Aus der Klinik für Allgemein-, Viszeral-, und Gefäßchirurgie

(Direktor: Prof. Dr. Dr. h.c. H. Lippert)

der Medizinischen Fakultät

der Otto-von-Guericke-Universität Magdeburg

in Zusammenarbeit mit dem

VA Medical Center/UT Southwestern in Dallas / Texas (USA)

Effects of Deoxycholic Acids and Urodeoxycholic Acids on Cancerogenic Progression in Barrett's Esophagus[1]

DISSERTATION

(written in English / geschrieben in Englisch)

zur Erlangung des Doktorgrades

Dr. med.

(doctor medicinae)

an der Medizinischen Fakultät

der Otto-von-Guericke-Universität Magdeburg

vorgelegt von Stefanie Jürgens

aus Herford

Magdeburg 2013

[1] Gefördert durch finanzielle Unterstützung vom Referat für Forschung durch die Leistungsorientierten Mittelvergabe (LOM) / Mittel zur Förderung von Doktoranden der Otto-von-Guericke-Universität Magdeburg

Dokumentationsblatt

Bibliographische Beschreibung:

Jürgens, Stefanie

Effects of Deoxycholic Acids and Urodeoxycholic Acids on Cancerogenic Progression in Barrett's Esophagus. *-2013. - 76 Pages, 28 Figures, 1 Table*

The University of Texas Southwestern Medical Center and University of Magdeburg

Supervising Professor: Rhonda F. Souza, MD AGAF, FASGE Professor of Medicine (USA); Professor Dr. Frank Meyer (Germany)

Abstract:

Background: Barrett's Esophagus (BE) is a pre-malignant metaplasia of the esophageal mucosa and can often be found in patients with gastro-esophageal reflux disease (GERD). Although most of the Barrett's patients are under acid suppressing therapy, the rate of adenocarcinoma of the esophagus has been steadily increasing during the last 30 years. So, bile salts presented to the esophagus, especially the higher proportion of unconjugated bile acids including Deoxycholic-Acid (DCA), found in BE patients, are believed to play an important role in the progression to the esophageal adenocarcinoma. Urodeoxycholic Acid (UDCA), a therapeutic drug for cholestatic liver diseases, might also have a chemopreventive effect in BE.

Methods: Barrett's Bar-T and Bar-T10 epithelial cell lines and biopsies taken from Barrett's patients were treated with either 250 μM of DCA or UDCA for 5, 10 and 30 minutes *in vivo* or *in vitro*. Subsequently, DNA damage and activation of NF-κB pathway proteins were detected by Western blots. Apoptosis and cell number were evaluated by Cell Death ELISA and cell counts.

Results: DCA induced DNA damage and phosphorylation of NF-κB pathway proteins, but not apoptosis in BAR-T cell. In contrast, UDCA did not cause DNA damage, phosphorylation of NF-κB pathway proteins, or apoptosis in BAR-T cells.

Conclusions: In BAR-T cells, DCA induces DNA damage but fails to induce apoptosis, events that may contribute to carcinogenesis. It can be suggested that bile acid-induced resistance to apoptosis may be mediated via activation of the NF-κB pathway. In addition, UDCA is less genotoxic than DCA and may be a potential chemopreventive drug to prevent the progression of Barrett's Esophagus to esophageal adenocarcinoma.

Key words: Barrett's Esophagus, DCA, UDCA, bile acids

This study is based on the following publications

"The Role of Bile Acids in the Neoplastic Progression of Barrett's Esophagus – a short representative overview""

Jürgens S, Meyer F, Spechler SJ, Souza RF

Zeitschrift für Gastroenterologie, Thieme Verlag. 2012 Sep;50(9):1028-34

„**Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF-κB activation in benign Barrett's epithelial cells."**

Huo X, Juergens S, Zhang X, Rezaei D, Yu C, Strauch ED, Wang JY, Cheng E, Meyer F, Wang DH, Zhang Q, Spechler SJ, Souza RF.

Am J Physiol Gastrointest Liver Physiol. 2011 Aug;301(2):G278-86. Epub 2011 Jun 2.

CONTENTS

1. LIST OF ABBREVIATIONS

Abbreviations	Meaning
ABTS	2,2'-Azino-Bis(3-Ethylbenzthiazoline-6-Sulfonic Acid
AIF	Apoptosis Inducing Factor
APS	Ammonium-Per Sulfate
ATM	Ataxia Telangiectasia Mutated
ATR	ATM- and Rad3-related Kinases
BAR	Barrett's cell line
BE	Barrett's Esophagus
BPE	Bovine Pituitary Extract
BSA	Bovine Serum Albumin
°C	Degrees Celsius
C	Control
CA	Cholic Acid
CDCA	Chenodeoxycholic Acid
CDX	Caudal Drosophila Homobox Gene
CHK	Checkpoint Kinase
cm	Centimetre
COX	Cyclooxygenase
D	Duplicate
DCA	Deoxycholic Acid
DISC	Death Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Desoxyribonucleic Acid
DSB	Double Strand Breaks
E-	Ethanol
EDTA	Ethylene Diamine Tetra-Acetate
E.G.	Example given
EGF	Epidermal Growth Factor
EGTA	Ethylene-Glycol-Bis(2-Aminoethylether)-N,N,N',N'-Tetraacetic Acid

ELISA	Enzyme-Linked Immunosorbent Assay
FADD	Fas-associated death domain
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
Fig	Figure
g	Gramm
GERD	Gastro-Esophageal Reflux Disease
H	Histone
h	Hour
HOX	Homobox
hrs	Hours
IAP	Inhibitors of Apoptosis
I–κK	I– kappa Kinase
IL	Interleukin
KBM	Keratinocyte Basal Medium
KGM	Keratinocyte Growth Medium
M	Mol
MAPK	Mitogen- Activated Protein Kinase
min	Minutes
mm	Millimeter
ml	Milliliter
µ	Micro
MP	Microplate
MUC	Mucin
MW	Molar Weight
n	Nano
NEMO	NF- kappa B Essential Modulator
NF-κB	Nuclear Factor - kappa B
OD	Optical Density
P	Protein
p-	phosphorylated
PBC	Primary Biliary Cirrhosis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline plus Tween 20

PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl Fluoride
POD	Peroxidase
PPI	Proton Pump Inhibitor
PSC	Primary Sclerosing Cholangitis
ROS	Reactive Oxygen Species
S-	Sodium
SCJ	Squamocolumnar Junction
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
SER	Serine
SMAC	Second Mitochondria-derived Activator of Caspase
SSB	Single Strand Breaks
t-	Total
Tab	Table
TBST	Tris-buffered Saline plus Tween 20
TEMED	N, N, N´, N´-Tetramethylethylendiamin
TG	Tri-Glycin
TGS	Tri-Glycin Sulfate
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis Inducing Ligand
Tris	Tris(hydroxymethyl)-aminomethan
UDCA	Urodeoxycholic Acid
V	Volt
VIL	Villin
XSG	Certain Typ of Growth Medium
‘	Minute

2. INTRODUCTION

Gastro-esophageal reflux disease (GERD) and Barrett's Esophagus (BE) are the main risk factors for developing the esophageal adenocarcinoma, a deadly malignancy whose incidence has been increasing tremendously during the last decades. Currently, it is not clear which components of the refluxed juice contribute to the progression to the adenocarcinoma. The application of proton pump inhibitors (PPIs) to suppress the acidic reflux is a common and widely spread strategy for Barrett's and GERD patients; however, the incidence of adenocarcinoma is still rising. The reasonable use of PPIs is limited by the fact that they are not able to eliminate toxic bile salts and their reflux into the esophagus. So, it can strongly be suggested that acidic reflux alone cannot be responsible for the progression to cancer; a more important contribution of bile salts that has been ignored until now, has to be considered.

2.1. Barrett's Esophagus

The replacement of the normal esophageal epithelium by an epithelium of specialized intestinal-like columnar cells is a metaplastic condition which is called *Barrett's Esophagus*.

The specialized intestinal metaplasia characteristic of BE has an increased risk of neoplastic progression to esophageal adenocarcinoma. [86] During the last 30 years, the incidence of squamous cell carcinoma of the esophagus has declined, especially in the United States of America, while the rate of adenocarcinoma of the esophagus has been steadily increasing during this same time span. [24] [65]

The interest on this kind of cancer is illustrated by its incidence, which has increased by 600 % in Western countries and the fact that it has been the most abundant esophageal cancer since 1995. [64] [83] Its aggressivity is huge and with a 5-years survival rate of less than 15%, BE mostly ends deadly in only a little while [25]

GERD and Barrett's Esophagus are known as the main risk factors in the development of esophageal adenocarcinoma.

Patients, suffering only from GERD already have a 30 times higher risk, but for patients with GERD and BE the risk of degeneration is even 125 times higher than for those in the general population. [62]

"However, recent data indicate that the risk as reported above has most likely been tremendously overestimated. In this context, Hvid-Jensen *et al*. [39] have found out in a large, nation-wide cohort study that the annual risk of Barrett's adenocarcinoma is only 0.12% and the incidence of the adenocarcinoma is 4- to 5-fold lower than previously reported. (Own publication) [45] Other risk factors for esophageal adenocarcinoma, apart from GERD and BE, are obesity, dietary agents, alcohol and tobacco use. [100]

The repeated exposure of refluxed gastric and bile acids to the esophagus can constitute the beginning of the metaplastic progression. The pathomorphological changes are believed to be triggered by GERD-induced esophagitis. Usually, this kind of injury heals through squamous cell regeneration. In some patients who have chronic GERD, metaplastic, intestinal-like cells instead of normal esophageal ones are produced to repair the injury. The benefit of this metaplasia is that these cells are more resistant against the damaging, chronic reflux. [100]

The origin of the "Barrett's cells" is still slightly explored, but there are a few theories that try to give answers about them. One of the older and no longer substituted attempts to explain misconceived that columnar cells from the stomach are able to migrate to the injured esophagus. It has been suggested that these cells can recover the refluxate-damaged mucosa and, by this way, protect the esophagus mucosa against the continuing reflux. However, later it was found out that the metaplastic epithelium is different from the gastric mucosa and Barrett's metaplasia was able to develop in experimental models where esophagus and stomach were disconnected. More current studies discovered embryonic stem cells, remaining in the squamo-columnar junction (SCJ) in adult mice as well as in humans, which are believed to be the origin of BE. In mouse models, genetically engineered, the adult squamous epithelium was destroyed by targeted expression of diphtheria toxin A. The discovered embryonic cells migrated from the SCJ to the destroyed regions of the esophagus and, interestingly, they manifested markers and properties of Barrett's epithelium. [96]

Another theory is that reflux-induced damage to the esophageal squamous cells in the superficial cell layer of the stratified squamous epithelium stimulates the stem

cells, located in the basal cell layer of the epithelium, leading to their abnormal differentiation into metaplastic "Barrett's cells" rather than normal esophageal squamous cells. The so called *Transdifferentiation Theory* describes the differentiation of basal esophageal cells into intestinal-type cells. It assumes that GERD creates inflammation. Hereby, the permeability of the esophageal cell layer is disturbed and gastric acids, bile acids and other components of the reflux can directly impact on undifferentiated basal esophageal cells. Those substrates are believed to interact with the DNA and activate special promoters and their morphogenetic factors.

Cells which are stimulated by this way show typical properties of small intestine cells and result in Barrett's metaplasia. [85]

A very recent study emanates from multipotential adult progenitor cells. Those cells hail from the bone marrow and are believed to play a role in regeneration of the refluxed-damaged cells and contribute to the generation of Barrett's epithelium.
In rat models bone marrow of female rats had been destroyed by radiation. Thereupon, bone marrow of male rats was transplanted; 8 weeks after the transplantation Y chromosomal material could be found in the squamous esophageal cells as well as in the intestinal metaplasia of the female rats. Hutchinson *et al.* irradiated the bone marrow of mice and transplanted it with total bone marrow of ROSA 26 mice. At an age of 12 weeks, mice were esophago-jejunostomized. At week 22, the animals were euthanized and esophagi were examined. Interestingly, some of the mice developed columnar epithelium next to squamous epithelium at the anastomosis area. These changes were consistent to Barrett's metaplasia in a mouse model. [38] However, these results have only been demonstrated in rat models, yet. [85]

Interestingly, Barrett's metaplasia involves the expression of transcription factor proteins, for example the HOX family, which were present in the esophagus during its embryonic development. HOX DNA sequences are genes that induce shifts in the development of organs; they encode transcription factors, important for the intestinal cell developing and are responsible for the gut developing from the endoderm. [9] A very important HOX gene is the *Caudal Drosophila Homobox gene 2*, Cdx2. Normally its expression is confined in the small and large intestine, but it is also expressed in the embryonic esophagus, as well as in the Barrett's Esophagus. Cdx2 causes the transcription of proteins, for example mucin 2 (*MUC2*) and villin 1 (*VIL1*), which can

be found in the intestine and hereby it is important for the development of the intestinal mucosa. In the esophagus, Cdx2 function gets lost by conditional deletion of that gene in the developing endoderm, which effects a replacement of the embryonic, esophageal columnar cells with squamous cells. Another theory suggested that this replacement is incomplete and islands of columnar cells are remaining in the esophagus after birth. [7] In Barrett's Esophagus, Cdx2 expression is increased again and hereby Muc2 (a goblet cell marker), cytokeratin 20 and other intestine proteins can also be verified. [17] Due to the fact that the mechanisms underlying the development of BE are not completely understood and the therapies to eliminate Barrett's Esophagus are still under investigation, it is important to treat GERD in order to prevent the development of Barrett's metaplasia.

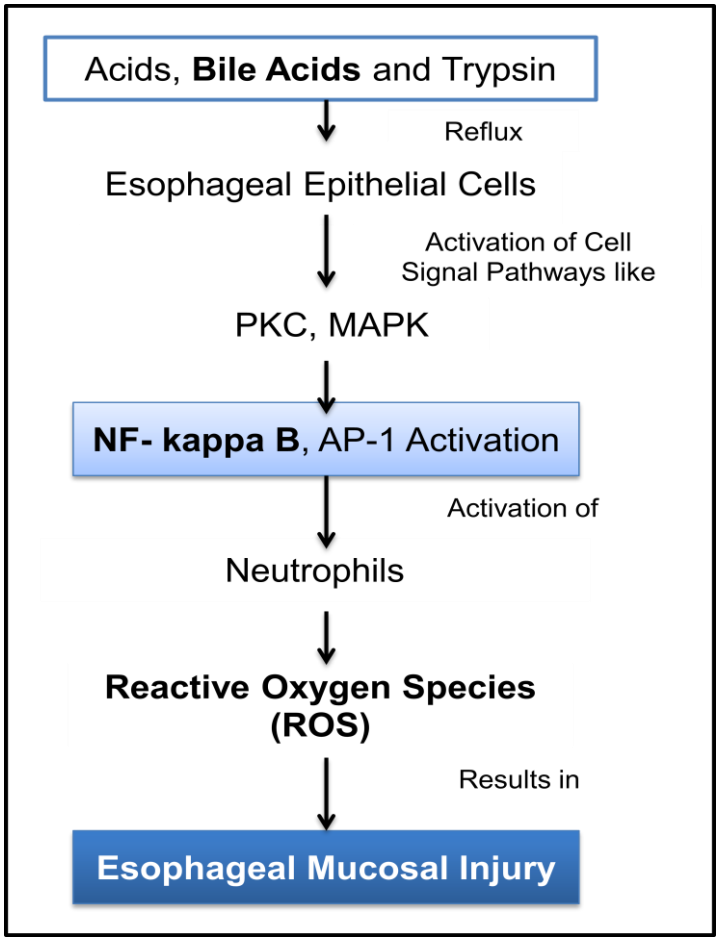
In patients with Barrett's Esophagus, therapies are targeted to prevent the progression to esophageal adenocarcinoma. The use of anti-secretory therapy with proton pump inhibitors (PPIs) and COX-2 inhibitors, as potential chemopreventive agents, is one approach. [35] Other potential endoscopic therapies are ablation of the metaplasia by laser or photodynamic therapy as well as anti-reflux surgery. [18] However, the use of these agents to ablate non-dysplastic, metaplastic Barrett's Esophagus is still under investigation. Currently, patients with Barrett's Esophagus are treated with PPIs in doses that relieve their symptoms of GERD or if they have no symptoms, with once daily dosing. In addition, endoscopic surveillance with biopsies for the early detection of dysplasia and/or cancer has been recommended by the American College of Gastroenterology. [95] Thus, in order to spare a patient all these invasive procedures, it is important to understand how Barrett's Esophagus develops so that better methods to prevent and treat this precancerosis can be developed.

2.2. The Role Of GERD, Acid And PPIs In Barrett's Esophagus

A serious complication of chronic gastro-duodenal reflux in the esophagus is the developing of the specialized, intestinal metaplasia. Although the whole development of Barrett's Esophagus is not completely understood right now, it can be suggested that acid is an important etiological factor in its pathogenesis. [28]

During a period of reflux, a combination of hydrochloric acid and pepsin attains the esophagus; after repeated exposure, this may lead to mucosal injuries and inflammation. The damaged epithelium is believed to be predisposed to heal by replacement of the squamous cells by metaplastic, columnar cells to form a protection against the irritating agents. [16] [97] Refluxed gastric juice, especially, which contains the digestive protein pepsin, is thought to destroy the tight junctional proteins between esophageal cells. The disturbed mucosal permeability facilitates penetration of hydrogen ions to the deeper cell layer and makes these cells vulnerable for further acid attack. [89] Acid also damages the surface of the epithelial layer and if the damage is too destructive the cells will die. Stimulated by the death of those cells, the basal cell layer proliferates and renews the squamous epithelium. A consecutive reaction is the hyperplasia of the basal cells and papillae. [41] The acid damaged cells may also cause an inflammatory reaction, by which inflammatory cells, like T-Lymphocytes and Neutrophils, infiltrate the mucosa and submucosa and release cytokines. [60] This cytokine secretion is believed to influence and initiate the cell proliferation. [84] Interleukins found in esophagitis patients have the ability to enhance the epithelial turnover and to accelerate the regeneration of cells. [70]

Another fact is that immune cells produce *Reactive Oxygen Species* (ROS). The released ROS are able to produce membrane and/ or DNA damage, mostly double strand breaks. [21] [98] This can enhance the esophageal mucosal injury. Figure 1 shows the development of mucosal injury. Hydrochloric acids, bile acids, and digestive enzymes damage the epithelial cells. Intracellular signaling pathways, such as PKC and MAPK, are activated leading to the activation of transcription factors, such as NF-κB, AP-1, and NF-IL-6, which cause Interleukin-8 (IL-8) transcription and, subsequently their production and release. IL-8, a chemokine, attracts immune cells, like Neutrophils and activates them. ROS are produced by those infiltrating immune cells and, further, injuring of the epithelium.



[98]

Figure 1: *Developing of mucosal injury*. Acids, bile acids and trypsin are interfering with esophageal epithelium which activates intracellular pathways (PKC and MAPK). NF-κB and other transcription factor proteins are also enabled. The NF-κB pathway leads to IL-8 production, a chemokine, which attracts immune cells like Neutrophils. Those Neutrophils are able to produce ROS. ROS interact with cell structures like membranes, destroy them and cause mucosal injury.

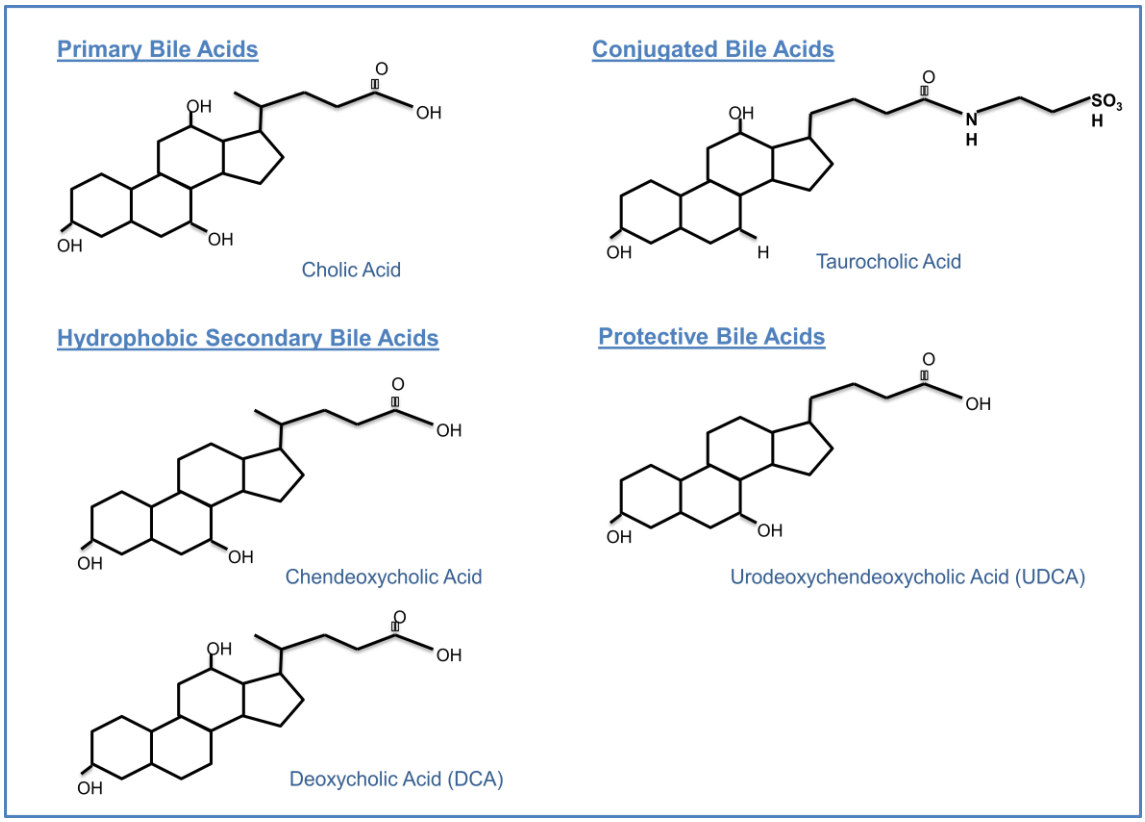
So, of course the acidic, gastric reflux is harmful, but it alone does not explain the mutation and progression to Barrett's Esophagus. It should not be forgotten that it is not only the acidic gastric juice with its components, like hydrochloric acid and pepsin, alone, which are regurgitated; it is also duodenal reflux that contains the conjugated and unconjugated bile acids, trypsin and lysolecithin.

A common applied therapy to normalize the esophageal pH value is the treatment with PPIs like Omeprazole. Even though, the therapy with PPIs is widely used, the incidence rate of adenocarcinoma continues to rise. [64] So, there have to be used other substances, which also have effects on the cell differentiation and proliferation that cannot be inhibited by PPIs. Several prior studies found out that bile acid exposure in Barrett's patients is significantly higher than in patients with esophagitis.

This results on one hand from more periods of acid reflux and on the other hand, patients with BE are exposed to longer periods of acid exposure, often more than 5 minutes. [27] [75] Indeed acid suppressive agents can regulate the pH environment, reduce the inflammation and prevent against peptic ulcerations, but their shifting of the pH value has several other consequences. At a pH of 3-5, which is generated by treatment with PPIs, the unconjugated, duodenal-esophageal bile acids become predominant in the gastric juice. In the generated neutral environment they have carcinogenetic effects in contrast to conjugated bile acids, which are damaging in lower pH levels and dominant in untreated patients. [59] In PPI treated patients the unconjugated bile acids are able to transmigrate through the esophageal mucosal or mitochondrial membranes and damage mucosal cells and interfere with intracellular functions and pathways. [16] [55] [50] This damage might also cause changes in intracellular pathways and could be an important fact in the developing and progression of BE. Future studies have to be made to find out more about the role of those bile acids and the role they are playing in carcinogenesis.

2.3. Bile Acids In Barrett's Patients

All important endogenous bile acids in the human body are derivates of cholesterol. They are synthesized in the liver by hepatocytes as the two primary bile salts Cholic Acid (CA) and Chenodeoxycholic Acid (CDCA). [69] The bile acids are secreted into the bile tract and have a functional exercise in digestion of fat soluble substrates. CA (40%), CDCA (40%) and Deoxycholic Acid (DCA 20%), a secondary bile acid are composing the majority (67%) of the human bile. [63] To increase their solubility, bile acids can be conjugated, especially with taurine or glycine. During their transit through the intestine, bacteria cause modifications, like de-conjugation and hydroxylation. A product of those modifications on CA represents the hydrophobic DCA; by epimerization of CDCA, the hydrophilic UDCA (3α, 7β-dihydroxy-5β-cholanoic acid) is synthesized. [58] The conjugated acids like glycocholate and taurocholate and the unconjugated and secondary DCA, the most damaging bile salt for the esophageal mucosa, are the major constituents of bile acids that are exposed to the esophagus during a period of duodenogastro-esophageal reflux. [22] Figure 2 shows the structures of the most important bile acids.

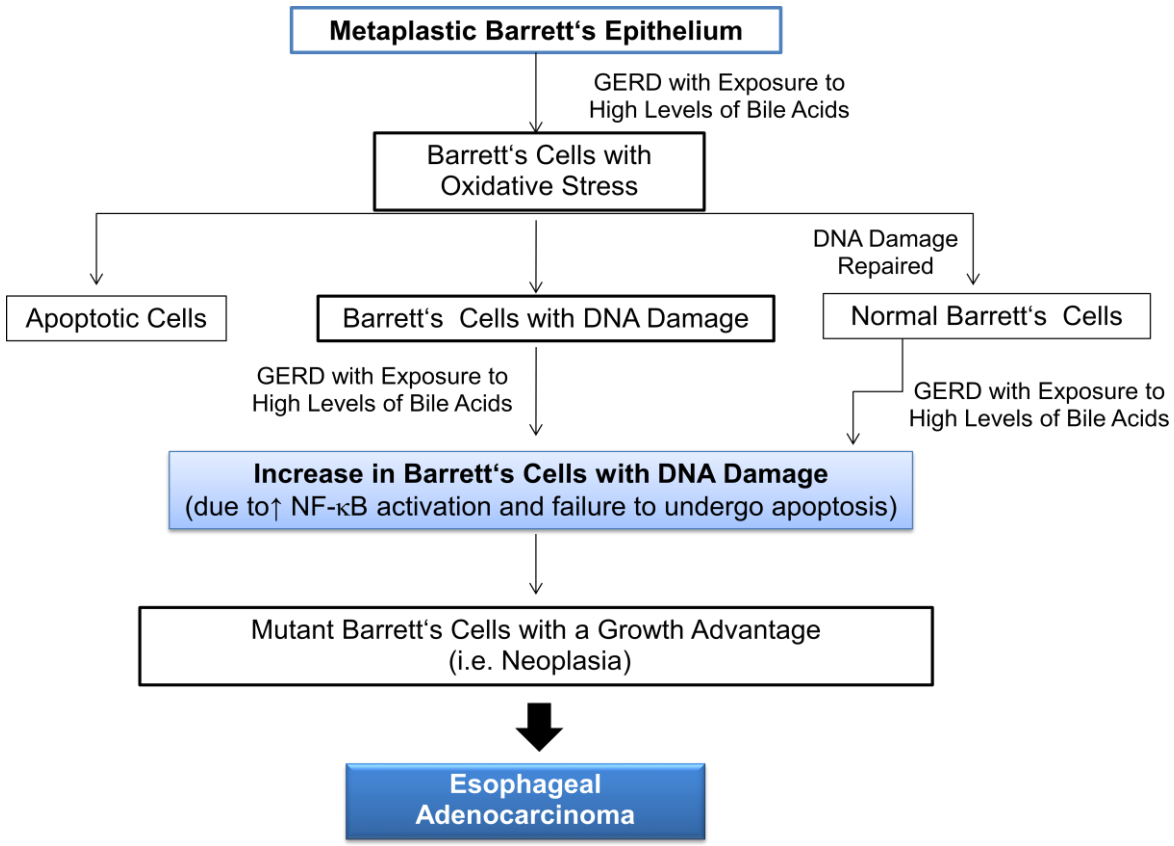


[63]

Figure 2: ***Structures of the most important bile acids.*** The picture illustrates the chemical structure of the primary bile acids such as Cholic Acid (CA) and Chenodeoxycholic Acid (CDCA), as well as Deoxycholic Acid and one of their conjugated metabolites, Taurocholic Acid, all synthesized out of a Cholesterol frame. Also the structure of the protective, hydrophilic bile acid Urodeoxycholic Acid UDCA can be seen.

Patients, who are under PPI medication against the acidity of the reflux, still proceed to the adenocarcinoma. So, digestive enzymes and gastric acid cannot be the only harmful components, presented to the esophagus. Recent clinical studies have shown that a significant higher concentration of bile acids is refluxed into the esophagus in BE patients in contrast to GERD patients. [87] Over and above, in those patients bigger amounts of duodeno-gastro-esophageal reflux, especially postprandially or at night can be verified. [48] Those patients show higher levels of DCA, the most damaging secondary bile acid, too. [59] So, it can strongly be suggested that bile salts also might be implicated in the carcinogenesis. In this context, it is important to know that secondary bile acids, like DCA, in their unconjugated state are the more damaging ones. [43] For instance, it could be shown that DCA is able to increase the production of pro-inflammatory gene products, like COX-2 and NF-κB pathway proteins in esophageal adenocarcinoma cells, hepatocytes and colon cancer cells. [15] [20] [34] Experiments were done in

neutral pH environment, so acid reflux was stopped, but bile salts were still presented to the esophagus; the same conditions can frequently be found in patients with GERD under acid- suppressing therapy. [29] It might be possible that NF-κB, a cell survival pathway protein is triggered by the same mechanisms under the influence of bile acids in Barrett's Esophagus. For a long time, bile acids have been known to be extremely cell toxic; they can damage cell or mitochondrial membranes and then interfere with intracellular pathways and functions. [13] [31] [92] Studies with DCA in Barrett's Esophagus are extremely rare. There are some data for biopsies of patients suffering from familial adenomatous polyposis (FAP) that demonstrate the DNA and chromosomal damaging and single strand breaks- inducing effects of bile acids. [14] [77] In other cells, hepatocytes for example, bile salts can release ROS and may induce oxidative DNA damage. [26]



[11] [45]

Figure 3: ***Development from normal esophageal squamous cells to adenocarcinoma***. Esophageal squamous cells are exposed to high levels of bile acids. They are stressed and generate DNA damage. Some of them die, in some of them DNA damage can be repaired, and some cells survive with DNA damage. Those cells with DNA damage can acquire growth advantages as reflux continues and further DNA damage ensues. By natural selection, pre-malignant Barrett's cells with growth advantages due to DNA damage can progress to adenocarcinoma.

Figure 3 illustrates three consequences for ROS-stressed cells:

1. cells die by inducing apoptosis in consequence of the huge cell injury, or
2. cells repair the DNA damage and survive, or
3. cells survive with unrepaired DNA damage.

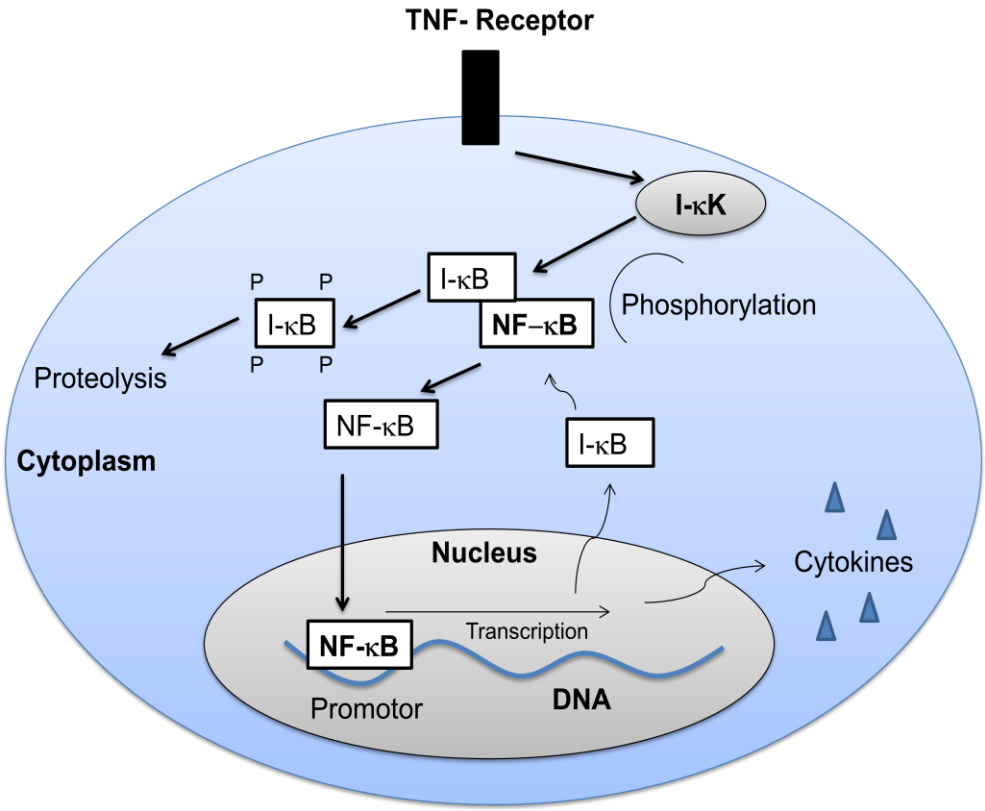
If these DNA-damaged cells undergo replication, then an increased number of mutated cells arises. These pre-malignant cells might now progress to cancer due to their growth advantages. In contrast to adenocarcinoma, DCA exposure in normal, squamous esophageal cells is cytotoxic and induces apoptosis; damaged cells are showing typical signs of apoptosis, like margination and membrane blebbing, condensation of chromatin, and cell shrinkage; but "Barrett's cells" did not react with apoptosis. [23] In "Barrett's cells" repeated exposure creates an apoptotic-resistant metaplastic epithelium, which is more resistant to apoptosis and the toxic, refluxed bile acids. Those morphogenetic changes might abet the progression of the disease and can lead to generalization of esophageal adenocarcinoma. [47]

But, of course, not all bile acid species cause the same disruptive effects like DCA does. The hydrophilic UDCA is known to have anti-apoptotic and cell protective effects in patients with increased levels of apoptosis, like primary biliary cirrhosis and primary sclerosing cholangitis (PSC). [94] [88] In patients with ulcerative colitis, UDCA even reduces the rate of cancer progression. [19] It is assumed that these favorable effects are triggered by stabilization of the mitochondrial membrane permeability and the reduced production of ROS; hereby UDCA can inhibit the swelling of liver mitochondria and prevent apoptosis. [73] So, if UDCA reacts in a similar way in Barrett's Esophagus, it might be a possible drug to prohibit the progression to adenocarcinoma. It is necessary to research the effects that UDCA can trigger, because most of them are still unknown and uninvestigated.

2.4. NF-κB – A Main Figure In Barrett's Esophagus

Nuclear Factor-κB is one of the most important proteins in an anti-apoptotic survival pathway of cells and has the ability to regulate the balance between cell death and survival. It is known that NF-κB is up-regulated in many kinds of cancers. High expression of this transcription factor can also be demonstrated in esophageal adenocarcinoma and in Barrett's Esophagus. [1] [36] The NF-κB belongs to the Rel-transcription factor family, consisting of the five members NF-κB1 (p50/p105), NF-κB2 (p52/p100), c-Rel, RelA (p65) and RelB. The inactive NF-κB complex is arranged in the cytoplasm and as soon as it is activated, it can be translocated to the nucleus. Normally, proteins of the I-κB family, composed of I*κ*B*α*, I*κ*B*β*, IκB*γ*, IκB*ε*, BCL-3 and 2 precursor proteins, bind to NF-κB dimers and prevent its translocation to the cell nucleus. [33] To enable the initiation of NF-κB activation, the protein kinase I-κK plays an instrumental role. I-κB kinase (I-κK) is an enzyme with 2 determining catalytic subunits (I-κKα and I-κKβ) and gets stimulated by NF-κB activating substrates. This kinase phosphorylates I-κB subunits and initiates its relief from the NF-κB complex and hereby NF-κB's nuclear translocation. A further regulatory subunit, I-κKγ, also known as NEMO (NF-κB essential modulator) fulfills a function in pro-inflammatory effects and is needed when cytokines like TNF-α stimulate cells, for example in immunological processes. [79] The up-regulation of transcription starts to as soon as NF-κB binds to specific promoters on the DNA; proteins that are generated are cytokines like IL1, IL-6, TNF-α, cell adhesion molecules, chemokines, growth factors, pro- and anti- apoptotic proteins (Bcl-xL, Fas Ligand) [30]; even angiogenesis can be initiated. [68]

Figure 4 illustrates the above explained pathway.



[44]

Figure 4: *NF-κB pathway*. I-κB binds to NF-κB dimers and forms inactive complexes in the cytoplasm. As soon as activating subtrates, like TNF-α interfer with cell receptors, the I-κK kinase is activated and phosphorylates I-κB subunits which initiate proteolysis and release the NF-κB complex. The now activated complex is able to pass the nuclear membrane and binds on specific promotor regions on the DNA that leads to expression of genes, like cytokines etc.

To stop the expression of those proteins, NF-κB is down-regulated by negative feedback mechanisms in which I-κB is new-synthesized, translocated to the nucleus and attaches to NF-κB dimers again. These NF-κB - I-κB-complexes are exported to the cytoplasm. [52] Depending on the cause of its activation, there are 3 kinds of NF-κB pathways that can be initiated. The first one is called "*classical*" (canonical), the second is known as the "*alternative*" (non-canonical) and the last one is an *atypical* pathway. The classical pathway is triggered by bacterial or viral infections and inflammatory cytokines. It is I-κKβ mediated; I-κB becomes phosphorylated by the kinase and induces I-κB degradation. NF-κB1/REL A (p50/p65) and cREL are activated and used to initiate immunity and to block apoptosis in response to cell injury and to ensure survival of the cells. NF-κB inducing kinase mediates the non-canonical pathway, activated by growth factors and TNF, cytokines. This kinase activates I-κKα homodimers. P52:RelB and NF-κB are generated to NF-

κB2 p52/RelB heterodimers by involving the precursor protein p100. In the cells this signaling pathway is always important for the cell survival of premature B-Lymphocytes and the adaptive immune system. The third pathway can be activated by UV-light or oxidative stress, which also induces DNA damage and does not need the I-κK. It causes I-κBα degradation and hereby nuclear NF-κB activity. [79] [93] So, there is a close link between NF-κB activation, the immune system and inflammation.

Chronic inflammation, caused by GERD, activates the NF-κB pathway and induces the expression of anti-apoptotic proteins (Bcl2), a condition that may favor development of cell cancer. [46] Because of its anti-apoptotic qualities, NF-κB activity can be observed in many pre-malignant and malignant cells. In colon cancer, for example, NF-κB suppresses on one hand cells undergoing apoptosis and on the other hand it can increase the expression of growth factors, like IL-6, which contributes to the progression of cell growth. [10] Furthermore it has been found out in current studies that NF-κB in Barrett's Esophagus regulates two caudal type homobox transcription factors 1 and 2 (Cdx1/2). These homoboxes can initiate gene expression for intestinal mucins and cytokeratins in immortalized esophageal cells and esophageal adenocarcinoma cell lines and so they are believed to play an important role in the development of intestinal metaplasia. [81] [85]

2.5. DNA-Damage And Apoptosis

Apoptosis is a genetically programmed form of cell death and it is necessary for maintaining the homeostasis between cell death and survival. In contrast to necrosis, it is an active process and is characterized by morphological correlates, like membrane blebbing, chromatin condensation, forming of "apoptotic bodies" and organelle compaction. If DNA of cells gets damaged by toxic reagents, oxidative stress, UV-light or other conditions, the cell has two opportunities to manage that damage: the first one is to repair the harm; the second is the induction of cell death, mediated via apoptosis. These mechanisms are essential for physiological cell homeostasis and, in particular, to ensure the survival of healthy cells, because DNA damage can lead to chromosomal changes, mutations and transformation to a pre-malignant cell type. [74] Finally, those changes in balance between death and survival might lead to the development of an immortal, apoptosis resistant cell type

and, in conclusion, to cancer. There are two different groups of proteins for the regulation of apoptosis: the apoptosis suppressing ones (Bcl-2, Bcl-xL, mcl-1, etc.) and the apoptosis promoting ones (Bax, Bcl-x, Bak). [56]

It has been established that apoptosis can be initiated by the intrinsic, mitochondrial pathway or the extrinsic, death receptor pathway; between these mechanisms, cross talks are existing. Toxic agents, members of the TNF family, but also TRAIL (TNF-related apoptosis inducing ligand), and antibodies can figure ligands to activate the cell death receptor. Their binding to the receptor leads to aggregation and recruitment of the Fas-associated death domain (FADD), which recruits and activates caspase-8. In combination with caspase-8, FADD forms DISC, the death inducing signaling complex. [5] The protein caspase-8 stands at the beginning of a caspase-cascade and cleaves other caspases, like caspases-3, -6 and -7. These execute many regulatory proteins, which, at last, activate the caspase-activated DNase, a downstream effector caspase, and hereby the cell death via apoptosis. [82] The mitochondrial pathway is triggered by intracellular and oxidative stress as well as extensive DNA damage. A major role in this signaling way is the release of pro-apoptotic factors from the mitochondrial membrane, like Cytochrome C, apoptosis inducing factor (AIF) and second mitochondria-derived activator of caspase (SMAC). [51] Cytochrome C is able to activate caspase-3 by forming the Cytochrome C/Apaf-1/caspase-9-containing apoptosome complex. [101] Thus, a protein cascade is activated that also leads to DNase activation, DNA nicking, and the induction of cell suicide.

Early phenomena of DNA damage are double strand breaks (DSBs) or single strand breaks (SSBs). If DSBs are generated, an ATM protein complex (ataxia teleangiectasia mutated) is activated. Its purpose is to phosphorylate cell cycle regulating enzymes, like checkpoint kinase 2 (Chk2) and p53, a major tumor suppressor protein, to stop the cell cycle and provide time for DNA repair; if the DNA damage is too extensive, the cell goes to apoptosis. A very sensitive marker to demonstrate DSBs is the detection of the phosphorylated histone H2, especially p-H2AX. As soon as the DNA is damaged, the chromatin structure is changed and ATM and ATM- and Rad3-related (ATR) kinases are activated and accomplish the phosphorylation of the histone, as well as of p53, Chk-2 and other cell cycle regulating proteins. [8] [102]

If DNA cannot be repaired and the mechanism of apoptosis is disturbed, because of further DNA mutations, the resistance to apoptosis would allow the persistence of cells with DNA damage and mutations and thereby promote cancer formation. It has to be found out which of the above described changes can be found in Barrett's Esophagus and if there is a resistance to apoptosis as well as, finally, which role this plays in the progression to esophageal adenocarcinoma.

2.6. Aims

The aim of this study is to determine the effects of two relevant bile acids, Deoxycholic Acid and Urodeoxycholic Acid on metaplastic Barretts' cell lines and Barretts' patients' tissues.

Special attention will be focused on molecular mechanisms initiated by exposure to the different kinds of bile acids and the possible induction of DNA damage.

Further on, this study explores additional effects of DCA and UDCA on activation of cell survival pathway proteins and the generation of apoptosis resistant cells.

It will be looked at the possible role of the bile acids in contribution to the malignant process by causing the induction of DNA damage without the initiation of growth arrest or apoptosis of the metaplastic Barrett's epithelium.

Additionally, it is tried to figure out the different behavior of cells after exposure to the hydrophilic or hydrophobic acids and it will even be tried to illustrate possible positive and cytoprotective effects the UDCA might have in BE.

3. MATERIALS AND METHODS

All studies were approved by the institutional review board of the Dallas VA Medical Center at Dallas/ Tx (USA). Experiments were performed according to the guidelines of the Declaration of Helsinki from 1964 for Biomedical Research and its up-dated versions.

3.1. Cell Culture

Barrett's Cells: Biopsies of non-dysplastic Barrett's mucosa were obtained from patients with Barrett's Esophagus (average age 64.5 ± 2.8 SEM years, 10 patients: 9 male, 1 female, all on PPI medication). The non-transformed cell lines (BarT and BarB10T) created from adequate biopsies, were telomerase-immortalized by infecting them with a retroviral construct that contained the catalytic component of telomerase expression (hTERT), as described by Jaiswal *et al*. [42] [67] [76]

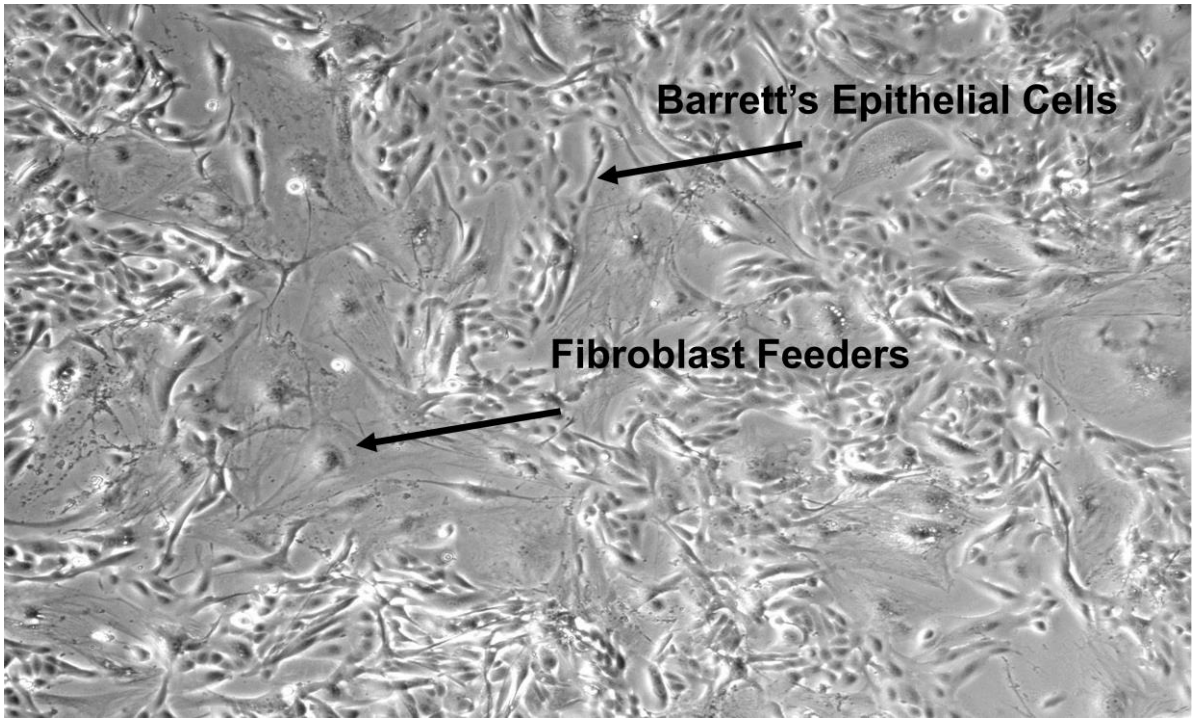


Figure 5: ***Barrett's Epithelial Cells in co-culture with Fibroblast Feeder Layer Cells***. The created Barrett's cell line is shown growing in co-cultivation with Fibroblast cells.

Figure 5 shows the created Barrett's cell line in co- culture with fibroblasts. The long and thin morphology of the "Barrett's cells" draws a clear dividing line between them and the more spherical and wider fibroblasts. All pictures of cellular morphology were documented by using the Metamorph imaging system (Universal Imaging Cooperation, Downingtown/ PA, USA).

Also, Barrett's tissues from patients with Barrett's Esophagus, all male gender, who exhibited long-segment (> 3 cm) of Barrett's Esophagus, involving the distal esophagus, were used (with generous help of Dr. Stuart J. Spechler, VA Medical Center Dallas/TX, USA). GERD patients with BE, scheduled for elective endoscopy with clinical indication, were asked whether some additional biopsies of the Barrett's mucosa could be taken. During the routine endoscopies, biopsies were taken with a jumbo biopsy forceps (Olympus FB-50K-1; Olympus, Tokyo, Japan). During endoscopy, a perfusion catheter was passed through the channel of the endoscope and Barrett's mucosa was perfused for 5 minutes with either 250 μM DCA or UDCA. Tissue samples were snap frozen immediately and stored at -80 °C.

Cells were cultured in KGM-2, Keratinocyte growth media (Cambrex Biologicals, East Rutherford/ NJ, USA) and maintained in monolayer culture at 37 °C in humidified air with 5 % CO_2. They were co-cultured with a fibroblast feeder layer cells (Swiss 3T3 cells). For individual experiments, cells were seeded into collagen-IV-coated wells (BD Biosciences, San Jose/ CA, USA).

Cell Culture: *XSG-Medium* has been the nutrition and growth medium for the fibroblast feeder layer cells. It consists of 10 % Cosmic Calf Serum (CCS) (Hydoclone Laboratories, East Greenbush/ NY, USY) and X-Medium (DMEM (Life Technologies, Grand Island/ NY, USA), Medium 199 (Life Technologies, Grand Island/ NY, USA), Sodium pyruvate (Sigma, St. Louis/ MO, USA), Sodium bicarbonate (Sigma, St. Louis/ MO, USA), adjusted to pH of 7.2-7.3); 25 μg/ml Gentamicin (Sigma, St. Louis/ MO, USA), has been added. $1x10^6$ feeder layer cells (Swiss 3T3 Cells, ATCC, Manassas/ VA, USA) were placed on a 1000mm-dish with 10 ml XSG- Medium, $1,5x10^5$ cells and 5 ml XSG for 600 mm-dishes and $0,7x10^5$ cells and 1 ml XSG for 24 well plates and kept at 37 °C in humidified air with 5 % CO_2 for 2-4 hours. Before their use as feeder cells, the fibroblasts were treated with 10 μg/ml Mitomycin C (Sigma, St. Louis/ MO, USA) to stem proliferation. Hereto, XSG-

Medium was removed and the dishes were washed with Solution A (destilled water, Hepes buffer, Phenol Red, Sodium Hydroxide (10N) (pH, 7.0), Glucose, Potassium Chloride, Sodium Chloride, Sodium Phosphate, all brought to pH 7.5) twice to remove all detaching cells and all remaining traces of XSG medium.

Barrett's cells were placed on the dishes ($1x10^6$ for 1 000 mm-dishes, $1,5x10^5$ for 600 mm-dishes and $0,7x10^5$ for 24-well plates) and cultured in *KGM-2 Medium* in co-culture with the Fibroblasts. KBM-2 (Keratinocyte basal media, Clonetics, San Diego/ CA, USA) has been supplemented with 5 % FBS (Atlanta Biologicals, Lawrenceville / GA, USA , 400 ng/ml Hydrocortisone (Sigma, St. Louis/ MO, USA), 20 ng/ml EGF (Sigma, St. Louis/ MO, USA), 0,1 mM Cholera Toxin (Calbiochem, Darmstadt, Germany), 200 μg/ml Adenine (Sigma, St. Louis/ MO, USA), 100 U/ml Penicillin-Streptomycin (Life Technology, Grand Island / NY, USA), 5 μg/ml Insulin (Sigma, St. Louis/ MO, USA), 5 μg/ml Transferrin (Life Technology, Grand Island / NY, USA) and 70 μg/ml BPE (Hammond Cell Tech., Windsor/ CA, USA). For some experiments, serum free medium (Reduced medium) was needed to grow Barrett's cells, consisting of KBM-2 with all ingredients listed above with exception of FBS. Medium was changed regularly every second day. After cells were at 80-90 % confluence, cells were spit and subcultured. The dishes with the cells were rinsed with Solution A and washed with 10 ml of EDTA 2 times to remove the feeder cells. Then, dishes were washed 3 times with Solution A. 0.5-2 ml of 0.05 % trypsin was added to detach cells from dish and cells were placed for 5 min in the incubator. Cells were collected with 5 ml of their medium and 250 μg/ml of trypsin inhibitor (Sigma, St. Louis/ MO, USA). One fiftieth of this solution was mixed with 1 ml saline solution and cells were counted in a Z1 Particle Counter (Z1 Cell Counter; Beckman, Coulter; Chicago/ IL, USA). Cell morphology was documented by using the Metamorph imaging system (Universal Imaging Cooperation, Downingtown/ PA, USA).

3.2. Cell Treatment

Bile Acid Exposure: For individual experiments, Barrett's cell lines were placed on collagen-IV-coated dishes; $1x10^6$ cells on 100 mm-dishes, $0,7x10^6$ on 60 mm-dishes and $1,5x10^5$ on 24-well plates. The subconfluent cells were incubated in their growth media, KGM-2 and for some experiments in the minimal reduced media (serum-free) for 48 hours or until they were 80 % confluent. Then, Barrett's cell (Bar-T and Bar-10T) were treated with either 250 μM or UDCA (DCA and UDCA from Sigma, St. Louis/ MO, USA). For cell treatment, a 100x solution had been made of 414.455 g/mol Sodium Deoxycholate, solved in PBS (phosphate-bufferd saline) or a 100x stock solution of DCA (MW, 414) solved in water. Shortly before use, DCA had been thawed and diluted to a 1x solution in KGM-2 or KBM-2. The cold DCA solution was added and left on the plates for either 5, 10 or 30 min and immediately removed, or fresh media was put on the plates for additional 24 hours. All experiments were done in duplicates and an untreated dish was used as a control. The exposure time of 5 min was chosen to simulate a physiological episode of reflux in GERD patients. After media were removed, dishes were washed with Solution A and 200 μl ice cold cell lysis buffer (Cell Signaling Technology Inc., Danvers/ MA, USA) was added on the plates and incubated for 5 min on ice. 1x cell lysis buffer contained of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM Sodium Pyrophosphate, 1 mM beta-Glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml Leupeptin (Cell Signaling Technology Inc., Danvers/ MA, USA). The buffer was diluted in Milli Q water; 0.5 mM PMSF (phenylmethylsulfonyl fluoride) was added in a 1.5 ml microcentrifuge tube.

Then, cell lysates were collected immediately, sonicated and centrifuged for 10 min at 4 ºC and 14000 g. Supernatant was removed and used to measure the protein concentration.

3.3. Protein Concentration Detection

For detecting the protein concentration, the BCA-200 Protein Assay kit (Pierce, Rockford/ IL, USA) was used. A working reagent made of 50 parts BCA A Reagent and 1 part BCA B Reagent, was produced. Reagent A contained Sodium Carbonate, Sodium Bicarbonate, Bicinchroninic Acid, Sodium Tartrate in 0.1 M Sodium Hydroxide; Reagent B contained 4 % Cupric Sulfate. Then, 2 ml working reagent and a 20x protein sample dilution (0.05 ml protein and 0.95 ml H_2O) were mixed and incubated in a 37 °C-warm water bath for 30 min. After incubation, tubes were cooled down to room temperature and the protein concentration was measured in an UV spectrometer (Spectronic genesys5/ Spectronic instruments, Garforth Leeds, UK) with an OD at 562 nm. The standard curve was calculated by blotting the 562 nm measurement for BSA (bovine serum albumin) against its concentration in μg/ml. Table 1 shows the mixture ratio for the Pierce standard protein, which consists of BSA, H_2O and lysis buffer.

Table 1: ***Mixing ratios*** for producing the Pierce standard protein are shown in this table. The protein mixture consists of BSA, H_2O and lysis buffer

Standard Protein Concentration	**BSA /μl**	**H_2O/μl**	**Lysis buffer/μl**
0	0	3232	170
5	86	3146	170
10	170	3060	170
15	258	2974	170
20	340	2890	170
25	430	2801	170
30	510	2720	170

.

3.4. Western Blot

Expression of p-H2AX, p-p65, p-IκB, Bcl-2, Bax and Tubulin were detected by using Western blots. Proteins were separated based on their weight and electrical properties.

Gel preparation for electrophoresis: Resolving gels were made of Milli Q water, 29 % Bis/Acryamid, 2 M Tris-HCl pH 8.9, 200 mM EDTA, 10 % SDS, 10 % APS and TEMED. 8 ml of that mixture were filled between two glasses and the gel was consolidated for 20 min. For detection of p-H2AX and Tubulin, 12 % gels were used, for p-p65 and p-I-κB 10 % gels and for Bax and Bcl-2 15 % gels were produced.
For the upper 4 % stacking gel, Milli Q water, 29 % Bis/Acryamid, 1 M Tris-HCl pH 6.8, 200 mM EDTA, 10 % SDS, 10 % APS and TEMED were mixed and filled between the glasses.

Electrophoresis: Proteins were heated at 100 °C for 10 min and diluted with 4x DYN and balanced with 1x DYN to get a final amount of 50 µl, the limit of content for the gel chambers. Electrophoresis chamber was filled up with 1x TGS running buffer. 6 µl marker (Precision Plus Protein Standards, Bio-Rad Laboratories, Hercules/ CA, USA) running along with the protein mixtures were filled in the gel chambers. Electrophoresis was running for 15 min at 100 V and for 75 min at 140 V.

Transfer: After electrophoresis, the proteins were transferred from the SDS gel to a nitrocellulose membrane. Proteins were transferred after running for 1 hour at 100 V with TG Transfer buffer (TG, 20 % methanol, H_20). The nitrocellulose membranes were washed in PBST, membranes for I-κB were washed in TBST and put into blocking solution 5 % carnation non fat milk solved in PBST (or TBST for I-κB) for 1 hour at room temperature. Membranes were washed again 3 times for 5 min with BPST / TBST. After washing, membranes were incubated with antibodies.

Antibody incubation: Western blot membranes were then incubated with a 1:1000 dilution polyclonal rabbit–anti-p-H2AX (Ser139), anti-phospho-p65 (Ser536), rabbit monoclonal anti Bcl-2 or 1:500 dilution of mouse anti-human phospho-I-κBα (Ser32/36) (Cell Signaling Inc., Beverly/ MA, USA) or 1:3000 mouse-anti-β Tubulin (Sigma, St. Louis/ MO, USA). A 1:3000 solution was also used for detecting

t-p65 (rabbit AB) (Cell Signaling Inc., Beverly/ MA, USA). All blots were performed in duplicates.

On the following day, the membranes were washed 3 times with PBST, I-κB in TBST, for 5 min.

For 1 h at room temperature, they were incubated with the secondary antibody: Mouse–anti rabbit antibody (Cell Signaling Inc., Beverly/ MA, USA) and anti-mouse Antibody (Santa Cruz Biotechnologies, Santa Cruz/ CA, USA), for β Tubulin, t-p65 and p-I-κB.

After this, membranes were washed again 3 times with PBST/TBST for 5 min.

Development of the Blots: For detecting t-p65 and Tubulin, Pierce ECL Western Blotting Substrate (Pierce, Rockford/ IL, USA) was used. Same amounts of Detection Reagent 1 (Peroxide Solution) and Detection Reagent 2 (Luminol Enhancer Solution) were mixed and added on the membrane for 2 min.

For detecting the other proteins, described above, SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford/ IL, USA) was used. Luminol/ Enhancer Solution and Stable Peroxide Buffer were mixed and put on the membrane for 4 min. Membranes were then stripped using Restore Stripping Buffer (Pierce, Rockford/ IL, USA) and re-probed.

3.5. Detection Of Apoptosis- ELISA

Apoptosis rates were detected using the Cell Death Detection ELISA^{Plus} kit (Roche Applied Science, Indianapolis/ IN, USA).

1,2x10^5 cells were plated in one well on a collagen-IV-coated 24-well plate. Cells were incubated for 24 hours in 1 ml of their full nutrition medium, maintained in monolayer culture at 37 °C in humidified air with 5 % CO_2. To measure cell death rates in the minimal media, reduced media had been given on the wells and cells were also incubated over night for 12 hours.

The next day, media was removed and cells were treated with either 250 μM DCA or UDCA. After 5-, 10- and 30- min exposures, plates were rinsed with Solution A and fresh media was given on the plates for 24 or 48 hours. All experiments were done in 2 duplicates and repeated 3 times. Figure 6 shows the design of an ELISA plate used in individual experiments. Untreated cells were used as a control.

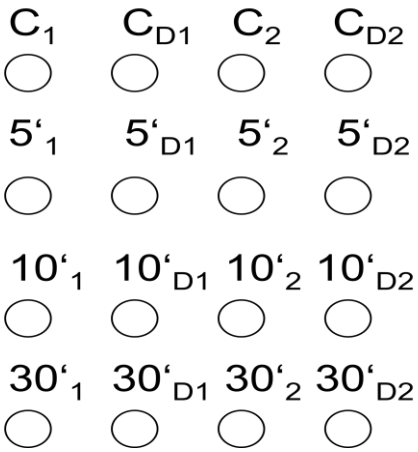


Figure 6: ***Experimental design of an ELISA plate.*** In each case duplicate 1 and 2 were treated. Experiment has been done in double duplicates D_1 and D_2. All cells were treated for 5, 10 and 30 min, respectively. Four wells were used for the untreated control C.

The supernatant contained necrotic cells and was removed and thrown away. Then, the plate was washed with 1x PBS. PBS was removed and cells were incubated with 500 µl cell lysis buffer at room temperature for 30 min.

20 µl of each sample had been extracted and were given on a Streptavidin-coated microplate (MP) and 80 µl Immuno-Reagent, contained of Incubation buffer, Anti-histone-biotin and Anti-DNA-POD, was added and samples were covered with a foil and incubated on a MP shaker for 2 hours at room temperature. The mouse antibody anti-Histone biotin was used for binding the histone and capturing to the micro plate on Streptavidin. ABTS tablets were solved in substrate buffer.

Mouse Anti-DNA-POD Antibody was conjugated with a Peroxidase. It bound components of the DNA and was accountable for the color reaction of this kit, as well as ABTS. For positive control, a DNA histone complex was used.

Cells were washed 3 times with 300 µl Incubation buffer and 100 µl ABTS Start Solution was added; the plate was shook again for 10-20 min until color developing was sufficient. Once the color had turned to blue, 100 µl ABTS stop solution were added and tissues were analyzed with Multimode Analysis Beckman-Coulter Software (Beckman-Coulter, Brea/ CA, USA). The samples were measured at 405 nm against ABST and ABST stop solution as a blank. Another series of measurements was made with a reference wavelength of 490 nm. Values were averaged, background (incubation buffer and ABTS stop solution) subtracted and

absorbance at 405 nm minus absorbance at 490 nm were blotted against time of cell treatment.

3.6. Cell Count

For counting cells, $0{,}75x10^6$ cells were placed on a collagen-coated 600mm-dish. Cells were grown in KGM-2 medium for 48 hours, treated with either DCA or UDCA for 5, 10 or 30 min, the medium was removed and new, fresh KGM-2 medium was added on the plates, which were then incubated for 24 hours in 37 °C and 5 % CO_2.

Then, KGM-2 was sucked up and plates were washed with Solution A. 1 ml 0.5 % trypsin- EDTA (1x) was added on the dish to detach cells from the bottom and plates were incubated again for 5 min.

The detached cells were collected with trypsin and placed into a tube, filled with 2 ml KGM-2. 140 µl of that mixture were added to 1 ml saline (pH, 7.0-7.2). Cells were finally counted with the Coulter Z1 Particle Counter (Z1 Cell Counter; Beckman, Coulter, Chicago/ IL, USA) based on a flow cytometry technique.

3.7. Statistical Analysis

The data were collected from at least two independent experiments, which were always done in duplicates.

Quantitative data are expressed as the mean plus (±) the standard error of the mean (SEM). Statistical analysis was performed using ANOVA and the Student-Newman-Keuls multiple-comparison test with the Instat for Windows statistical software package (GraphPad Software, San Diego/ CA, USA). *P* values of < 0.05 were considered significant for all analyses.

4. RESULTS

4.1. Effects Of Deoxycholic Acid In Barrett's Esophagus

4.1.1. Effect Of DCA On DNA- Damage In BAR-T Cells Is Not Consistent On Non-Collagen-Coated Dishes

Barrett's cell lines were treated on non collagen-coated dishes with Deoxycholic Acid, solved in Sodium-PBS or in ethanol and diluted in KGM-2 at a physiological concentration of 250 μM. The cells were exposed to the DCA solution for 5 min and collected immediately (5 min), or 1 hour later (5 min 1 hr), 4 hours later (5 min 4 hrs) or 24 hours later (5 min 24 hrs). Only DCA that was dissolved in Sodium-PBS could induce a clear effect (Fig. 7). By looking at the phosphorylated core protein H2AX, an increase in expression could be observed starting immediately after treatment. The protein level was still elevated 24 hrs after treatment. In contrast to that, the p-H2AX level for Bar-T cells already went down 24 hrs after treatment with Ethanol-DCA.

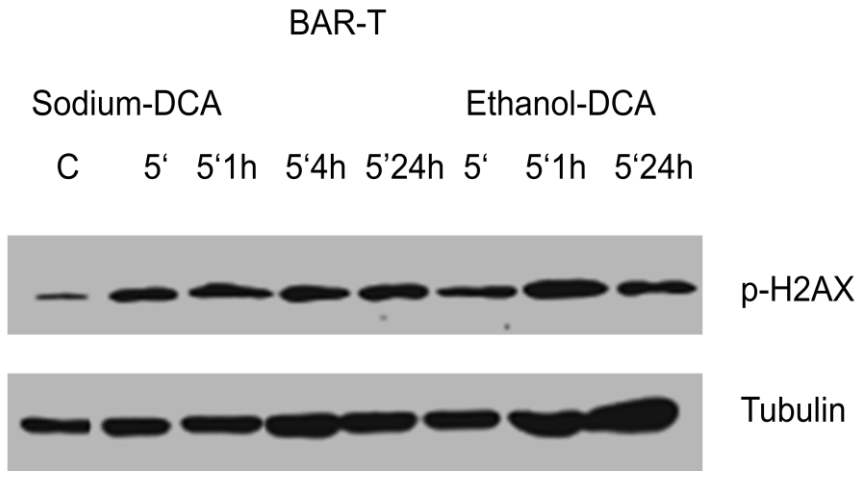


Figure 7*: Western blot for Sodium- and Ethanol-DCA.* BarT cells were treated for 5 min with S-DCA or E-DCA and collected immediately, or 1 and 24 hrs after treatment, S-DCA additionally 4 hrs after treatment. An increase in expression could be detected either for p-H2AX compared to the untreated control. p-H2AX with S-DCA treatment showed a constantly elevated level that remained elevated until 24 hrs after treatment, whereas it already decreased after E-DCA exposure at the same point of time. Tubulin was used as house-keeping gene as also shown in Fig. 8

Based on these data, Sodium-DCA was used for all further experiments, because it could show clear effects that persisted till 24 hours after treatment for both BAR-T cell lines.

After figuring out the optimal solution medium for DCA, the experiment was modified. To simulate more physiological conditions, the exposure time of DCA to the cells was changed. In Barrett's patients a period of gastric reflux lasts about 5 to 10 min. To keep up with this condition, in all further experiments cells were treated for 5, 10 or 30 min with DCA and collected immediately or 24 hours after that exposure.

Figure 8 shows the Western blots for phosphorylated histone H2AX. The effect of DCA on DNA damage, symbolized through phosphorylation of the histone in Barrett's cells was not consistent on non-collagen-coated dishes.

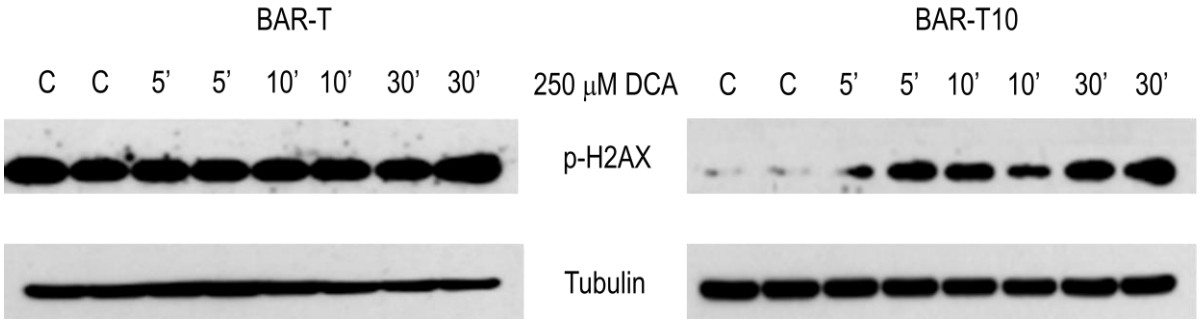


Figure 8: ***Western blot for Sodium-DCA treatment on non-collagen-coated dishes***. BarT cells did not show an increase of p-H2AX and hereby of DNA damage. The protein level seemed to be steady to all time points. Bar-T10 cells showed an increase of p-H2AX level, beginning immediately after treatment. The highest expression could be observed after an exposure time of 30 min.

Bar-T cells did not show a change in p-H2AX expression after treatment on non-collagen-coated-dishes. In contrast to that, Bar-T10 cells were presenting a different behavior. Compared to the untreated control, level of p-H2AX expression was clearly increased and reached its highest point at an exposure time of 30 min. The longer the DCA had been presented to the cells, the higher was the DNA damage caused by DCA.

Because the two metaplastic cell lines had shown an inconstant reaction to DCA, conditions were changed. In prior experiments, it could be shown that the more sensitive Bar-T cells could grow better and without showing signs of cell stress on collagen-IV-coated dishes, which also present a more physiological environment; thus cells were cultured on collagen-coated dishes for all further experiments.

4.1.2. DNA Damage Is Induced Immediately Following DCA Exposure In Barrett's Cells As Well As Activation Of The NF-κB Pathway

After DCA exposure to both cell lines, Bar-T and Bar-T10, for 5, 10 and 30 min, which were treated in duplicates, p-H2AX had been focused. Interestingly, DNA damage could be detected in both cell lines. Phosphorylation of histones was induced immediately after DCA treatment and exhibited the highest level after 30-min exposure (Fig. 9). This effect is consistent for the two Barrett's lines on collagen-coated dishes.

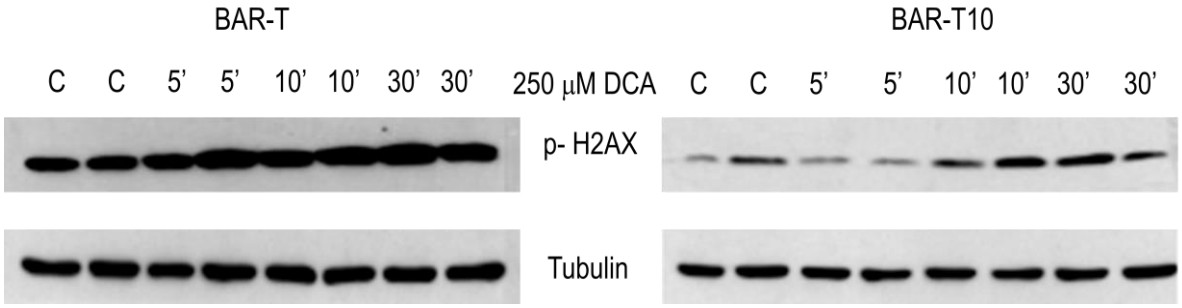


Figure 9: ***Western blot for Sodium-DCA treatment on collagen-coated dishes***. DNA was damaged in Bar-T and Bar-T10 cells. It was induced immediately following DCA exposure and reached its highest point after a 30-min treatment.

Further effects of the DCA were figured out by looking at the cell survival pathway. After treatment, NF-κB pathway was activated following immediately DCA exposure. The phosphorylation of I-κB started, as well as the H2AX phosphorylation, directly after DCA treatment. The longer the cells had been treated, the higher was the following expression level of that protein, with a peak after a 30-min treatment. So, a time dependency could be seen. Also p-p65 increased in the same way. That there was no new synthesis of that protein could be observed regarding the total amount of p65. The band illustrated a constant activation level; there was no increase (Fig. 10).

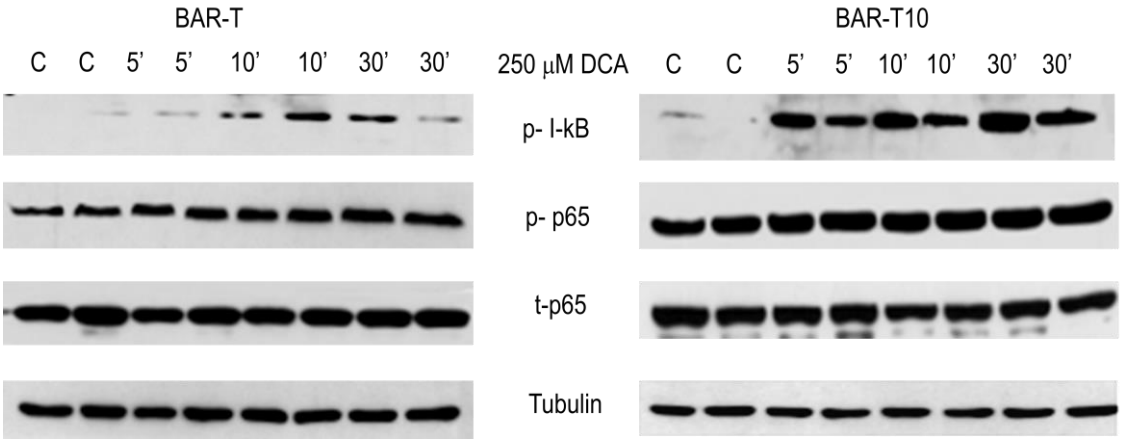


Figure 10: ***Western blot for DCA treatment on collagen-coated dishes***. DCA exposure influenced the NF-κB cell survival pathway. I-κB was phosphorylated and activated immediately following DCA exposure in both cell lines. Also p-p65 levels increased. The expression of t-p65 remained constant, which means that there was no indication of new-synthesis of that protein.

4.1.3. DCA-Induced DNA Damage Returned To Baseline After 24 hrs

After treating the cell lines again for 5, 10 and 30 min in duplicates, cells were incubated in KGM-2 and collected after 24 hours.

The p-H2AX expression went down to the baseline level, which was presented by the untreated control, after 5- and 10-min DCA exposure and after 24 hours. For cells that were treated for 30 min, there was still an elevation (Fig. 11), but clearly lower than the immediately collected cells showed (Fig. 9). Barrett's cells were able to repair their DNA damage within 24 hours.

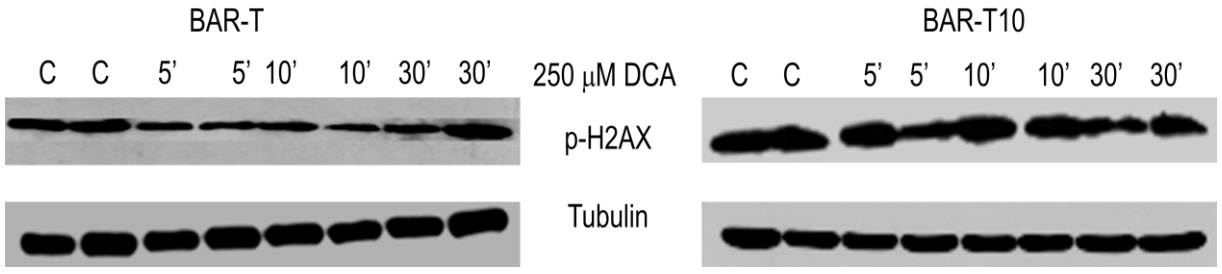


Figure 11: ***Western blot for Barrett's Cells on collagen-coated dishes 24 hours after DCA treatment.*** Expression of p-H2AX went down to a baseline level 24 hours after a 5- and 10-min treatment. Cells that were treated with DCA for 30 min were still showing a moderate elevated band after 30 min and 24 hrs, meaning DNA damage could be repaired.

DCA-induced NF-κB pathway protein activity also returned to baseline after 24 hrs. The protein expression of I-κB and p-p65 went back to baseline and showed no differences in expression compared to the untreated control. After 24 hours the protein level seemed to be steady again (Fig. 12).

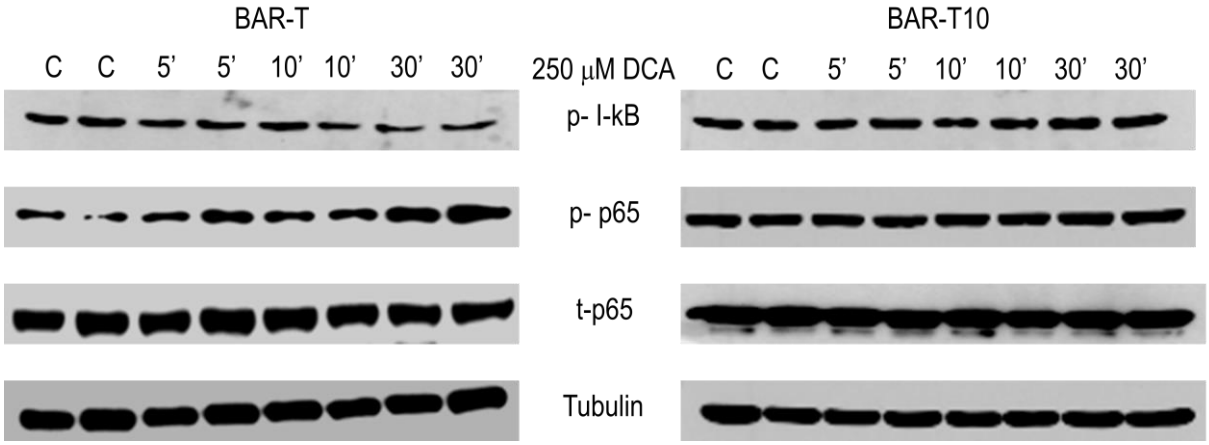


Figure 12: ***Western blot for Barrett's Cells on collagen-coated dishes 24 hours after DCA treatment.*** DCA-induced NF-κB protein activity returned to baseline after 24hs. There was no elevated band either for p- I-κB, or for p-p65 in Bar-T and Bar-T10 cell lines. There was no newly synthesized p-65; total-p65 stayed constant.

4.1.4. Barrett's Cells Did Not Change Their Morphology 24 Hours After DCA Treatment

The typical apoptic signs like cell shrinkage and membrane blebbing could not be observed for the Barrett's cell lines 24 hours after treatment. Cells were looking healthy. Dead and floating cells could not be seen; they did not change their morphology (Fig. 13 A, B).

For comparison, cells appeared small and shrunken, phenotypic changes suggestive of apoptosis, after they had been treated with 200 J/m^2 UV-B irradiation, a well known inducer of apoptosis (Fig. 13 C). [99]

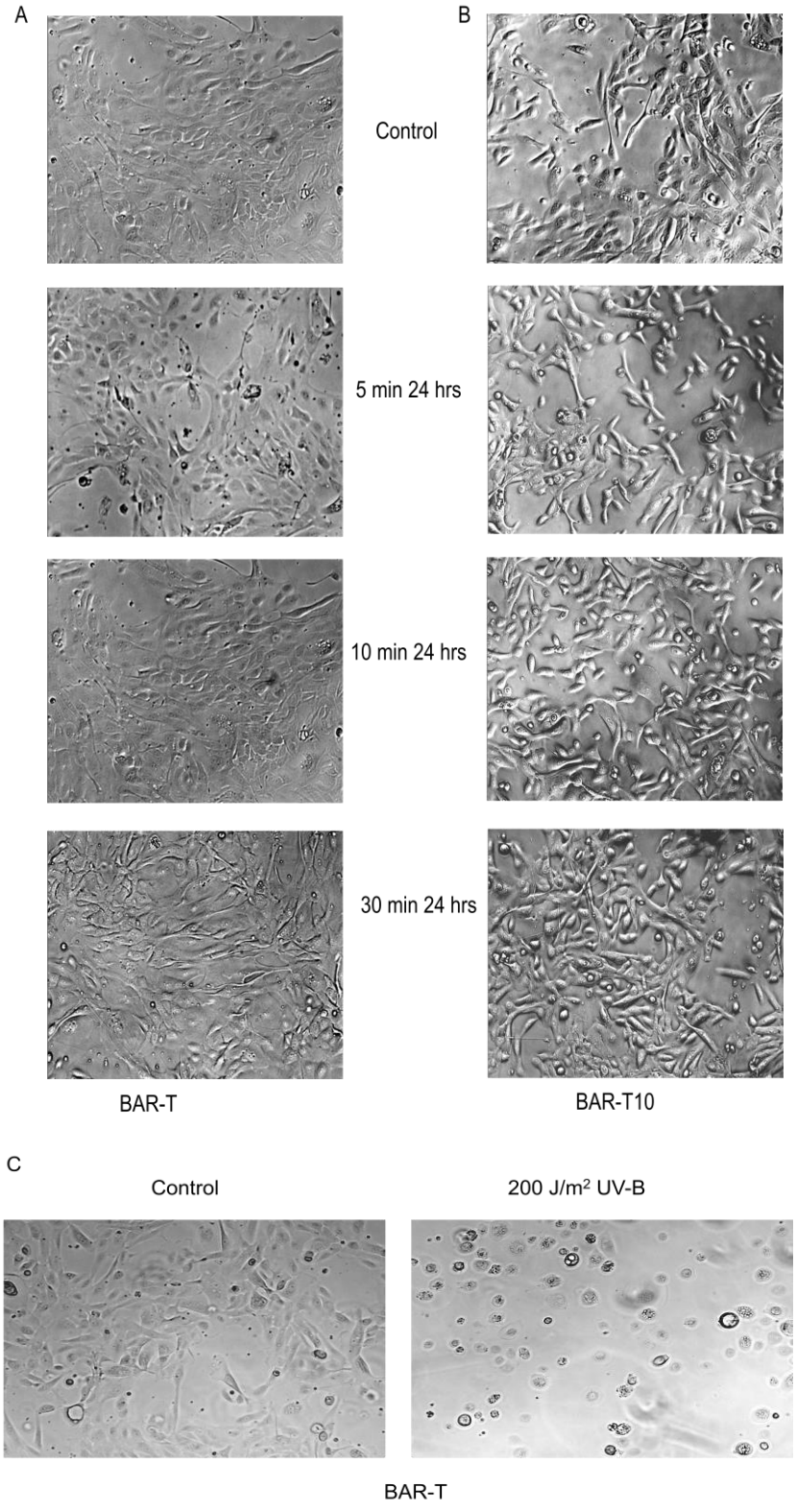


Figure 13: ***Microscopic pictures of Bar-T and Bar-T10 cell lines 24 hours after DCA treatment.*** Bar-T **(A)** and Bar-T10 **(B)** cell lines were looking healthy; there were no floating or dead cells and cell morphology did not change at any time point 24 hours after treatment. Furthermore, cells could grow and proliferate. **(C)** Bar-T cell line as a representative example was treated with 200 J/m^2 UV-B light. UV-B light of that intensity is a well know inducer of DNA damage and apoptosis. Compared to the control or to all other cell lines in **A** and **B**, Bar-T cells looked small and shrunken. A lot of floating and dead cells could be detected. They demonstrated the typical signs of apoptosis.

4.1.5. DCA Did Not Induce Apoptosis In Barrett's Cell Lines And Did Not Change Number Of Cells

To get biological data about the growth and death rates of the BAR-T cells, cell counting and cell death ELISA were performed. Results of these experiments confirmed the observations, ascertained by the microscope (Fig. 13 A/B).

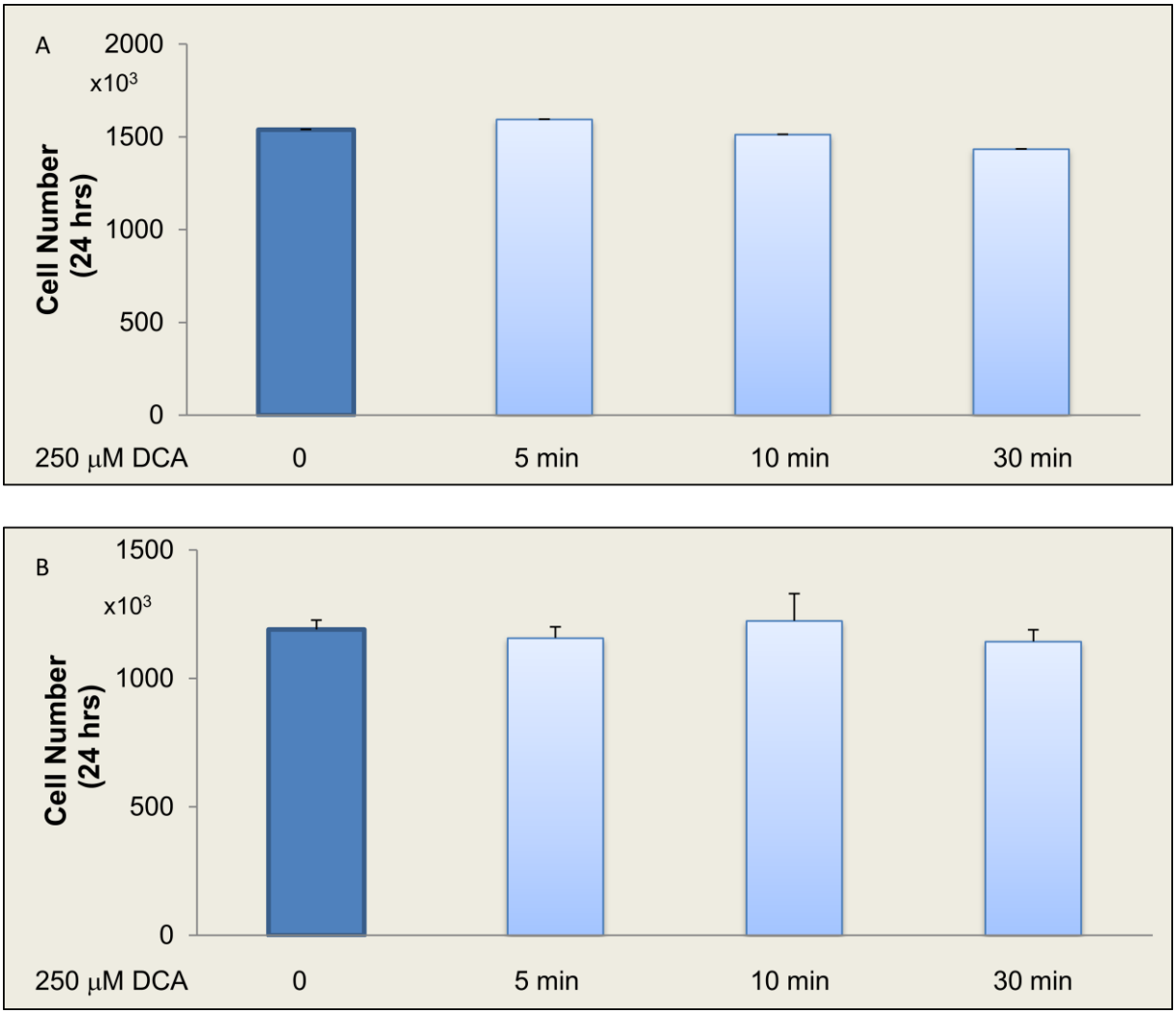


Figure 14: ***Cell Count.*** **A**: Number of cells of *Bar-T* cell line. The cell number stayed almost stable without big variations. The total number remained around $1{,}5x10^6$. No significant changes could be found ($p > 0.05$). **B**: Number of cells of *Bar-T10* cell line. Cell number was stable; there were no big differences in cell number and no significant changes ($p > 0.05$). The bar graphs depict the mean ± SEM.

Cells were grown to 65 % confluence and placed in KGM-2 media overnight after treatment for 5, 10 and 30 min with DCA. Cells were counted the next day. No increase or decrease in cell number could be determined (Fig. 14). Cell number remained stable for all time points of treatment and did not result in significant changes ($p > 0.05$).

The cell death ELISA was performed 24 hours (Fig. 15) after treatment of Bar-T and Bar-T10 cells and 48 hours (Fig. 16) after DCA exposure of Bar-T cell lines.

Interestingly, no significant differences could be detected, that would show that cells were dying. That also confirms the prior data on cell morphology.

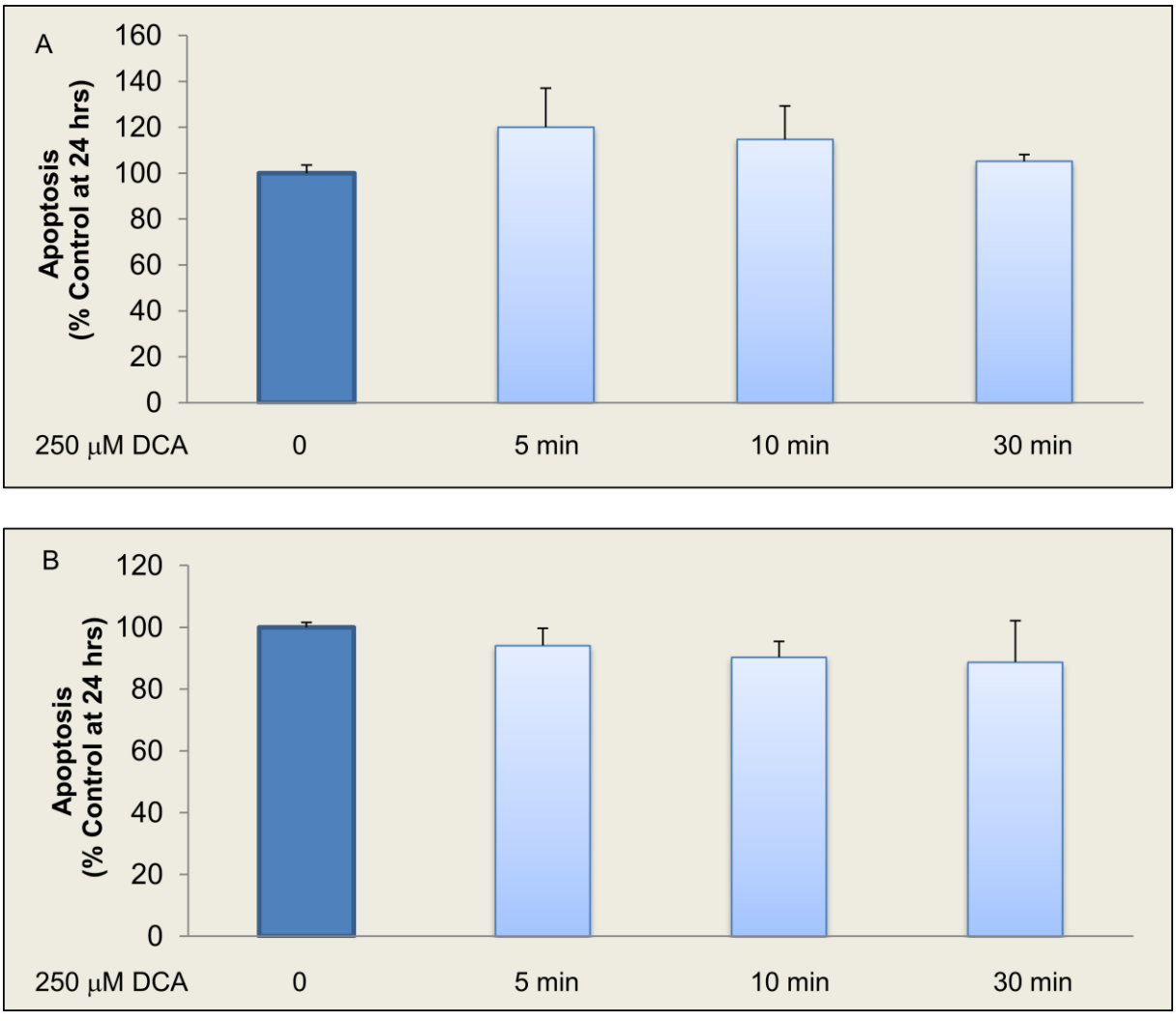


Figure 15*: Cell Death ELISA* **A**: The figure shows the calculated rate of apoptosis in percent for Bar-T. Cells are not dying; there are no significant changes ($p > 0.05$). **B**: Rate of apoptosis for Bar-T10 cells. It also cannot be observed significant changes in number of apoptotic Bar-T10 cells. Interestingly, cell death even seems to decrease and runs tightly under the baseline level of the untreated control. The bar graphs depict the mean ± SEM.

Even 48 hours after DCA exposure, there were no signs for an apoptotic activity (Fig. 16). They were still growing, not dying. Summarized, the cells recovered from the bile acid exposure.

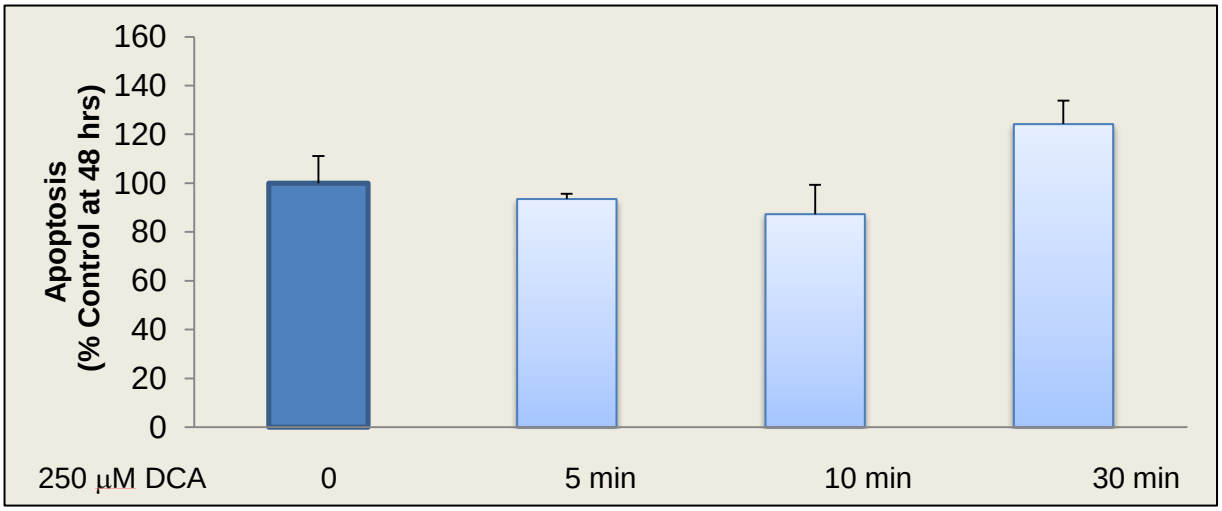


Figure 16: ***Cell Death ELISA for Bar-T cells 48 hours after treatment.*** The picture is showing the rate of apoptosis for Bar-T cells after 48 hours. Cells were still not dying. Their apoptotic rates even run under the baseline level. Cells completely recovered from the bile acid irritation. No significant data could be found for apoptosis ($p > 0.05$). The bar graphs depict the mean ± SEM.

Therefore, it can be concluded that Barrett's cells may resist apoptosis in the face of bile-acid induced DNA damage through activation of NF-κB.

4.1.6. The Decrease Of The Pro-Apoptotic Protein Bax And The Increase Of The Anti-Apoptotic Protein Bcl-2 Might Emphasize The Imbalance Between Cell Death And Cell Survival

When NF-κB binds to the specific promoters on the DNA strand, anti-apoptotic genes are read and proteins are expressed that allow the cells to resist apoptosis. Bcl-2 is one of the target proteins, which are expressed after NF-κB activation.
Bar-T cell line has been exposed to 250 μM DCA again and a Western blot for the anti-apoptotic protein Bcl-2 was made (Fig. 17). The bands for Bcl-2 already began to increase after a 5-min treatment and reached their highest expression after the 30-min exposure. The pro-apoptotic protein Bax showed, in contrast, an opposing behavior. Expression went down. So, there was an imbalance between the pro- and anti-apoptotic proteins, with an overexpression on the anti-apoptotic side.

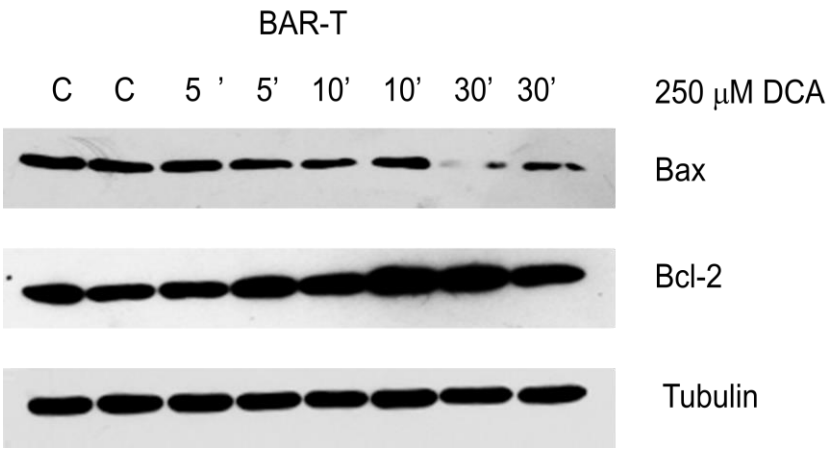


Figure 17: ***Western blot for the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax.*** An overexpression of the anti-apoptotic protein Bcl-2 could be found in Bar-T cell line and a decrease of the pro-apoptotic Bax following exposure to DCA. Protein expression indicated an imbalance between cell death and survival.

4.2. Effects Of Urodeoxycholic Acid In Barrett's Esophagus

4.2.1. UDCA Does Not Induce DNA Damage Or NF-κB Pathway Activation In Barrett's Cell Lines

The hydrophilic bile acid UDCA is in clinically use to treat chronic liver diseases and has been shown to reduce the frequency of colon cancer in patients who have ulcerative colitis. Effects of UDCA on "Barrett's cells" are extensively unexplored.

Barrett's cell lines were treated with 250 μM Sodium-UDCA, following the same conditions and background used in all prior experiments to find out in which way Barrett's cells react after UDCA exposure.

Cell lines were treated in duplicates for 5, 10 and 30 min on collagen-coated dishes and collected immediately after exposure and Western blots were performed.

Interestingly, cells did not show an increase in phosphorylation of H2AX (Fig. 18). Protein expression remained stable and constant for all time points. So, UDCA was not able to induce DNA damage in the Barrett's cell lines.

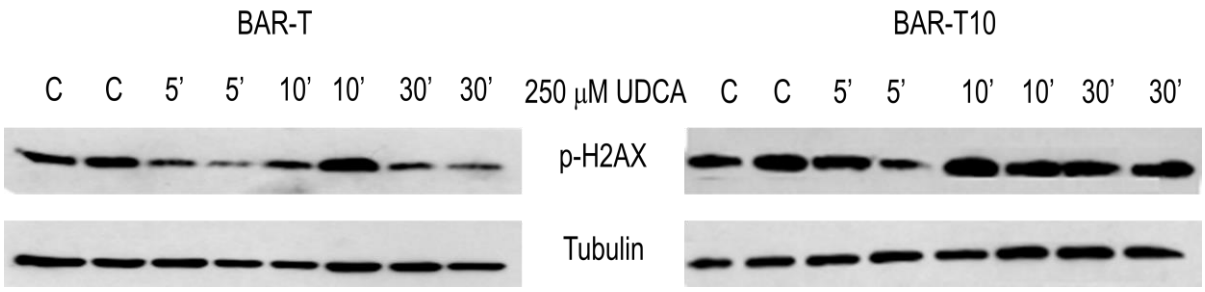


Figure 18: ***Western blot for Bar-T and Bar-T10 cell lines collected immediately after UDCA exposure.*** UDCA was not able to induce an increase of p-H2AX and did not cause DNA damage. Protein expression remained stable and constant for all time points.

Furthermore, UDCA did not influence the phosphorylation of p65, nor its new-synthesis. Bands for p-p65 did not vary at any time points. The NF-κB pathway could not be activated by UDCA; p-I-κB could not be detected (Fig. 19).

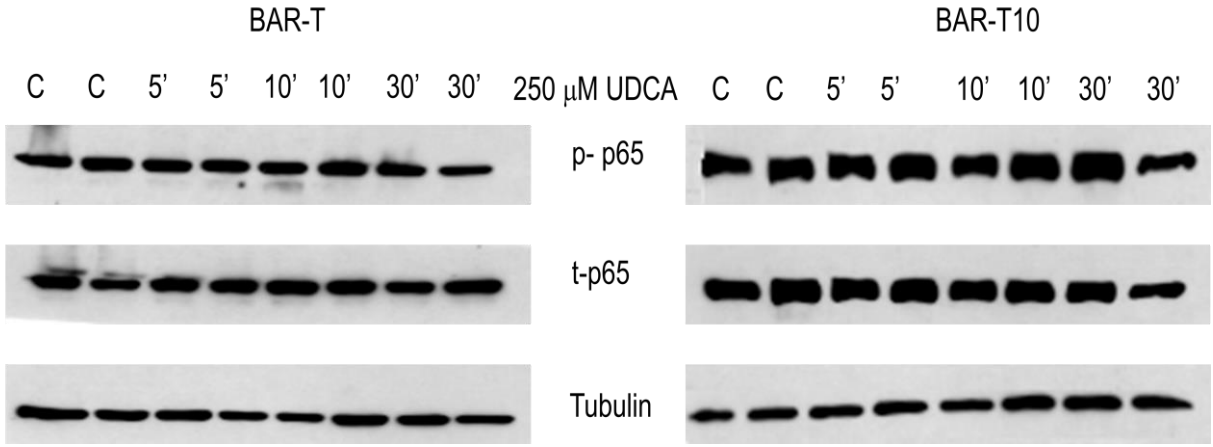


Figure 19: ***Western blot for Bar-T and Bar-T10 Cells after UDCA exposure.*** The NF-κB pathway could not be activated; p-p65 did not increase. A protein expression of I-κB could even not be detected.

4.2.2. UDCA Treatment Did Not Alter Morphology Of Barrett's Cells

After UDCA treatment, cells were assessed under the microscope. Cells looked healthy and no signs of apoptosis could be seen. They did not seem to die. UDCA did not change the cell morphology or altered growths or apoptosis (Fig. 20).

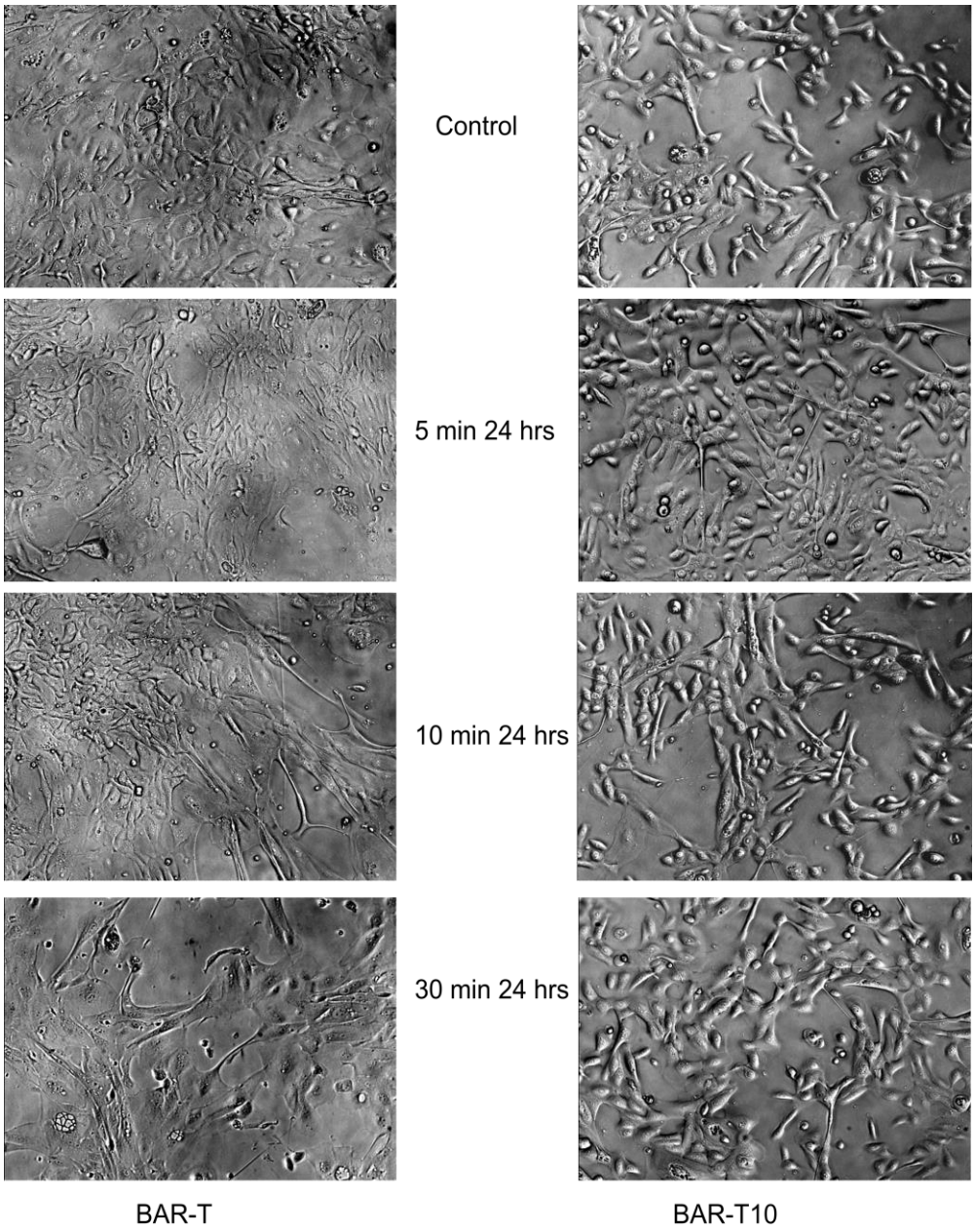


Figure 20: ***Microscopic picture of Barrett's Cells after UDCA treatment.*** Cells looked healthy at all time points; there were no signs of apoptosis.

4.2.3. Numbers Of Cells Did Not Change After UDCA Treatment And UDCA Did Not Induce Apoptosis In Barrett's Cell Lines

Cells were placed on 60 mm-dishes and grown to 65 % confluence over night in KGM-2 media. Cells were counted the following day. Figure 21 demonstrates that cell numbers did not change after UDCA treatment; cell number remained stable and no significant changes could be detected ($p > 0.05$).

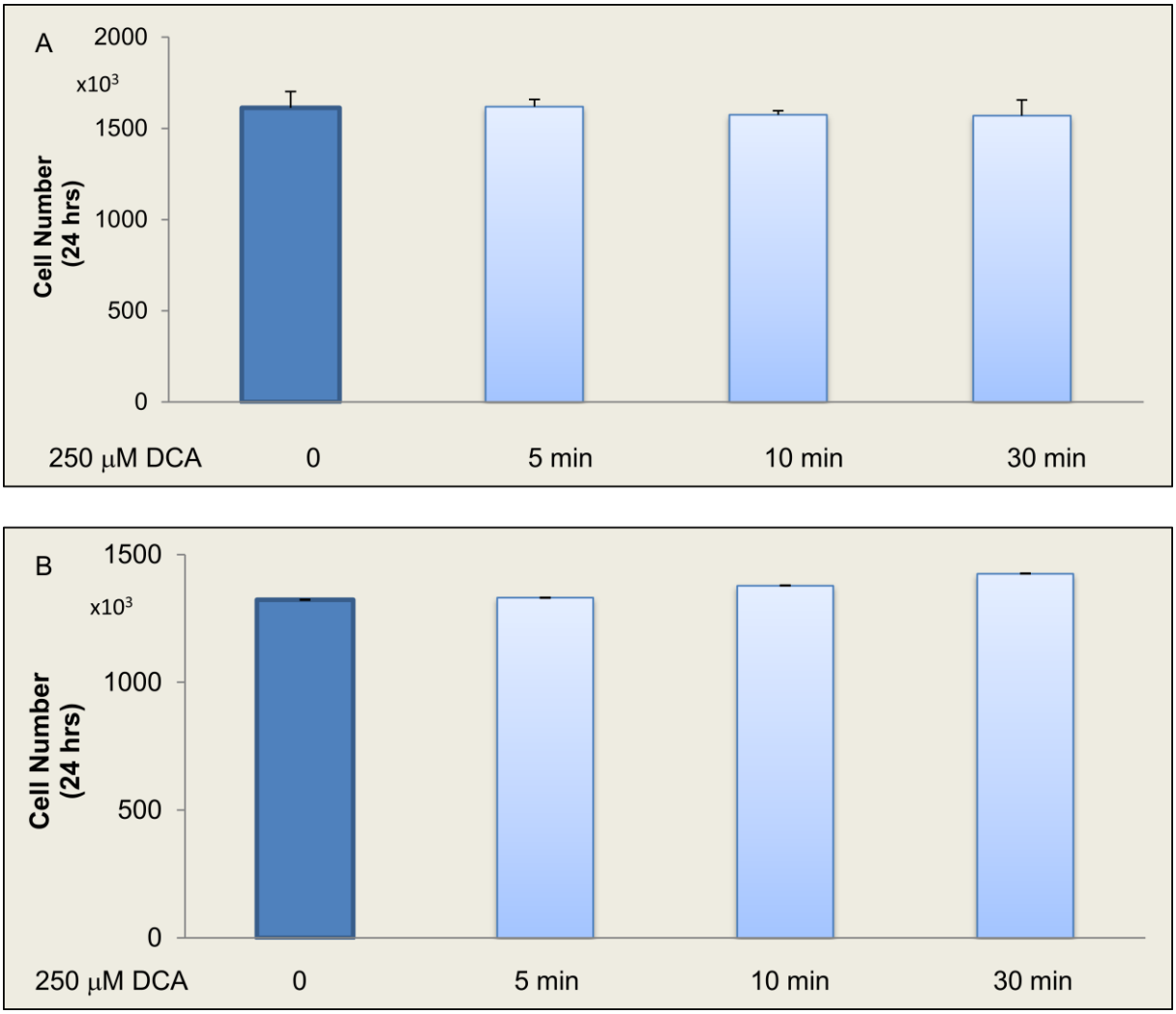


Figure 21: ***Cell count 24 hours after UDCA treatment.*** Bar-T cells **(A)** and Bar-T10 cells **(B)** did not show a change in cell number at any time point. The bar graphs depict the mean ± SEM.

The cell death ELISA, shown in Figure 22, performed 24 hours after UDCA exposure, did not show significant data for cell death. Barrett's cell lines did not respond with apoptosis to UDCA exposure.

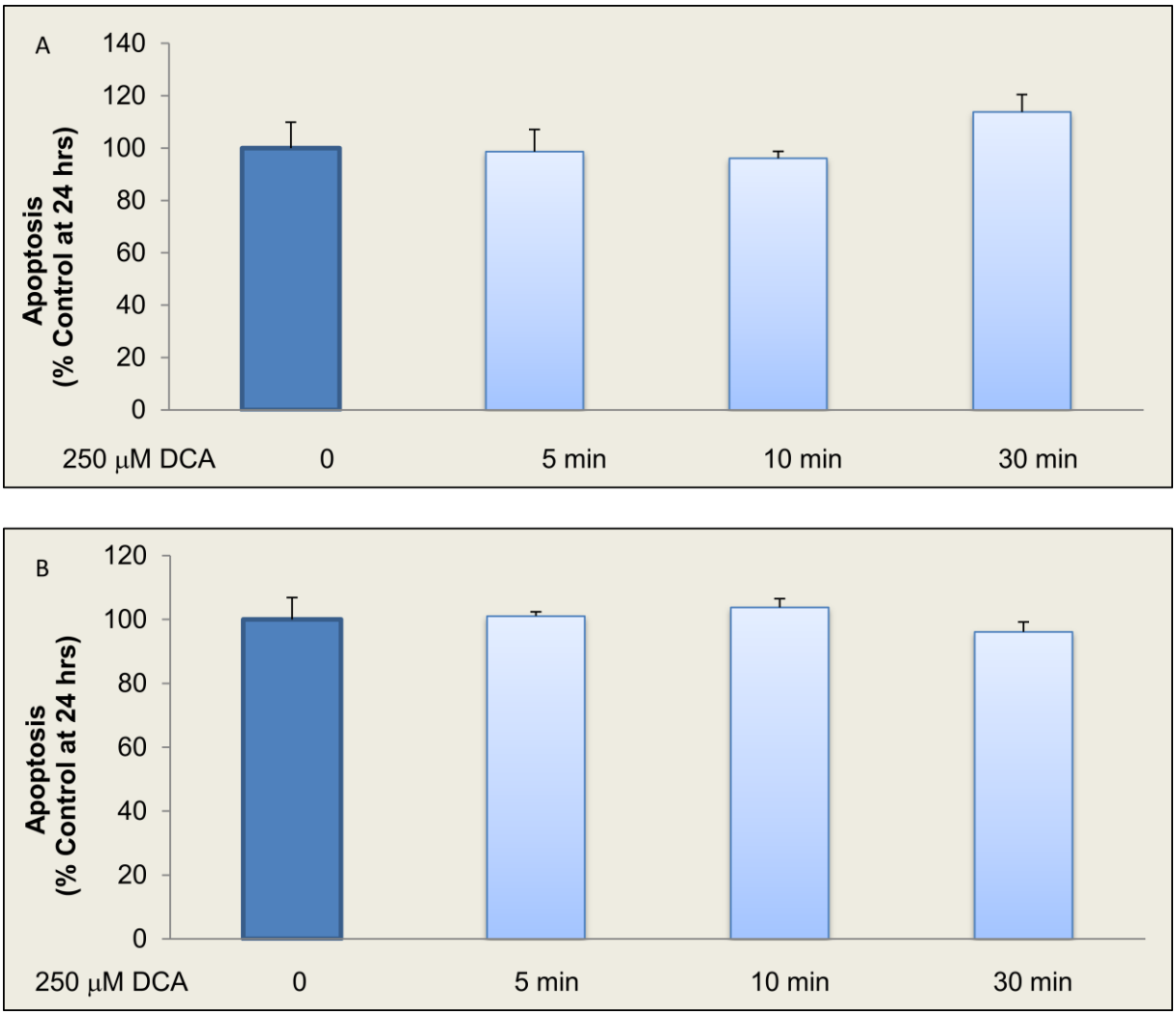


Figure 22: ***Cell death ELISA.*** Bar-T **(A)** and Bar-T10 **(B)** do not show a statistical relevant rate of apoptosis 24 hours after UDCA treatment. The bar graphs depict the mean ± SEM.

This demonstrates that the resistance against apoptosis followed after DCA or UDCA exposure was not just a phenomenon of a single Barrett's cell lines. It seems to be a common property of Barrett's epithelial cells. It could be proved, that the bile acid UDCA has a non toxic effect on Barrett's epithelial cells. The clinical use of UDCA to alter the components of the duodeno-gastric reflux to the esophagus may not cause further DNA damage and thus may help to prevent the progression to esophageal adenocarcinoma.

4.3. The Effect Of DCA And UDCA On The Barrett's Mucosa *In Vivo*

4.3.1. DCA Induced DNA Damage And NF-κB Activation In Barrett's Tissues

Biopsies of the Barrett's mucosa were taken from GERD patients with Barrett's Esophagus, who were scheduled for endoscopy for a clinical indication. These biopsies were taken before and after a perfusion with either DCA or UDCA. During endoscopy, a perfusion catheter was passed through the channel of the endoscope (Olympus FB.50K-1) and the bile acids were given for a 5-min perfusion with a concentration of either 250 μM DCA or UDCA to the esophageal mucosa. Six biopsies were taken before and six after the bile acid perfusion. Figure 23 shows a model of the clinical endoscopic investigation. Then, Western blots for p-H2AX and p-p65 expression were performed on the biopsies (Figs. 24 and 25).

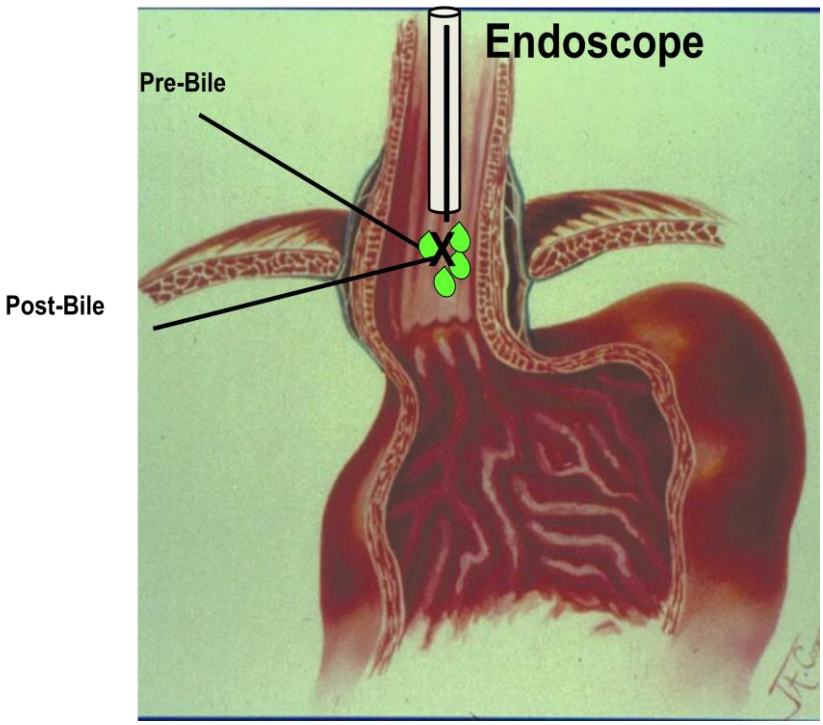


Figure 23*:* ***Getting the biopsies of the Barrett's Mucosa.*** During an endoscopy, biopsies were taken from patients with GERD and Barrett's Esophagus. After taking those biopsies, the esophagus was perfused through the channel of the endoscope with either UDCA or DCA. After a 5-min bile acid perfusion (concentration of 250 μM), biopsies were taken again.

The Western blot for p-H2AX showed that DCA was also able to elevate the level of p-H2AX, an indicator of DNA damage, *in vivo* (Fig. 24). Blots were made for the tissues of two patients, whereby Patient A demonstrated a more obvious increase in p-H2AX.

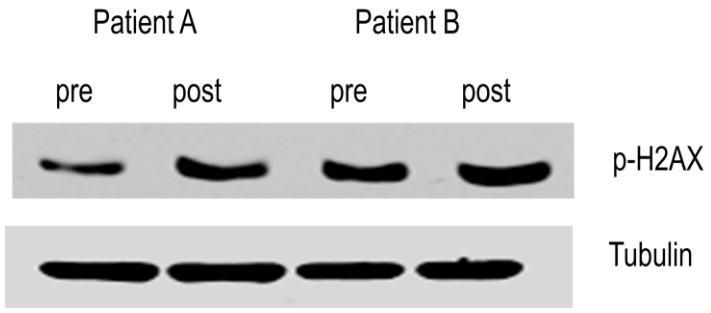


Figure 24: *Representative Western blots for Barrett's Esophagus biopsies* in vivo. Barrett's mucosa was perfused with 250 μM DCA for 5 min *in vivo*. p-H2AX was clearly elevated in Patient's A biopsy, but also for Patient B a slight elevation could be observed.

So, causing the DNA damage by DCA is not just a phenomenon *in vitro*. When the Western blot for p-p65 was performed, it could also be seen, that the NF-κB pathway was activated (Fig. 25). p-p65 increased, but it was impossible to detect and get a clear band for p-I-κB. As with p-H2AX expression, the increase in p-p65 was more pronounced in patient A than in patient B.

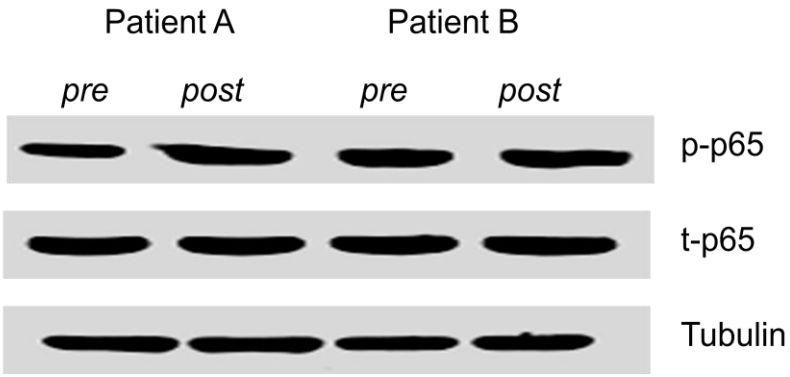


Figure 25: Representative *Western blots for p-p65 activity for Barrett's mucosa tissues* in vivo. p-p65 was increasing, especially in the more sensitive mucosa from Patient A, but also for Patient B suggesting activation of the NF-κB pathway.

4.3.2. UDCA Did Not Induce DNA Damage Or NF-κB Activation In Barrett's Tissues

After patient's tissues had been perfused with 250 μM UDCA for 5 min *in vivo*, Western blots for p-H2AX and p-p65 were performed.

Interestingly, UDCA was not able to induce DNA damage and activation of the NF-κB pathway *in vivo*. That supports earlier results of this study. Patient C and Patient D did not show elevated bands for either p-H2AX, or p-p65 (Fig. 26). It was impossible to get a clear band for p-I-κB.

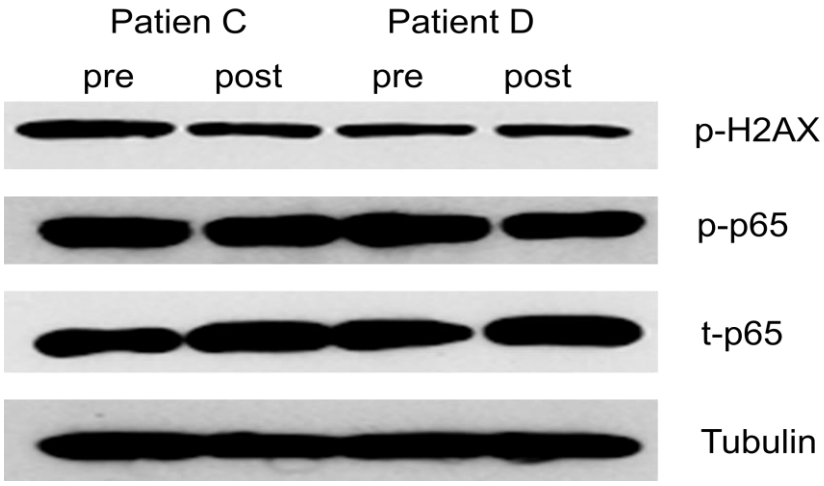


Figure 26: ***Representative Western blots for* in vivo *tissues after UDCA treatment.*** No elevation for p-H2AX or p-p65 could be seen. The cell survival pathway was not activated; DNA was not damaged *in vivo*, too.

5. Discussion

5.1. DCA Induced Apoptotic Resistance - The Step To Promote Progression To Esophageal Adenocarcinoma?

In this study it could be shown that Barrett's cell lines and Barrett's patients' mucosa were resistant to apoptosis caused by DCA despite the induction of DNA damage. The mechanism, why cells can withstand the toxic effect of the bile acid is established in activating a cell survival pathway. By phosphorylation and activation of the NF-κB subunit p-65 and I-κB, phosphorylation of I-κB leads to its destruction which frees the NF-κB subunit p65 so that it can move into the nucleus and bind to its DNA binding sites located in the promoter region of a number of anti-apoptotic genes, like Bcl-2. The toxic and pro-apoptotic effects of the bile acids can be counterbalanced by expression of the anti-apoptotic Bcl-2.

So, Barrett's cells are resistant to apoptosis.

Prior studies, using esophageal biopsies cultured *ex vivo* also suggested that the Barrett's epithelium is more resistant to apoptosis than normal esophageal squamous cells. Problems that those studies afforded were that those biopsies contained not only Barrett's epithelium, but also inflammatory and stromal cells. So, the direct effect of the used agent onto the epithelial cells is difficult to distinguish because inflammatory and stromal cells could have influenced or mediated the observed results. To eliminate those disruptive factors, benign, telomerase-immortalized epithelial cells were cultured and used in this study.

The response to DCA exposure, by activating the NF-κB pathway was observed in two different Barrett's cell lines (Bar-T and Bar-T10) and in patient tissues, so it can be suggested that this effect is not restricted to only one cell line, but rather that it is a common property of non-neoplastic "Barrett's cells" and it is not influenced or effected by inflammatory or stromal cells. As Hanahan and Weinberg [32] previously established, resistance to apoptosis can be considered as the most important alteration of cancer cells.

Further effects of the bile acid DCA that highlight its geno-toxicity were to cause DNA damage in cell lines as well as in the biopsy specimens. All the experiments were based on physiological conditions, which mean a concentration of DCA of 250 μM for

an exposure time of 5 min, which has been found by other investigators to represent the duration of a reflux event in Barrett's patients. [49] [59] There was also a time-dependent increase in phosphorylation of H2AX (Fig. 9) followed by DCA. Mah *et al*. [54] showed that H2AX is a very sensitive marker for double strand breaks. Measured by expression of this marker, DNA damage could be demonstrated immediately after bile salts exposure. Activation of the NF-κB pathway enabled the cells not to die and likely to be able to repair the DNA damage within 24 hours (Fig. 11).

"If Barrett's epithelial cells get DNA damage, and they can repair the injury while resisting apoptosis, then there would not be any clinical consequences; but if one of those apoptosis-resistant Barrett's cells fails to repair the DNA damage, then the mutated cell could be replicated and the damaged DNA would be given to all future daughter cells. If the mutated cells have a growth advantage, they could expand over the esophageal mucosa and progress to cancer. Thus, this mechanism may underlie the malignant predisposition of the Barrett's cells." (Own publication [45])

In their experiments with "Barrett's cells" and normal esophageal cells, Hormi-Carver *et al*. [36] exposed the cells to UV-B irradiation. After low dose UV-B irritation (50 and 100 J/m^2) DNA damage occurred in "Barrett's cells" and further, they reacted with an induction of the NF-κB pathway - results very similar to this study.

They also worked with Bar-T and Bar-10T cell lines, those that were used in these experiments. One problem of their test conditions was that UV-B irradiation, in contrast to exposure to the bile acid DCA, is not a physiological inducer of DNA damage in the esophagus. Nevertheless, UV-B irritation and DCA seem to trigger similar intracellular pathways. Both conditions can make the cells sustainable to DNA damage and reduce apoptosis in consequence of expression of anti-apoptotic proteins induced by activation of the NF-κB pathway. But not only in Barrett's metaplasia, also in esophageal adenocarcinoma cell lines (OE33) DCA leads to an increase of I-κB expression and induces significantly DNA damage. [43]

Interestingly, it has been shown by Jenkins *et al*. [44] that DCA is active only at a neutral pH value, for example in patients with non-acidic reflux. This illustrates a clinical problem, because patients on acid suppressing therapy may consequently have a higher risk of degeneration to cancer. In patients with extensive mucosal injury and esophagitis dehydroxylated taurodeoxycholic acid, unconjugated cholic

acid and especially DCA are the predominant bile salts causing the most damage in the ultrastucture of the esophageal squamous epithelium. [59]

It emanates from studies from Armstrong *et al*. that DCA is also the bile acid with the most gastrotoxic effects. [4]

Dvorakova *et al*. [23] found that squamous esophageal epithelium, treated with an unphysiologically high concentration of DCA (1 mM) for 3 hours, demonstrated a change in cell morphology and the typical signs of apoptosis, like chromatin margination and condensation. When they treated Barrett's biopsy tissues *ex vivo* with this high concentration of DCA, the Barrett's biopsy tissues did not show any signs of apoptosis.

Summarized, these data suggest that "Barrett's cells" are more resistant to the bile acid induced apoptosis in contrast to normal esophageal mucosa that reacts very sensitively to the toxic effects of bile DCA.

But it should not be forgotten, that DCA is just one component of the reflux in the esophagus. The refluxate consists of further agents, other bile acids, the gastric acid and enzymes, which can enhance or inhibit each other's effects if used in combination. So, this area needs to be investigated in future studies, particularly under more complex conditions that mean the combination of all these gastric components.

5.2. Differences of Barrett's Specialized Intestinal Epithelium to Normal Intestine Mucosa

Far not all kinds of gastrointestinal cells react with alteration of their cell properties after DCA exposure. In the small intestine, for example, the incidence of cancer is extremely rare, although bile acids are abounding constantly and in high concentrations. To determine whether the observed differences in resistance to apoptosis and DNA damage followed by DCA exposure are primarily due to their differences in cell phenotype or whether these are common properties of all intestinal cells, Huo and Juergens *et al*. [37] treated rodent intestinal cells with DCA and UDCA. The cells, they used were normal rat intestine epithelial cells (IEC-6).

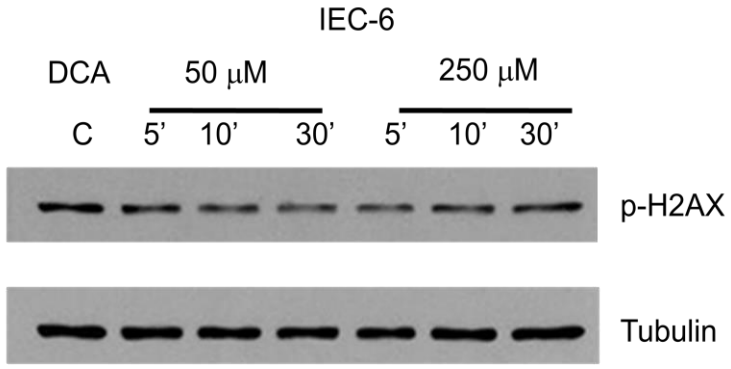


Figure 27: ***Representative Western blots for rats' IEC-6 cells after DCA exposure.*** There is no change in expression of phosphorylated H2AX after exposure to DCA at a concentration of 50 μM or 250 μM DCA.

There was no increase in expression for phosphorylated H2AX after the exposure to DCA with a concentration of 50 μM or 250 μM after an exposure time of 5, 10 or 30 min (Fig. 27). So, unlike the "Barrett's cells", there was no induction of DNA damage for the rat intestinal cells.

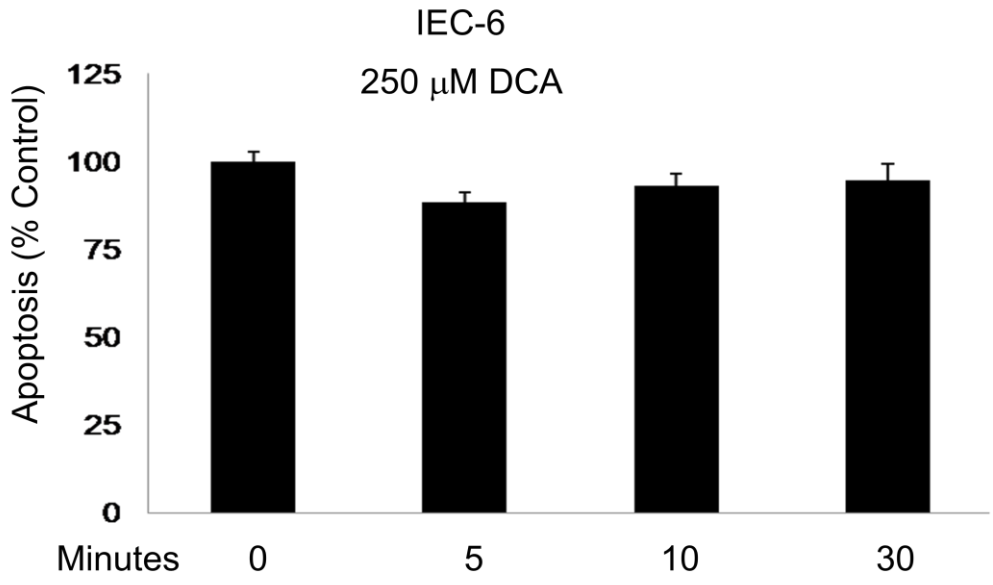


Figure 28: ***Annexin V Assay detecting representative rates of apoptosis for IEC-6 after DCA exposure.*** There are no changes in apoptosis for any cell line after 50 μM and 250 μM DCA exposure. Bar graphs depict the mean ± SEM of at least 3 individual experiments.

As expected, since DCA was not able to induce DNA damage in rodent intestinal cells, there were no changes in rates of apoptosis (Fig. 28).

Summarized, resistance to apoptosis cannot only be due to their intestinal phenotype, as the experiment illustrates. Barrett's metaplasia constitutes a special kind of columnar cells with properties different from normal intestinal cells.

In contrast to intestinal cells they activate the NF-κB pathway and resist apoptosis even though their DNA gets damaged. This condition could explain their potential to undergo transformation to cancer.

The explanations above highlight different effects of DCA dependent on the cell type.

DCA in colonic cells has similar effects on the NF-κB pathway and induced the expression of anti-apoptotic proteins as Lee *et al*. [53] could find out. So, it can implicate colon cancer growth and progression.

Other investigators [61] also performed research with DCA previously and confirmed that the bile salt is able to interfere with cell cycle enzymes like Cyclin D1 and contribute to cancer progression. The effects of DCA seem to be similar in Barrett's and colon cancer cells, despite the fact that there are a lot of differences between the cell types and other unexplored factors.

5.3. Therapeutic and Cytoprotective Meaning of UDCA

One of the most important therapeutic conclusions of these studies is the fact that UDCA offers less geno-toxicity than DCA. Barrett's cell lines and patient tissues did not show an increase in phosphorylated H2AX after exposure to UDCA (Fig. 18). The meaning of this observation is that cells did not acquire DNA damage after UDCA treatment, which is different from the situation after DCA exposure. With the less DNA damaging effect of UDCA the chance of genetic mutations is less and consequently the progression to cancer might be less as well. This is not the only effect that differs to DCA; UDCA did not provoke the activation of the NF-κB pathway proteins (Fig. 19). There was no increase in p-p65 levels or in I-κB phosphorylation. So, the process of apoptosis was not disturbed after cells had been exposed to UDCA.

Subsumed, UDCA seems to be less genotoxic to the Barrett's cell lines than its hydrophobic partner.

These effects could have therapeutic consequences. UDCA is already used as a therapeutic drug for different diseases, but at this time it is not used to treat patients suffering from Barrett's Esophagus.

Amaral *et al*. [3] could demonstrate that hydrophilic bile acids acted cytoprotective in stressed hepatocytes, while accumulation showed converse effects.

Patients that nowadays benefit from UDCA`s protective properties suffering from cholestatic liver diseases, like primary biliary cirrhosis. Here, UDCA has been clinically widely used for a long time and, while it has been in use, an increase of liver cancer has not been observed. [12] The effects of UDCA are multifold in these diseases. On the one hand the bile acid is able to initiate apoptosis; but on the other hand it can be found similarities to what was found out for Barrett's metaplasia. Activation of the NF-κB pathway was demonstrated in hepatocytes and hereby expression of anti-apoptotic proteins (Bcl-2 and Bcl-X). So, cells were protected against cell death. [78] By treatment with UDCA, cholangiocytes become more resistant against the DNA damaging effects of DCA and apoptosis of hepatocytes can be inhibited.

An explanation of the intracellular interaction of UDCA was established by Rodrigues *et al*. [72] In their experiments with rat models, they exposed rat livers to DCA after they fed the animals with UDCA. Hereby, they observed an inhibition of ROS production and of the release of the pro-apoptotic Bax protein from the mitochondrial membrane.

Even for patients with colorectal adenocarcinoma in association with primary sclerosing cholangitis, UDCA is an often used chemopreventive agent, because it is able to decrease the rates of adenocarcinoma recurrence. Furthermore, it can figure in prevention of progression to colorectal carcinoma in patients with PSC. These observations are particular important for patients with ulcerative colitis and PSC, where DCA lowers the rate of dysplasia, as Tung *et al*. [90] and Serfaty *et al*. [80] showed. Inflammatory activity is reduced by UDCA triggered suppression of NF-κB pathway protein expression. Above all, these effects occur in liver cells.

In patients with colonic cancer, DCA is a cytotoxic reagent. Im *et al*. [40] presented data for colonic cancer cells (HCT116), which showed that UDCA can prevent the toxic effect of DCA like destroying the plasma membrane function or activating pro-oncogenes. Their experiments were similar to the ones of Rodrigues *et al*. [72],

described above. They also pretreated cells with UDCA before they were exposed to DCA and showed that UDCA could inhibit the cancer development.

5.4. Clinical Relevance and Problems of the Current Treatment

For several diseases, like the colorectal adenocarcinoma, PSC and PBC, the cytoprotective UDCA is a routinely used therapeutic option. In contrast to DCA, which can cause degradation and progression to cancer, it does not show those cytotoxic effects and is even able to protect against cell damage.

Nevertheless, UDCA treatment is currently not a common therapy for Barrett's patients; it is only in use in clinical research studies.

It can considerably be seen that a necessity exists to perform further investigations with knowing of this background. It is not much known about UDCA treatment in human Barrett's patients, but some experiments in rat models have been done. In those rats esophagojejunostomy was made. This operation should create reflux injury, Barrett's metaplasia and adenocarcinoma. Interestingly, the incidence of developing an esophageal adenocarcinoma was significantly lower for rats that were fed with Aspirin and UDCA compared to an untreated control. Subsequently, BAR-T cell lines were exposed to the UDCA-Aspirin mixture *in vitro* and the supplementation could reduce cell proliferation (but not apoptosis), an important step in development of carcinoma. [71]

If UDCA would interact in human BE patients, like it does in rat models and cholestatic diseases (PSC, PBC), it would deliver a therapeutic option with immense potential.

The fact, that Barrett's patients have significantly higher concentration of DCA and also have quantitatively more bile reflux than patients with GERD and without BE, gives an explanation, why DCA, and not gastric acid, might be responsible for cell degeneration and transformation to cancer. [59] [91]

An idea to capitalize the potential chemopreventive power of UDCA may be to alter the composition of the duodeno-gastric juice presented to the esophagus in BE patients. The strategy, standing behind the clinical UDCA application, pursues the goal to increase the less toxic bile acid and to reduce the concentration of the toxic

DCA in the refluxates. To realize this therapeutic strategy large prospective clinical studies are needed to be conducted to find out more about the UDCA effects *in vivo*.

Presently, a common pharmacological therapy for patients with GERD, but also for patients with Barrett's metaplasia is the administration of PPIs. As described above, bile salts, like DCA are particularly damaging and destructive in neutral pH environment created by PPIs. In addition, it should not be forgotten that Barrett's patients have higher concentrations of DCA in their refluxates. [15]

So, if neutral pH levels would increase the toxicity of DCA and thereby its detrimental effect of increasing DCA-induced damage, this problem might be the weak point of this therapeutic strategy. Some present studies clearly demonstrate that special bile salts, like DCA cause their deleterious biological effects in neutral pH, and BE patients definitely have higher concentrations of Deoxycholic Acid in their refluxates than patients with GERD.

These studies demonstrate that PPIs might not be a sufficient treatment for Barrett's patients and could conceivably even promote the progression to cancer. [66] Jenkins *et al*. [43] offered an approach to improve the treatment of BE patients. They studied the effects of antioxidant supplements, such as Vitamin C and suggested that those antioxidants may protect against the DCA–induced DNA damage and NF-κB activation.

Other detergents, the non-steroidal COX-2 inhibitors and n-3 fatty acids with their anti-inflammatory and eicosanoid suppressing properties, might also act chemopreventive and are standing in the focus of interest of further investigations. [2] [57]

Summarized, with the knowing of this background new therapeutic strategies for Barrett's patients may be the introduction of UDCA to alter the bile acid composition, in combination with the antioxidant Vitamin C, COX-2 inhibitors or n-3 fatty acids to prevent the progression to esophageal adenocarcinoma.

5.5. Future Aspects and Further Experiments

From this study some of the basic effects of DCA and UDCA exposure in Barrett's esophageal cell lines and in patient tissues could be inferred. Deoxycholic Acid seems to inhibit apoptosis for those cells by activating the NF-κB cell survival pathway. The phosphorylated I-κB, as well as the subunit p65 began to increase, while the total amount of p65 stayed constant. Also, there was no new synthesis of the mentioned protein. To confirm these findings, it is necessary to design future experiments, where NF-κB inhibitors are used to demonstrate that DCA does in fact induce apoptosis through activation of the NF-κB pathway. Possible inhibitors include Bay 11-7085 (Sigma), which is a pharmacological inhibitor that is able to block the phosphorylation of I-κB. The problem with such pharmacological inhibitors is that they can cause non-specific effects. For that reason, a specific siRNA against NF-κB/ p-65 should also be used, as previously described by Souza *et al*. [36] It can be anticipated that apoptosis rates increase and the expression levels of p-p65 and p-I-κB do not change after exposure to DCA under such experimental conditions.

Furthermore it should be determined whether DCA affects anti- and pro-apoptotic proteins. It should be shown, how other NF-κB downstream target proteins react and how they influence resistance to apoptosis by using Western blotting and by inhibiting those proteins. Particularly, the anti-apoptotic proteins Bcl-2, Bcl-xL that build a complex and inactivate the pro-apoptotic protein Bax and inhibit the release of cytochrome C from the mitochondria should be investigated. [6] Inhibitors of apoptosis (IAP) proteins might also be interesting to understand the mechanism of anti- apoptosis and could be assessed by Western blotting.

A very important aim for further studies is to determine whether UDCA is able to protect against the DCA-induced DNA damage.

Two models for such a study could be:

1. Barrett's cell lines should be pre-treated with UDCA
   a) for a short time,
   b) for a long time.

   After UDCA pre-treatment, the media should be changed and DCA should be added to the cells. To simulate a physiological reflux period, cells may be

treated with DCA for 5, 10 and 30 minutes. Western blots for p-H2AX, for p-p65 and p-I-κB should be performed, to determine whether cells acquire DNA damage in the setting of UDCA pre-treatment. The results should be compared with the data of this study.

2. Another model might be designed in such a way that DCA and UDCA are mixed together and then added to the Barrett's cells. This experimental design would simulate physiological conditions, because there would always be an amount of DCA in the reflux. Again, the prior mentioned proteins should be observed and compared with the data from the current study.

These kinds of experiments should examine and also take a look at the pro- and anti- apoptotic proteins, as well as at the total rates of cell death/ apoptosis and cell survival.

After the basics are uncovered in *in-vitro* experiments, a clinical study should follow to research the effects of UDCA *in vivo* and to determine the role of UDCA as a therapeutic drug for Barrett's patients.

To understand the differences between mutated cancer cells and normal esophageal epithelium, these cells have to be compared. Therefore, the response of both cell types to treatment with DCA, UDCA or a mixture under the same experimental conditions should be investigated.

Another interesting question is to find out whether DCA is able to affect other proteins that were found to play an important role in the progression from Barrett's Esophagus to adenocarcinoma, proteins like cyclo-oxygenase-2, c-myc and mitogen-activated protein kinase.

6. SUMMARY

In these studies it could be shown that the potentially cancerogenic bile acid DCA was able to induce DNA damage *in vivo* and *in vitro* immediately after exposure to two Barrett's cell lines (Bar-T and Bar-T10) and to Barrett's patients' tissues.

Detected by Western blotting and using monoclonal antibodies, phosphorylated core protein H2AX, a marker for DNA double strand breaks increased significantly compared to the untreated control.

Interestingly, the NF-κB pathway proteins I-κB and p65 also became phosphorylated and activated detected by an increase in corresponding bands in representative Western blots. So, despite DNA was damaged the cell survival pathway was activated

24 hours after DCA exposure, protein bands were decreased almost to baseline (control) levels. Performed cell counts resulted in no significant changes in cell numbers and optical cellular morphology did not give evidence of apoptotic signs and activity. By using cell death ELISA, results could be quantified. Indeed, it could be found a maximum increase in rates of apoptosis of about 20% after 5-min exposure and further 24 hours of cell culture, but the Student- Newman-Keuls multicomperisson test, again, did not show statistical relevant data ($p > 0.05$) for induction of apoptosis. It can strongly be suggested that the activation of the NF-κB pathway leads to this observed behavior in "Barrett's cells".

UDCA treatment did not affect the protein expression of H2AX, p65 and I-κB. Exposure could not change cell morphology, cell numbers and rate of apoptosis. The Student's test resulted in numbers for $p > 0.05$ and so data have not been statistically relevant. So, the role of UDCA as a therapeutic drug for Barrett's and GERD patients seems to be more important than accepted. An alteration of the refuxates with the less toxic bile acid UDCA could present a new strategy in treatment of BE that might also have cytoprotective effects and might prevent progression to the esophageal adenocarcinoma.

ZUSAMMENFASSUNG

Im Rahmen dieser Arbeit konnte gezeigt werden, dass die in ihrer Wirkung als potentiell kanzerogen geltende Gallensäure DCA in schädigender Weise auf die Zell-DNA einwirkt. Diese Wirkung wurde nicht nur für die immortalisierte Barrett Zelllinien (Bar-T und Bar-T10) *in vitro* erzielt, sondern konnte auch für Biopsien von Patienten, die an Barrett Esophagus leiden, *in vivo* reproduziert werden. Methodisch wurde dazu die Phosphorilierung des Histons H2AX - eines äußerst sensitiven Markers für Doppelstrangbrüche - durch Westernblots und Interaktion mit monoklonalen Antikörpern nachgewiesen, wobei die relevanten Proteinbanden in den Blots im direkten Vergleich mit der unbehandelten Probe einen signifikanten Anstieg zu verzeichnen hatten. Interessanterweise wurden zusätzlich die NF-κB Signalweg Proteine I-κB und p65 phosphoriliert und hiermit aktiviert. Auch für diese ließen sich – abhängig von der Expositionsdauer und verglichen mit der Kontrolle - deutlich ansteigende Banden im Westernblot nachweisen. Diese molekularbiologischen Veränderungen haben große Bedeutung, denn trotz Schädigung der DNA wurde ein Cell Survival Pathway aktiviert, der die geschädigten Zellen befähigte sich der natürlichen Apoptose zu widersetzen. Etwa 24 Stunden nach DCA-Kontakt ließ sich durch Wiederholung der Westernblots beobachten, dass die entsprechenden Proteinbanden wieder nahezu auf das Aufgangslevel abgefallen waren. Weder das Auszählen der Zellen, noch ihre morphologische Erscheinung gaben Hinweise auf die typischen Zeichen des Zelltods. Diese qualitativen Beobachtungen konnten im Weiteren durch die Anwendung eines Cell Death ELISAs auch quantifiziert werden. Zwar zeigte sich tatsächlich ein Anstieg der Apoptose auf ca. 20 % nach einer 5-minütigen DCA Exposition und weiteren 24 Stunden des Cell Culturing, aber ein angewandter Student-Newman-Keuls Test erwies keine statistische Relevanz dieses Anstiegs ($p > 0.05$). Es ist anzunehmen, dass die Aktivierung des NF-kB Signalweges sowie dessen Folgen für die an den „Barrett Zellen" getätigten Beobachtungen verantwortlich waren.

Im Gegenteil dazu, konnte eine, unter gleichen Versuchsbedingungen durchgeführte, UDCA Exposition keine molekularbiologische Veränderung, wie Phosphorilierung, hiermit Aktivierung, bewirken. Die Morphologie der Zellen blieb unverändert, es stellten sich keine Apoptosezeichen dar. Auch Zellzählungen und Cell Death ELISA ergaben keine statistisch relevanten Ergebnisse ($p > 0.05$).

Schlussfolgernd scheint die Rolle, die UDCA als therapeutische Option auch beim Barrett Esophagus und GERD Patienten spielen könnte, einen größeren Stellenwert einzunehmen als bisher angenommen. Durch Alteration der Refluxate mit der weitaus ungefährlicheren Gallensäure UDCA könnte sich ein neuer, chemoprotektiver Behandlungsansatz auch gegen das Voranschreiten des BE zu dem gefährlichen Adenokarzinoms ergeben.

7. REFERENCES

[1] Abdel-Latif MMM, Kelleher D & Reynolds JV 2009, 'Potential role of NF-kappaB in esophageal adenocarcinoma: as an emerging molecular target', *The Journal of surgical research,* vol. 153, no. 1, pp. 172-80.

[2] Abrams JA 2008, 'Chemoprevention of esophageal adenocarcinoma', *Therapeutic advances in gastroenterology,* vol. 1, no. 1, pp. 7-18.

[3] Amaral JD, Viana RJS, Ramalho RM, Steer CJ & Rodrigues CMP 2009, 'Bile acids: regulation of apoptosis by ursodeoxycholic acid', *Journal of lipid research,* vol. 50, no. 9, pp. 1721-34.

[4] Armstrong D, Rytina ER, Murphy GM & Dowling RH 1994, 'Gastric mucosal toxicity of duodenal juice constituents in the rat. Acute studies using ex vivo rat gastric chamber model', *Digestive diseases and sciences,* vol. 39, no. 2, pp. 327-39.

[5] Ashkenazi A 2008, 'Targeting the extrinsic apoptosis pathway in cancer', *Cytokine growth factor reviews,* vol. 19, no. 3-4, pp. 325-31.

[6] Baldwin AS Jr 1996, 'The NF-kappa B and I kappa B proteins: new discoveries and insights', *Annual review of immunology,* vol. 14, pp. 649-83.

[7] Bani-Hani KE & Bani-Hani BK 2006, 'Pathogenesis of columnar-lined esophagus', *World journal of gastroenterology : WJG,* vol. 12, no. 10, pp. 1521-8.

[8] Bartek J & Lukas J 2007, 'DNA damage checkpoints: from initiation to recovery or adaptation', *Current opinion in cell biology,* vol. 19, no. 2, pp. 238-45.

[9] Beck F & Stringer EJ 2010, 'The role of Cdx genes in the gut and in axial development', *Biochemical Society transactions,* vol. 38, no. 2, pp. 353-7.

[10] Becker C, Fantini MC, Schramm C, Lehr HA, Wirtz S, Nikolaev A, Burg J, Strand S, Kiesslich R, Huber S, Ito H, Nishimoto N, Yoshizaki K, Kishimoto T, Galle PR, Blessing M, Rose-John S & Neurath MF 2004, 'TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling', *Immunity,* vol. 21, no. 4, pp. 491-501.

[11] Bernstein H, Bernstein C, Payne CM & Dvorak K 2009, 'Bile acids as endogenous etiologic agents in gastrointestinal cancer', *World journal of gastroenterology: WJG,* vol. 15, no. 27, pp. 3329-40.

[12] Beuers U, Boyer JL & Paumgartner G 1998, 'Ursodeoxycholic acid in cholestasis: potential mechanisms of action and therapeutic applications', *Hepatology,* vol. 28, no. 6, pp. 1449-53.

[13] Billington D, Evans CE, Godfrey PP & Coleman R 1980, 'Effects of bile salts on the plasma membranes of isolated rat hepatocytes', *The Biochemical journal,* vol. 188, no. 2, pp. 321-7.

[14] Booth LA, Gilmore IT & Bilton RF 1997, 'Secondary bile acid induced DNA damage in HT29 cells: are free radicals involved?', *Free radical research,* vol. 26, no. 2, pp. 135-44.

[15] Burnat G, Majka J & Konturek PC 2010, 'Bile acids are multifunctional modulators of the Barrett's carcinogenesis', *Journal of physiology and pharmacology: an official journal of*

the Polish Physiological Society, vol. 61, no. 2, pp. 185-92.

[16] Chen X & Yang CS 2001, 'Esophageal adenocarcinoma: a review and perspectives on the mechanism of carcinogenesis and chemoprevention', *Carcinogenesis,* vol. 22, no. 8, pp. 1119-29.

[17] Colleypriest BJ, Farrant JM, Slack JMW & Tosh D 2010, 'The role of Cdx2 in Barrett's metaplasia', *Biochemical Society transactions,* vol. 38, no. 2, pp. 364-9.

[18] Contini S & Scarpignato C 2003, 'Endoscopic treatment of gastro-oesophageal reflux disease (GORD): a systematic review', *Digestive and liver disease: official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver,* vol. 35, no. 11, pp. 818-38.

[19] Croog VJ, Ullman TA & Itzkowitz SH 2003, 'Chemoprevention of colorectal cancer in ulcerative colitis', *International journal of colorectal disease,* vol. 18, no. 5, pp. 392-400.

[20] Crowley-Weber CL, Payne CM, Gleason-Guzman M, Watts GS, Futscher B, Waltmire CN, Crowley C, Dvorakova K, Bernstein C, Craven M, Garewal H & Bernstein H 2002, 'Development and molecular characterization of HCT-116 cell lines resistant to the tumor promoter and multiple stress-inducer, deoxycholate', *Carcinogenesis,* vol. 23, no. 12, pp. 2063-80.

[21] Dabbagh K, Takeyama K, Lee HM, Ueki IF, Lausier JA & Nadel JA 1999, 'IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo', *Journal of immunology*, vol. 162, no. 10, pp. 6233-7.

[22] Dvorak K, Chavarria M, Payne CM, Ramsey L, Crowley-Weber C, Dvorakova B, Dvorak B, Bernstein H, Holubec H, Sampliner RE, Bernstein C, Prasad A, Green SB & Garewal H 2007, 'Activation of the interleukin-6/STAT3 antiapoptotic pathway in esophageal cells by bile acids and low pH: relevance to barrett's esophagus', *Clinical cancer research: an official journal of the American Association for Cancer Research,* vol. 13, no. 18 Pt 1, pp. 5305-13.

[23] Dvorakova K, Payne CM, Ramsey L, Bernstein H, Holubec H, Chavarria M, Bernstein C, Sampliner RE, Riley C, Prasad A & Garewal H 2005, 'Apoptosis resistance in Barrett's esophagus: ex vivo bioassay of live stressed tissues', *The American journal of gastroenterology,* vol. 100, no. 2, pp. 424-31.

[24] El-Rifai W & Powell SM 2002, 'Molecular biology of gastric cancer', *Seminars in radiation oncology,* vol. 12, no. 2, pp. 128-40.

[25] Eloubeidi MA, Mason AC, Desmond RA & El-Serag HB 2003, 'Temporal trends (1973-1997) in survival of patients with esophageal adenocarcinoma in the United States: a glimmer of hope?', *The American journal of gastroenterology,* vol. 98, no. 7, pp. 1627-33.

[26] Fang Y, Han SI, Mitchell C, Gupta S, Studer E, Grant S, Hylemon PB & Dent P 2004, 'Bile acids induce mitochondrial ROS, which promote activation of receptor tyrosine kinases and signaling pathways in rat hepatocytes', *Hepatology (Baltimore, Md.),* vol. 40, no. 4, pp. 961-71.

[27] Fitzgerald RC 2005, 'Barrett's oesophagus and oesophageal adenocarcinoma: how does acid interfere with cell proliferation and differentiation?', *Gut,* vol. 54 Suppl 1, pp. i21-6.

[28] Fitzgerald RC, Lascar R & Triadafilopoulos G 2001, 'Review article: Barrett's oesophagus, dysplasia and pharmacologic acid suppression', *Alimentary pharmacology*

therapeutics, vol. 15, no. 3, pp. 269-76.

[29] Frazzoni M, Conigliaro R & Melotti G 2011, 'Weakly acidic refluxes have a major role in the pathogenesis of proton pump inhibitor-resistant reflux oesophagitis', *Alimentary pharmacology therapeutics,* vol. 33, no. 5, pp. 601-6.

[30] Ghosh S & Karin M 2002, 'Missing pieces in the NF-kappaB puzzle', *Cell,* vol. 109 Suppl, pp. S81-96.

[31] Gores GJ, Miyoshi H, Botla R, Aguilar HI & Bronk SF 1998, 'Induction of the mitochondrial permeability transition as a mechanism of liver injury during cholestasis: a potential role for mitochondrial proteases', *Biochimica et biophysica acta,* vol. 1366, no. 1-2, pp. 167-75.

[32] Hanahan D & Weinberg RA 2000, 'The hallmarks of cancer', *Cell,* vol. 100, no. 1, pp. 57-70.

[33] Hatada EN, Nieters A, Wulczyn FG, Naumann M, Meyer R, Nucifora G, McKeithan TW & Scheidereit C 1992, 'The ankyrin repeat domains of the NF-kappa B precursor p105 and the protooncogene bcl-3 act as specific inhibitors of NF-kappa B DNA binding', *Proceedings of the National Academy of Sciences of the United States of America,* vol. 89, no. 6, pp. 2489-93.

[34] Hellerbrand C, Hoeger S, Muelbauer M, Thasler W & Lock G 2000, 'Bile acids induce proinflammatory cytokine expression in activated hepatic stellate cells vie NFKB activation', *Hepatology,* no. 32, pp. 156.

[35] Hillman LC, Chiragakis L, Shadbolt B, Kaye GL & Clarke AC 2004, 'Proton-pump inhibitor therapy and the development of dysplasia in patients with Barrett's oesophagus', *The Medical journal of Australia,* vol. 180, no. 8, pp. 387-91.

[36] Hormi-Carver K, Zhang X, Zhang HY, Whitehead RH, Terada LS, Spechler SJ & Souza RF 2009, 'Unlike esophageal squamous cells, Barrett's epithelial cells resist apoptosis by activating the nuclear factor-kappaB pathway', *Cancer research,* vol. 69, no. 2, pp. 672-7.

[37] Huo X, Juergens S, Zhang X, Rezaei D, Yu C, Strauch ED, Wang J, Cheng E, Meyer F, Wang DH, Zhang Q, Spechler SJ & Souza RF 2011, 'Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF-kappaB activation in benign Barrett's epithelial cells', *American journal of physiology. Gastrointestinal and liver physiology,* vol. 301, no. 2, pp. G278-86.

[38] Hutchinson L, Stenstrom B, Chen D, Piperdi B, Levey S, Lyle S, Wang TC & Houghton J 2011, 'Human Barrett's adenocarcinoma of the esophagus, associated myofibroblasts, and endothelium can arise from bone marrow-derived cells after allogeneic stem cell transplant', *Stem cells and development,* vol. 20, no. 1, pp. 11-7.

[39] Hvid-Jensen F, Pedersen L, Drewes AM, Sorensen HT & Funch-Jensen P 2011, 'Incidence of adenocarcinoma among patients with Barrett's esophagus', *The New England journal of medicine,* vol. 365, no. 15, pp. 1375-83.

[40] Im E & Martinez JD 2004, 'Ursodeoxycholic acid (UDCA) can inhibit deoxycholic acid (DCA)-induced apoptosis via modulation of EGFR/Raf-1/ERK signaling in human colon cancer cells', *The Journal of nutrition,* vol. 134, no. 2, pp. 483-6.

[41] Ismail-Beigi F, Horton PF & Pope CE2 1970, 'Histological consequences of

gastroesophageal reflux in man', *Gastroenterology,* vol. 58, no. 2, pp. 163-74.

[42] Jaiswal KR, Morales CP, Feagins LA, Gandia KG, Zhang X, Zhang H, Hormi-Carver K, Shen Y, Elder F, Ramirez RD, Sarosi GAJ, Spechler SJ & Souza RF 2007, 'Characterization of telomerase-immortalized, non-neoplastic, human Barrett's cell line (BAR-T)', *Diseases of the esophagus : official journal of the International Society for Diseases of the Esophagus / I.S.D.E,* vol. 20, no. 3, pp. 256-64.

[43] Jenkins GJS, Cronin J, Alhamdani A, Rawat N, D'Souza F, Thomas T, Eltahir Z, Griffiths AP & Baxter JN 2008, 'The bile acid deoxycholic acid has a non-linear dose response for DNA damage and possibly NF-kappaB activation in oesophageal cells, with a mechanism of action involving ROS', *Mutagenesis,* vol. 23, no. 5, pp. 399-405.

[44] Jenkins GJS, Harries K, Doak SH, Wilmes A, Griffiths AP, Baxter JN & Parry JM 2004, 'The bile acid deoxycholic acid (DCA) at neutral pH activates NF-kappaB and induces IL-8 expression in oesophageal cells in vitro', *Carcinogenesis,* vol. 25, no. 3, pp. 317-23.

[45] Jürgens S, Meyer F, Spechler SJ & Souza R 2012, 'The Role of Bile Acids in the Neoplastic Progression of Barrett's Esophagus - a short representative overview', *Zeitschrift für Gastroenterologie,* vol. 50, no. 09, pp. 1028-34.

[46] Karin M, Cao Y, Greten FR & Li Z 2002, 'NF-kappaB in cancer: from innocent bystander to major culprit', *Nature reviews. Cancer,* vol. 2, no. 4, pp. 301-10.

[47] Katada N, Hinder RA, Smyrk TC, Hirabayashi N, Perdikis G, Lund RJ, Woodward T & Klingler PJ 1997, 'Apoptosis is inhibited early in the dysplasia-carcinoma sequence of Barrett esophagus', *Archives of surgery (Chicago, Ill. : 1960),* vol. 132, no. 7, pp. 728-33.

[48] Katz PO 2000, 'Review article: the role of non-acid reflux in gastro-oesophageal reflux disease', *Alimentary pharmacology therapeutics,* vol. 14, no. 12, pp. 1539-51.

[49] Kauer WK, Peters JH, DeMeester TR, Feussner H, Ireland AP, Stein HJ & Siewert RJ 1997, 'Composition and concentration of bile acid reflux into the esophagus of patients with gastroesophageal reflux disease', *Surgery,* vol. 122, no. 5, pp. 874-81.

[50] Kauer WK, Peters JH, DeMeester TR, Ireland AP, Bremner CG & Hagen JA 1995, 'Mixed reflux of gastric and duodenal juices is more harmful to the esophagus than gastric juice alone. The need for surgical therapy re-emphasized', *Annals of surgery,* vol. 222, no. 4, pp. 525-31; discussion 531-3.

[51] Kroemer G, Galluzzi L & Brenner C 2007, 'Mitochondrial membrane permeabilization in cell death', *Physiological reviews,* vol. 87, no. 1, pp. 99-163.

[52] Lee CH, Jeon Y, Kim S & Song Y 2007, 'NF-kappaB as a potential molecular target for cancer therapy', *BioFactors (Oxford, England),* vol. 29, no. 1, pp. 19-35.

[53] Lee DK, Park SY, Baik SK, Kwon SO, Chung JM, Oh E & Kim HS 2004, 'Deoxycholic acid-induced signal transduction in HT-29 cells: role of NF-kappa B and interleukin-8]', *The Korean journal of gastroenterology = Taehan Sohwagi Hakhoe chi,* vol. 43, no. 3, pp. 176-85.

[54] Mah L, El-Osta A & Karagiannis TC 2010, 'gammaH2AX: a sensitive molecular marker of DNA damage and repair', *Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, U.K,* vol. 24, no. 4, pp. 679-86.

[55] Mahmoud NN, Dannenberg AJ, Bilinski RT, Mestre JR, Chadburn A, Churchill M,

Martucci C & Bertagnolli MM 1999, 'Administration of an unconjugated bile acid increases duodenal tumors in a murine model of familial adenomatous polyposis', *Carcinogenesis,* vol. 20, no. 2, pp. 299-303.

[56] McConkey DJ & Orrenius S 1996, 'Signal transduction pathways in apoptosis', *Stem cells (Dayton, Ohio),* vol. 14, no. 6, pp. 619-31.

[57] Mehta SP, Boddy AP, Cook J, Sams V, Lund EK, Johnson IT & Rhodes M 2008, 'Effect of n-3 polyunsaturated fatty acids on Barrett's epithelium in the human lower esophagus', *The American journal of clinical nutrition,* vol. 87, no. 4, pp. 949-56.

[58] Monte MJ, Marin JJG, Antelo A & Vazquez-Tato J 2009, 'Bile acids: chemistry, physiology, and pathophysiology', *World journal of gastroenterology: WJG,* vol. 15, no. 7, pp. 804-16.

[59] Nehra D, Howell P, Williams CP, Pye JK & Beynon J 1999, 'Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity', *Gut,* vol. 44, no. 5, pp. 598-602.

[60] Orlando RC 2008, 'Pathophysiology of gastroesophageal reflux disease', *Journal of clinical gastroenterology,* vol. 42, no. 5, pp. 584-8.

[61] Pai R, Tarnawski AS & Tran T 2004, 'Deoxycholic acid activates beta-catenin signaling pathway and increases colon cell cancer growth and invasiveness', *Molecular biology of the cell,* vol. 15, no. 5, pp. 2156-63.

[62] Pascu O & Lencu M 2004, 'Barrett's Esophagus', *Romanian journal of gastroenterology,* vol. 13, no. 3, pp. 219-22.

[63] Perez M & Briz O 2009, 'Bile-acid-induced cell injury and protection', *World journal of gastroenterology: WJG,* vol. 15, no. 14, pp. 1677-89.

[64] Pohl H & Welch HG 2005, 'The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence', *Journal of the National Cancer Institute,* vol. 97, no. 2, pp. 142-6.

[65] Quaroni L, Zhao R & Casson AG 2009, 'Shining light on Barrett's esophagus', *Expert review of gastroenterology hepatology,* vol. 3, no. 6, pp. 577-80.

[66] Raj A & Jankowski J 2004, 'Acid suppression and chemoprevention in Barrett's oesophagus', *Digestive diseases (Basel, Switzerland),* vol. 22, no. 2, pp. 171-80.

[67] Ramirez RD, Morales CP, Herbert BS, Rohde JM, Passons C, Shay JW & Wright WE 2001, 'Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions', *Genes development,* vol. 15, no. 4, pp. 398-403.

[68] Ravi R & Bedi A 2004, 'NF-kappaB in cancer--a friend turned foe', *Drug resistance updates: reviews and commentaries in antimicrobial and anticancer chemotherapy,* vol. 7, no. 1, pp. 53-67.

[69] Ridlon JM, Kang D & Hylemon PB 2006, 'Bile salt biotransformations by human intestinal bacteria', *Journal of lipid research,* vol. 47, no. 2, pp. 241-59.

[70] Rieder F, Biancani P, Harnett K, Yerian L & Falk GW 2010, 'Inflammatory mediators in gastroesophageal reflux disease: impact on esophageal motility, fibrosis, and carcinogenesis', *American journal of physiology. Gastrointestinal and liver physiology,* vol. 298, no. 5, pp. G571-81.

[71] Rizvi S, Demars CJ, Comba A, Gainullin VG, Rizvi Z, Almada LL, Wang K, Lomberk G, Fernandez-Zapico ME & Buttar NS 2010, 'Combinatorial chemoprevention reveals a novel smoothened-independent role of GLI1 in esophageal carcinogenesis', *Cancer research,* vol. 70, no. 17, pp. 6787-96.

[72] Rodrigues CM, Fan G, Ma X, Kren BT & Steer CJ 1998, 'A novel role for ursodeoxycholic acid in inhibiting apoptosis by modulating mitochondrial membrane perturbation', *The Journal of clinical investigation,* vol. 101, no. 12, pp. 2790-9.

[73] Rodrigues CM, Fan G, Wong PY, Kren BT & Steer CJ 1998, 'Ursodeoxycholic acid may inhibit deoxycholic acid-induced apoptosis by modulating mitochondrial transmembrane potential and reactive oxygen species production', *Molecular medicine,* vol. 4, no. 3, pp. 165-78.

[74] Roos WP & Kaina B 2006, 'DNA damage-induced cell death by apoptosis', *Trends in molecular medicine,* vol. 12, no. 9, pp. 440-50.

[75] Salminen JT, Tuominen JA, Ramo OJ, Farkkila MA & Salo JA 1999, 'Oesophageal acid exposure: higher in Barrett's oesophagus than in reflux oesophagitis', *Annals of medicine,* vol. 31, no. 1, pp. 46-50.

[76] Sarosi G, Brown G, Jaiswal K, Feagins LA, Lee E, Crook TW, Souza RF, Zou YS, Shay JW & Spechler SJ 2008, 'Bone marrow progenitor cells contribute to esophageal regeneration and metaplasia in a rat model of Barrett's esophagus', *Diseases of the esophagus: official journal of the International Society for Diseases of the Esophagus / I.S.D.E,* vol. 21, no. 1, pp. 43-50.

[77] Scates DK, Venitt S, Phillips RK & Spigelman AD 1995, 'High pH reduces DNA damage caused by bile from patients with familial adenomatous polyposis: antacids may attenuate duodenal polyposis', *Gut,* vol. 36, no. 6, pp. 918-21.

[78] Schoemaker MH, Gommans WM, Conde de la Rosa L, Homan M, Klok P, Trautwein C, van Goor H, Poelstra K, Haisma HJ, Jansen PLM & Moshage H 2003, 'Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to nuclear factor-kappa B activation', *Journal of hepatology,* vol. 39, no. 2, pp. 153-61.

[79] Senftleben U & Karin M 2002, 'The IKK/NF-kappa B pathway', *Critical care medicine,* vol. 30, no. 1 Suppl, pp. S18-26.

[80] Serfaty L, De Leusse A, Rosmorduc O, Desaint B, Flejou J, Chazouilleres O, Poupon RE & Poupon R 2003, 'Ursodeoxycholic acid therapy and the risk of colorectal adenoma in patients with primary biliary cirrhosis: an observational study', *Hepatology*, vol. 38, no. 1, pp. 203-9.

[81] Silberg DG, Sullivan J, Kang E, Swain GP, Moffett J, Sund NJ, Sackett SD & Kaestner KH 2002, 'Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice', *Gastroenterology,* vol. 122, no. 3, pp. 689-96.

[82] Sola S, Aranha MM, Steer CJ & Rodrigues CMP 2007, 'Game and players: mitochondrial apoptosis and the therapeutic potential of ursodeoxycholic acid', *Current issues in molecular biology,* vol. 9, no. 2, pp. 123-38.

[83] Souza RF & Spechler SJ 2005, 'Concepts in the prevention of adenocarcinoma of the distal esophagus and proximal stomach', *CA: a cancer journal for clinicians,* vol. 55, no. 6, pp. 334-51.

[84] Souza RF, Huo X, Mittal V, Schuler CM, Carmack SW, Zhang HY, Zhang X, Yu C, Hormi-Carver K, Genta RM & Spechler SJ 2009, 'Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury', *Gastroenterology,* vol. 137, no. 5, pp. 1776-84.

[85] Souza RF, Krishnan K & Spechler SJ 2008, 'Acid, bile, and CDX: the ABCs of making Barrett's metaplasia', *American journal of physiology. Gastrointestinal and liver physiology,* vol. 295, no. 2, pp. G211-8.

[86] Spechler SJ, Sharma P, Souza RF, Inadomi JM & Shaheen NJ 2011, 'American Gastroenterological Association medical position statement on the management of Barrett's esophagus', *Gastroenterology,* vol. 140, no. 3, pp. 1084-91.

[87] Stein HJ, Kauer WK, Feussner H & Siewert JR 1998, 'Bile reflux in benign and malignant Barrett's esophagus: effect of medical acid suppression and nissen fundoplication', *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract,* vol. 2, no. 4, pp. 333-41.

[88] Stiehl A 1992, 'Die Behandlung cholestatischer Lebererkrankungen; zur Rolle der Ursodesoxycholsaure', *Zeitschrift für Gastroenterologie,* vol. 30, no. 10, pp. 743-7.

[89] Tobey NA, Hosseini SS, Caymaz-Bor C, Wyatt HR, Orlando GS & Orlando RC 2001, 'The role of pepsin in acid injury to esophageal epithelium', *The American journal of gastroenterology,* vol. 96, no. 11, pp. 3062-70.

[90] Tung BY, Emond MJ, Haggitt RC, Bronner MP, Kimmey MB, Kowdley KV & Brentnall TA 2001, 'Ursodiol use is associated with lower prevalence of colonic neoplasia in patients with ulcerative colitis and primary sclerosing cholangitis', *Annals of internal medicine,* vol. 134, no. 2, pp. 89-95.

[91] Vaezi MF & Richter JE 1996, 'Role of acid and duodenogastroesophageal reflux in gastroesophageal reflux disease', *Gastroenterology,* vol. 111, no. 5, pp. 1192-9.

[92] Vaezi MF & Richter JE 2000, 'Duodenogastro-oesophageal reflux', *Bailliere's best practice research. Clinical gastroenterology,* vol. 14, no. 5, pp. 719-29.

[93] Van Waes C 2007, 'Nuclear factor-kappaB in development, prevention, and therapy of cancer', *Clinical cancer research: an official journal of the American Association for Cancer Research,* vol. 13, no. 4, pp. 1076-82.

[94] Van de Meeberg PC, van Erpecum KJ & van Berge-Henegouwen GP 1993, 'Therapy with ursodeoxycholic acid in cholestatic liver disease', *Scandinavian journal of gastroenterology. Supplement,* vol. 200, pp. 15-20.

[95] Wang KK & Sampliner RE 2008, 'Updated guidelines 2008 for the diagnosis, surveillance and therapy of Barrett's esophagus', *The American journal of gastroenterology,* vol. 103, no. 3, pp. 788-97.

[96] Wang X, Ouyang H, Yamamoto Y, Kumar PA, Wei TS, Dagher R, Vincent M, Lu X, Bellizzi AM, Ho KY, Crum CP, Xian W & McKeon F 2011, 'Residual embryonic cells as precursors of a Barrett's-like metaplasia', *Cell,* vol. 145, no. 7, pp. 1023-35.

[97] Xu X, Li Z, Zou D, Xu G, Ye P, Sun Z, Wang Q & Zeng Y 2006, 'Role of duodenogastroesophageal reflux in the pathogenesis of esophageal mucosal injury and gastroesophageal reflux symptoms', *Canadian journal of gastroenterology = Journal canadien de gastroenterologie,* vol. 20, no. 2, pp. 91-4.

[98] Yoshida N 2007, 'Inflammation and oxidative stress in gastroesophageal reflux disease', *Journal of clinical biochemistry and nutrition,* vol. 40, no. 1, pp. 13-23.

[99] Zhang H, Zhang X, Hormi-Carver K, Feagins LA, Spechler SJ & Souza RF 2007, 'In non-neoplastic Barrett's epithelial cells, acid exerts early antiproliferative effects through activation of the Chk2 pathway', *Cancer research,* vol. 67, no. 18, pp. 8580-7.

[100] Zhang HY, Spechler SJ & Souza RF 2009, 'Esophageal adenocarcinoma arising in Barrett esophagus', *Cancer letters,* vol. 275, no. 2, pp. 170-7.

[101] Zou H, Li Y, Liu X & Wang X 1999, 'An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9', *The Journal of biological chemistry,* vol. 274, no. 17, pp. 11549-56.

[102] von Holzen U, Chen T, Boquoi A, Richter JE, Falk GW, Klein-Szanto AJ, Cooper H, Litwin S, Weinberg DS & Enders GH 2010, 'Evidence for DNA damage checkpoint activation in barrett esophagus', *Translational oncology,* vol. 3, no. 1, pp. 33-42.

8. ACKNOWLEDGEMENTS

Thank you first to Dr. Souza who helped me a lot to design and implement this project and who introduced me to the topic of Barrett's Esophagus and has been patient again and again in explaining much about the background.

Thank you to Xiaofang Huo who taught me most of the techniques that I used and gave me a hand in cell culturing and who was instrumental in helping to solve all problems with the experimental conditions.

Thank you to Xi Zhang for her help with orders of reagents and who taught me the basic functions and how to use the microscope and for refurbishing the Barrett's cell lines.

Thank you to Dr. Spechler for introducing me into the clinical side of research, for collaborating and helping me to get the patients' tissues.

Furthermore thank you to the "Referat für Forschung" of the University Hospital at Magdeburg Saxony-Anhalt (Germany) that gave me financial support for the time I spent in the United States.

Thank you to Prof. Dr. Meyer, including his boss, Prof. Dr. Lippert, Heat, Dept. of Surgery, without their support this project never could have been started. Thanks especially to Prof. Dr Meyer for his constant help and his encouragement of medical student research.

9. DECLARATION

Hiermit erkläre ich, Stefanie Jürgens, dass ich die der Medizinischen Fakultät der Otto-von-Guericke-Universität Magdeburg zur Promotion eingereichte Dissertation mit dem Titel:

Effects of Deoxycholic Acids and Urodeoxycholic Acids on Cancerogenic Progression in Barrett's Esophagus

in der Klinik für Allgemein-, Viszeral-, und Gefäßchirurgie

mit Unterstützung durch Associate Professor of Medicine at Dallas Rhonda Souza and Prof. Dr. Frank Meyer

ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu verbreiten.

Bielefeld, den 15.02.2013

Stefanie Jürgens

10. CURRICULUM VITAE

Persönliche Daten

Name	Jürgens
Vorname	Stefanie
Geburtsdatum	20.08.1985
Geburtsort	Herford
Familienstand	ledig
Staatsangehörigkeit	deutsch

Schule und Ausbildung

Ab 02/2013	Assistenzarzt Innere Medizin/ Pneumologie im evangelischen Krankenhaus Bielefeld
11/2012-01/2013	Überarbeitung und Vervollständigung der medizinischen Dissertation
10/2012	2. Staatsexamen und Approbation als Arzt
08/2011- 08/2012	Praktisches Jahr in den Fächern: • Chirurgie im Harzklinikum Wernigerode • Anästhesie und Intensivmedizin im Klinikum Magdeburg GmbH • Innere Medizin – Pneumologie/ Onkologie in der Lungenklinik Lostau
04/2011	Fortführung des Medizinstudiums
11/2010- 03/2011	Beginn mir Verfassung der Dissertation
03/2010- 10/2010	Forschungsaufenthalt für die medizinische Doktorarbeit an der Southwestern University Dallas/ Tx; VA Medical Center, Dallas/Tx, USA
09/2007- 02/2010	Medizinstudium bis einschließlich 9. Fachsemester
08/2007	Physikum
10/2005	Studium der Humanmedizin an der

	Otto- von Guericke Universität in Magdeburg
08/1996- 07/2005	Immanuel Kant Gymnasium in Bad Oeynhausen Abschluss: Abitur
08/1992- 08/1996	Grundschule Werste in Bad Oeynhausen

Bielefeld, den 15.02.2013

Stefanie Jürgens

11. PUBLIKATIONSVERZEICHNIS

- Vortrag September 2010: „**Effects of Deoxycholic Acids and Urodeoxycholic Acids on Barrett's Esophagus**"
  Stefanie Jürgens
  Interne Fortbildung des VA Southwestern Medical Center Dallas, Texas , USA; Arbeitsgruppe R Souza und S Spechler

- Paperpublikation: „**Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF-κB activation in benign Barrett's epithelial cells."**
  Huo X, Juergens S, Zhang X, Rezaei D, Yu C, Strauch ED, Wang JY, Cheng E, Meyer F, Wang DH, Zhang Q, Spechler SJ, Souza RF. Am J Physiol Gastrointest Liver Physiol. 2011 Aug;301(2):G278-86. Epub 2011 Jun 2.

- Posterpräsentation: „**Differenzierte Effekte verschiedener Gallensäuren im kanzerogenen Progressionsgeschehen beim Barrett-Esophagus – experimentelle in-vitro-Studien"**
  Jürgens S, Meyer F, Souza RF
  15. September 2011, DGAV Leipzig 2011

- Abstractpublikation: **„Differenzierte Effekte verschiedener Gallensäuren im kanzerogenen Progressionsgeschehen beim Barrett-Esophagus – experimentelle in-vitro-Studien"**
  Jürgens S, Meyer F, Souza RF
  Z Gastroenterol 2011;49:1050 (Abstract P002 - Abstracts of the 66th Congress of the German Society of Gastroenterology and Metabolism & 5th Autmn Congress of the German Society of Greneral and Abdominal Surgery, September 14-17, 2011, Leipzig [Germany]).

- Paperpublikation: **"The Role of Bile Acids in the Neoplastic Progression of Barrett's Esophagus – a short representative overview""**
  Jürgens S, Meyer F, Spechler SJ, Souza RF
  Zeitschrift für Gastroenterologie, Thieme Verlag. 2012 Sep;50(9):1028-34