant pharmacological properties have been demonstrated for many of them [18, 19]. Most herbal preparations are mixtures of a few herbs. They consist of one main herb that normalizes the major imbalance. The rest of the herbs in the recipe reinforce the effect of the main herb. An herbal preparation typically consists of four or more herbs, but the total number can vary from one to about a hundred. Herbal medicines that are manufactured and administered must meet certain standards in China. These can be found in the State Food and Drug Administration (SFDA), where almost 1500 herbal medicines are registered. In addition, the Chinese Pharmacopoeia 2015 lists more than 600 types of herbs and describes, for example, their functions and recommended dosages [20].

1.3.1 *Zeng Ye Tang*

Zeng Ye Tang is an important herbal formula of TCM that is often used to relieve constipation due to body fluid deficiencies. According to the traditional theory it generates fluids, moistens dryness and nourishes yin. It was first noted by the Chinese physician Tang Wu (AD 1758–1836) in the book *Wen-Bing-Tiao-Bian*, which dates back to the Qing Dynasty of ancient China. *Zeng Ye Tang* is also used in TCM to treat diabetes [22, 23, 24, 25, 26].

It is an extract of three *yin*-nourishing herbs with the ratio of 5:4:4: *Scrophulariae radix* (*Xuan Shen*), *Ophiopogonis radix* (*Mai Dong*) and *Rehmanniae radix* (*Di Huang*) [23, 24]. According to the traditional theory *yin* represents feminine forces, for example water, softness, contraction or intuition [27]. The components of *Zeng Ye Tang* and their quantities are listed in table 1.

Table 1: Ingredients of *Zeng Ye Tang*

Chinese name	Latin name	Species	English name of species	Quantity
Xuan Shen	*Scrophulariae radix*	*Scrophularia ningpoensis*	Ningpo figwort	30g
Mai Dong	*Ophiopogonis radix*	*Ophiopogon japonicus*	Dwarf Lilyturf Mondo Grass	24g
Di Huang	*Rehmanniae radix*	*Rehmannia glutinosa*	Chinese foxglove	24g

The main herb is the *yin*-nourishing *Xuan Shen* (*Scrophulariae radix*), one of the most popular traditional Chinese medicines, which tones the viscera, promotes glandular secretion and removes toxins. It is the root of *Scrophularia ningpoensis* (Ningpo figwort), a perennial herb native to Southeastern China, which belongs to the *Scrophulariaceae*. Different pharmacological studies have demonstrated that *Scrophulariae radix* can be used to treat constipation, rheumatism, arthritis and conjunctival congestion. It contains catalpol, harpagide, harpagoside, phenylpropanoid glycosides, iridoid glycosides, acteoside, angoroside C and cinnamic acid [24, 28, 29, 30].

Figure 4: *Scrophularia ningpoensis* [31]

The root of *Scrophularia ningpoensis* (*Xuan Shen*) is a popular Chinese medicine.

Mai Dong is the root of *Ophiopogon japonicus* (Dwarf Lilyturf), an evergreen perennial, which belongs to the Liliaceae and is widely located in Southeast Asia. In TCM, it is classified as a yin tonic. *Ophiopogon radix* promotes the production of body fluids and moisturizes the lung and intestine. It is used to treat hyperglycaemia, chronic inflammation, cardiovascular diseases and heartburn. It contains homoisoflavonoids, saponins, amino acids, polysaccharides and nucleosides [24, 32, 33, 34].

Figure 5: *Ophiopogon japonicus* [35]

The dried root of *Ophiopogon japonicus* (*Mai Dong*) is used to promote the body fluid production.

Di Huang (*Rehmanniae radix*) is the root of *Rehmannia glutinosa* (Chinese foxglove), which also belongs to the *Scrophulariaceae*. *Rehmannia glutinosa* is a perennial herbaceous plant, which is widely cultivated in China. According to the traditional theory, it supplements, boosts and cools the blood and fills the marrow. It also promotes the production of body fluids. *Rehmanniae radix* is used to treat diabetes, senile osteoporosis, haematological and gynaecological diseases. It contains phenethyl alcohol glycosides, monoterpenoids and triterpenes [36, 37].

Figure 6: *Rehmannia glutinosa* [38]
According to TCM the dried root of *Rehmannia glutinosa* (*Di Huang*) also promotes the body fluid production.

The Chinese formula *Zeng Ye Tang* is made by decoction, which is the most common form of application of herbs in TCM. It is usually taken orally. A decoction is created by placing dry herbs into cold water, letting it soak for at least one hour and boiling it for 20 minutes. The decoction is then poured through a sieve in another vessel, fresh water is added to the herbs in the pot and boiled again for 20 minutes. Finally, the two decoctions are mixed together [39].

1.4 Aim of the Study

Chronic constipation is a common gastrointestinal disorder worldwide. It affects many people and can be very stressful for patients. As conventional symptomatic treatments are often unsatisfactory, more and more patients seek help from the traditional Chinese medicines [26].

A popular Chinese herbal formula to treat constipations is *Zeng Ye Tang*. The aim of this study is to examine the influence of the *Zeng Ye Tang* on the intestinal epithelium in a cell culture model of the intestine. A study conducted at the Department of Cell Biology, Histology and Embryology in 2018 examined the development of ALI cultures for the colon epithelium as well as the influence a Chinese formula on the intestinal epithelium. Using two different cell lines for the ALI culture (CaCo2 and HT29) in a ratio of 3:1, a structured cell layer with mucus production was developed [16]. Since this showed the best results, we use the same method for this study. The Chinese formula *Zeng Ye Tang* is added to the cells in different concentrations. To evaluate changes in the epithelium, it is examined with histological techniques and computer-based automated analysis.

2 Materials and Methods

2.1 List of Materials

Materials for Cell Culture

CaCo2 cells (*Department of Medicine III, Division of Gastroenterology and Hepatology, Graz, Austria*)

HT29 cells (*Amin El-Heliebi, Department of Cell Biology, Histology and Embryology, Graz, Austria*)

Minimum Essential Medium (*Gibco, ThermoFisher Scientific, Massachusetts, USA*)

HyClone™ Fetal Bovine Serum (*ThermoFisher Scientific, Massachusetts, USA*)

Penicillin/Streptomycin (*Gibco, ThermoFisher Scientific, Massachusetts, USA*)

Minimum Essential Medium Non-Essential Amino Acids 100X (*Gibco, ThermoFisher Scientific, Massachusetts, USA*)

Sodium Pyruvate 100 mM (*Gibco, ThermoFisher Scientific, Massachusetts, USA*)

Trypsin-EDTA 10X (*Biowest, Nuaillé, France*)

Trypsin-EDTA 1X, diluted in Phosphate-buffered saline (*Biowest, Nuaillé, France*)

Phosphate-buffered saline, pH 7.4 (*Gibco, ThermoFisher Scientific, Massachusetts, USA*)

Bovine Fibronectine, diluted in Phosphate-buffered saline, final concentration 2 µg/ml (*Sigma, Missouri, USA*)

CASY Model TT (*Schärfe System, Reutlingen, Germany*)

Zeng Ye Tang, hydrophilic concentrate, 50 g (*Apotheke Boznerplatz, Innsbruck, Austria*)

Transwell plates (*Corning, New York, USA*)

Materials for Histological Techniques

Ethanol 100% (*MERCK, Vienna, Austria*)

Acetic acid 100% (*MERCK, Vienna, Austria*)

Chloroform (*MERCK, Vienna, Austria*)

Paraffin (*MERCK, Vienna, Austria*)

Excelsior Tissue Processor (*ThermoFisher Scientific, Massachusetts, USA*)

Rotation Microtome HM355S (*ThermoFisher Scientific, Massachusetts, USA*)

Superfrost Plus Microscope Slides (*ThermoFisher Scientific, Massachusetts, USA*)

Cover glasses, 24 x 24 mm (*ThermoFisher Scientific, Massachusetts, USA*)

Tissue Clear (*Histolab Products AB, Askim, Sweden*)

Aqua destillata (self-produced in the laboratory)

Hematoxylin, Mayer's hemalum solution (*MERCK, Vienna, Austria*)

Ammoniac 25% (*MERCK, Vienna, Austria*)

Eosin 1% (*Sigma-Aldrich, Vienna, Austria*)

Alcian blue (*Sigma-Aldrich, Vienna, Austria*)

Nuclear fast red (*MERCK, Vienna, Austria*)

Other Materials

peqGOLD Total RNA Kit (*Peqlab, Erlangen, Germany*)

NanoDrop™ 1000 Spectrophotometer (*ThermoFisher Scientific, Massachusetts, USA*)

GOLD cDNA Kit (*Peqlab, Erlangen, Germany*)

cDNA Cycler, DNA Engine Dyad, Peltier Thermal Cycler (*Bio-Rad, Hercules, USA*)

Primer: MUC2, MUC5AC, MUC5B (*Microsynth, Vienna, Austria*)

iTaq Universal SYBR Green Supermix (*Bio-Rad, Hercules, USA*)

qPCR cycler, CFX96 Real-Time System, C1000 Thermal Cycler (*Bio-Rad, Hercules, USA*)

EZ4U Nonradioacitve cell proliferation and cytotoxicity assay, BI-5000 (*Biomedica, Vienna, Austria*)

2.2 Colon Cells and Cell Culture

2.2.1 Cultivation of CaCo2 and HT29 Cells

The colon carcinoma cell lines CaCo2 and HT29 were cultivated in a growth medium and incubated at a temperature of 37°C and 5% CO_2. The medium consisted of Minimum Essential Medium (MEM) with 10% Fetal Bovine Serum (FBS), 1% Non-Essential Amino Acids (NEAA), 1% Penicillin/Streptomycin (P/S) and 1% sodium pyruvate. It was changed two times a week.

When the cells reached 90% confluence, they were separated using 10X Trypsin for the CaCo2 cells and 1X Trypsin for the HT29 cells. The splitting ratio for both cell lines was 1:3. As a first step, the medium was removed from the cell culture flask. 10 ml Phosphate-buffered saline (PBS) with 1% P/S was used to wash the cells. Afterwards 2 ml Trypsin was added. The cells were then incubated at 37°C for five minutes. As a next step the cells were resuspended into 8 ml medium. After a centrifugation at 1200 U/min for five minutes, the medium was removed again. The cells were then transferred in a growth medium and seeded in 75 cm^2 culture bottles, which were filled up with medium up to 12 ml.

2.2.2 Air-Liquid Interface Culture

In order to measure the cell concentration in the CaCo2 and HT29 culture flask, 50 µl of each cell line was removed and placed in a CASY cell counter. Using the results, the required volumes of both culture bottles were calculated. The cells were mixed in a ratio of 3:1 with three parts of CaCo2 cells and one part of HT29 cells. Growth medium was then added to the colon cells, using 500 µl medium for each well.

For the ALI culture 12-well transwell plates were used, which were provided with permeable polycarbonate membrane inserts. These inserts had a diameter of 12 mm and a pore size of 0.4 µm. The wells were coated with bovine fibronectin and air dried under a laboratory fume hood for one hour.

The cell suspension was transferred into the upper compartment of 15 inserts. In each well 3 x 10^5 cells were seeded and the basal compartment was filled with 1 ml medium. The cells were then placed in an incubator for three days.

After three days of incubation the medium in the upper compartments was removed. The inserts were washed with PBS. The apical chamber was left without medium and exposed to the air. In the basal compartments the medium was aspirated and refilled with fresh medium. The plates were then incubated again. The medium change of the basal compartment was constantly repeated every second day for ten days.

2.3 Treatment with *Zeng Ye Tang*

To investigate the impact of the Chinese herbal preparation *Zeng Ye Tang* on the intestinal epithelium, the formula was added to the cells in different concentrations. The cells were incubated for three or seven days. Six of the 15 wells were treated with 0.3% *Zeng Ye Tang*, of which one half was incubated for three days and the other half for seven days. Six other wells were treated with 0.6% *Zeng Ye Tang*. Three of which were incubated for three days and the others for seven days. The remaining three wells were not treated with the Chinese formula and used as a control. An overview of the concentration of *Zeng Ye Tang* and the time of incubation of the 15 wells is given in table 2.

Table 2: Overview of the concentration of *Zeng Ye Tang* and the time of incubation

	Time of incubation				
	3 days	7 days	3 days	7 days	3 days
#1	CaCo2/HT29 (3:1) **0.3% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0.3% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0.6% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0.6% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0% *Zeng Ye Tang***
#2	CaCo2/HT29 (3:1) **0.3% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0.3% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0.6% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0.6% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0% *Zeng Ye Tang***
#3	CaCo2/HT29 (3:1) **0.3% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0.3% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0.6% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0.6% *Zeng Ye Tang***	CaCo2/HT29 (3:1) **0% *Zeng Ye Tang***

2.4 Histological Techniques

2.4.1 Fixation and Paraffin Embedding

After three or seven days of incubation with the Chinese formula *Zeng Ye Tang* the membranes were removed from the wells with a scalpel and cut into halves. One part of each membrane was put in 400 µl of RNA Lysis Buffer T and stored at -80°C to analyse the RNA of the colon epithelium at a later time.

The other part of the membranes was given in a Carnoy fixing solution, which consisted of six parts of 100% ethanol, three parts of chloroform and one part of glacial acetic acid (100%). After 20 minutes at room temperature the tissue was placed in cassettes and washed with PBS. To embed the membranes in paraffin the tissue processor Excelsior^{TM} was used. The cassettes were placed in the tissue processor during the night, where the membranes were paraffin embedded. The paraffined membranes were then placed vertically in a square form, poured with warm paraffin and hardened on a cooling plate.

Using a rotation microtome, the resulting paraffin blocks were subsequently cut in tissue slices with a thickness of 5 µm. The slices were put on microscope slides and dried at 53°C for one night.

Before staining with hematoxylin/eosin and alcian blue the tissue slices were deparaffinated. As a first step they were consecutively put in four different containers with Tissue Clear solutions (Histolab Clear) for five minutes in each. The tissue slices were then placed in a 1:1 mixture of Tissue Clear and 100% ethanol for three minutes. Afterwards the slides were immersed shortly in four containers with a descending ethanol content (100%, 96%, 70%, 50%) and in a last container with aqua destillata, that was changed several times. The different steps of the procedure of deparaffinization and dehydration are listed in table 3.

Table 3: Deparaffinization and Dehydration

Tissue Clear 1a & 1b	5 minutes each
Tissue Clear 2a & 2b	5 minutes each
Tissue Clear / 100% Ethanol mixture 1:1	3 minutes
100% Ethanol	1 minute
96% Ethanol	1 minute
70% Ethanol	1 minute
50% Ethanol	1 minute
Aqua destillata	Rinse and change several times

2.4.2 Hematoxylin and Eosin Staining

A proportion of the deparaffinated and dehydrated specimens were stained with hematoxylin and eosin in order to see the overall effect of *Zeng Ye Tang* on the intestinal epithelium. The different steps of the procedure are listed in table 4. After drying, the slides were permanently covered with a cover glass.

Table 4: Hematoxylin and Eosin staining

Hematoxylin, Mayer's hemalum solution	10 minutes
Aqua destillata	rinse and change 2-3 times
NH_3-Water (2.5 ml Ammoniac / 1 l Aqua destillata)	a few seconds
Aqua destillata	rinse and change 2 times
1% Eosin	5-60 seconds
96% Ethanol (2 charges)	rinse
100% Ethanol	rinse
100% Ethanol / Tissue Clear (1:1)	rinse
Tissue Clear	10 minutes

2.4.3 Alcian blue and Nuclear fast red Staining

The other proportion of the deparaffinated and dehydrated specimens were stained with alcian blue and nuclear fast red to see the effect of *Zeng Ye Tang* on the mucus in the intestinal epithelium. The different steps of the staining procedure are listed in table 5. After drying, the specimens were permanently covered with a cover glass.

Table 5: Alcian blue and Nuclear fast red staining

3% Acetic acid	3 minutes
1% Alcian blue in 3% acetic acid	30 minutes
3% Acetic acid	rinse
Aqua destillata	rinse
0.1% Nuclear fast red in 5% aqueous aluminium sulphate	5 minutes
Aqua destillata	rinse and change 2-3 times
50% Ethanol	rinse
70% Ethanol	rinse
100% Ethanol	rinse
96% Ethanol	rinse
100% Ethanol /Tissue Clear (1:1)	rinse
Tissue Clear	10 minutes

2.5 Cell Viability Assay

The EZ4U Nonradioactive cell proliferation and cytotoxicity assay is a test for the measurement of cell proliferation and cell toxicity. It is based on the reduction of tetrazolium salt to coloured formazan, which requires living cells with functional mitochondria [40]. The EZ4U test was used to determine the cytotoxic effect of the Chinese medicine *Zeng Ye Tang* on the colon epithelial cells.

The CaCo2 and HT29 cells were seeded into 15 wells of a 96-well plate in a ratio of 3:1. Each well contained $5x10^4$ cells cultivated in 200 µl medium. After one week, the medium was removed and 200 µl of a growth medium spiked with the Chinese formula was added to each well. The growth medium contained different concentrations of *Zeng Ye Tang*: 0%, 0.3%, 0.6%, 1.25% and 5%.

After 24 hours the medium was aspirated and replaced by 200 µl of fresh medium. As a control, three wells were prepared, which contained only medium without cells. The substrate was dissolved in 2.5 ml activator. 20 µl of the substrate solution were added into each well and swirled gently. The cells were then placed in an incubator at 37°C for two hours. The plate was shaken on a vibrating plate. Finally, the absorbance was read at 492 nm with 620 nm as reference.

2.6 RNA Isolation

For the isolation of RNA of the part of each membrane, which was put in 400 µl of RNA Lysis Buffer T and stored at -80°C, the peqGOLD Total RNA Kit was used. This kit is based on the reversible binding properties of PerfectBind RNA Columns combined with the speed of minicolumn spin technology [41]. The RNA isolation was carried out according to the protocol of the peqGOLD Total RNA Kit. The most important steps are described in table 6.

Table 6: Overview of the RNA isolation process [41]

1. Lysis: - adding of 400 µl RNA Lysis Buffer T
   - centrifugation
2. Load and Bind: - adding of an equal volume 70 % ethanol
   - pipetting of the lysate directly to a PerfectBind RNA Column
   - centrifugation
3. Wash I: - adding of 500 µl RNA Wash Buffer I to the PerfectBind RNA Column
   - centrifugation
4. DNase Digestion (optional)
5. Wash II: - adding of 600 µl completed RNA Wash Buffer II to the PerfectBind RNA Column
   - centrifugation
6. Dry: - centrifugation for 2 min at 10,000 x g*
7. Elution: - adding of 50 to 100 µl sterile RNAse-free dH_2O directly to the binding matrix
   - centrifugation

After the RNA isolation the absorption of the samples was determined at 260 nm and 280 nm, using Nanodrop to measure the concentration of RNA. The RNA samples were then stored at –80 °C in sterile RNase-free dH_2O.

2.7 cDNA Synthesis

After the RNA isolation and the nanodrop measurement, the RNA was transcribed through reverse transcription into cDNA since DNA is more stable and easier to work with. For the synthesis of cDNA, the peqGOLD cDNA kit was used. According to the calculations for 1 µg RNA, the RNA was diluted with sigma water to a total volume of 10 µl. 1 µl of a random primer and 1 µl of an oligo primer were added to each sample. After a short centrifugation the samples were placed in a thermocycler for five minutes at 65°C. A mixture of 4 µl buffer solution, 1 µl RNAse Inhibitor, 2 µl dNTP mixture and 1 µl reverse transcriptase was then pipetted in each sample and briefly centrifugated. The samples were again placed in the thermocycler for 70 minutes at 65°C. After the cDNA synthesis the samples (which contained 20 µl in total) were diluted with 980 µl H2O to receive a final concentration of 1 µg/ml. They were then centrifugated and stored at -20°C.

2.8 Real-Time Quantitative PCR

The real-time quantitative PCR (qPCR) is a method to amplify nucleic acids. It is similar to the conventional polymerase chain reaction (PCR), but additionally allows the quantification of the DNA obtained in a sample. This technique was used to determine the mucin production of the intestinal cells. Since these are the most common mucins, primers for MUC2, MUC5AC and MUC5B were applied.

To perform the qPCR the iTaq Universal SYBR Green Supermix and a CFX96 Real-Time System was used. To receive a total reaction, mix volume of 10 µl, 5 µl SYBR Green supermix, 2 µl cDNA template, 2 µl Primer (forward and reverse primer) and 1 µl sigma water were mixed. The first step was mixing the SYBR Green Supermix with the primers and sigma water and pipetting the mixture into the wells of a qPCR plate. Then the cDNA samples were added to the wells containing the master mix. After sealing the plate, it was centrifuged and placed into the qPCR cycler. The thermal cycling protocol was programmed and started. It was running at 95°C for 15 minutes, at 95°C for 15 seconds, then at 60°C (for MUC5B 58°C) for one minute and finally at 72°C for one minute. This was repeated 40 times. The process is shown in figure 7. The results were then analysed with a Bio-Rad CFX manager software.

It was necessary to run through two protocols with different temperatures as the used primers for MUC2, MUC5AC and MUC5B had different melting points. This is shown in figure 7. Beta-actin was chosen as positive control, using two primers with different melting points (58°C and 60°C). As negative control, a master mix without DNA template was used.

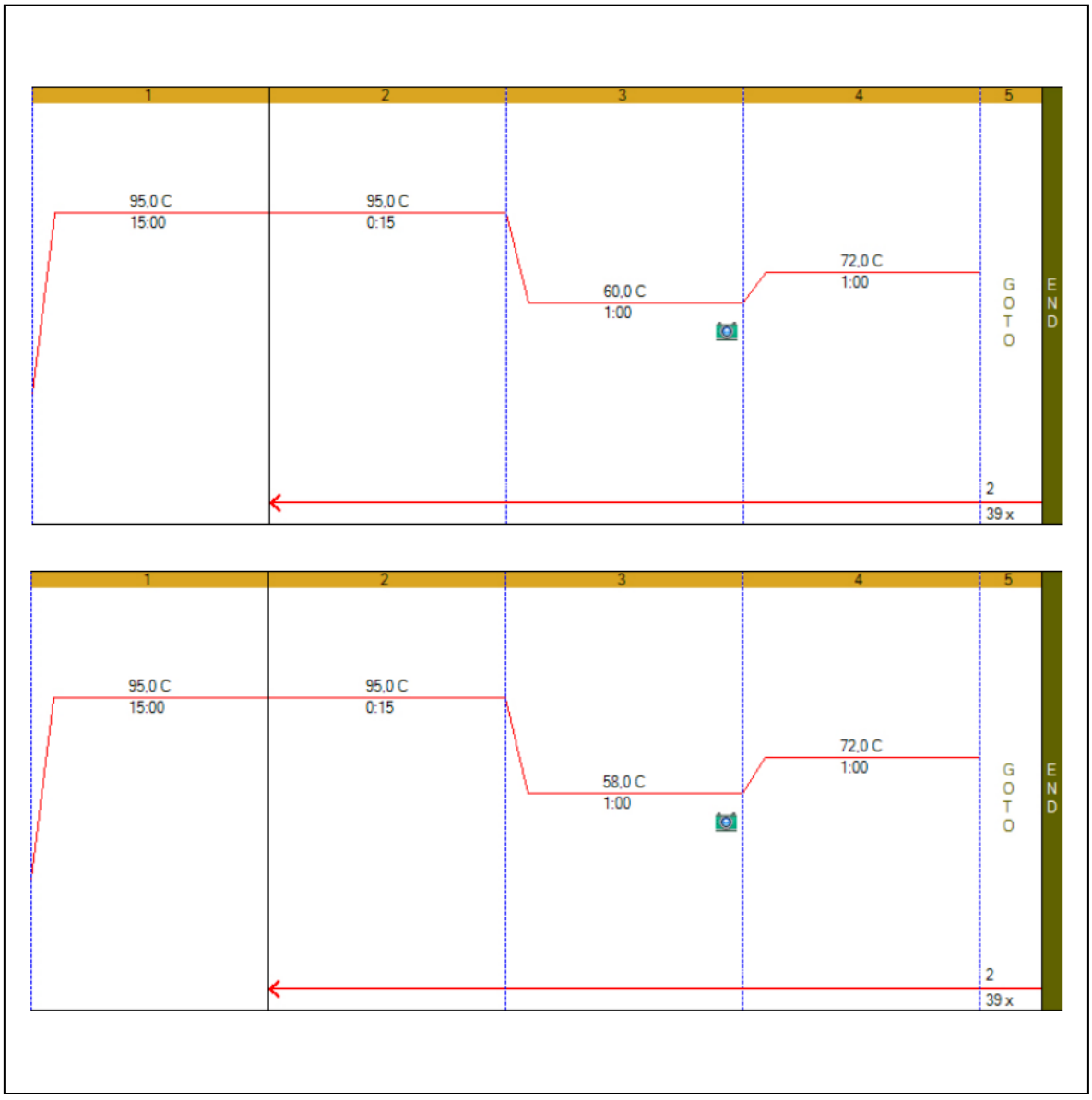

Figure 7: qPCR protocols for the used primers with different melting points [16]

Each protocol was running 40 cycles. Upper illustration qPCR protocol for MUC2 and MUC5AC. Lower illustration qPCR protocol for MUC5B.

3 Results

3.1 The Influence of *Zeng Ye Tang* on CaCo2/HT29 ALI Cultures

To examine the impact of the Chinese formula *Zeng Ye Tang* on the intestinal epithelium, CaCo2 cells and HT29 cells were seeded in a ratio of 3:1 in wells for an ALI culture. After three weeks, *Zeng Ye Tang* was added to the cells in different concentrations (0.3% and 0.6%) with some samples left untreated as a control. Half of the cells were incubated for three days and the other half for seven days. Afterwards half of each sample was used for a qPCR analysis (see Chapter 3.3). The other part of the membranes was fixed, embedded in paraffin, cut with a rotation microtome in tissue slices and applied to microscope slides. The specimens then were deparaffinated, stained with hematoxylin/eosin or alcian blue and finally visualised digitally with an Olympus microscope.

Figures 8 and 9 show the intestinal epithelium after an incubation of three days with different concentrations of the Chinese formula. The tissue in figure 8 is stained with hematoxylin and eosin. The hematoxylin stains the cell nuclei blue, whereas the eosin stains the cytoplasm and the extracellular matrix pink. The tissue appears to increase in thickness with a higher concentration of *Zeng Ye Tang*. The blue stained cell nuclei also seem to be bigger in the sample with 0.6% Chinese medicine than in the one without treatment.

The tissue in figure 9 is stained with alcian blue and nuclear fast red. Alcian blue stains acidic polysaccharides (such as glycosaminoglycans) blue, while nuclear fast red stains the cell nuclei red. Therefore, in this picture the mucus is coloured blue. It is noticeable that the mucus production seems to be higher with an increasing concentration of *Zeng Ye Tang*. The blue coloured cells represent the goblet cells, which produce the mucus. The goblet cells in the tissue treated with 0.6% Chinese medicine are much bigger than in the untreated control tissue.

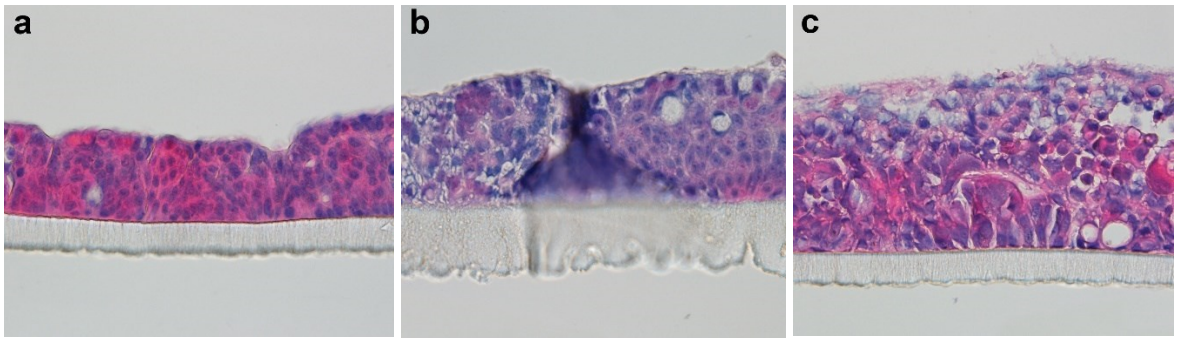

Figure 8: Incubation of the epithelium with *Zeng Ye Tang* for three days (Hematoxylin/eosin staining)
Hematoxylin and eosin stained CaCo2/HT29 cells (ratio 3:1) ALI culture after an incubation of three days with a) 0%, b) 0.3% and c) 0.6% Chinese medicine.

Figure 9: Incubation of the epithelium with *Zeng Ye Tang* for three days (Alcian blue staining)
Alcian blue and nuclear fast red stained CaCo2/HT29 cells (ratio 3:1) ALI culture after an incubation of three days with a) 0%, b) 0.3% and c) 0.6% Chinese medicine. The arrows indicate the goblet cells.

Figures 10 and 11 show the intestinal epithelium treated with different concentrations of the Chinese formula after an incubation of seven days. For comparison, the untreated tissue, which was incubated for three days, is also shown. The tissue in figure 10 is stained with hematoxylin and eosin. As also seen in figure 8 the tissue is increasing in thickness with a higher concentration of *Zeng Ye Tang*. The tissues treated with the Chinese medicine is detached from the membrane. This is due to the cutting process.

The tissue in figure 11 is stained with alcian blue and nuclear fast red. There appears to be an increase in thickness between the tissues treated with 0.3% and 0.6% *Zeng Ye Tang* and the sample without treatment. There also might be slightly more mucus, but the difference is not significant. The goblet cells in the sample treated with 0.6% *Zeng Ye Tang* have about the same size as the one without treatment.

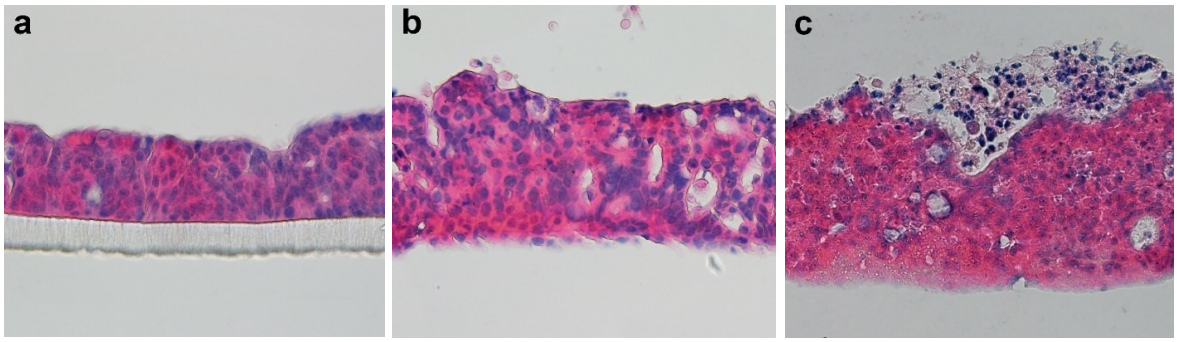

Figure 10: Incubation of the epithelium with *Zeng Ye Tang* for seven days (Hematoxylin/eosin staining)
Hematoxylin and eosin stained CaCo2/HT29 cells (ratio 3:1) ALI culture after an incubation of a) three days with 0% and seven days with b) 0.3% and c) 0.6% Chinese medicine.

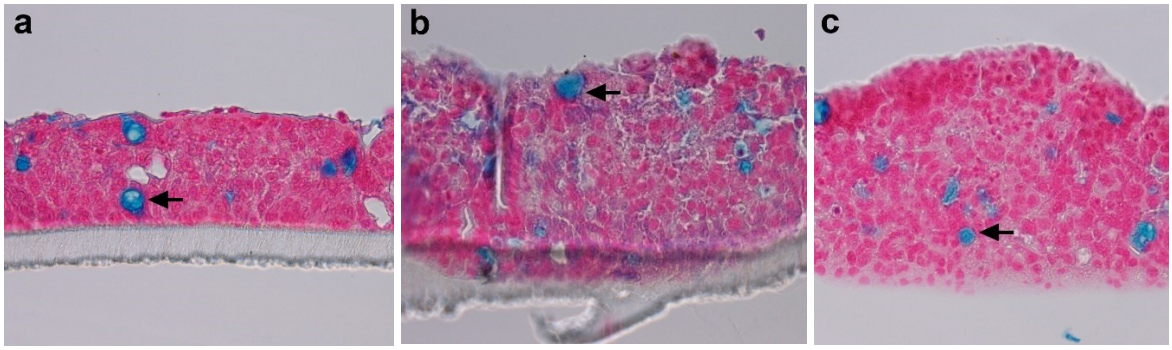

Figure 11: Incubation of the epithelium with *Zeng Ye Tang* for seven days (Alcian blue staining)
Alcian blue and nuclear fast red stained CaCo2/HT29 cells (ratio 3:1) ALI culture after an incubation of a) three days with 0% and seven days with b) 0.3% and c) 0.6% Chinese medicine. The arrows indicate the goblet cells.

The goblet cells are responsible for the production of mucins, the structuring components of the mucus. The mucus is an important part of the intestinal barrier function, which prevents a strong immune reaction with the intestinal flora and lubricates intestinal contents during transport and excretion. An increase in mucus could improve constipation, which *Zeng Ye Tang* is normally used for.

To get a more accurate result concerning the mucus, we further analysed the alcian blue stained images with the cell image analysis software CellProfiler. The software is used for measuring the amount, size and intensity of a cell or other biological object in a microscopy image. We used 13 appropriate images from 9 different samples with a magnification of 10x (4x control, 3x 0.3% TCM for three days, 3x 0.6% TCM for three days, 2x 0.3% TCM for seven days and 1x 0.6% TCM for seven days). With the software, all blue-coloured areas representing the mucus were marked as seen in figure 12.

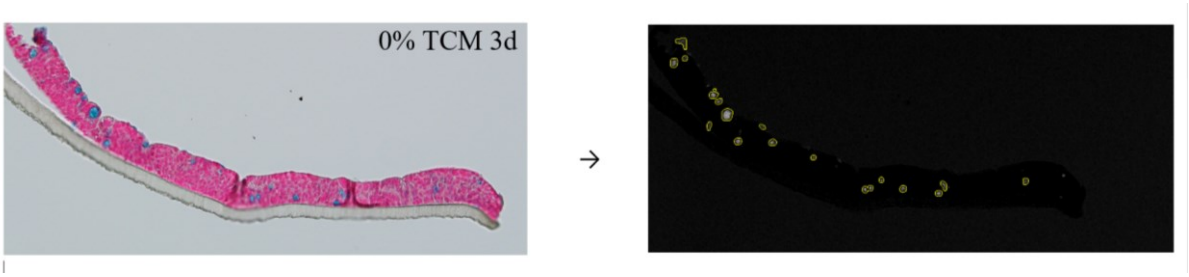

Figure 12: Sample for marking the mucus with the software CellProfiler

With the software the mucus in the microscopy images with a magnification of 10x of all 15 samples was marked. The blue coloured areas represent the mucus.

The program then calculated the mean intensity of the mucus, the size and the amount of the goblet cells of each sample. The results are shown in figures 13 to 15. The first graphic (figure 13) demonstrates the mean intensity of the mucus in the different samples. The intensity of the mucus per primary object in a tissue treated with 0.3% *Zeng Ye Tang* for three days, appears to be slightly less than the intensity of the one, which is not treated with the Chinese formula, while the tissue treated with 0.6% *Zeng Ye Tang* shows no difference to the one which is not treated. However, the intensity of the mucus of the tissue treated for seven days is much lower. For the tissue treated with 0.6% Chinese medicine there is about half as much intensity as for the one which is not treated with *Zeng Ye Tang*. The difference is significant (**). Since a less intensity of the mucus represents a higher mucus production, this underlines the hypothesis that a treatment with *Zeng Ye Tang* of seven days can cause a positive change in the mucus production of the intestinal epithelium.

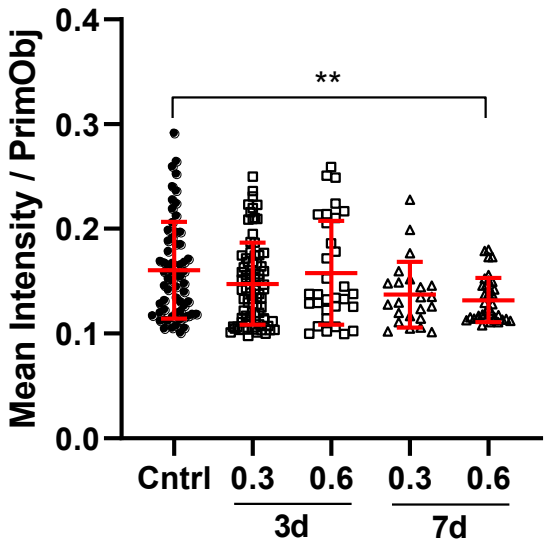

Figure 13: The mean intensity of the mucus in the different samples

A significant difference in the mucus intensity compared to the control is seen in the samples treated with *Zeng Ye Tang* for seven days. Cntrl = Control (0% TCM).

Figure 14 shows the differences in the size of the goblet cells of the samples. The size is roughly the same for the tissue that has been treated with *Zeng Ye Tang* for three days and the sample, which has not been treated. The Chinese formula does not seem to have an influence on the size of the goblet cells. After a treatment of seven days, the size seems to even decrease for both concentrations of *Zeng Ye Tang*.

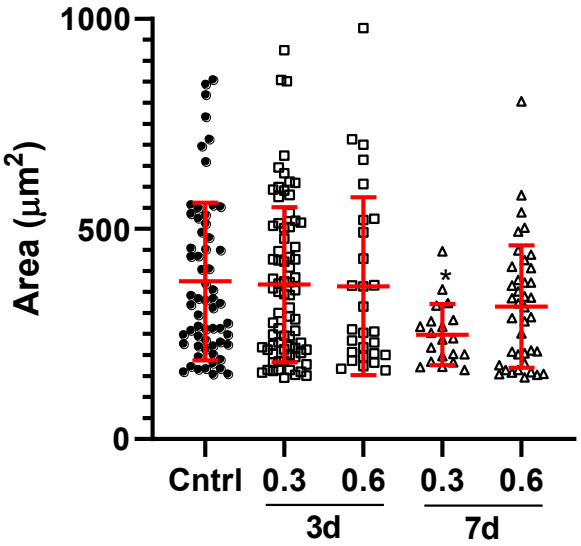

Figure 14: Size of the goblet cells in the different samples

Zeng Ye Tang does not seem to have had any influence on the size of the goblet cells. The size of the cells even seems to decrease after a treatment for seven days. Cntrl = Control.

Figure 15 shows the average amount of goblet cells for each combination of *Zeng Ye Tang* concentration and incubation time. Samples of the tissue, which are treated with 0.3% *Zeng Ye Tang* for three days, have slightly more goblet cells in average (19.3) than the ones not treated with the Chinese herbal preparation (15.8). However, there are less goblet cells in the tissue treated with 0.6% *Zeng Ye Tang* and the one treated with 0.3% for seven days. On the other hand, with an amount of 41 the tissue treated with 0.6% for seven days shows more than twice as many goblet cells.

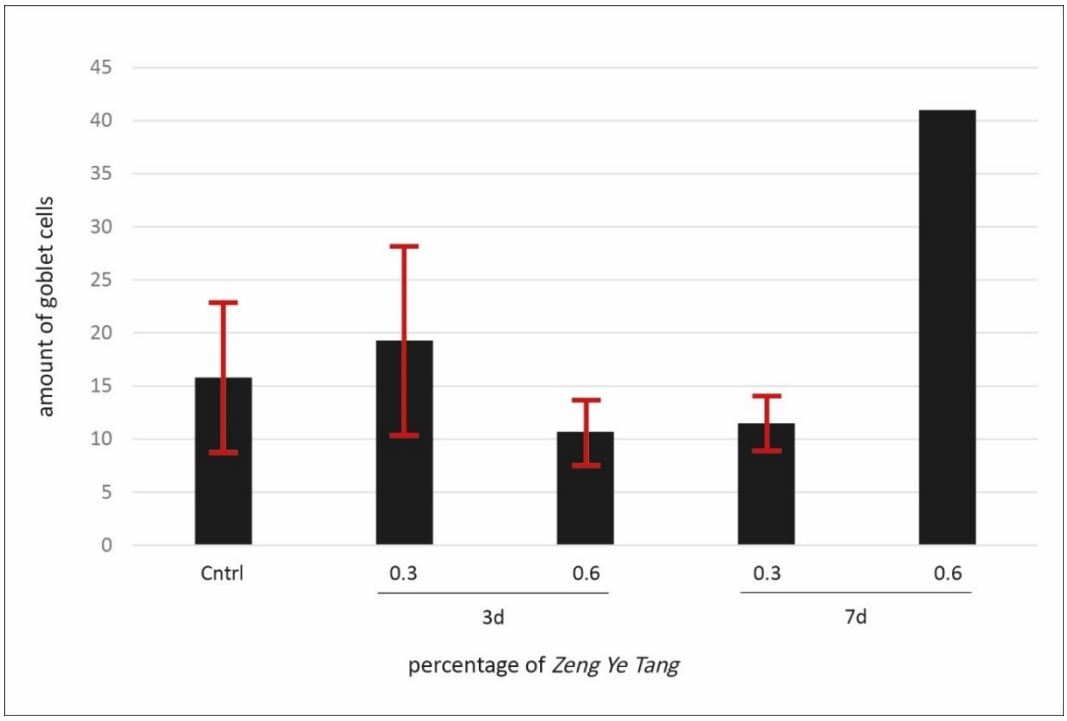

Figure 15: Amount of goblet cells in the different samples

The samples treated with 0.3% *Zeng Ye Tang* for three days and with 0.6% for seven days showed a higher amount of goblet cells compared to the control, while in the others there was a lower amount. Cntrl = Control.

3.2 The Viability of the CaCo2 and HT29 Cells after Treatment with *Zeng Ye Tang*

The EZ4U assay was used to determine the cytotoxic effect of the Chinese formula *Zeng Ye Tang* on the colon epithelial cells. CaCo2 and HT29 cells were seeded in a ratio of 3:1 and after growing one week the Chinese formula was added in different concentrations: 0%, 0.3%, 0.6%, 1.25% and 5%. The wells were incubated for 24 hours and afterwards the viability and proliferation of the colon cells were assessed.

Figure 16 shows the mean viability of the cells after a treatment with *Zeng Ye Tang* in different concentrations. The control (0%) was set to 100%. The samples that were treated with the Chinese formula all showed about 10 to 20% more viability than the control. Therefore, the treatment of the cells with *Zeng Ye Tang* had no negative impact on their viability. It does not have a cytotoxic effect.

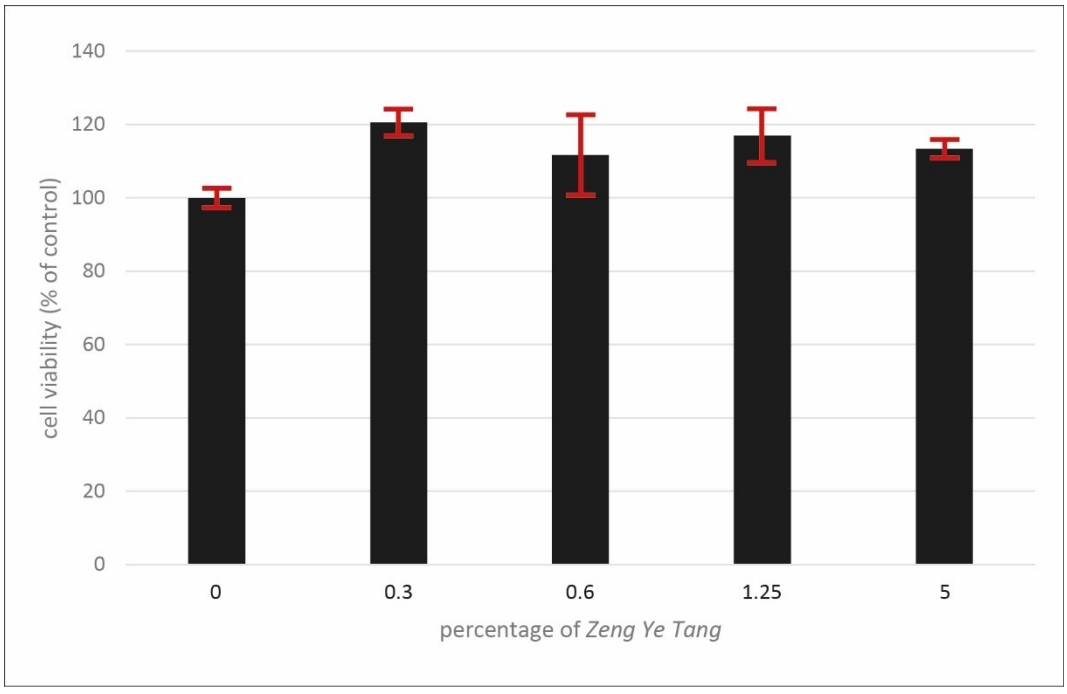

Figure 16: Mean cell viability after a treatment with *Zeng Ye Tang* in different concentrations
A treatment with the Chinese medicine showed a higher viability of the cells than the control (0%).

3.3 The Effect of *Zeng Ye Tang* on the MUC mRNA Expression

After three or seven days of incubation with the Chinese formula *Zeng Ye Tang*, the ALI culture membranes were removed from the wells and cut into halves. One part of each membrane was used for an RNA isolation. Afterwards, the RNA was transcribed into cDNA and analysed with qPCR (real-time quantitative PCR) in order to determine the mucin production of the intestinal cells. Since these are the most common mucins, primers for MUC2, MUC5AC and MUC5B were used.

Figure 17 shows the fold change in the MUC2 mRNA expression after treating the cells with *Zeng Ye Tang* in comparison to the samples without treatment. The samples, which were treated for three days with the Chinese medicine, have a significantly higher mRNA expression then the ones not treated. As MUC2 is the most common mucin in the intestinal mucus, this indicates that *Zeng Ye Tang* could lead to an increased mucus production in the colon. The samples treated for seven days show only a little increase in the MUC2 mRNA expression. Both, the samples treated for three days and the samples treated for seven days, showed no difference for the different concentrations.

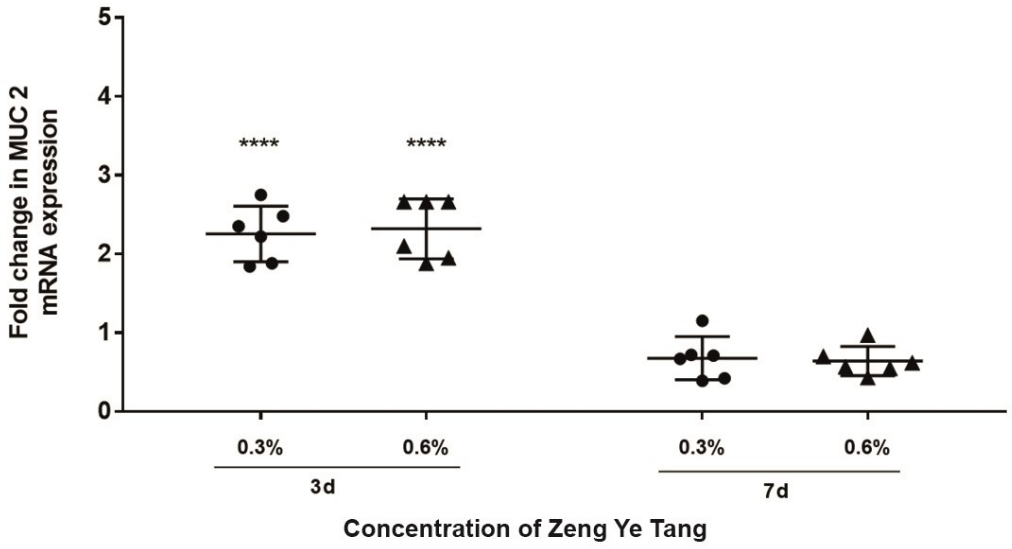

Figure 17: Fold change in MUC2 mRNA expression compared to the control (0%)
The samples treated for three days with *Zeng Ye Tang* show a significant higher MUC2 mRNA expression compared to the control.

A similar trend is seen in figure 18, which shows the fold change in the MUC5AC mRNA expression after treating the cells with *Zeng Ye Tang*. The samples, treated for three days, show a significantly higher mRNA expression than the untreated samples. The samples treated for seven days only show a little increase in the MUC5AC mRNA expression.

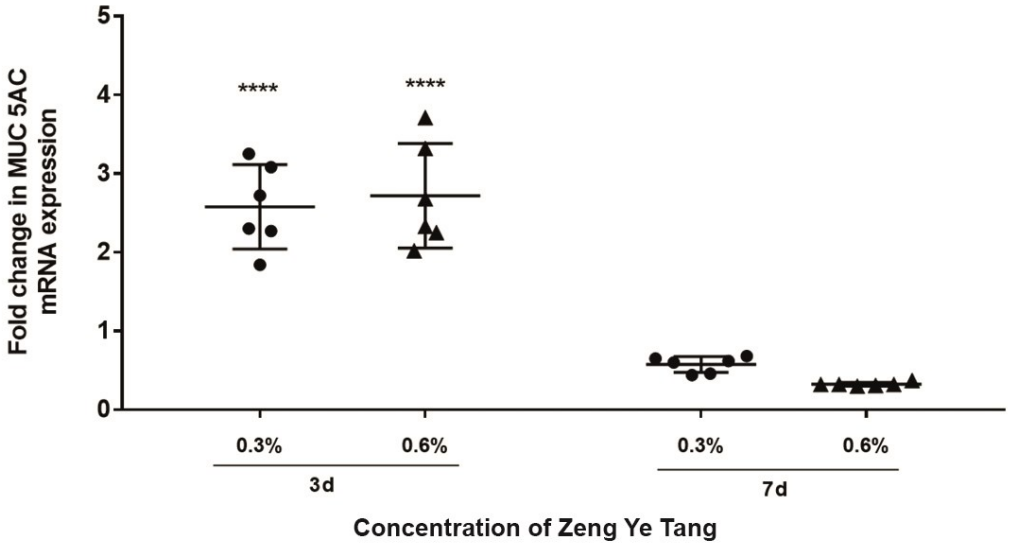

Figure 18: Fold change in MUC5AC mRNA expression compared to the control (0%)
The samples treated for three days with *Zeng Ye Tang* show a significant higher MUC5AC mRNA expression compared to the control.

Figure 19 demonstrates the fold change in the MUC5B mRNA expression. All samples treated with *Zeng Ye Tang* have a higher mucin expression than the untreated ones. Unlike the expression of the other mucin primers the mRNA expression of the samples treated with 0.6% *Zeng Ye Tang* for three days is much higher than the ones treated with 0.3% *Zeng Ye Tang*. Also, the samples treated for seven days show a higher MUC5B mRNA expression, but little difference between the concentrations can be observed.

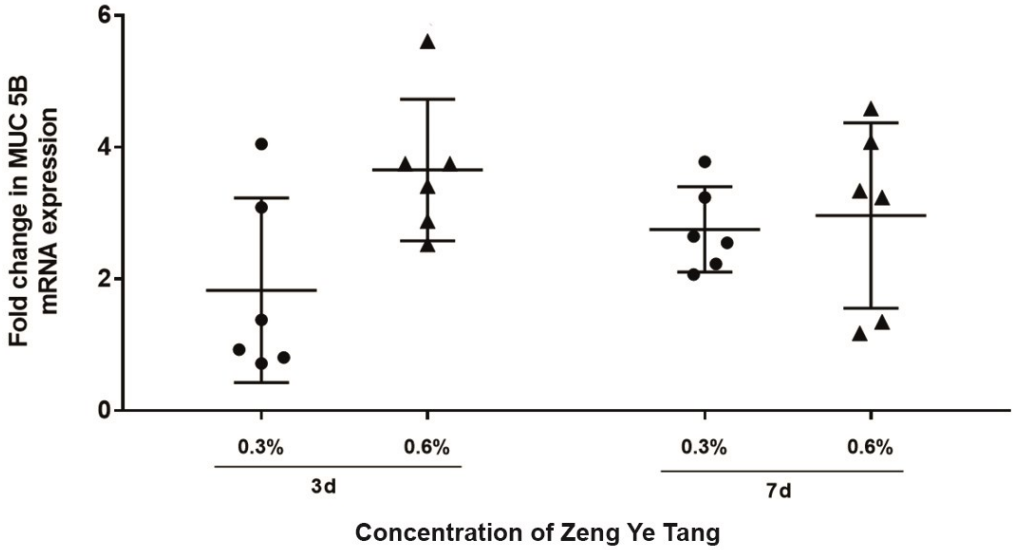

Figure 19: Fold change in MUC5B mRNA expression compared to the control (0%)
All samples show a higher MUC5B mRNA expression compared to the control.

In summary, it could be said that a treatment with the Chinese medicine *Zeng Ye Tang* has a beneficial effect on the mRNA expression of different mucins and therefore could lead to a higher mucus production in the colon.

4 Discussion

Chronic constipation is a common gastrointestinal disorder with an estimated worldwide prevalence of 12 to 19 percent. The exact pathogenesis is still unclear, but it is mainly related to diet type, colon motility and absorption, as well as genetic, pharmacological and behavioural factors. Especially in Europe and North America, many people are affected by constipations. It can be very stressful for patients and lead to a reduction of life quality, as they suffer not only from physical symptoms but also psychological distress. This can limit their social activities and work productivity. Many also suffer from sexual dysfunction and urinary retention. Constipation is typically treated with fibre intake, stimulant or osmotic laxatives, stool softeners or enemas. However, this symptomatic therapy is often unsatisfactory, which is why more and more patients seek help from TCM, particularly Chinese herbal medicine [26, 42, 43].

Several studies have examined the relationship between Chinese medicine and chronic constipation. A good therapeutic effect on chronic constipation could be proven for different Chinese medicines. In some cases, the Chinese medicine was even more effective than conventional medicines. A combined application of Chinese medicine and Western medicine for constipation could also be promising. An advantage of a therapy with Chinese medicine could be the usually lower rate of side effects [26, 43, 44, 45]. Since many people suffer from constipation and the conventional therapy is often unsatisfactory, it is important to investigate alternative treatments like Chinese herbal medicine more closely, also in terms of safety and side effects.

In TCM, many different herbs and formulas are used to treat constipation. *Zeng Ye Tang* is one of the most used classical formulas, along with *Ma Zi Ren Wan* (mainly consisting of Cannabis seeds and rhubarb root). The most frequently used proprietary Chinese herbal medicine is *Run Chang Wan* (mainly consisting of Cannabis seeds, honey and peach kernel). When combining four different Chinese medicines for constipation, the most prescribed drugs are *Ophiopogon japonicus*, *Rehmannia glutinosa*, *Scrophularia ningpoensis* (all found in the formula *Zeng Ye Tang*) and *Run Chang Wan*. *Rehmanniae radix* is one of the ten most frequently used herbs for constipation, while all three of the herbs featured in this formula *Zeng Ye Tang* are among the most prescribed single herbs for constipation [26, 34]. Since *Zeng Ye Tang* and the herbs it contains are used particularly often to treat constipation, this study may help to provide more information on how this formula works in the intestine.

Studies have already confirmed the beneficial effect of *Zeng Ye Tang* on the treatment of constipation [25]. In addition, studies with formulas containing the components of *Zeng Ye Tang*, such as the *Zengyechengqi* decoction, which in addition to *Rehmanniae*, *Scrophulariae* and *Ophiopogonis radix* also contains rhubarb and mirabilite, also showed a clear therapeutic effect on constipation. It also has a preventive effect in patients with thoracolumbar vertebral compression fractures who often develop constipation [46]. However, the exact mechanisms of *Zeng Ye Tang* and its containing herbs in the treatment of constipation remains unclear.

In the present work, we looked at the changes in the intestinal mucosa and mucus production in a CaCo2 and HT29 cell ALI culture after a treatment with *Zeng Ye Tang*. The hematoxylin/eosin and alcian blue stained microscopy images of the CaCo2 and HT29 cell ALI culture showed an increase in the thickness of the tissue with a higher concentration of *Zeng Ye Tang*. The cell proliferation seems to be increasing due to this Chinese herbal preparation. As seen in figure 9, the mucus production appears to increase with a higher concentration of *Zeng Ye Tang* after a treatment of three days. Nevertheless, no difference could be observed after a treatment with *Zeng Ye Tang* for seven days (see figure 11).

The evaluation of the computer software CellProfiler did not show clear results regarding the amount and size of the goblet cells (see figures 14 and 15). However, the mean intensity of the mucus was - with one exception - lower or even significant lower in the treated samples (especially the ones incubated for seven days) compared to the untreated control (see figure 13). With a low mucus intensity, the mucus is less viscous and the mucus production is higher.

With the EZ4U-Test, we were able to determine that *Zeng Ye Tang* is not cytotoxic and does not have a negative impact on the viability of the CaCo2 and HT29 cells when used in the analysed concentrations.

To investigate the amount of mucin mRNA in the samples we used primers for the mucins MUC2, MUC5AC and MUC5B. After transcribing the RNA into cDNA, the samples were analysed with qPCR. The evaluation showed that the mucin mRNA expression of the samples after treating the cells with *Zeng Ye Tang* was higher compared to the untreated control (especially for the samples treated for three days, see figures 17 to 19). The Chinese herbal preparation has a positive effect on the mRNA expression of different mucins and could therefore lead to a higher mucus production in the colon.

In summary, it could be said that a treatment with the Chinese formula *Zeng Ye Tang* of CaCo2 and HT29 cell ALI cultures appears to have a positive effect on the intestinal mucosa and mucus production. However, this could not be confirmed in all experiments. While the mRNA determination showed clear results regarding the mucus production, overall, these were not so clear for the microscopy images.

As in the present study, a study investigating the effect of MDG, a polysaccharide derived from *Ophiopogon japonicus*, on mice with high-fat diet-induced obesity also examined the gene expression of MUC2. It was found that the mice fed a high-fat diet had a significantly higher MUC2 mRNA level when they got MDG than the mice that did not receive MDG. However, unlike in the present study, the MUC2 gene expression was slightly lower compared to the control (mice on a low-fat diet without MDG) as seen in figure 20 [47].

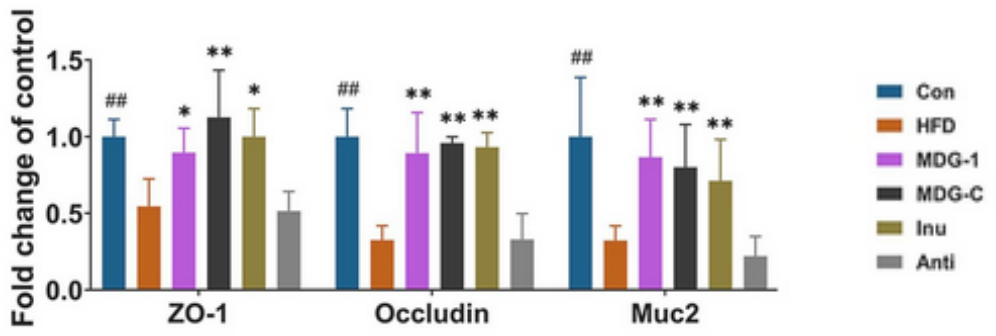

Figure 20: Intestinal barrier related gene expression in the diet-induced obesity mice [47]
Con = control, HFD = high-fat died, MDG-1 = purified MDG, MDG-C = coarse MDG, Inu = Inulin, Anti = MDG-C and antibiotics

In another study with diabetic mice, after a treatment with Stachyose, an oligosaccharide occurring in *Rehmannia glutinosa*, a slightly lower MUC2 gene expression was shown compared to the control [48]. The results of the MUC2 gene expression are thus partly opposing in different studies. The effect of the Chinese formula *Zeng Ye Tang* and its ingredients on the mucin production therefore needs to be further investigated.

In addition to the effect on the intestinal mucosa and the mucus production, the positive effect of *Zeng Ye Tang* in the treatment of constipation could also be based on other mechanisms. For example, another study examined the efficacy and mechanism of action of the *Yangyin Runchang* decoction, which consists of a *Zengye* decoction and other herbs, in treating slow transit constipation in mice. A positive effect was found: after the treatment

with the *Yangyin Runchang* decoction the fecal volume and the intestinal transit rate increased. Based on further investigation, the researchers hypothesized that this effect could be due to an increase in the number of interstitial cells of Cajal and an improvement in their function. A decrease in interstitial cells of Cajal may play an important role in the pathogenesis of the slow transit constipation. Interstitial cells of Cajal have a pacemaker function in the gastrointestinal tract and stimulate the intestinal motility. The slow waves they generate cause rhythmic phasic smooth muscle contractions [43, 49]. Hence, the improvement of the number and function of the interstitial cells of Cajal would be another possible mechanism of action of *Zeng Ye Tang* in the treatment of constipation.

Another possibility by which mechanism of action *Zeng Ye Tang* could have a beneficial effect on constipation is by regulating the intestinal microbiome. Various studies have found an altered composition of the intestinal microbiome in people with constipation. The studies showed a decrease in beneficial bacteria and a reduced species richness. Conversely, changes in the intestinal microbiome can also lead to constipation, so regulating the intestinal microbiome has a positive effect on constipation. Some studies have shown that after treating constipation with Chinese medicine, the intestinal flora was restored. Ingredients that may play an important role are polysaccharide saponins and flavonoids, which are also found in *Ophiopogon japonicus* [43].

A study by Deliang Liu et al. examined the effect of *Zeng Ye Tang* on the gastrointestinal tract, specifically the gut microbiome, in 2019. Elderly constipated rats were treated with *Zeng Ye Tang*. The results showed that after the treatment with *Zeng Ye Tang*, the rats' gut microbiome was restored. More specifically, the level of harmful bacteria in the gut (such as Prevotella, Desulfovibrio, Ruminococcus, and Dorea) that can produce harmful substances have decreased, and the level of beneficial bacteria that can protect the body (such as Oxalobacter, Clostridium, and Roseburia) have increased. Clostridium produces butyric acid, which most likely stimulates the growth of the intestinal mucosa and inhibits mucosal inflammation in the colon. Roseburia probably produces short chain fatty acids. These provide energy for the intestinal mucosa cells and promote the growth and metabolism of colon cells. The short chain fatty acids also lower the pH in the colon and reduce the growth of harmful bacteria. In addition, the treatment with *Zeng Ye Tang* revealed several other metabolic changes in the elderly constipated rats, most of which are related to gut microbiota function. Thus, the administration of *Zeng Ye Tang* led to the regulation of carbohydrates, amines, amino acids and short-chain fatty acids. Also, the energy reserves increased, the

methane metabolism was inhibited, bacterial toxins were reduced, the amino acid metabolism was regulated, and the function of glutathione improved [25, 43].

A positive effect on the intestinal microbiome could also be demonstrated in other studies. For example, diabetic mice with hindlimb ischemia fed with the *BuZangTongLuo* formula, which also contains *Scrophularia ningpoensis* and *Ophiopogon japonicus*, showed an increased abundances of beneficial bacteria while the abundances of harmful bacteria decreased [50].

In particular, the positive effect of *Ophiopogon japonicus* and its ingredients, such as Ophiopogon polysaccharide, Ophiopogonin D and MDG-1, a water-soluble ß-d-fructan, on the intestinal microbiome has already been examined and confirmed in several studies. There have been changes in the abundance of beneficial and harmful bacteria, as well as in diversity. The polysaccharide of *Ophiopogon radix* has a probiotic effect and may prevent dysbiosis. However, most of the studies have been conducted in mice. Therefore, further investigations are necessary [47, 51, 52, 53, 54, 55, 56].

Studies have also shown that *Rehmannia glutinosa* and its components, such as the oligosaccharide stachyose, can modulate the gut microbiota and promote the activity and growth of beneficial bacteria [48, 57].

It is therefore likely that *Zeng Ye Tang* may have a therapeutic effect on constipation by influencing the gut microbiota. However, further studies on the effect of *Zeng Ye Tang* on the intestinal microbiome are necessary. In addition, the exact role of intestinal bacteria in the treatment of constipation should be examined more closely. For example, the bacterium Akkermansia muciniphila has an impact on mucin secretion and could therefore have a positive effect on constipation [48].

However, since no intestinal bacteria were used in the laboratory work for this diploma thesis, *Zeng Ye Tang* seems to have a positive effect on constipation via various mechanisms. These may include the effect on the intestinal mucosa and mucus production, a change or restoration of the intestinal microbiome, an increase in the number and function of the interstitial cells of Cajal and possibly other mechanisms.

In addition, the individual plants and their ingredients should be examined more closely. For example, the herbs *Rehmannia glutinosa* and *Scrophularia ningpoensis* found in the formula *Zeng Ye Tang* contain iridoids which may possess a purgative effect [34]. Furthermore, the

question should be investigated whether the positive effect of *Zeng Ye Tang* in the treatment of constipation can be attributed to individual components of the formula. If this should be the case, it would be sufficient to limit the therapy to individual components. However, it may also be a complex interaction of the ingredients and the formula can only be administered as a whole.

In summary, one can say that since the Chinese formula *Zeng Ye Tang* has been used in TCM for centuries to treat constipations, the benefits have already been confirmed in studies and this study showed a beneficial effect on the intestinal mucosa and mucus production, it could also play a role in the therapy of constipation in the Western medicine (among other general recommendations, such as change in diet). For this, however, further in vitro and in vivo studies are necessary to prove the positive effects as well as to identify possible side effects of the formula and to get more information about the effects of the individual herbs. Interactions with Western medicines should also be investigated.

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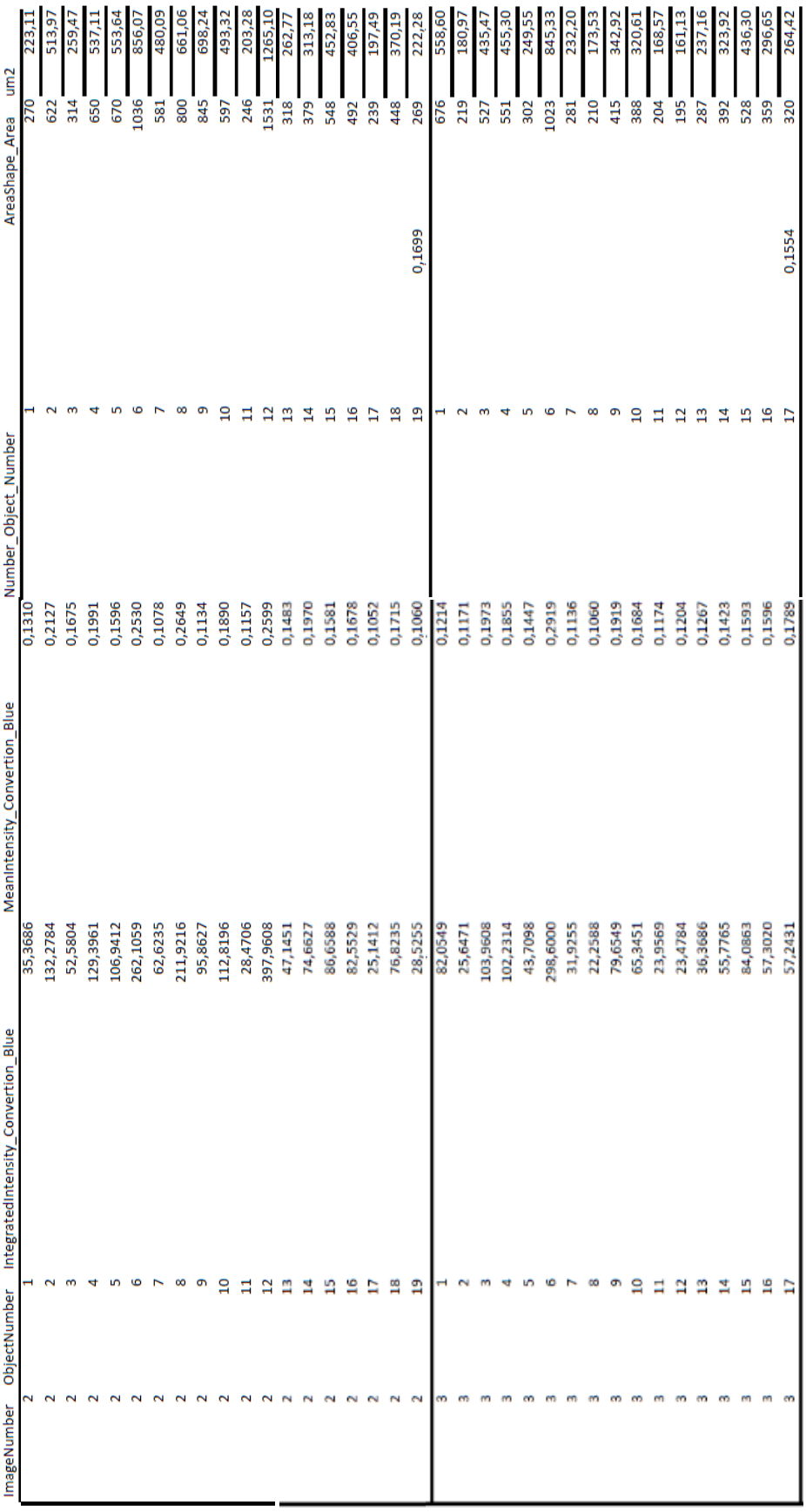
Appendix

Cell Profiler: mean intensity of the mucus, size and amount of the goblet cells



ImageNumber	ObjectNumber	IntegratedIntensity_Convertion_Blue	MeanIntensity_Convertion_Blue	Number_Object_Number	AreaShape_Area um2
2	1	35,3686	0,1310	1	270 223,11
2	2	132,2784	0,2127	2	622 513,97
2	3	52,5804	0,1675	3	314 259,47
2	4	129,3961	0,1991	4	650 537,11
2	5	106,9412	0,1596	5	670 553,64
2	6	262,1059	0,2530	6	1036 856,07
2	7	62,6235	0,1078	7	581 480,09
2	8	211,9216	0,2649	8	800 661,06
2	9	95,8627	0,1134	9	845 698,24
2	10	112,8196	0,1890	10	597 493,32
2	11	28,4706	0,1157	11	246 203,28
2	12	397,9608	0,2599	12	1531 1265,10
2	13	47,1451	0,1483	13	318 262,77
2	14	74,6627	0,1970	14	379 313,18
2	15	86,6588	0,1581	15	548 452,83
2	16	82,5529	0,1678	16	492 406,55
2	17	25,1412	0,1052	17	239 197,49
2	18	76,8235	0,1715	18	448 370,19
2	19	28,5255	0,1060	19	269 222,28
				0,1699	
3	1	82,0549	0,1214	1	676 558,60
3	2	25,6471	0,1171	2	219 180,97
3	3	103,9608	0,1973	3	527 435,47
3	4	102,2314	0,1855	4	551 455,30
3	5	43,7098	0,1447	5	302 249,55
3	6	298,6000	0,2919	6	1023 845,33
3	7	31,9255	0,1136	7	281 232,20
3	8	22,2588	0,1060	8	210 173,53
3	9	79,6549	0,1919	9	415 342,92
3	10	65,3451	0,1684	10	388 320,61
3	11	23,9569	0,1174	11	204 168,57
3	12	23,4784	0,1204	12	195 161,13
3	13	36,3686	0,1267	13	287 237,16
3	14	55,7765	0,1423	14	392 323,92
3	15	84,0863	0,1593	15	528 436,30
3	16	57,3020	0,1596	16	359 296,65
3	17	57,2431	0,1789	17	320 264,42
				0,1554	

Image Numbers:

2-5: 3 days, 0% TCM

6-9: 3 days, 0.3% TCM

10-11: 7 days, 0.3% TCM

12-14: 3 days, 0.6% TCM

15: 7 days, 0.6% TCM

4	1	61,8980	0,1544	1		401	331,36
4	2	35,4353	0,1189	2		298	246,24
4	3	45,5020	0,1507	3		302	249,55
4	4	54,2471	0,1695	4		320	264,42
4	5	25,8784	0,1125	5		230	190,05
4	6	21,9961	0,1164	6		189	156,18
4	7	39,1412	0,1201	7		326	269,38
4	8	55,5333	0,1658	8		335	276,82
4	9	226,7177	0,2283	9		993	820,54
4	10	101,2118	0,2066	10		490	404,90
4	11	22,3333	0,1188	11		188	155,35
4	12	408,6706	0,2195	12		1862	1538,62
4	13	84,2471	0,1323	13		637	526,37
4	14	159,7216	0,2370	14		674	556,94
4	15	67,4314	0,1661	15		406	335,49
4	16	149,8510	0,2250	16		666	550,33
4	17	35,5451	0,1288	17		276	228,07
4	18	48,4627	0,1124	18		431	356,15
4	19	34,0471	0,1310	19		260	214,84
4	20	223,6471	0,2407	20		929	767,66
4	21	27,7608	0,1013	21		274	226,41
4	22	83,4000	0,1530	22		545	450,35
4	23	90,6863	0,1402	23	0,1587	647	534,63
5	1	59,3961	0,1463	1		406	335,49
5	2	160,4000	0,1854	2		865	714,77
5	3	27,4667	0,1346	3		204	168,57
5	4	24,9098	0,1233	4	0,1474	202	166,92
6	1	31,5255	0,1061	1		297	245,42
6	2	58,6824	0,1133	2		518	428,04
6	3	249,8784	0,2231	3		1120	925,48
6	4	33,0941	0,1345	4		246	203,28
6	5	53,7020	0,1557	5		345	285,08
6	6	48,1059	0,1436	6		335	276,82
6	7	127,7922	0,1668	7	0,1490	766	632,96

7	1	151,7255	0,2090	1		726	599,91
7	2	83,8667	0,1379	2		608	502,41
7	3	90,7216	0,1641	3		553	456,96
7	4	29,9608	0,1161	4		258	213,19
7	5	67,9961	0,1295	5		525	433,82
7	6	37,0235	0,1327	6		279	230,54
7	7	84,7412	0,1875	7		452	373,50
7	8	25,7529	0,1150	8		224	185,10
7	9	94,6706	0,1522	9		622	513,97
7	10	47,4431	0,1252	10		379	313,18
7	11	391,4745	0,2096	11		1868	1543,57
7	12	105,5922	0,1515	12		697	575,95
7	13	31,6000	0,1339	13		236	195,01
7	14	90,7137	0,1951	14		465	384,24
7	15	65,4510	0,1566	15		418	345,40
7	16	446,6745	0,2198	16		2032	1679,09
7	17	32,6706	0,1219	17		268	221,46
7	18	21,1647	0,1064	18		199	164,44
7	19	87,8667	0,1775	19		495	409,03
7	20	29,4275	0,1136	20		259	214,02
7	21	113,6824	0,1746	21		651	537,94
7	22	138,7137	0,1869	22		742	613,13
7	23	74,8549	0,1745	23	0,1561	429	354,49

9	1	20,3098	0,1110	1		183	151,22
9	2	103,6706	0,1686	2		615	508,19
9	3	45,6196	0,1516	3		301	248,72
9	4	170,4588	0,2310	4		738	609,83
9	5	29,3529	0,1328	5		221	182,62
9	6	25,8667	0,1064	6		243	200,80
9	7	26,9020	0,1196	7		225	185,92
9	8	34,6314	0,1391	8		249	205,75
9	9	33,3098	0,1267	9		263	217,32
9	10	50,8353	0,1594	10		319	263,60
9	11	31,0667	0,1146	11		271	223,93
9	12	57,1569	0,1119	12		511	422,25
9	13	315,2431	0,3049	13		1034	854,42
9	14	66,8000	0,1575	14		424	350,36
9	15	22,1333	0,1112	15		199	164,44
9	16	374,0314	0,2363	16		1583	1308,07
9	17	33,9373	0,1281	17		265	218,98
9	18	379,1020	0,1686	18		2249	1858,40
9	19	473,7216	0,2230	19		2124	1755,11
9	20	76,7020	0,1418	20		541	447,04
9	21	180,2275	0,1750	21		1030	851,11
9	22	57,7961	0,1332	22		434	358,62
9	23	131,7765	0,2095	23		629	519,76
9	24	717,8040	0,2498	24		2874	2374,86
9	25	113,9333	0,1585	25		719	594,13
9	26	33,2824	0,1197	26		278	229,72
9	27	20,5686	0,1071	27		192	158,65
9	28	168,8392	0,2159	28	0,1612	782	646,19
10	1	30,3608	0,1058	1		287	237,16
10	2	53,0196	0,1353	2		392	323,92
10	3	33,1529	0,1348	3		246	203,28
10	4	49,8706	0,1295	4		385	318,13
10	5	127,6039	0,1770	5		721	595,78
10	6	62,2039	0,1443	6		431	356,15
10	7	49,2353	0,1520	7		324	267,73
10	8	46,4157	0,1595	8		291	240,46
10	9	23,8157	0,1197	9		199	164,44
10	10	44,6196	0,1458	10		306	252,86
10	11	30,2627	0,1235	11		245	202,45
10	12	23,1686	0,1048	12		221	182,62
10	13	47,9177	0,1479	13		324	267,73
10	14	30,1569	0,1142	14	0,1353	264	218,15

11	1	35,0196	0,1021	1		343	283,43
11	2	137,9569	0,1991	2		693	572,64
11	3	24,1647	0,1167	3		207	171,05
11	4	80,4314	0,1487	4		541	447,04
11	5	22,6118	0,1014	5		223	184,27
11	6	42,7137	0,1260	6		339	280,12
11	7	26,4745	0,1108	7		239	197,49
11	8	167,3804	0,2280	8		734	606,52
11	9	26,5882	0,1278	9	0,1401	208	171,88
12	1	41,5255	0,1340	1		310	256,16
12	2	311,0941	0,2241	2		1388	1146,94
12	3	34,1098	0,1287	3		265	218,98
12	4	92,7059	0,1783	4		520	429,69
12	5	90,3765	0,2054	5		440	363,58
12	6	86,7647	0,1375	6		631	521,41
12	7	752,4549	0,2591	7		2904	2399,65
12	8	76,9216	0,1291	8		596	492,49
12	9	203,2824	0,1718	9	0,1742	1183	977,54
13	1	28,9294	0,1226	1		236	195,01
13	2	50,6235	0,1325	2		382	315,66
13	3	214,9373	0,2488	3		864	713,94
13	4	159,4078	0,2169	4		735	607,35
13	5	29,8510	0,1070	5		279	230,54
13	6	39,1765	0,1379	6		284	234,68
13	7	25,5216	0,1257	7		203	167,74
13	8	169,3020	0,2106	8	0,1627	804	664,37
14	1	22,6706	0,1145	1		198	163,61
14	2	181,6275	0,2142	2		848	700,72
14	3	43,5922	0,1379	3		316	261,12
14	4	21,9412	0,0997	4		220	181,79
14	5	46,7098	0,1526	5		306	252,86
14	6	25,3294	0,1025	6		247	204,10
14	7	25,6353	0,1021	7		251	207,41
14	8	20,8784	0,0994	8		210	173,53
14	9	337,6941	0,2140	9		1578	1303,94
14	10	82,4510	0,1861	10		443	366,06
14	11	84,3059	0,1328	11		635	524,72
14	12	408,4549	0,2510	12		1627	1344,43
14	13	35,2235	0,1450	13		243	200,80
14	14	24,0353	0,1064	14		226	186,75
14	15	55,4863	0,1255	15	0,1456	442	365,24

15	1	21,6588	0,1171	1		185	152,87
15	2	56,2157	0,1131	2		497	410,68
15	3	96,8980	0,1380	3		702	580,08
15	4	63,5529	0,1495	4		425	351,19
15	5	336,9294	0,1404	5		2400	1983,18
15	6	95,5098	0,2085	6		458	378,46
15	7	23,3686	0,1174	7		199	164,44
15	8	97,8000	0,1801	8		543	448,69
15	9	94,1059	0,2087	9		451	372,67
15	10	28,1569	0,1149	10	0,1488	245	202,45
15	11	60,3020	0,1733	11		348	287,56
15	12	60,7882	0,1375	12		442	365,24
15	13	21,3098	0,1140	13		187	154,52
15	14	41,2353	0,1216	14		339	280,12
15	15	137,7922	0,2110	15		653	539,59
15	16	103,5059	0,1731	16		598	494,14
15	17	20,1961	0,1135	17		178	147,09
15	18	23,4941	0,1217	18		193	159,48
15	19	22,1137	0,1152	19		192	158,65
15	20	33,0392	0,1291	20		256	211,54
15	21	44,1490	0,1129	21	0,1384	391	323,09
15	22	90,9765	0,2017	22		451	372,67
15	23	27,5020	0,1109	23		248	204,93
15	24	24,6588	0,1158	24		213	176,01
15	25	25,7490	0,1181	25		218	180,14
15	26	51,5686	0,1469	26		351	290,04
15	27	58,0039	0,1526	27		380	314,00
15	28	161,2627	0,1558	28		1035	855,25
15	29	51,0235	0,1241	29		411	339,62
15	30	22,7216	0,1142	30		199	164,44
15	31	32,4627	0,1283	31		253	209,06
15	32	20,2745	0,1078	32		188	155,35
15	33	45,1608	0,1107	33		408	337,14
15	34	77,3647	0,1457	34		531	438,78
15	35	136,4118	0,2240	35		609	503,23
15	36	55,3765	0,1123	36		493	407,38
15	37	35,0667	0,1154	37		304	251,20
15	38	35,8039	0,1421	38		252	208,23
15	39	72,5882	0,1788	39		406	335,49
15	40	133,4980	0,1372	40		973	804,01
15	41	77,9294	0,1510	41	0,1397	516	426,38

EZ4U Test

Method name: EZ4U
Application: SparkControl V1.0
Device: SPARK 10M Serial number: 1505001434
Firmware: ABS:V3.1.2|ABS_MEX:V3.3.2|MTP:V8.2.29

Date: 2019-11-22
Time: 09:48
System PCSPARKREADER
User NT-AUTORITÄT\NETZWERKDIENST
Plate [NUN96ft]
Lid lifter No lid
Humidity Cassette No humidity cassette

List of actions in this measurement script:
Plate
Absorbance EZ4U

Mode Absorbance
Name EZ4U
Measurement wavelength 492 nm
Reference wavelength 620 nm
Number of flashes 10
Settle time 0 ms
Part of Plate A1-H12

Start Time 2019-11-22 09:47:56
Temperature 24,37 °C

EZ4U

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,057079	0,057894	0,057239	0,056489	0,056826	0,057168	0,057303	0,057134	0,056171	0,058197	0,055496	0,05584
B	2,536257	2,222941	2,509558	2,137141	2,544292	0,569538	0,056989	0,05549	0,056597	0,056232	0,058723	0,057382
C	2,714351	2,737634	2,742648	2,270188	2,513829	0,573749	0,058658	0,057173	0,055814	0,055639	0,056375	0,056608
D	2,593551	2,277546	2,380614	2,164904	2,402445	0,588426	0,055752	0,057898	0,055904	0,056172	0,056775	0,056623
E	0,056242	0,056193	0,056551	0,060885	0,060251	0,056035	0,057381	0,055741	0,056498	0,055644	0,05666	0,056824
F	0,057102	0,056656	0,056733	0,056315	0,056083	0,056395	0,055596	0,055718	0,055977	0,05558	0,056165	0,056677
G	0,058302	0,056894	0,056287	0,055934	0,056043	0,055717	0,056051	0,055869	0,056547	0,056783	0,056093	0,058135
H	0,057174	0,057887	0,05689	0,058251	0,056883	0,056161	0,05665	0,056574	0,056672	0,057515	0,056756	0,060563

Reference

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,055571	0,055178	0,054611	0,055047	0,053784	0,0544	0,054177	0,053989	0,053705	0,05623	0,052743	0,05384
B	0,270167	0,236941	0,276436	0,24083	0,31026	0,188955	0,054011	0,05366	0,053042	0,053227	0,05525	0,055287
C	0,288876	0,282832	0,299286	0,268493	0,305163	0,191271	0,057022	0,053972	0,05351	0,053082	0,053512	0,054566
D	0,305395	0,250463	0,282322	0,273607	0,282685	0,199198	0,053733	0,055752	0,053576	0,053478	0,05404	0,053785
E	0,053923	0,054068	0,054066	0,058008	0,057249	0,05323	0,054723	0,05316	0,053785	0,053004	0,053971	0,053952
F	0,053443	0,05393	0,053974	0,053515	0,053199	0,053478	0,053018	0,053034	0,05305	0,052933	0,053274	0,053926
G	0,05593	0,054339	0,053833	0,053631	0,053799	0,053456	0,053734	0,05342	0,053665	0,054105	0,053392	0,054609
H	0,054807	0,054973	0,053983	0,055601	0,05405	0,053581	0,053779	0,053842	0,053915	0,054651	0,054269	0,057425

Difference 0,3 0,6 1,25 0 5 blank

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	0,001508	0,002716	0,002628	0,001442	0,003041	0,002768	0,003126	0,003145	0,002466	0,001966	0,002754	0,002001
B	2,26609	1,985999	2,233122	1,896311	2,234031	0,380583	0,002979	0,00183	0,003554	0,003005	0,003472	0,002096
C	2,425475	2,454802	2,443362	2,001695	2,208666	0,382479	0,001637	0,003202	0,002304	0,002557	0,002864	0,002041
D	2,288156	2,027084	2,098292	1,891296	2,11976	0,389228	0,002019	0,002146	0,002328	0,002694	0,002735	0,002839
E	0,002318	0,002125	0,002485	0,002877	0,003001	0,002805	0,002658	0,002581	0,002713	0,00264	0,002689	0,002872
F	0,003659	0,002725	0,002759	0,0028	0,002884	0,002918	0,002578	0,002684	0,002927	0,002647	0,002891	0,002752
G	0,002372	0,002555	0,002453	0,002303	0,002244	0,002261	0,002317	0,002449	0,002882	0,002678	0,002701	0,003525
H	0,002368	0,002913	0,002907	0,00265	0,002833	0,00258	0,002871	0,002733	0,002758	0,002864	0,002487	0,003138

End Time 2019-11-22 09:48:32

MUC mRNA expression

3d	ACTB60	MUC 2	dCP	ddCP		Ratio	mean	SD	
0%	18,76	28,9							
0%	18,76	28,51	9,75						
0%	17,95	28,35	10,40						
0%	18,03	27,87	9,84						
0%	18,02	28,49	10,47						
0%	18,06	28,2	10,14					10,12	
0.3 %	19,02	27,83	8,81	-1,31	1,31	2,48			
0.3 %	19,14	28,03	8,89	-1,23	1,23	2,35			
0.3 %	18,73	27,39	8,66	-1,46	1,46	2,75			
0.3 %	18,45	27,69	9,24	-0,88	0,88	1,84			
0.3 %	18,82	28,03	9,21	-0,91	0,91	1,88			
0.3 %	18,99	27,96	8,97	-1,15	1,15	2,22		2,25	0,32
0.6 %	18,89	27,60	8,71	-1,41	1,41	2,66			
0.6 %	18,70	27,86	9,16	-0,96	0,96	1,95			
0.6 %		27,75	9,05	-1,07	1,07	2,10			
0.6 %	19,10	27,81	8,71	-1,41	1,41	2,66			
0.6 %	20,37	29,08	8,71	-1,41	1,41	2,66			
0.6 %	20,06	29,27	9,21	-0,91	0,91	1,88		2,34	0,32
7d									
0	18,76	28,90							
0	18,76	28,51	9,75						
0	17,95	28,35	10,40						
0	18,03	27,87	9,84						
0	18,02	28,49	10,47						
0	18,06	28,20	10,14					10,12	
0.3 %	20,09	30,70	10,61	0,49	-0,49	0,71			
0.3 %	20,09	30,01	9,92	-0,20	0,20	1,15			
0.3 %	18,51	29,11	10,60	0,48	-0,48	0,72			
0.3 %	18,79	29,49	10,70	0,58	-0,58	0,67			
0.3 %	18,38	29,87	11,49	1,37	-1,37	0,39			
0.3 %	18,44	29,82	11,38	1,26	-1,26	0,42		0,68	0,25
0.6 %	18,85	29,49	10,64	0,52	-0,52	0,70			
0.6 %	18,87	29,04	10,17	0,05	-0,05	0,97			
0.6 %	18,35	29,16	10,81	0,69	-0,69	0,62			
0.6 %	18,25	29,17	10,92	0,80	-0,80	0,57			
0.6 %	18,05	29,04	10,99	0,87	-0,87	0,55			
0.6 %	17,94	29,29	11,35	1,23	-1,23	0,43		0,71	0,15

3d	ACTB60	MUC 5AC	dCP	ddCP		Ratio	mean	SD	
0%	18,76	27,63							
0%	18,76	27,19	8,43						
0%	17,95	26,04	8,09						
0%	18,03	25,92	7,89						
0%	18,02	26,36	8,34						
0%	18,06	26,47	8,41					8,23	
0.3 %	19,02	25,55	6,53	-1,70	1,70	3,25			
0.3 %	19,14	25,75	6,61	-1,62	1,62	3,08			
0.3 %	18,73	25,52	6,79	-1,44	1,44	2,72			
0.3 %	18,45	25,63	7,18	-1,05	1,05	2,07			
0.3 %	18,82	25,85	7,03	-1,20	1,20	2,30			
0.3 %	18,99	26,04	7,05	-1,18	1,18	2,27		2,62	0,44
0.6 %	18,89	25,70	6,81	-1,42	1,42	2,68			
0.6 %	18,70	25,76	7,06	-1,17	1,17	2,25			
0.6 %		25,71	7,01	-1,22	1,22	2,33			
0.6 %	19,10	25,60	6,50	-1,73	1,73	3,32			
0.6 %	20,37	26,71	6,34	-1,89	1,89	3,71			
0.6 %	20,06	27,28	7,22	-1,01	1,01	2,02		2,65	0,42
7d									
0	18,76	27,63							
0	18,76	27,19	8,43						
0	17,95	26,04	8,09						
0	18,03	25,92	7,89						
0	18,02	26,36	8,34						
0	18,06	26,47	8,41					8,23	
0.3 %	20,09	29,06	8,97	0,74	-0,74	0,60			
0.3 %	20,09	29,02	8,93	0,70	-0,70	0,62			
0.3 %	18,51	27,37	8,86	0,63	-0,63	0,65			
0.3 %	18,79	27,57	8,78	0,55	-0,55	0,68			
0.3 %	18,38	27,74	9,36	1,13	-1,13	0,46			
0.3 %	18,44	27,87	9,43	1,20	-1,20	0,44		0,57	0,09
0.6 %	18,85	28,52	9,67	1,44	-1,44	0,37			
0.6 %	18,87	28,76	9,89	1,66	-1,66	0,32			
0.6 %	18,35	28,29	9,94	1,71	-1,71	0,31			
0.6 %	18,25	28,14	9,89	1,66	-1,66	0,32			
0.6 %	18,05	28,00	9,95	1,72	-1,72	0,30			
0.6 %	17,94	27,80	9,86	1,63	-1,63	0,32		0,33	0,02

3d	ACTB60	MUC 5B	dCP	ddCP		Ratio	mean	SD	
0%	18,58	31,55							
0%	18,53	32,03	13,50						
0%	17,49	31,56	14,07						
0%	17,49	32,24	14,75						
0%	17,85	30,65	12,80						
0%	18,04	30,86	12,82				13,59		
0.3 %	19,16	30,73	11,57	-2,02	2,02	4,05			
0.3 %	19,15	31,11	11,96	-1,63	1,63	3,09			
0.3 %	18,87	32,57	13,70	0,11	-0,11	0,93			
0.3 %	18,56	32,45	13,89	0,30	-0,30	0,81			
0.3 %	19,13	32,25	13,12	-0,47	0,47	1,38			
0.3 %	19,16	33,22	14,06	0,47	-0,47	0,72	1,83	1,28	
0.6 %	18,87	30,69	11,82	-1,77	1,77	3,41			
0.6 %	19,10	30,78	11,68	-1,91	1,91	3,75			
0.6 %	19,10	31,16	12,06	-1,53	1,53	2,88			
0.6 %	19,16	31,41	12,25	-1,34	1,34	2,53			
0.6 %	20,49	31,59	11,10	-2,49	2,49	5,61			
0.6 %	20,52	32,20	11,68	-1,91	1,91	3,75	3,14	0,47	
7d			0,00	-13,59	13,59	12313,90			
0	18,58	31,55	12,97	-0,62	0,62	1,53			
0	18,53	32,03	13,50						
0	17,49	31,56	14,07						
0	17,49	32,24	14,75						
0	17,85	30,65	12,80						
0	18,04	30,86	12,82				13,49		
0.3 %	20,27	32,70	12,43	-1,16	1,16	2,23			
0.3 %	20,22	32,46	12,24	-1,35	1,35	2,55			
0.3 %	18,32	30,21	11,89	-1,70	1,70	3,24			
0.3 %	18,62	30,29	11,67	-1,92	1,92	3,78			
0.3 %	18,33	30,51	12,18	-1,41	1,41	2,65			
0.3 %	18,14	30,68	12,54	-1,05	1,05	2,07	2,75	0,59	
0.6 %	18,79	30,18	11,39	-2,20	2,20	4,59			
0.6 %	18,64	30,49	11,85	-1,74	1,74	3,34			
0.6 %	18,49	30,05	11,56	-2,03	2,03	4,08			
0.6 %	18,53	30,42	11,89	-1,70	1,70	3,24			
0.6 %	18,32	31,67	13,35	-0,24	0,24	1,18			
0.6 %	18,44	31,60	13,16	-0,43	0,43	1,35	3,81	0,55	