INFLUENCE OF TEA CATECHINS ON THE VIABILITY, IL-8 SYNTHESIS AND SECRETION, AND NF-κB ACTIVATION OF GASTRIC EPITHELIAL AGS CANCER CELLS

THESIS

Presented in Partial Fulfillment of the Requirements for
the Degree Masters of Science in the Graduate
School of The Ohio State University

By
Fabiola Gutierrez Orozco, B.S.

*****

The Ohio State University
2009

Thesis Committee:  Approved by
Dr. Joshua A. Bomser, Advisor
Dr. Mark L. Failla  Advisor
Dr. Martha A. Belury  Human Ecology Graduate Program
ABSTRACT

Chronic inflammation is involved in the development of gastric cancer, the second leading cause of cancer-related death worldwide. Epidemiological evidence suggests that increased consumption of catechin rich tea may be associated with reduced risk of gastrointestinal cancers. The goal of this study was to evaluate the effects of catechin-rich extracts of green (GT) and black tea (BT) and individual tea catechins (EGC, EGCG, EGC/EGCG) on cell viability, intracellular oxidation and inflammation in the AGS gastric cancer cell line. Cell viability was measured using the MTT assay. Intracellular oxidation was evaluated using the probe dichlorofluorescin. Inflammatory markers included measurement of intracellular and secreted IL-8 protein and the activation of nuclear factor-kappa B (NFkB). Treatment of AGS cells (48h) with EGC, EGC/EGCG and EGCG, reduced cell viability by 36%, 31% and 19%, respectively. Interestingly, a similar reduction in cell viability (27%) was observed upon treatment of cells with BT and GT extracts. Treatment of cells with GT (1.5 mg/ml), BT (1.5 mg/ml) and EGCG (1.5 mg/ml) also inhibited cytokine-induced IL-8 production and subsequent secretion. These anti-inflammatory effects are due, in part, to a reduced activation of NFkB in the AGS cell line. This study demonstrates a potential mechanism by which tea catechins may reduce inflammation associated with gastric cancer.
To my parents, for their love,
unconditional support and encouragement.

To my brothers and sisters,
for supporting me throughout my career in their own way.

To the love of my life for finding me

To all my friends
I would like to thank my advisor, Joshua Bomser, for his support and guidance throughout my master’s career.

I would like also to thank Mark Failla for his scientific guidance, interest and mentorship during the most important stage of my research. Thank you to Dr. Belury for her useful suggestions and encouragement as well.

Many thanks to Dr. Mario Ferruzzi and his lab at Purdue University for providing the samples used in this research.

I am very thankful to Elizabeth Clubbs for her patience, knowledge and friendship. Her advice and support has been a very important part of this process.
VITA

September 17, 1981……………………………….Born - San Luis Potosi, Mexico

2003……………………………………………..B.S. Agro-industrial Engineering
                Universidad Autonoma Chapingo–Mexico

2006-Present………………………….Graduate Teaching and Research Associate
                The Ohio State University

FIELDS OF STUDY

Major Field: Human Nutrition
# TABLE OF CONTENTS

Abstract....................................................................................................................... ii  
Dedication.................................................................................................................. iii  
Acknowledgments...................................................................................................... iv  
Vita............................................................................................................................... v  
List of Tables............................................................................................................... ix  
List of Figures........................................................................................................... x  

## Chapters

1. Literature Review....................................................................................................... 1  
   1.1. Stomach Anatomy and Physiology................................................................. 1  
   1.2. Cancer............................................................................................................... 4  
   1.3. Stomach cancer cell lines............................................................................... 7  
   1.4. Stomach Cancer.............................................................................................. 8  
      1.4.1. Stomach cancer development................................................................. 11  
      1.4.2. Stomach cancer and inflammation....................................................... 14  
      1.4.3. Signaling during stomach carcinogenesis.......................................... 16  
      1.4.4. Diagnosis and treatments.................................................................... 19  
      1.4.5. Diet and stomach cancer...................................................................... 20  
   1.5. Tea.................................................................................................................... 21  
      1.5.1. Tea polyphenols...................................................................................... 22  
      1.5.2. Tea polyphenols digestion and absorption......................................... 24  
      1.5.3. Tea and stomach cancer........................................................................ 26  
      1.5.4. Epidemiological evidence..................................................................... 26  
      1.5.5. *In vitro* and *in vivo* evidence............................................................. 27  
      1.5.6. Clinical evidence..................................................................................... 27  
   1.6. Present study..................................................................................................... 28  

2. Tea catechins reduce cell viability, IL-8 synthesis and secretion, and NFkB activation in AGS gastric cancer cells......................................................... 31  
   2.1. Introduction...................................................................................................... 31  
   2.2. Methods and materials................................................................................... 33
2.2.1. Cell line and cell culture ........................................... 33
2.2.2. Reagents ............................................................. 33
2.2.3. Tea Extracts and Sample preparation ............................. 33
2.2.4. Treatments ............................................................ 34
2.2.5. AGS cell viability .................................................... 34
2.2.6. Intracellular oxidation ............................................. 35
2.2.7. IL-8 assays ........................................................... 35
  2.2.7.1. MTT analysis ................................................... 37
  2.2.7.2. Effect of medium pH ......................................... 37
2.2.8. NFkB activation ..................................................... 37
2.2.9. HPLC sample preparation ......................................... 38
2.2.10. HPLC-MS analysis ................................................. 39
2.2.11. Statistical analysis ............................................... 40

2.3. Results .................................................................................. 41
  2.3.1. Effect of tea/catechin extracts on AGS cell viability .............. 41
  2.3.2. Effects of tea/catechins extracts on AGS gastric cells intracellular oxidation .............................................. 44
  2.3.3. GT, BT and EGCG inhibited IL-1β-induced production and secretion of IL-8 protein in AGS gastric epithelial cells .............................................. 46
    2.3.3.1. Cell viability on AGS gastric cells treated for IL-8 analysis ........................................................................ 49
    2.3.3.2. Dose dependent IL-8 production and secretion inhibition by green tea ................................................ 51
    2.3.3.3. Effect of time of addition of tea/catechin extracts on IL-8 production and secretion ................................. 51
    2.3.3.4. Production of IL-8 protein at a low pH in AGS cells ................................................................................. 54
  2.3.4. NFkB activation assay .................................................. 57
    2.3.4.1. Competitive binding experiments ............................. 57
    2.3.4.2. IL-1β-induced NFkB activation is reduced by green tea, black tea and EGCG ........................................ 57
  2.3.5. Identification of tea extracts and catechin degradation products ................................................................. 60

2.4. Discussion ............................................................................. 62

2.5. Conclusion ............................................................................ 72
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Estimated new cancer cases</td>
<td>9</td>
</tr>
<tr>
<td>Table 1.2</td>
<td>Estimated cancer deaths</td>
<td>9</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Analysis of tea solutions by HPLC-PDA</td>
<td>42</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Tea catechins and their degradation products</td>
<td>61</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Anatomy of the stomach and representation of the oxyntic glands of the body of the stomach.</td>
<td>2</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>The multistage process of carcinogenesis, initiation, promotion, and progression.</td>
<td>5</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Hallmarks of cancer.</td>
<td>6</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Schematic representation of factors which interact to determine the clinical outcome of <em>Helicobacter pylori</em> infection.</td>
<td>10</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Multistep model of gastric precancerous process.</td>
<td>13</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>The balance of pro-inflammatory and anti-inflammatory cytokines produced in the immune response.</td>
<td>15</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>Nuclear factor (NF-κB) activation of the gastric mucosal cell in response to <em>Helicobacter pylori</em>.</td>
<td>18</td>
</tr>
<tr>
<td>Figure 1.8</td>
<td>Structure of major catechins from green tea.</td>
<td>23</td>
</tr>
<tr>
<td>Figure 1.9</td>
<td>Auto-oxidation reactions of EGCG at near-neutral pH.</td>
<td>25</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Effects of tea extracts and their catechins on AGS gastric epithelial cell viability.</td>
<td>43</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Intracellular oxidation on AGS cells after treatment with tea extracts and their catechins.</td>
<td>45</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>(A) Intracellular and (B) secreted IL-8 protein (% relative to IL-1β) in AGS cells.</td>
<td>48</td>
</tr>
</tbody>
</table>
Figure 2.4  Cell viability assays on cells treated for IL-8 analysis.................50
Figure 2.5  Dose dependency green tea-mediated inhibition of (A) Intracellular
and (B) Secreted IL-8 protein (pg/mL) in IL-1β-treated AGS cells........52
Figure 2.6  Effect of time of addition of tea extracts and EGCG on (A) Intracellular
and (B) secreted IL-8 protein (pg/mL) in AGS cells .........................53
Figure 2.7  Intracellular IL-8 protein (pg/mL) in AGS cells at pH 6.0..............55
Figure 2.8  Effects of low pH on morphology of AGS gastric epithelial cells......56
Figure 2.9  NFκB activation in AGS cells: competitive binding experiments......58
Figure 2.10 NFκB activation in AGS gastric cells......................................59
CHAPTER 1

LITERATURE REVIEW

1.1 Stomach Anatomy and Physiology

The stomach is localized just below the diaphragm in the upper part of the abdominal cavity mainly to the left of the midline under a portion of the liver. The stomach is composed of five regions: (a) the cardia and gastroesophageal (GE) junction, (b) the fundus, (c) the corpus, (d) the antrum, and (e) the pylorus (Figure 2.1). The fundus and corpus harbor acid-secreting glands, whereas alkaline-secreting surface epithelium and endocrine, gastrin-secreting G-cells are found in the antrum. The pylorus is a ring of muscle separating the stomach from the duodenum. The stomach is richly vascularized [3]. The right side of the stomach shown in Figure 1.1 is called the greater curvature and that on the left the lesser curvature [4].
Figure 1.1: Anatomy of the stomach and representation of the oxyntic glands of the body of the stomach. Adapted from Saunders Co [5].
The stomach has a roughly J shape at rest; its size and shape varies with the volume of food or fluids it contains, the position of the body and the phase of respiration [6].

The gastric mucosa is divided into acid-secreting and non-acid secreting regions. The first region is found in the corpus and fundus. The gastric gland is the acid secreting unit of the mucosa. Pepsinogen-secreting chief cells are found at the base of the gastric gland, while the HCl-secreting parietal cells are localized in the middle of the gastric gland. Toward the lumen, at the neck, mucus neck cells are found; mucus cells secrete alkaline mucus protecting the epithelium against shear stress and acid and then, near the opening, the mucosa is mainly populated with surface epithelial cells [3].
1.2 Cancer

Cancer is a major public health problem in the United States and many other parts of the world. Currently, one in 4 deaths in the United States is due to cancer. A total of 1,437,180 new cancer cases and 565,650 deaths from cancer are expected to occur in the US in 2008 [7].

The carcinogenic process is a multistep process of initiation, promotion and progression as depicted in Figure 1.2. In the initiation stage, a normal cell is transformed to an initiated cell when DNA damage occurs. This initiated cell is then altered, allowing it for clonal expansion into a population of pre-neoplastic cells, a stage termed promotion. Additional genetic alterations lead to the transformation of the pre-neoplastic cells to a malignant tumor, a final stage called progression [8].

The genetic alterations that lead a normal cell through the different stages in cancer development have been proposed to be group into six categories or hallmarks of cancer as shown in Figure 1.3. These six basic alterations are thought to be shared by most and probably all types of cancer tumors. These hallmarks are described as the following: evading apoptotic signals; self-sufficiency in growth signals via oncogenic activation; sustaining angiogenesis; tissue invasion and metastasis; insensitivity to anti-growth signals via tumor suppressor inactivation; and limitless replicative potential [9].
Figure 1.2: The multistage process of carcinogenesis, initiation, promotion, and progression. Taken from Hursting et al [8].
Figure 1.3: Hallmarks of cancer. Taken from Hanahan and Weinberg [9].
1.3 Stomach cancer cell lines

The cell line used in the studies described later on is the human gastric cancer cell line AGS. These cells were isolated from a surgically resected human adenocarcinoma of the stomach. The parent cancer cell line was shown to grow in vivo in athymic mice, and the presence of mucin confirms that they are epithelial [10]. AGS cells exhibit a polygonal phenotype forming dense mono-layers containing a proportion (~10%) of flattened multinuclear cells. Absence of cell-cell junctions is characteristic of these cells. This cell line arises from moderate to well differentiated gastric cancers. The lack of E-cadherin in these cells might explain the loss of integrity after confluence is reached. They present tyrosine kinase receptors [11]. Further studies have confirmed that these cells are poorly differentiated and do not polarize or for proper cell tight junctions [12, 13, 14].
1.4 Stomach Cancer

Although the overall incidence of gastric cancer has declined in the past 75 years, it still remains as the fourth most common type of cancer and the second leading cause of cancer related death just behind lung cancer (Table 1.1 and 1.2) [1, 2]. It is estimated that in 2008, 21,500 men and women will be diagnosed with stomach cancer in the US and 10880 will die from this disease. Furthermore, 1 in 113 men and women will be diagnosed with stomach cancer during their lifetime; and most people affected by gastric cancer are over 65 years of age. Asians and Pacific Islanders have the highest mortality rates, followed by African Americans, Hispanics, American Indians and Alaskan Natives, and whites. As in many other types of cancer, men have higher stomach cancer incidence and mortality rates than women [15].

It has been proposed that the gastric carcinogenesis process represents the interaction of three major sets of factors: the agent (*Helicobacter pylori*), the host, and the external environment as depicted in Figure 1.4.
<table>
<thead>
<tr>
<th>Cancer</th>
<th>New cancer cases</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>1239000</td>
<td>12.3</td>
</tr>
<tr>
<td>Breast</td>
<td>1050000</td>
<td>10.4</td>
</tr>
<tr>
<td>Colon/Rectum</td>
<td>945000</td>
<td>9.4</td>
</tr>
<tr>
<td>Stomach</td>
<td>876000</td>
<td>8.7</td>
</tr>
<tr>
<td>Liver</td>
<td>564000</td>
<td>5.6</td>
</tr>
<tr>
<td>Prostate</td>
<td>543000</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Table 1.1: Estimated new cancer cases. Ten most common sites, world 2000. Adapted from Parkin et al. [1]

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Deaths</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>1103000</td>
<td>17.8</td>
</tr>
<tr>
<td>Stomach</td>
<td>647000</td>
<td>10.4</td>
</tr>
<tr>
<td>Liver</td>
<td>549000</td>
<td>8.8</td>
</tr>
<tr>
<td>Colon/Rectum</td>
<td>492000</td>
<td>7.9</td>
</tr>
<tr>
<td>Breast</td>
<td>373000</td>
<td>6.0</td>
</tr>
<tr>
<td>Esophagus</td>
<td>338000</td>
<td>5.4</td>
</tr>
</tbody>
</table>

Table 1.2: Estimated cancer deaths. Ten most common sites, world 2000. Adapted from Parkin et al. [1]
Figure 1.4: Schematic representation of factors which interact to determine the clinical outcome of *Helicobacter pylori* infection. Taken from Correa et al [16].
1.4.1 Stomach cancer development

From all gastric cancers, approximately 90% are adenocarcinomas [17] that can be further classified as intestinal and diffuse subtypes [18]. The early stages of the disease usually remain silent until the time of diagnosis when the patients have advanced stages and the prognosis is poor. For instance, a 5-year survival rate of around 20% in the US has been reported [19]. It has been well characterized that the precancerous stages usually takes decades to develop into gastric cancer [18].

A sequence of events in the gastric mucosa has to occur before invasive neoplasia occurs as shown in Figure 1.5 [16]. Each of these lesions may present different types of severity and phenotypic characteristics. Development of multifocal atrophic gastritis, which is most prevalent in populations with an increased risk of developing gastric cancer [20], is characterized by the loss of specialized glandular tissue, for instance, loss of oxyntic glands, which produce parietal cells (Figure 1.1) [21].
A critical step in the development of gastric cancer is the presence of atrophic gastritis as shown in mapping studies of resected stomachs of patients with intestinal-type gastric cancer. Every case in this study showed that atrophic gastritis was present in these patients but not intestinal metaplasia [22]. As mentioned before, atrophic gastritis involves the loss of parietal cells which in turn, is associated with a reduction on the secretion of signaling molecules that regulate the growth and differentiation of gastric progenitors, giving rise to an increase on proliferation and accumulation of undifferentiated progenitors. Therefore, the loss of parietal cells might lead to the emergency of metaplasia [23].

Atrophic gastritis usually progress to intestinal metaplasia. Type I or complete intestinal metaplasia is characterized by a phenotype resembling the small intestine mucosa with absorptive enterocytes alternating with goblet cells [24, 25]. Further transformation leads to the so-called Type III or incomplete intestinal metaplasia, usually seen in older patients, where the metaplastic cells present a “colonic” phenotype. This phenotype is characterized by columnar cells without a defined brush border and is found near the areas of dysplasia or in patients with “early” carcinomas [25]. This condition predispose for a higher risk for gastric cancer than complete metaplasia [26]. In general, the precancerous process has shown a slow progression to more advances lesions [27] and therefore it is possible to take preventative actions to decrease the risk of developing gastric cancer.
Figure 1.5: Multistep model of gastric precancerous process. Taken from Correa et al. [16].
As mentioned previously, a series of factors might be involved in the development of gastric cancer; however, according to several studies it is clear that chronic inflammation plays a crucial role in this malignancy [28]. For instance, early studies analyzing surgical resection specimens suggested stronger association between advanced chronic gastritis and gastric cancer than duodenal ulceration. Stomach tumors have also been found in areas of chronic inflammation and atrophic gastritis [29, 30].

1.4.2 Stomach cancer and inflammation

Extensive epidemiological evidence has identified chronic inflammation as a critical factor in the development of intestinal metaplasia and mutations in oncogenes that precede the development of gastric cancer.

The balanced production of inflammatory and anti-inflammatory molecules during an immune response is crucial for protection against the environment without producing excessive inflammation. On the contrary, an imbalance in the immune response may lead to chronic inflammation. In response to infection, the body produces pro-inflammatory cytokines which is accompanied by a compensatory induction of the anti-inflammatory response to maintain an appropriate balance (Figure 1.6) [31].
During infection, epithelial cells transduce signals for the engagement of receptors that can stimulate the synthesis of molecules such as interleukin-8 (IL-8) which then, activates and recruits neutrophils, as well as mast cells, macrophages, etc. to the gastric mucosa. The increase in inflammatory molecules such as IL-8 leads to a further raise in oxidative stress damage and consequent epithelial cell damage, including DNA damage and cell death. Damage to the epithelium causes disruption of its physiological barrier function and increased cell turnover. This damage tissue can eventually be replaced by fibrosis or metaplastic tissue [31].
In addition, inflammatory conditions are characterized by activation of the transcription factor nuclear factor kappa B (NF-κB), which leads to the expression of NF-κB-dependent inflammatory-related genes, such as inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2). Inhibition of programmed cell death (apoptosis) by these genes leads to cell survival [32]. For instance, it was shown that exposure to pro-inflammatory cytokines in the absence of NF-κB activation lead to apoptosis [33]. Furthermore, chronic inflammation leads to constitutive activation of NF-κB and thus, to the continuous expression of pro-survival genes, as proved in chronic gastritis [34].

Although the survival of cells during inflammation can be beneficial, constitutive activation of NF-κB and anti-apoptotic cells may promote the continuous proliferation of cells and therefore give raise to malignant tissues [32].

1.4.3 Signaling during stomach carcinogenesis

Not only IL-8 is a chemo-attractant for neutrophils and lymphocytes [35] but also an inducer of tumor cells migration [36], proliferation of keratinocytes and melanoma cells [37, 38] and angiogenesis [39, 40].

The gene encoding for the synthesis of IL-8 contains several binding regions within its promoter region (Figure 1.7). A binding motif for NF-κB is present in the IL-8 gene at nucleotides -80 to -70. Additional binding sites exist for transcription factors such as c-fos and c-jun, which comprise AP-1, found at nucleotides -126 to -120 [41].
It has been confirmed that the production of IL-8 involves tyrosine phosphorylation and NF-κB activation [42]. Recent studies using gastric epithelial cells further confirmed the presence of tyrosine phosphorylation when the cells were stimulated with inflammatory cytokines [43].

It has been largely known that activation of NF-κB by pro-inflammatory cytokines involve the activation of the inhibitory κB kinases (IKK). IKKβ phosphorylates IκBα at serine 32 and 36, resulting in poly-ubiquitination and subsequent degradation by the proteosomal pathway. The release of NF-κB from its inhibitor IκBα allows NF-κB to translocate to the nucleus and start the transcription of genes containing an NF-κB binding site in their promoter region, such as IL-8 [44].

Furthermore, NF-κB activation has been linked to the proliferation of cells and tumor growth [45]. More importantly, the role of NF-κB activation in H. pylori infection of gastric mucosa has been documented before [46, 47]. The activation of NF-κB in gastric epithelial cells has been found to increase the IL-8 mRNA and protein levels [48, 49].

In addition, other transcription factors might be involved in the regulation of IL-8 gene expression. For instance, gastric epithelial cells infected with H. pylori induce a stress signal leading to AP-1 activation and IL-8 release [50].
Figure 1.7: Nuclear factor (NF-κB) activation of the gastric mucosal cell in response to *Helicobacter pylori*. Taken from Naito and Yoshikawa [41].
1.4.4 Diagnosis and treatments

Growth factors, cytokines, and angiogenic factors have been proposed as molecular-pathological prognostic factors of gastric cancer. Since gastric cancer cells express a wide range of growth factors and their correspondent receptors, they could be used as markers for this disease. These factors induce not only cell growth but also extracellular matrix degradation leading to metastasis, and angiogenesis which helps with cell proliferation. For instance, deep invasion, advanced stage and poor prognosis have been associated with simultaneous expression of epidermal growth factor (EGF), transforming growth factor (TGF-α) and EGF-receptor [51, 52].

In order for the tumor to grow and metastasize, angiogenesis must be present. Neovascularization promotes the growth of primary tumors and provides a path for hematogenous metastasis. Angiogenic factors produced by gastric cancer cells include endothelial growth factor (VEGF), IL-8, basic fibroblast growth factor (bFGF), among others [53-55]. It has been found that increased vascularization correlates with lymph node metastasis, hepatic metastasis, and poor prognosis, all of these may serve as prognostic factors of gastric cancer. For instance, patients displaying high expression tumor levels of VEGF and IL-8, have a significantly poorer prognosis than those patients with low expression levels [56].

Since both inflammation and angiogenesis are promoted by increased production of chemokines and cytokines, growth factors, etc., that provide the conditions necessary for the progression of cancer cells, it could be expected that anti-cancer therapies have
these factors as targets. For example, in a cancer xenograft model has been showed that inflammatory stimuli by IL-1β, produced a raise in tumor growth and angiogenesis and this inflammatory angiogenesis was, in part, mediated by NF-κB and AP-1. Therefore, synthetic or natural compounds that could block both inflammation and angiogenesis could be used as anticancer therapeutic agents [57].

1.4.5 Diet and stomach cancer

It has been shown that diet plays an essential role in the prevention and development of cancer. For instance, it is estimated that nutritional factors account for 80 percent of the gastrointestinal (GI) cancers and that an appropriate modification of the diet might prevent 30 percent of the cancer mortality and morbidity [58-60].

Various dietary components have been thought to have either a causative or a protective role with respect to gastric cancer. For example, there is strong evidence that consumption of salty foods, such as salted pickles and salted fish, increases the risk of gastric cancer [61].

The association between excess salt intake and gastric cancer was first presented in 1965. It was suggested that continuous consumption of high amounts of salt would result in early atrophic gastritis, and thus, the risk of developing gastric cancer. Subsequent ecologic and analytical studies confirmed this association [62, 63].
On the other hand, consumption of vegetables and fruits has protective effects. In addition, epidemiologic and scientific research has suggested that green tea may have a protective effect against gastric cancer [64-66].

1.5 Tea

Tea is the second most consumed beverage in the world only preceded by water. The consumption of tea has been steadily increasing during the last years which can be attributed to several factors: the convenience and availability of ready-to drink tea products, the increased health concern of consumers along with new scientific findings supporting the health benefits of tea consumption and the marketing strategies that have helped to communicate this information [67].

Tea is derived from *Camellia sinensis*, a warm-weather evergreen. Depending on the process followed by the fresh leaves, different types of tea will result: green, black and oolong tea which comprise 75, 23 and 2 percent of the world production, respectively [68]. Black tea, for instance is produced by fermentation and oxidation of the leaves and differs from green tea, in whose process, leaves are steamed and dried out after picking to prevent oxidation of the catechins present in them. Oolong tea is produced by a partial oxidation of the leaves and thus, is a chemical mixture of green and black tea [69]. Green tea is mostly consumed in eastern Asian countries, China and Japan, while black tea is most popular in Europe and India [70].
Because of these differences in processing, black and green tea differ in their chemical composition. For instance, black tea is richer in complex antioxidant such as theaflavins and thearubigins, while green tea contains a higher amount of simpler antioxidants known as catechins [71].

1.5.1 Tea polyphenols

It is estimated that 30% w/w of the dried leaves of green tea is represented by polyphenolic compounds including flavanols, flavandiols, flavonoids and phenolic acids. The predominant polyphenolic compounds in green tea are known as monomeric flavan-3-ols or catechins, which include catechin (C), epicatechin (EC), gallocatechin (GC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), which together comprise 60 percent of the total catechins content [72]. Other compounds present in green tea are caffeine, theanine, flavor compounds and some flavanols compounds such as quercetin, keempferol and rutin [73]. EGCG is probably the most studied tea constituent since it accounts for more than 50 percent of the polyphenolic fraction and it is estimated that up to 200mg of EGCG are present in a single cup of green tea [74].
Figure 1.8: Structure of major catechins from green tea. Taken from Green et al [75]
1.5.2 Tea polyphenols digestion and absorption

A large part of tea catechin ingested orally is distributed in the intestinal mucosa, and is excreted finally into feces. Tea catechins are subject of conjugation reactions (glucoronide and sulfate formations) in the intestinal mucosa, liver, and kidney, and substantial levels of catechin is incorporated into human body in the non-conjugated forms. For instance, EGCG is incorporated into human plasma after drinking green tea [76]. Glucoronidation of EGCG and EGC has been also characterized not only in humans, but also in mice and rats [77]. Methylated EGCG has also been detected in human plasma and urine following green tea consumption [78]. Another study examined the pharmacokinetic parameters of EGCG, EGC, and EC after oral administration of a single dose of green tea (20mg tea solids/kg) or EGCG (2mg/kg). The maximum plasma concentrations of EGCG, EGC, and EC were 77.9, 223.4, and 124.03ng/mL respectively. The peak concentrations were reached after 1.3 to 1.6 h [79].

Tea catechins have been shown to be stable at acidic pH but they are extensively degraded in fluids of near neutral or greater pH, such as intestinal juice and plasma bile. Among tea catechins, EGCG and EGC are the most sensitive to higher pH, ECG has intermediate stability, and EC and C are relatively stable. Degradation of EGCG includes epimerization and auto-oxidation reactions involving the B-ring leading to the formation of homocatechin dimers such as theasinensins (THSN) A, THSN D, and P-2 (Figure 1.9), which are also minor constituents of oolong and black teas [80].
Figure 1.9: Auto-oxidation reactions of EGCG at near-neutral pH. Taken from Neilson et al [80].
1.5.3 Tea and stomach cancer

Since chronic inflammation is a key feature of gastric cancer development, the ability of green tea catechins to decrease not only the proliferation of tumor cells, but also to prevent or inhibit inflammation is an important aspect when considering green tea as a possible chemopreventative therapy in gastric cancer.

1.5.4 Epidemiological evidence

Epidemiological studies have shown an inverse association between green tea consumption and risk of gastric cancer. It has been suggested that gastric cancer risk decreases with the amount of tea consumed [81-83]. Gao et al reported that drinking green tea was associated with reduced esophageal cancer risk in women [81]. Inoue et al reported that increased green tea intake was associated with decreased risk of gastric cancer among Japanese subjects [82]. In addition, Nakachi et al observed lower total cancer incidence among Japanese subjects who consumed 10 cups of green tea per day compared with subjects who consumed fewer than 3 cups per day [83]. However, another large prospective cohort study from Japan failed to show that green tea offers any protective effects against gastric cancer [84, 85].

To our knowledge, no prospective studies of green tea consumption and gastric cancer risk have been reported to date from European or American subject populations.
1.5.5 *In vitro and in vivo* evidence

EGCG has been shown to induce apoptosis in the MKN45 gastric cancer cell line by activating the mitochondrial pathway [86]. Another study, examining the effects of EGCG on the carcinogenesis of the glandular stomach in rats found that EGCG inhibits the cellular kinetics of the gastric mucosa during the promotion stage of the gastric carcinogenesis process [87]. Furthermore, green tea was tested for their ability to exert anti-*H pylori* activity in vivo in a mouse model of experimental infection and it was found that green tea significantly prevented gastritis and limited the localization of bacteria to the surface of the gastric epithelium and prevented its damage [88].

In relation to inflammation, green tea and EGCG were found to inhibit the secretion of the inflammatory chemokine IL-8 in Caco-2 cells. However, in the same study, green tea and EGCG it induced IL-8 mRNA and protein expression [89]. In the HT-29 colon cancer cell line, EGCG was also shown to inhibit the activation of NF-κB by reducing IκBα phosphorylation [90].

1.5.6 Clinical evidence

Data from green tea chemopreventative trials are limited. Studies analyzing the basic distribution patterns, pharmacokinetic parameters, and preliminary safety profiles for short-term oral administration of various green tea preparations are available [76, 79, 91, 92]. Results from these studies have been mentioned above. Nevertheless, no clinical studies analyzing the effects of green tea on gastric cancer exist to this date.
1.6 Present Study

The present study was designed to examine the effects of green (GT) and black tea (BT) and the tea catechins (EGC, EGCG, EGC/EGCG) on gastric cancer in an in vitro model using the AGS gastric epithelial cancer cell line. The central hypothesis is that whole green tea extracts and their individual catechins will exert chemopreventative activity in gastric cancer. This hypothesis is based on previous in vitro models where tea catechins have shown anti-cancer activity. To test this hypothesis the following specific aims are proposed:

Specific Aim 1: Establish the effects of whole green (GT) and black tea (BT) extracts and the individual tea catechins (EGC, EGCG, EGC/EGCG) on cell viability.

Research outlined in specific aim 1 will examine the ability of individual and combined tea catechins and whole tea extracts to influence gastric cancer cell viability using an in vitro model. The working hypothesis is that whole tea extracts rather than individual tea catechins will exert cancer growth inhibitory effects using the AGS gastric epithelial cell line. This hypothesis is based on preliminary data from our lab. This portion of the research will provide evidence that complex catechins mixtures are more effective at decreasing cancer cell viability than individual catechins.
Specific Aim 2: Evaluate the effects of whole green (GT) and black tea (BT) extracts and the individual tea catechins (EGC, EGCG, EGC/EGCG) on modulation of intracellular oxidation. This research will evaluate the effects of tea extracts and catechins in modulating intracellular oxidation using the AGS gastric epithelial cancer cell line. The working hypothesis is that tea extracts and their catechins will decrease intracellular oxidation using a gastric cancer in vitro model. This hypothesis is based on previous results from our lab, where intracellular oxidation was decreased by tea extracts when an in vitro model using a colon cancer cell line was investigated. Results from this specific aim will contribute to explain the anti-cancer effects of tea.

Specific Aim 3: Determine the effects of whole green (GT) and black tea (BT) extracts and the individual tea catechins (EGC, EGCG, EGC/EGCG) on the inflammatory cytokine IL-8 production and secretion. Given that chronic inflammation is one of the main factors leading to the development of gastric cancer, research outlined in this specific aim will examine the anti-inflammatory properties of tea extracts and their catechins. The working hypothesis is that tea extracts and their catechins will decrease the secretion of the inflammatory cytokine IL-8 in the AGS gastric cancer cell line, while having the opposite effect on IL-8 intracellular production. This hypothesis is based on a previous research using a colon cancer in vitro cell model where IL-8 secretion was decreased by applying green tea extracts and the individual tea catechins EGCG. This portion of the research will provide more evidence explaining the anti-inflammatory effects of tea on gastric cancer.
Specific Aim 4: Determine the effects of whole green (GT) and black tea (BT) extracts and the tea catechins EGCG on the activation of NF-κB. Given that NF-κB activation might be responsible, at least in part, for the induction of the inflammatory chemokine IL-8, research outlined in this specific aim will examine the effects of tea extracts and EGCG on the NF-κB pathway. The working hypothesis is that tea extracts and EGCG will inhibit the activation NF-κB in the AGS gastric cancer cell line. This hypothesis is based on a previous research using a colon cancer in vitro cell model where inhibition of NF-κB activation by EGCG was observed. This portion of the research will provide more evidence explaining the anti-inflammatory effects of tea on gastric cancer.
CHAPTER 2

TEA CATECHINS REDUCE CELL VIABILITY, IL-8 SYNTHESIS AND SECRETION, AND NF-KB ACTIVATION IN AGS GASTRIC CANCER CELLS

2.1 Introduction

Although gastric cancer incidence and mortality has declined during the last several decades, it still remains the second leading cause of cancer related death just behind lung cancer [1]. Inflammation has been implicated in the development of intestinal metaplasia, and mutations in oncogenes that precede the development of gastric cancer [28]. Gastric epithelial cells secrete chemokines, such as IL-8 that contribute to the inflammatory response [31]. It has been shown that diet plays an important role in modulating the inflammatory process associated with cancer development. It is estimated that dietary modification alone may lead to a 30% decrease in gastric cancer mortality and morbidity [58-60].
Previous studies suggest that tea consumption reduce cancer risk and positively affects outcomes in the GI tract [86, 93-95]. Catechins, the main tea component, are thought to be responsible, in part, for these anticancer effects. Some proposed mechanisms by which tea catechins exert these anticancer effects include modulation of cell proliferation, scavenging of reactive oxygen species, inhibition of transcriptional factors such as NF-κB, inducement of apoptosis, and regulation of metabolizing enzymes. However, it is still not clear by which mechanisms tea catechins exert chemopreventative activity in gastric cancer. In addition, research on tea catechins have focused mainly on the study of the individual tea catechin EGCG, rather than other catechins or their mixtures. Furthermore, black tea, the main form in which tea is consumed in the western world has not been examined for its chemopreventative effects.

Understanding the factors responsible for the apparent protective effects of tea consumption, including identification of the compound or compounds responsible for these effects is crucial. The overall objective of this study is to evaluate the effects of green (GT) and black tea (BT) and the tea catechins (EGC, EGCG, EGC/EGCG) on gastric cancer in an in vitro model using the AGS gastric epithelial cancer cell line. Effects of tea catechins on cell viability and intracellular oxidation in AGS cells were evaluated. The effect of tea catechins on the production and secretion of the inflammatory cytokine IL-8 and NF-κB activation was also examined. The central hypothesis is that whole green tea extracts and their individual catechins will exert chemopreventative activity in gastric cancer. This hypothesis is based on previous in vitro models where tea catechins have shown anti-cancer activity.
2.2 Methods and materials

2.2.1 Cell line and cell culture.

The human gastric epithelial cell line AGS (American Type Culture Collection, Manassas, VA) was cultured in F-12K medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cell cultures were incubated in humidified atmosphere (5%CO₂, 95%O₂) at 37°C.

2.2.2 Reagents.

All reagents were obtained from Sigma-Aldrich, St. Louis, MO, unless otherwise stated.

2.3.3 Tea Extracts and Sample preparation.

Powdered green tea extract (a gift from Nestle R&D, Marysville, OH, USA); black tea extract (PlanetExtrakt, Germany) and the individual catechins standards of EGCG, and EGC (Sigma-Aldrich, St. Louis, MO) were used in this study. Samples were prepared using a previous methodology [75] with minor modifications. Tea infusions were prepared by dissolving 170mg of extract into 100mL of deionized-distilled water (RT) to result in ~100mg total catechins in green tea formula. Individual catechins (EGCG, EGC, and EGCG/EGC combination) were dissolved in deionized-distilled water (adjusted to pH 5.5 to mimic acidity of a tea infusion) at RT to create a 0.1mg/mL solution. Each tea infusion and catechin standard solution (20mL) was transferred into triplicate 50mL tubes. Samples were placed immediately on ice, acidified with 200μL of
2.0N HCl, sampled for HPLC analysis, and triplicate digestions were pooled. Aliquots (10mL) were freeze dried, and samples flushed with nitrogen and sealed prior to freezing (-80°C). Catechin profiles for the tea and catechin standard solutions were obtained by HPL-PDA analyses and are given in Table 2.1

2.2.4 Treatments.

Green tea (GT), black tea (BT), individual catechins EGCG, EGC, and recombinant EGCG/EGC were dissolved in respective media. For proliferation and intracellular oxidation analysis samples were applied at a concentration of 3, 1.5, 0.75 and 0.375mg of extract per mL of media. For IL-8 and NF-κB assays, GT, BT and EGCG were used at a concentration of 1.5mg/mL.

2.2.5 AGS cell viability.

AGS cells were plated in 96-well cell microtiter plates at a density of 5 x 10⁴ cells/mL. After a 24h-incubation, AGS cells’ growth medium was aspirated and cells were treated with the different tea extracts for 48h. After that, cell viability was measured using the MTT assay. Briefly, cells were incubated with 15μL of MTT (5mg/mL) for 3h. Then, 150 μL of 0.04N HCl-isopropanol were added and within 30min, absorbance was recorded at 610nm. Media alone and water were used as a no-vehicle and vehicle control respectively. Changes in cell viability are normalized to the control.
2.2.6 Intracellular oxidation.

AGS cells were plated at an initial concentration of $1.5 \times 10^5$ cells/mL. Confluent cell cultures were tested. Following 24h incubation, growth medium was removed and cells were washed with PBS. DCF assay was performed using AAPH (Cayman, Ann Arbor, MI) as radical generator at 1.25mM concentration. Dichlorofluorescin (DCF) solution was prepared to give a final 40µM concentration in each well. Test media was prepared to give a final well concentration of 3mg of tea/catechin extract per mL of media. Media containing either DCF or AAPH was used as a control. Test media (100uL) and DCF solution (100uL) were applied at the same time. Plates were incubated at 37°C for 1h. Then the medium was removed and cells were washed with PBS. 150uL of 1.25mM AAPH were applied to each well and fluorescence was measured in a microplate reader set at 485/525nm for the excitation/emission filters respectively, with the temperature maintained at 37°C. Data points were taken every 90sec for 1h.

2.2.7 IL-8 assays.

AGS cells were plated at an initial concentration of $5 \times 10^5$ cells/mL in 60mm dishes and incubated for 48 h until confluence was reached. Growth medium was removed and cells were washed with 3mL of EBSS containing 10mM N-(2-hydroxyethyl)piperazine-N’-ethaesulfonic acid, (HEPES), pH 7.4 and 0.1% gelatin to remove any remaining antibacterial-containing media [43]. Control cells were incubated with media supplemented with 10% FBS. Cells were stimulated for one hour by adding media containing IL-1β (1ng/mL). After that, medium was removed and medium
containing green tea, black tea EGCG, EGC, and EGCC/EGC (1.5mg/mL) plus IL-1β (1ng/mL) extracts was applied and cells were incubated for an additional 23h. Brefeldin A (BFA) was used as a positive control at a concentration of 1.5ug per mL of media for both secreted and intracellular IL-8 assays. BFA is a fungal derivative and it has been previously proved to be a strong inhibitor of secretion by interfering with vesicular transport [89]. In order to determine IL-8 secretion, medium was collected and centrifuged to remove any non-adherent cells and the supernatant stored at -20°C until assayed. Intracellular concentration of IL-8 was determined by lysing the cells in 2mL protein extraction buffer and crude intracellular proteins were isolated. Cell lysates were stored at -20°C until assayed. Both intracellular and secreted IL-8 concentrations were assayed using a commercial ELISA kit (Pierce Biotech, Rockford, IL) which has a sensitivity of less than 2pg/mL. 50uL samples were applied to each well and samples were evaluated in triplicate. A standard curve was used to determine IL-8 concentrations.

Effects of dose on IL-1β-induced IL-8 production and secretion were examined by applying concentrations of GT ranging from 1.5 to 0.375mg/mL.

Effects of time of exposure were also evaluated by applying GT, BT and EGCG (1.5mg/mL) extracts containing IL-1β (1ng/mL) at the same time. After incubation for 24h, both secreted and intracellular IL-8 levels were measured as described before.
2.2.7.1 MTT analysis.

Cell viability was evaluated after applying the treatments for the IL-8 assays described above using the MTT assay.

2.2.7.2 Effect of medium pH.

Given that the gastric epithelium is exposed to an acidic pH (1.0-2.0), the effects of a low pH on the production of IL-8 protein were studied. Media containing 15mM 2-(N-Morpholino) ethanesulfonic acid (MES) was adjusted to a final pH of 6.0 using HCl. Cells were plated and incubated until confluent as mentioned above for the IL-8 assays using regular media at pH 7.4. Cells were washed and media was renewed using either media at pH 7.4 or pH 6.0, followed by incubation for 1h at 37°C. After that, media was removed and corresponding treatments were applied. Control cells were incubated with the media at regular pH (7.4) and pH 6.0. Treatments at the pH 6.0 were as follow: GT, BT and EGCG (1.5mg/mL) and GT (1.5mg/mL) containing IL-1β (1ng/mL). Cells were incubated for additional 4h and cell lysates were prepared as described before. IL-8 intracellular concentrations were measured using the same kit mentioned above.

2.2.8 NF-κB activation.

GT, BT, and EGCG extracts were tested for their effect on NF-κB activation. After a pre-incubation period with medium containing IL-1β (1ng/mL), cells were treated with the tea/catechin extracts GT, BT and EGCG (1.5mg/mL) containing IL-1β (1ng/mL). Cells were further incubated for 3h, after which, whole cell extracts were prepared using the Nuclear Extract Kit from Active Motif (Carlsbad, CA). Whole cell
lysates were stored at -20°C until assayed. Protein concentration was determined using the NI™ protein assay (G-Biosciences, St. Louis, MO). The amount of active NF-κB was measured using the TransAM™ ELISA kit (Active Motif, Carlsbad, CA) which specifically detects the activity of the p65 subunit. The kit contains a 96-well plate on which the oligonucleotide containing the NF-κB consensus site has been immobilized. The active form of NF-κB contained in the whole cell extracts specifically binds to this oligonucleotide. 2-20ug of protein was used for this assay. The Jurkat nuclear extract (TPA and CI stimulated) was used as a positive control for NF-κB p65 activation. This extract is optimized to give a strong signal when used at 2.5ug/well. In order to monitor the specificity of the assay, a mutated-type consensus oligonucleotide was used as a competitor for NF-κB binding. When used at 20pmol/well, the oligonucleotide prevents NF-KB binding to the probe immobilized on the plate. A wild consensus oligonucleotide was also used, which should have no effect on NF-κB binding. All the procedures were in accordance with the protocol. Samples were evaluated in triplicate. NF-κB activation was plotted as a percentage relative to IL-1β treatment.

2.2.9 HPLC sample preparation.

GT, BT and EGCG were dissolved in F-12K media at a 1.5mg/mL concentration and incubated at 37°C. Aliquots were taken at different times (0, 0.5, 1 and 3h) and dissolved in 1:1 2% acetic acid solution. Samples were stored at -80°C until assayed.
2.2.10. HPLC-MS analysis.

Samples of each media were diluted in 2% acetic acid (1 mL media + 0.25 mL acetic acid). Aliquots were filtered through 0.45 µm PTFE filters and 25 µL of each was injected onto HPLC-MS. HPLC separation was performed on a Waters 2695 separations module employing a Waters XTerra reverse-phase C$_{18}$ column (2.1x100 mm, 3.5 µm) maintained at 35°C. Binary linear gradient elution was performed over 12 min at a flow rate of 0.3 mL/min, employing 0.1% aqueous formic acid and 0.1% formic acid in acetonitrile as the mobile phases based upon the following gradient: initial concentrations of 90:10 A/B, followed by a linear gradient to 30:70 A/B (5.5-7 min) and an immediate linear gradient back to initial conditions (7.5-12 min). Following separation, HPLC column effluent was introduced into a Waters ZQ2000 single-quadrupole mass spectrometer by (−)-mode electrospray ionization (ESI). The capillary voltage was -3.5 kV, the source and desolvation temperatures were 150 and 300°C, respectively, and the nebulizer and cone gases were N$_2$ at flow rates of 400 and 60 L/h, respectively. Compounds were detected by obtaining selected ion response (SIR) chromatograms of the deprotonated pseudomolecular ions ([M−H]$^-$). Standard curves comprising authentic reference material in 2% acetic acid at various concentrations were analyzed as described above, and linear regression lines relating sample concentration to SIR peak areas calculated from these curves were used to quantify species in media samples.
2.2.11. Statistical analysis.

Statistical significance between treatments was determined using one-way analysis of variance (ANOVA) with Tukey’s comparisons (Minitab 15.1.20.0 software; State College, PA). Data was plotted using SigmaPlot 11.0 (Systat software; Chicago, IL). Data are presented as means ± standard error of the mean (S.E.M) with alpha p<0.05 considered significant.
2.3 Results

2.3.1. Effect of tea/catechin extracts on AGS cell viability

Catechin profiles of the different extracts are provided in Table 2.1. The total catechin content in green tea was higher than that of black tea extracts as it was expected. This is due to the oxidation step that black tea is subjected to during its manufacture. Catechins are oxidized during this step producing polymerization products such as theaflavins. The effects of green tea and black tea extracts, or the tea catechins EGCG, EGC and the combined EGCG/EGC on cell viability were tested on the gastric epithelial cell line AGS. No significant differences were found between black and green tea extracts when cell viability was evaluated (Figure 2.1A). When used at the highest concentration (3mg solids/mL), both, black and green tea reduced cell viability by 23%, 48h post treatment (p<0.05). Lower concentrations (<3mg solids/mL) did not significantly affect cell viability. While the tea catechin EGC (3mg solids/mL) caused the greatest reduction (36%), EGCG reduced cell viability by 29% (p<0.05). When EGCG/EGC were used in combination, the reduction on cell viability was lower (31%) than when EGC was used alone (Figure 2.1B) and this difference was significant.
<table>
<thead>
<tr>
<th>Extract/Catechin</th>
<th>Caffeine</th>
<th>EGC</th>
<th>EC</th>
<th>EGCG</th>
<th>ECG</th>
<th>Total Catechin (mg/100mL)</th>
<th>Gallic Acid Equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT</td>
<td>10.2</td>
<td>20.9</td>
<td>53.4</td>
<td>25.8</td>
<td>4.2</td>
<td>104.3</td>
<td>42.2</td>
</tr>
<tr>
<td>BT</td>
<td>11.9</td>
<td>2.4</td>
<td>23.8</td>
<td>3.2</td>
<td>2.2</td>
<td>31.6</td>
<td>34.2</td>
</tr>
<tr>
<td>EGCG</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>9.7</td>
<td>0.2</td>
<td>9.9</td>
<td>4.4</td>
</tr>
<tr>
<td>EGC</td>
<td>0.0</td>
<td>8.4</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>8.4</td>
<td>2.8</td>
</tr>
<tr>
<td>EGCG/EGC</td>
<td>0.0</td>
<td>7.8</td>
<td>0.0</td>
<td>9.8</td>
<td>0.2</td>
<td>17.8</td>
<td>8.5</td>
</tr>
</tbody>
</table>

**Table 2.1.** Analysis of tea and catechin solutions by HPLC-PDA. Catechin and caffeine contents quantified with authentic standards represented as mg/100mL in solution and Folin-Ciocalteu method presented as gallic acid equivalents indicating total phenolic content. n=3
Figure 2.1: Effects of tea extracts and their catechins on AGS gastric epithelial cell viability. AGS cells were treated for 48h with 3, 1.5, 0.75 and 0.375 mg solids per mL of media of (A) green or black tea or (B) EGCG, EGC or the combination of EGCG/EGC. Control cells were incubated with media alone. All experiments were performed independently at least 3 times with an n=6/experiment. Significant differences from control are indicated by * (p<0.05).
2.3.2. Effects of tea/catechins extracts on AGS gastric cells intracellular oxidation

Intracellular oxidation was measured on AGS gastric cells treated with the different tea/catechin extracts GT, BT, EGCG, EGC and EGCG/EGC. None of the extracts was able to reduce intracellular oxidation at the different concentrations used (3, 1.5, 0.75 and 0.375 mg solids/mL) after 1h of treatment. GT and EGCG seemed to increase the generation of radical species when used at the highest concentration (3mg solids/mL) (Figure 2.2A-E).
Intracellular oxidation on AGS cells after treatment with tea extracts and their catechins. Tea/catechin extract concentration is given as mg of solids per milliliter. AAPH concentration was 1.25mM. DCF was used at 40µM. Test media and DCF solution were applied at the same time and cells were incubated for 1h at 37°C. After that, medium was removed and AAPH applied. Fluorescence was measured after 1h. Data is representative of 3 independent experiments with an n=6/experiment.
2.3.3 GT, BT and EGCG inhibited IL-1β-induced production and secretion of IL-8 protein in AGS gastric epithelial cells

Intracellular concentration of IL-8 protein was 202.3±27.6pg/mL (mean ± SEM) in control AGS cells. This concentration of IL-8 was considerably increased after stimulation with the cytokine IL-1β (1ng/mL) to 4873.7±633.1 pg/mL. Similarly, secreted levels of IL-8 protein were 777.3±10.0pg/mL and 10492.3±285.6pg/mL in control and IL-1β-treated cells respectively (data not shown). When IL-1β-stimulated AGS cells were treated with the tea extracts GT and BT and the tea catechin EGCG (1.5mg solids/mL), both intracellular and secreted IL-8 levels were significantly decreased (p<0.05). GT, BT, and EGCG reduced the intracellular concentration of IL-8 protein by 69%, 60% and 32%, respectively, in relation to the treatment with IL-1β after 24h (Figure 2.3A). Secreted levels of IL-8 protein after 24h were also reduced by 86%, 82% and 73% when AGS were treated with green tea, black tea and EGCG respectively (Figure 2.3B). The same tea/catechin extracts did not decrease intracellular and secreted IL-8 levels in untreated cultures (data not shown).

The tea catechins EGC and the combination of EGCG/EGC (1.5mg solids/mL) were also tested for their effect on IL-8 secretion. A significant reduction of 24% and 21% in IL-8 secretion respectively was observed after 24h of treatment (Figure 2.3C).
Brefeldin A (BFA) was used as positive control for the inhibition of IL-8 secretion. BFA (1.5µg/mL) inhibited IL-8 secretion by 95% after 24h in cultures exposed to IL-1β (Figure 2.3B). The amount of secreted IL-8 protein after BFA treatment was similar to that in control cells (media alone). As a consequence, IL-8 protein accumulated inside the cell, causing a 4-fold increase in intracellular IL-8 concentration as compared to IL-1β alone (Figure 2.3A). The reduction in IL-8 protein secreted caused by exposure to GT, BT and EGCG did not differ from that caused by BFA.
Figure 2.3: (A) Intracellular and (B) secreted IL-8 protein (% relative to IL-1β) in AGS cells. Cells were pre-incubated with IL-1β (1ng/mL) for 1h followed by application of GT, BT and EGCG (1.5mg/mL) or BFA ((1.5ug/mL) containing IL-1β for 23h. (C) Secreted IL-8 protein after EGC and EGC/EGCG (1.5mg/mL) treatment. IL-8 protein concentration was determined by ELISA. Control cells were incubated with media alone. Data in A and B represent the pooled results of 4 separate experiments (n=3). Treatments not sharing common letter superscript differ significantly (p<0.05). C: Results from a single experiment.
2.3.3.1 Cell viability on AGS gastric cells treated for IL-8 analysis.

The viability of cells treated for IL-8 assays was tested after 24h of incubation with the different treatment media. When used at 1.5mg/mL concentration, none of the tea/catechin extracts or IL-1β (1ng/mL) affected cell viability, except for the BFA-treated cells (Figure 2.4A-C) where a decrease of approximately 80% was observed as compared to the control (p<0.05).
Figure 2.4: Cell viability assays on cells treated for IL-8 analysis. Confluent cells were incubated for 24h with the following treatments (A) C: media only; IL-1β (1ng/mL); BFA: brefeldin A (1.5ug/mL); BFA+IL-1β and the tea extracts GT, BT and EGCG (1.5mg/mL).(B) 1h pre-stimulation period with IL-1β (1ng/mL) followed by treatment with GT, BT, and EGCG or (C) EGC and EGCG/EGC containing IL-1β for 23h. All experiments were performed independently at least 3 times with an n=3/experiment. Significant differences from control are indicated by * (p<0.05).
2.3.3.2 Dose dependent IL-8 production and secretion inhibition by green tea

Different concentrations of green tea (1.5, 0.75 and 0.375mg solids/mL) were tested on IL-1β-stimulated AGS cells. Intracellular and secreted IL-8 levels were measured after 24h. Green tea concentrations of 1.5 and 0.75mg solids/mL caused a significant reduction in the amount of secreted IL-8 protein (p<0.05). In contrast, a concentration of 0.75 mg solids/mL did not affect intracellular IL-8 but significantly reduced the amount of secreted protein (p<0.05) after 24h of treatment (Figure 2.5A and B).

2.3.3.3 Effect of time of addition of tea/catechin extracts on IL-8 production and secretion.

AGS cells were treated with either IL-1β alone, (1 ng/mL) or in combination with the tea/catechin extracts GT, BT and EGCG (1.5 mg solids/mL) for 24h. Intracellular and secreted IL-8 protein concentrations were significantly reduced (p<0.05) in response to the treatment with GT, BT and EGCG (Figure 2.6 A and B). The amount of produced and secreted IL-8 was decreased to a similar extent than when the cells were subjected to a pre-incubation period with IL-1β for 1h, before applying the tea/catechin extracts (Figure 2.3 A and B).
Figure 2.5: Dose dependency of green tea-mediated inhibition of (A) Intracellular and (B) secreted IL-8 protein (pg/mL) in IL-1β-treated AGS cells. Cells were pre-incubated with IL-1β (1ng/mL) for 1h followed by application of GT (0.375, 0.75, and 1.5mg/mL) containing IL-1β for 23h. IL-8 protein concentration was determined by ELISA. Data is representative of two independent experiments (n=2). Treatments not sharing common letter superscript differ significantly (p<0.05).
Figure 2.6: Effect of time of addition of tea extracts and EGCG on (A) Intracellular and (B) Secreted IL-8 protein (pg/mL) in AGS cells. IL-1β (1ng/mL) and tea/catechin extracts (GT, BT and EGCG, 1.5mg/mL)) were added at the same time to cultured cells. IL-8 protein concentration was determined by ELISA after 24h. Data is representative of two independent experiments (n=2).
2.3.3.4 Production of IL-8 protein at a low pH in AGS cells

The concentration of IL-8 protein produced after AGS cells were incubated in media at pH 6.0 was significantly lower as compared to that of cells maintained at pH 7.4 after 4h (Figure 2.7). In addition, pre-stimulation with IL-1β in cells maintained at pH 6.0 did not cause any increase in the amount of IL-8 protein produced, suggesting cytotoxicity. This was confirmed microscopically.

After being incubated for 7h at pH 6.0, AGS cells showed loss of cell junctions (Figure 2.8B) with a consequent change in cell shape from polygonal to rounded after 24h (Figure 2.8C).
Intracellular IL-8 protein (pg/mL) in AGS cells at pH 6.0. Media containing 15mM 2-(N-Morpholino) ethanesulfonic acid (MES) was adjusted to a final pH of 6.0 using HCl. Confluent cells were used. Incubation for 1h with either media at pH 7.4 or 6.0 was followed by treatments with GT, BT and EGCG (1.5mg/mL) and GT (1.5mg/mL) containing IL-1β (1ng/mL) at pH 6.0. Control cells were incubated with media at pH 7.4 and 6.0. Cells were treated for 4h and intracellular IL-8 concentrations were measured using an ELISA.
Figure 2.8: Effects of low pH on the morphology of AGS gastric epithelial cells. AGS gastric epithelial cells maintained at (A) pH 7.4 for 24h; (B) pH 6.0 for 7h; and (C) pH 6.0 after 24h. The morphology was examined by bright field microscopy (50X).
2.3.4 NF-κB activation assay

2.3.4.1 Competitive binding experiments

Competitive binding experiments were performed in order to monitor the specificity of the NF-κB assay. OD values at 450nm indicate the amount of active NF-κB p65. The positive control, Jurkat extract, produced an OD value of 0.953. When this extract was incubated in the presence of the wild oligonucleotide, the OD value was not affected (0.934). In contrast, incubation of the same extract in the presence of the mutated type oligonucleotide inhibited NF-κB binding and produced an OD value of 0.033. A constitutive NF-κB activation in AGS cells was observed as cells incubated in only media had an OD value of 0.482. When AGS cells were stimulated with IL-1β (1ng/mL), they had a higher activation of NF-κB, as reflected by an OD value of 0.619 (Figure 2.9).

2.3.4.2. IL-1β-induced NF-κB activation is reduced by green tea, black tea and EGCG

IL-1β-pre-stimulated AGS cells were further incubated for 3h with either IL-1β alone or in combination with one of the tea/catechin extracts GT, BT and EGCG (1.5mg/mL). A significant reduction in the amount of active NF-κB in AGS cells was observed (Figure 2.10). Green tea caused a 54% reduction in the amount of active NF-κB, while black tea and EGCG produced a 39% and 44% reductions, respectively, as compared to cells treated with IL-1β alone (p<0.05).
Figure 2.9: NF-κB activation in AGS cells: competitive binding experiments. Cells were pre-incubated with IL-1β (1ng/mL) for 1h. After that, media containing IL-1β (1ng/mL) with or without the following extracts was applied: GT, BT, and EGCG (1.5mg/mL). Cells were further incubated for 3h and whole cells extracts were prepared. PC: positive control: Jurkat nuclear extract (TPA and CI stimulated, 2.5µg/well); WT: wild type; M: mutated consensus oligonucleotide (20pmol/well); C: control cells incubated with media only. NF-κB activation was measured using an ELISA kit. Data are representative of 4 independent experiments and are presented as percentage relative to PC.
Figure 2.10: NF-κB activation in AGS gastric cells. Cells were pre-incubated with IL-1β (1ng/mL) for 1h. After that, media containing IL-1β (1ng/mL) with or without the following extracts was applied: GT, BT, and EGCG (1.5mg/mL). Cells were further incubated for 3h and whole cells extracts were prepared. NF-κB activation was measured using an ELISA kit. Data represent the pooled results of 4 independent experiments (n=2). Treatments not sharing common letter superscript differ significantly (p<0.05).
2.3.5 Identification of tea extracts and catechin degradation products

The tea/catechin extracts were incubated in F-12K medium and their degradation products were identified using HPLC-ESI-MS/MS analyses. Incubation of EGCG in the media resulted in the production of theasinensins (THSNs) A and D \((m/z\) 913) and P-2 \((m/z\) 883), its auto-oxidation homodimers after 30min. Degradation of the catechins GC, EGC, C, EC, EGCG, and ECG occurred as soon as 30min after and was almost total after 3h in both black tea and green tea extracts. However, formation of P-2 \((m/z\) 883), EGC dimers \((m/z\) 579, 609), and heterodimers \((m/z\) 731) was observed only in green tea containing media after 30min of incubation (Table 2.2).
Table 2.2: Tea catechins and their degradation products. GT, BT, and EGCG extracts were incubated in F-12K medium (pH 7.4) at different time points.

<table>
<thead>
<tr>
<th>Sample (min)</th>
<th>BT</th>
<th>BT</th>
<th>BT</th>
<th>BT</th>
<th>EGCG</th>
<th>EGCG</th>
<th>EGCG</th>
<th>EGCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>0.0</td>
<td>0.5</td>
<td>1.0</td>
<td>3.0</td>
<td>0.0</td>
<td>0.5</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Concentration (µM or %)</td>
<td>0.3</td>
<td>5.4</td>
<td>20.8</td>
<td>0.1</td>
<td>0.0</td>
<td>104.9</td>
<td>120.6</td>
<td>1.2</td>
</tr>
<tr>
<td>P-2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>EGC Dimer</td>
<td>0.3</td>
<td>0.1</td>
<td>1.4</td>
<td>2.9</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>EGC Dimer</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Heterodimer</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Heterodimer</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>[M−H] (m/z)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ret. Time</td>
<td>1.9</td>
<td>2.5</td>
<td>3.2</td>
<td>4.4</td>
<td>7.7</td>
<td>6.8</td>
<td>7.1</td>
<td>7.3</td>
</tr>
</tbody>
</table>

* Shaded values represent concentrations in µM, while the values under the shaded values represent the % relative to the shaded values above them for the same type of media.
2.4 Discussion

The most abundant tea catechin, EGCG has been extensively studied for its effects on the inhibition of cancer cell proliferation. For instance, EGCG has been shown to inhibit HT-29 colon cancer cells proliferation by inducing apoptosis [93]. Another study attributed this decrease in cell proliferation of HT-29 cells to the inhibition of Erk1/2 activation and VEGF expression [94]. Proliferation of other colon cancer cell lines such as CaCo2, HCT116, SW480, and SW837 has also been shown to be inhibited by EGCG and Polyphenol E, a decaffeinated extract of green tea that contains about 60% EGCG and lesser amounts of other tea catechins such as EGC, ECG, EC, and GCG [95].

In relation to gastric cancer, EGCG is, to the best of our knowledge, the only tea catechin that has been tested for its effects on gastric cancer cell proliferation given that it is the most abundant tea component. EGCG has been shown to induce apoptosis in the MKN45 gastric cancer cell line by activating the mitochondrial pathway [86]. In this study we examined the effects of green tea and black tea extracts and the individual tea catechins EGCG, EGC and the combination of EGCG/EGC on the viability of the AGS gastric cancer cell line. Contrary to what has been largely thought, the tea catechin EGC caused a higher reduction in cell viability than the extracts GT, BT and EGCG and this effect was attenuated when EGC was combined with EGCG, which might imply a possible competition on the uptake of EGC by EGCG when both catechins are present in the media. The fact that GT and BT caused less reduction in cell viability than the individual tea catechins EGCG and EGC, suggests interactions among the tea components that affect their bioactivity. Furthermore, the effect of green tea on cell
viability did not differ from that of black tea, even though the catechin profile in each is very different. Further research looking at the mechanisms by which these tea/catechin extracts exert their anti-proliferative effects on AGS gastric cancer cells is needed.

It should be noted that tea catechins have been proven to be extensively degraded near neutral or higher [96, 97]. In this study, culture of AGS cells was performed using F-12K medium (pH 7.4) and therefore, extensive degradation of tea catechins was expected. Degradation products from tea catechins have been identified before [75, 80]. Analyses of degradation products of green tea, black tea and EGCG extracts by HPLC-ESI-MS/MS confirmed the rapid transformation that these compounds suffer at neutral pH. The catechins GC, EGC, C, EC, EGCG, and ECG present in green tea and black tea were rapidly transformed in the media (starting as soon as 30min) and they were almost totally lost after 3h. Consequent formation of homodimers such as P-2 and EGC dimers, and heterodimers was observed in green tea, but not in black tea samples. Similarly, degradation of EGCG started as soon as 30min after and was almost lost after 1h. Concurrent formation of theasinensins (THSNs) and P-2 dimers was also observed after 30min and 1h, respectively. It is possible that the effect on cell proliferation of AGS cells incubated for 48h in presence of these tea/catechin extracts is the result of those degradation products rather than the initial catechins present in the extracts.

Free radicals and other reactive species are thought to play an important role in the development of many diseases such as cancer. Antioxidant activity of tea is perhaps its best known property and believed to convey protection by minimizing oxidative stress and subsequent risk of oxidative DNA damage through scavenging of radicals, reactive
oxygen species, reactive nitrogen species and free metal chelation [98-101]. Catechin metabolic conjugates have also demonstrated significant *in vitro* and *in vivo* antioxidant activity [102-104]. In addition, tea catechins have been shown to increase the cellular lipid antioxidant activity of vitamin C and vitamin E in human intestinal Caco-2 cells [105]. Therefore, we decided to study the effects of the tea/catechin extracts GT, BT, EGCG, EGC and the combined EGCG/EGC on intracellular oxidation of AGS cells using AAPH as a radical generator. The production of free radicals was monitored using the DCF-DA probe. The results from this study did not agree with those mentioned above, since none of the tea/catechin extracts were able to reduce intracellular oxidation at the different concentrations used. Moreover, GT and EGCG seemed to induce the generation of additional free radicals when used at the highest concentration (3mg solids/mL). The spontaneous generation of hydrogen peroxide caused by addition of tea catechins such as EGCG and EGC to cell culture media has been previously documented [106, 107]. Among the most common used cell culture media, such as McCoy’s 5A, DMEM, RPMI 1640, etc., F-12 medium has been shown to be the least prone to present spontaneous generation of hydrogen peroxide by EGCG [108]. Thus, it is possible that the presence of a higher free radical generation in AGS cells incubated in F-12K medium as a result of treatment with green tea or EGCG is due to this artifact.

Extensive epidemiological evidence has identified chronic inflammation as a critical factor in the development of intestinal metaplasia and mutations in oncogenes that precede the development of gastric cancer. As a result of infection, epithelial cells transduce signals for the engagement of receptors that can stimulate the synthesis of
molecules such as interleukin-8 (IL-8) which then, activates and recruits neutrophils, as well as mast cells and macrophages to the gastric mucosa [31]. IL-8 is a chemo-attractant for neutrophils and lymphocytes [35] and induces tumor cell migration [36], proliferation of keratinocytes and melanoma cells [37, 38] and angiogenesis [39, 40]. Expression of other pro-inflammatory cytokines, such as IL-1β, TNF-α, IFN-γ, and IL-6 has been associated with an increase in the development of gastritis [109-111]. Therefore, balanced tissue levels of inflammatory cytokines and chemokines are crucial for the maintenance of the normal gastric epithelium. Furthermore, there is some evidence suggesting that green tea may influence the inflammatory response. For instance, inhibitory effects of EGCG on TNF-α-induced IL-8 expression were observed in the intestinal cell lines HT-29 and T84 [112]. The same effect of EGCG was also observed in respiratory epithelial cells [113]. The complex green tea extract was also tested on intestinal epithelial cells Caco-2 and was shown to inhibit IL-8 secretion despite an increased intracellular IL-8 mRNA and protein [89]. To our knowledge, the effects of the more complex green tea and black tea extracts and the tea catechin EGCG on the production and secretion of IL-8 in the gastric epithelia have not been tested. Since the extracellular concentrations of these inflammatory molecules depend on both synthesis and secretion, we decided to study the effects of green tea, black tea and EGCG on the synthesis and secretion of IL-8 induced by IL-1β in AGS gastric cancer cells given the close proximity of these dietary compounds to the gastric epithelia. AGS cells had constitutive production and secretion of IL-8 protein (202.3±27.6 and 777.3±10.0pg/mL, respectively), which was considerably increased after stimulation with IL-1β (4873.7±633.1 and 10492.3±285.6 pg/mL, respectively) as previously reported [43].
When used at a concentration of 1.5mg solids per mL, green tea, black tea and EGCG extracts were able to decrease the intracellular and secreted levels of IL-8 after 24h of IL-1β stimulation. Green tea was the more potent inhibitor of IL-8 production and secretion (69% and 86%, respectively) followed by black tea (60% and 82%) and EGCG (32% and 73%). These results differ from those mentioned before, where an induction of IL-8 mRNA and protein was observed when Caco-2 cells were treated with green tea extract [89]. Furthermore, the inhibitory effects of green tea on IL-8 secretion in AGS cells were comparable to those of brefeldin A, a well known inhibitor of vesicular transport. The fact that green tea and black tea were more potent than EGCG at inhibiting IL-8 production and secretion suggests an important role of other compounds present in tea in conferring this effect. In this respect, it is also important to mention that another tea catechin, EGC, and the combination of EGCG/EGC were also tested and their effects on IL-8 protein synthesis and secretion were less than those caused by green tea, black tea, and EGCG.

The concentration of the IL-1β and the different tea/catechin extracts (1.5mg/mL) used in the IL-8 studies did not affect cell viability. The inhibitory effect of green tea extract on the production and secretion of IL-8 in AGS cells was found to be dose dependent. Green tea concentration of 1.5 mg solids/mL was able to cause a significant reduction in the amount of both intracellular and secreted IL-8 after IL-1β stimulation. A green tea concentration of 0.75 had only significant effects on reducing secreted IL-8 protein.
The effects of the tea/catechin extracts on IL-8 production and secretion mentioned above were observed when AGS cells were pre-stimulated with IL-1β for 1h and then incubated for an additional 24h in presence of both IL-1β and the different extracts. We wanted to test if these effects differed when there was no pre-stimulation period and the tea/catechin extracts were directly applied in the presence of IL-1β to AGS cells for 24h. Results showed that the amount of both intracellular and secreted IL-8 protein was decreased to a similar extent than when the IL-1β pre-incubation treatment was used. This would suggest that the tea/catechin extracts might not be interfering with IL-1β cell membrane receptors but rather affecting the transcription and/or translation of IL-8 protein.

Gastric epithelial cells are continuously exposed to acidic pH especially during fasting conditions. In response to acid, mammalian cells respond in different ways, including IL-8 secretion. Different pathways have been implicated in the induction of IL-8 expression as a result of acidic pH, such as AP-1, NF-κB, and MAPK [114-117].

Therefore, we decided to investigate the effects of acidic pH on the production and secretion of IL-8 protein in AGS gastric cells. AGS were incubated in media adjusted to a pH of 6.0 or 7.4 for 4h in the presence or absence of tea/catechin extracts and green tea plus IL-1β. The basal intracellular concentration of AGS cells maintained at pH 7.4 was similar to that observed earlier in this research. However, when the cells were incubated at pH 6.0 this basal IL-8 production was minimal and dismissible even after stimulation with IL-1β. Another study reported no change in IL-8 synthesis and cell viability when AGS cells were incubated at pH 6.0 [118]. Here we found that this pH
caused a minimal production of IL-8 and when AGS cells were observed under the microscope, a change in morphology and loss of cell junctions was found to be present after 7h of incubation at pH 6.0. After 24h, cell shape was completely changed from polygonal to round and most of cells were detached. These observations agree with those of another study showing rapid AGS cell lysis when the pH of the culture media was lower than 6.6 [119]. Thus, acidic pH was toxic to AGS cells in this study.

The observed reduction in intracellular IL-8 protein by green tea, black tea and EGCG extracts in the AGS cell line might suggest a potential anti-inflammatory role of these compounds in the gastric epithelium. A reduced IL-8 secretion by gastric epithelial cells may also cause less neutrophils and macrophages and therefore less free radicals in the stomach. This, in turn, may prevent chronic inflammation and DNA damage in the gastric epithelium, preventing in this way the initiation and promotion of the carcinogenic process.

The gene encoding IL-8 contains several binding regions within its promoter region. A binding motif for NF-κB is present in the IL-8 gene at nucleotides -80 to -70 [41]. It has been confirmed that the production of IL-8 involves NF-κB activation [42]. The translocation of NF-κB to the nucleus is followed by an increase in IL-8 mRNA and protein [42, 120]. Activation of NF-κB in gastric epithelial cell lines such as MKN45 and MKN28 has been related to increased production of IL-8 [120, 121]. Another study also showed that the increase in IL-8 production in gastric epithelial cells by the pro-
inflammatory cytokines TNF-α and IL-1β was associated with NF-κB activation [122]. AGS and KATOIII gastric cell lines were also found to present NF-κB activation after *H. pylori* infection (48). More recently, the role of NF-κB activation on *H. pylori*-induced IL-8 expression in AGS cell has been confirmed [118, 119, 123].

The effects of green tea catechins on NF-κB activation have not been fully characterized and are limited to cell lines such as HT-29 and HUVEC. For instance, EGCG was found to decrease NF-κB activation on HT-29 colon cancer cells [90], while green tea polyphenols exerted the same effect on HUVEC [124]. In this study, the ability of green tea, black tea and EGCG extracts to inhibit NF-κB activation in AGS gastric cells was evaluated. Our results showed that AGS cells have a constitutive NF-κB activation in agreement with what others have reported [125]. Stimulation of AGS cells with the pro-inflammatory cytokine IL-1β produced a significant increase in NF-κB activation as compared to control cells after 4h of treatment. Activation of NF-κB by pro-inflammatory cytokines involves the inhibitory kB kinases (IKK). IKKβ phosphorylates IkBα at serine 32 and 36, resulting in poly-ubiquitination and subsequent degradation by the proteosomal pathway. The release of NF-κB from its inhibitor IkBα allows NF-κB to translocate to the nucleus and enhances the transcription of genes containing an NF-κB binding site in their promoter region, such as IL-8 [44]. When tea/catechin extracts were applied, a significant reduction in the amount of active NF-κB was observed. In agreement with the effects shown by the same extracts on IL-8 production and secretion, green tea produced the highest inhibition of NF-κB activation (54%), followed by EGCG (4%) and black tea (44%) relative to treatment with IL-1β. These results are critically
important given that activation of NF-κB is well known to regulate the inflammatory response and apoptosis [118, 119, 126, 127]. Furthermore, NF-κB activation has been linked to the proliferation of cells and tumor growth [45]. More importantly, the role of NF-κB activation in *H. pylori* infection of gastric mucosa has been documented [46, 47]. The activation of NF-κB in gastric epithelial cells has been found to increase the IL-8 mRNA and protein [48, 49]. The activation of NF-κB also induced the synthesis of matrix metalloproteinases in the AGS gastric cell line (128). Matrix metalloproteinases are capable of degrading the extracellular matrix, thus giving cancer cells the ability of invade other tissues and metastasize. These molecules also induce the production of chemokines contributing to inflammation. Synthesis of other molecules is also up-regulated by activation of NF-κB in gastric epithelial cells. For instance, cyclooxygenase-2 and nitric oxide synthase 2, whose products (prostaglandins and nitric oxide) are important in the inflammatory response (129). As mentioned before, secretion of inflammatory chemokines induce the recruitment of neutrophils and macrophages to the site of infection. These cells will in turn secrete free radicals to fight the infection. However, continuous presence of free radicals in the gastric epithelium can induce DNA damage that if not repaired may initiate and promote the carcinogenic process. Therefore, the inhibitory effects of NF-κB activation shown by green tea, black tea and EGCG extracts might represent a preventative alternative to the development of gastric cancer since this down-regulation may prevent chronic inflammation in the gastric epithelium.
Although plasma levels of tea catechins such as EGCG after tea consumption are lower than those exerting biological activity in *in vitro* systems (130, 131), the gastric epithelium is exposed to dietary compounds and more likely to be exposed to high levels of green tea catechins that can affect cell activities [79]. Furthermore, these compounds would be less prone to degradation given the acidic pH of the stomach. Concentrations of green tea, black tea and EGCG extracts in this study exhibiting biological activity were 1.5mg/mL. Considering that a cup of tea contains 180mg of EGCG and that the volume of the stomach is 1L, the gastric EGCG concentration after consumption of 8oz of green tea would be 0.18mg/mL. This means that one would have to consume about 64oz of tea in order to reach a biological relevant concentration. However, from our studies, it was shown that green tea is more effective than EGCG at inhibiting both IL-8 production and secretion and NF-κB activation and therefore, a green tea intake half of that mentioned above might be enough to have effects *in vivo*. 
2.5 Conclusion

The results from this study provided evidence that green tea and black tea extracts and the tea catechin EGCG exert anti-proliferative effects on AGS gastric epithelial cancer cells, while intracellular oxidation was not affected. More importantly, these extracts were found to inhibit the IL-1β-induced production and secretion of the inflammatory chemokine IL-8. Green tea was found to produce the highest inhibitory effect, suggesting the important role of other tea catechins rather than EGCG itself. Furthermore, inhibition of both IL-8 secretion and production by green tea was found to be dose dependent. This inhibition was shown to be due, at least in part, to the inhibition of NF-κB activation. Possible in vivo relevance of these results is likely given that higher levels of tea catechins are present in the gastric mucosa and that the acidic pH of the stomach would prevent rapid tea catechin degradation. Since chronic inflammation is the key factor involved in the development of gastric cancer, consumption of green tea, black tea or EGCG might have preventative effects. These results provide support for further research exploring the role of these dietary components in gastric cancer prevention.
EPILOGUE

Epidemiological evidence has suggested that consumption of green tea and tea catechins may reduce the risk of gastric cancer development. *In vitro* studies have examined the anticancer and antioxidant role of these dietary compounds, focusing mainly in the most abundant tea catechin, EGCG. The anticancer and antioxidant activity of other individual tea catechins or complex catechin mixtures have not been evaluated. Furthermore, the anti-inflammatory activity of these compounds in gastric cancer has not been investigated. The objective of this study was to evaluate the anticancer, antioxidant, and anti-inflammatory effects of green tea, black tea, EGCG, EGC and the combined EGCG/EGC using the gastric epithelial cancer cell line AGS.

Data from the proliferation studies show that EGC exerted the highest antiproliferative effects in AGS cells. When used in combination, EGCG diminished the observed effect of EGC alone. No differences were found between black tea and green tea activities. In contrast, intracellular oxidation was not affected by any of the extracts. Furthermore, green tea, black tea and EGCG were shown to inhibit the production and secretion of the inflammatory cytokine IL-8. This effect was in part due to a reduced NF-κB activation caused by the same extracts.
The results from this study provide important insights for the anticancer and anti-inflammatory effects of green tea, black tea and EGCG in AGS gastric cells. This is important considering that chronic inflammation is the key factor involved in the development of gastric cancer. Furthermore, it was shown that more complex tea catechin mixtures might be more effective than the individual compounds.

The reduction in IL-8 synthesis was found to be due, at least in part, to a reduction in NF-κB activation. Further studies should confirm that this observation is exclusively due to a reduced activation of this signaling molecule. This can be done by measuring the total amount of NF-κB in the cell after the different treatments. The total amount of NF-κB should not be affected by any of the extracts. In addition, expression of other genes containing a binding site for NF-κB might also be affected by treatment with the different extracts. These genes may also have a role in inflammation and the effects on them should be studied as well.

It is also possible that other factors are involved in the synthesis of IL-8. For instance, AP-1 and MAPK signalling pathways might also be activated during the immune response. Additional studies should look at activation of these pathways during inflammation.
Infection of the gastric epithelium with *Helicobacter pylori* is involved in the development of gastric cancer. Improved *in vitro* models should take this factor into account. Induction of the inflammatory response by this bacterium in AGS cells instead of using the pro-inflammatory cytokine IL-1β may provide a better model of the *in vivo* situation.

Degradation of tea catechins readily occur at pH close to neutral. These studies were carried using cell culture media at pH 7.4 and therefore when analyzing our results, this factor will have to be taken into account. HPLC analyses showed that catechins are degraded as soon as 30min after being dissolved in the cell culture medium and therefore the observed effects may be the result of their degradation products. To confirm this, one could incubate the different extracts with medium only and then apply that medium to the cells and observe if the effects are similar to those observed when fresh extract solutions are apply.

*In vitro* studies using a cell line capable of growing at an acidic pH might be necessary to better mimic the conditions found *in vivo*. Analysis of cellular uptake of tea catechins would be also useful in determining which compounds and metabolites are responsible for the observed effects.

Answers to these questions may provide a better understanding as to how tea consumption may protect against gastric cancer.
LITERATURE CITED


43. Beales ILP, Calam J. Stimulation of IL-8 production in human gastric epithelial cells by Helicobacter pylori, IL-1b and TNF-a requires tyrosine kinase activity but not protein kinase C. Cytokine 1997;9:514-20.


94. Jung YD, Kim MS, Shin BA, Chay KO, Ahn BW, Liu W, Bucana CD, Gallick GE, Ellis LM. EGCG, a major component of green tea, inhibits tumour growth by


