Role of *Helicobacter pylori* Enriched Media in Inducing Oxidative Stress in Human Cell lines

Project submitted

For the award of

Master of Science

In

Life Sciences (Microbial Sciences)

By

Pallavi Samal

Supervisor

Dr. Monisha Dhiman

Department of Biochemistry and Microbial Sciences
School of Basic and Applied Sciences
Central University of Punjab
Bathinda, 151001

May, 2018

Declaration

I declare that the project report entitled "**Role of *Helicobacter pylori* Enriched Media in Inducing Oxidative Stress in Human Cell lines**" has been prepared by me under the guidance of Dr. Monisha Dhiman, Associate Professor, Department of Biochemistry and Microbial Sciences, School of Life Sciences,Central University of Punjab. No part of this dissertation has formed the basis for the award of any degree or fellowship previously.

Pallavi Samal
Department of Biochemistry and Microbial Sciences,
School of Life Sciences
Central University of Punjab, Bathinda - 151001.
Date:

Certificate

I certify that Pallavi Samal has prepared her project report entitled **"Role of *Helicobacter pylori* Enriched Media in Inducing Oxidative Stress in Human Cell lines,"** for the award of M.Sc degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Biochemistry and Microbial Sciences, School of life sciences.The Central University of Punjab.

Dr. Monisha Dhiman
Department of Biochemistry and Microbial Sciences
School of Life Sciences
Central University of Punjab, Bathinda - 151001.
Date:

ABSTRACT

"Role of *Helicobacter pylori* Enriched Media in Inducing Oxidative-Stress in Human Cell lines"

Name of student : Pallavi Samal
Registration number : 16mslsms05
Degree : M.Sc Microbial Sciences
Name of supervisor : Dr. Monisha Dhiman
Name of Department : Department of Biochemistry and Microbial Sciences
Name of school : School of Life Sciences

Keywords: *H. pylori,* VacA, ROS, RNS, HPEM, THP-1, AGS, Oxidative stress *Helicobacter pylori* is a gram-negative, helical, microaerophilic bacterium which colonizes the human gastrointestinal tract. Vacuolating cytotoxin A (VacA) is one of the major virulent factors. Reactive oxygen species (ROS) and Reactive nitrogen species (RNS) produced by the immune and epithelial cells damage the host cell thereby resulting in a persistent infection. The prolonged infection results in chronic inflammation, oxidative stress and DNA damage. The microbe affects the major macromolecules of the host tissues lipids, proteins and DNA which leads to lipid peroxidation, protein oxidation and DNA fragmentation hence making the oxidative stress a deleterious damage. Role of *H. pylori* enriched media (HPEM) in inducing oxidative stress in two human cell lines AGS (human gastric cell line) and THP-1(human monocytic cell line) was studied in present work. The AGS cells and THP-1 cells was treated with various concentrations of HPEM and oxidative stress was evaluated by examining the levels of protein carbonyls, TBARS (thiobarbituric acid reactive species) and nitric oxide by spectophotometric and Western blotting methods. The oxidative stress induced by HPEM showed damaging effects on the cell membrane, protein and produced significantly high nitric oxide (NO) when compared with the untreated controls. From the present work it can be concluded that HPEM exposure to THP-1 and AGS cells enhanced the oxidative stress which leads to cellular damage and is ultimately responsible for the severe *H. pylori* associated fatal complications during its pathogenesis.

Pallavi Samal **Dr. Monisha Dhiman**

ACKNOWLEDGMENT

In the name of almighty the creator of the universe before whom I bow my head and whose benign blessings gave me the required enthusiasm for the completion of this work.

It is a great fortune and a matter of pride and privilege for me to express my profound sense of gratitude and sincere thanks to my supervisor Dr.Monisha Dhiman, Associate Professor, Department of Biochemistry and Microbial sciences, Central University of Punjab,Bathinda who has been truly motivational and dynamic in her efforts as a guide.

I would also like to thankfully acknowledge Dr.Ramakrishna Wusirika, HOD of Department of Biochemistry and Microbial sciences who has been supportive, humble and inspirational.

I would like to convey my regards and admirations to all the PhD scholars of my department, specially Sandeep Kumar Sir, Sukhchain Ma'am, Kunj Bihari Sir and Shishir Upadhyay sir without their support the work would not have been a success.

My deepest sense of gratitude to my respected Parents for their affection, inspiration, motivation and encouragement during my whole studies. Their blessings, love and warm support has helped me in achieving my target. My heartful thanks to my friends and classmates Arti, Chayan, Riya, Ekta and the entire Department of Biochemistry and Microbial sciences for providing me all support needed for the completion of project.

Pallavi Samal

List of Contents

List of Tables

List of Figures

List of Chemicals and Reagents

Sr. No.	Chemical / Reagent	Abbreviation	Manufactured By
1.	Acrylamide-bis-acrylamide	--	SRL
2.	Ammonium Persulphate	APS	SRL
3.	Bovine Serum Albumin	BSA	Gen Nei
4.	Bromophenol Blue	--	Fisher Scientific
5.	Dimethylsulphoxide	DMSO	SDFCL
6.	Dinitrophenylhydrazine	DNPH	SRL
7.	Dulbecco's Modified Eagle medium	DMEM	HiMedia
8.	Fetal Bovine Serum	FBS	HiMedia
9.	Guanidium HCL	G-HCL	SRL
10.	2-Mercaptoethanol	--	SDFCL
11.	3-(4,5-dimethythiazol-2-yl)-2,5 diphenyltetrazolium bromide	MTT	SRL
12.	N(-1-Naphyl) ethylenediamine	NEDD	SRL
13.	Sodium Dodecyl Sulphate	SDS	SRL
14.	Sodium nitrate	$NaNO_2$	SDFCL
15.	Tetramethylethylenediamine	TEMED	Loba chemie
16.	Tris(hydroxymethyl)aminoethane	Tris-HCL	Loba chemie
17.	Thiobarbituric acid	TBA	Loba chemie
18.	Trichloroacetic acid	TCA	SRL

List of Abbreviations

Sr. No.	Full form	Abbreviation
1.	Blood group antigen binding adhesion	BabA
2.	Brain heart infusion broth	BHI
3.	Cytotoxin-associated gene A	CagA
4.	Gastric epithelial cells	GECs
5.	*Helicobacter pylori* enriched media	HPEM
6.	*Helicobacter pylori*-neutrophil-activating protein	HP-NAP
7.	Human gastric cells	AGS
8.	Human immune cells	THP-1
9.	Inducible NO synthase	iNOS
10.	Interleukin	IL
11.	L-arginine	L-Arg
12.	Lipopolysaccharide	LPS
13.	Mucosa associated lymphoid tissue	MALT
14.	Nitric oxide	NO
15.	Reactive nitrogen species	RNS
16.	Reactive oxygen species	ROS
17.	Sialic acid-binding adhesion A&B	SabA&B
18.	Thiobarbituric acid reactive species	TBARS
19.	Toll-like receptors	TLRs
20.	Tumor necrosis factor	TNF
21.	Vacuolating cytotoxin A	VacA

Standard abbreviation relating to Weights, Measures and Calendars

Sr. No.	Abbreviation	Name
1.	µL	microliter
2.	Mg	milligram
3.	G	gram
4.	hr(s	hour(s)
5.	M	minute(s)
6.	Nm	nanometer

CHAPTER -1

INTRODUCTION

Introduction

Helicobacter pylori is a gram-negative, microaerophilic bacterium that colonizes the human stomach and is the causative agent of various gastrointestinal diseases – stomach ulcers, chronic gastritis, Mucosa associated lymphoid tissue (MALT) lymphoma (Ding *et al.,* 2007). *Helicobacter pylori* is a common bacterium, and infects approximately 50% of the world's population (Algood and Cover, 2006). The prevalence of *H. pylori* infection is highly variable across different countries.

The bacterial colonization persists lifelong and the host is never able to clear the infection resulting in oxidative stress within the tissue hence leading to various gastrointestinal complications (Kuipers *et al.,* 2000).There are several *Helicobacter pylori* virulence factors that contribute to its ability to evade the immune system and disrupt the host's cell and helps in survival in the acidic environment of the stomach. After entering the host stomach, *H. pylori* utilizes its urease which catalyzes the production of ammonium ions and neutralize the acidic condition then moves into the host gastric epithelium cells, followed by specific interactions between bacterial adhesins with host cell receptors, which thus leads to successful colonization and establishment of infection (Montecucco and Rappuoli, 2001). After entering the stomach *H. pylori* acts as an initiating factor by up-regulating the inflammatory or immune response which is characterized by the expression of various pro-inflammatory cytokines, including IL-1b ,IL-6, IL-8 and TNF-α (Tang *et al.,* 2012).

Four steps are critical for *H. pylori* colonization and pathogenesis: (1) Survival under acidic stomach conditions; (2) movement toward epithelium cells through flagella-mediated motility; (3) attaching to host receptors by adhesins; (4) causing tissue damage by toxin release. The reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by the immune and epithelial cells damage the host cell thereby resulting in a persistent infection. The prolonged infection results in chronic inflammation, oxidative stress and DNA damage.

Treatment with completely extracellular active drugs, like amoxicillin, produces treatment success in only 50% to 80%. Therapies with reliable cure of the

infection are now accessible however need the utilization of multiple antimicrobial agents. Either metronidazole or a macrolide (e.g., clarithromycin) is enclosed in all therapies that give cure rates within the range of 90% (Björkholm, *et al*. 2000).

1.1 Knowledge gap(s)

Till date, although role of *H. pylori* induced oxidative stress is reported but the exact mechanism and their effect on host gastric cells and immune cells is not known.

1.2 Hypothesis

The virulent factors of *H. pylori* generate reactive oxygen species (ROS) and their accumulation induces oxidative stress which damages macromolecules like lipids, proteins and nucleic acids of the host, hence associated with disease severity.

1.3 Objective(s)

- Role of *H. pylori* enriched media (HPEM) in inducing oxidative and nitrosative stress in Human Gastric AGS Cell line.
- *H. pylori* enriched media (HPEM) in inducing oxidative and nitrosative stress in Human immune THP-1 Cell line.

CHAPTER -2

REVIEW OF LITERATURE

Review of Literature

Barry Marshall and Robin Warren isolated spiral-shaped bacteria called *Helicobacter pylori. Helicobacter pylori* is a Gram-negative, non-invasive, non-spore forming, spiral-shaped, microaerophilic bacterium (Atherton *et al.,* 1995). It is highly motile due to six flagella. It has major outer membrane proteins-putative adhesions, porins, iron transporters, flagellum associated proteins (flagellinA & flagellinB) (Celli *et al.,* 2009). *H. pylori* by its type IV secretion system inject proteins into the host cytosol and regulate the intracellular signal transduction in the host cell. During persistent gastrointestinal infections, chronic gastritis may remain asymptomatic or may evolve into more severe diseases, such as peptic ulcer or atrophic gastritis (Age-dependent)-55-80years (Kuipers *et al.,* 2000).*H. pylori* persistence is strongly influenced by the ability of the bacteria to evade, alter and manipulate the host's immune system.

When the *H. pylori* enters the stomach, it secretes urease enzyme which catalyzes the production ammonium ions and consequently neutralizes highly acidic pH(1-2) of the stomach (Montecucco and Rappuoli, 2001) which helps the bacterium to penetrate the gastric mucosa. The motility in a secretion layer is because of the presence of tuft flagella (Celli *et al.,* 2009). The helical shape of *H. pylori* promotes corkscrew like mechanism that eases the process of penetration into mucuous layer (Bonis *et al.,* 2010). In mucous layer, *H. pylori* live as free-swimming bacteria whereas few adhere to stomach epithelium surface and form microcolonies (Tang *et al.,* 2012). *H. pylori* in presence of gastric juices, bile, lysosome, antibiotics and oxygen develops plasma membrane deficiency and transform to coccoid type that is characterized by attenuated colonization and reduced adhesion to gastric membrane epithelial tissue, and low antigenicity (Yue *et al*., 2015). These features probably build coccoid type easier to escape from the host immune attack.

2.1 Factors Responsible for *H. pylori* Pathogenesis

H. pylori produces various virulent factors which helps it to survive the severe acidic conditions in stomach as well as can damage the host tissue (Tang *et al.,* 2012). One of the most studied factors is cytotoxin-associated gene A (CagA), which is injected into the host cell where it can affect the cell's shape, motility, and proliferation. The presence of *cagA* in a strain results in an increased risk of gastric carcinogenesis compared with individuals infected with CagA-negative strains (Tsugawa *et al.,* 2012). Increased hydrogen peroxide levels and oxidative DNA damage are seen with CagA positive strains (Handa *et al*., 2007).

In addition, there is an increase in tumor necrosis factor-α and IL-8, which are inflammatory and oxidative stress markers (O'Hara, Bhattacharyya *et al.* 2009). Although the precise mechanism CagA uses for carcinogenesis has not yet been defined, it is clear that these actions can contribute to the development of gastric cancer (Amieva and Peek 2016). Another virulence factor that may increase the chance for the development of gastric cancer is VacA. VacA is capable of inducing the generation of ROS that results in the activation of nuclear factor-κB, thereby increasing pro-inflammatory immune response (Kim *et al.,* 2007). *H. pylori* virulence factors urease, neutrophil activating factor A (Naf-A), and the enzyme catalase helps in the recruitment of neutrophils and protect itself from oxidative bursts. Urease and NapA recruit neutrophils to the site of infection and induce the oxidative burst from the neutrophils once they arrive (Wang, Hong *et al.* 2006).

Contributing to the survival of *H. pylori* while creating a chronic inflammatory state, the neutrophils are less likely to undergo apoptosis and *H. pylori* located in the lumen is protected from the oxy-radicals released by NapA and catalase. BabA is an adhesion protein that is well characterized. BabA-positive strains induce a strong IL8 and weak IL33 cytokine response (Shahi, Reiisi *et al.* 2015).This immune response drives a proinflammatory response without eventually killing the bacteria. Another adhesion protein is sialic acid–binding adhesion(SabA), which induces oxidative bursts in granulocytes (Unemo, Aspholm-Hurtig *et al.* 2005). γ-glutamyl transferase is a virulence factor that contributes to production of IL8 and activation of nuclear factor-κB

while stimulating the production of H_2O_2 from the gastric epithelium (Gong, Ling *et al.* 2010). It also is known that treatment of primary gastric cells and the AGS cancer cell line with γ-glutamyl transferase results in DNA damage from oxidative stress. The multiple ways of inducing the host immune response combined with the damage resulting from the oxidative stress response can initiate the steps toward carcinogenesis. Moreover, *H. pylori* also is able to protect itself from the host immune response by inducing apoptosis of macrophages. In vitro macrophages stimulated by the lipopolysaccharide of *H. pylori* produce polyamine, which suppress their iNOS and induces apoptosis (Bussière, Chaturvedi *et al.* 2005). Within the gastric epithelial cells, the polyamine is used to create H_2O_2. *H. pylori* is capable of inducing a host response and then manipulating it to create a tolerant, prosurvival environment for the bacteria, which produces a chronic inflammatory environment that is harmful to the host.

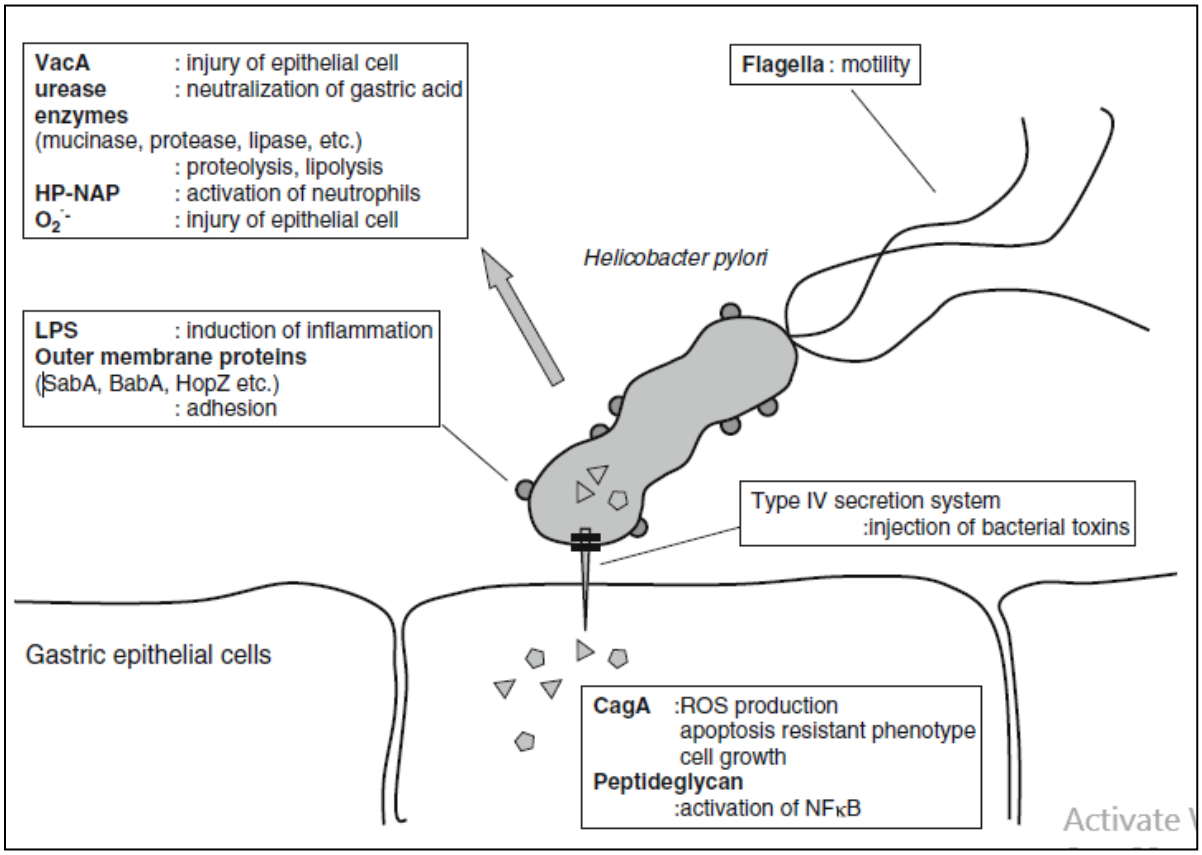


Figure 2.1: Various bacterial factors in *H. pylori*-induced gastric disease.

Source: Osamu Handa *et al.,* 2010

2.2 Pathogen and host cell interaction

The human body encounters several microrganisms daily , however most cannot with success colonize the stomach because of production of gastric acid within the stomach leading to a pH 1-2 that severely limits luminal colonization (Algood and Cover, 2006). Like intestine, the mucous layer in the stomach forms a physical barrier which limits bacterial penetration, In fact, *H. pylori* can only survive for minutes in the stomach lumen, and it quickly migrates to the gastric epithelial surface (Montecucco and Rappuoli, 2001).

Flagellar motility allow *H. pylori* to penetrate the gastric mucus layer whereas urease neutralizes the acidic environment of the stomach by converting urea to ammonia and helps the bacteria to survive in the acidic environment of the stomach and produce toxins, which degrades the mucus layer, bacteria enter the mucus layer and spread from top to bottom (Tang *et al.,* 2012). High acidity inside the stomach causes high inflammation and disease (ulcer) (Butcher *et al.,* 2017).*H. pylori* implant a toxin into the cell lining using the cytotoxin-associated gene A (CagA). Prolonged subjection to this toxin promotes chronic inflammation. In infectious cases, CagA-positive *H. pylori* inhibit tumor suppressor proteins and inactivate pathways that promote the production of pro-inflammatory cytokines (Yamaoka *et al.,* 2010). VacA and CagA are the main virulent factors produced by *H. pylori* both are secreted through type IV secretion mechanism. CagA produces IL-8 which interacts with neutrophils and causes inflammation that damages the stomach tissue (Leunk *et al.,* 1988).

2.3 Steps in bacteria and host cell interaction

- Attachment to the host cell
- Move towards epithelial cells by flagellar motility
- Survival in acidic pH of the stomach
- Damage to the host tissue by toxins released

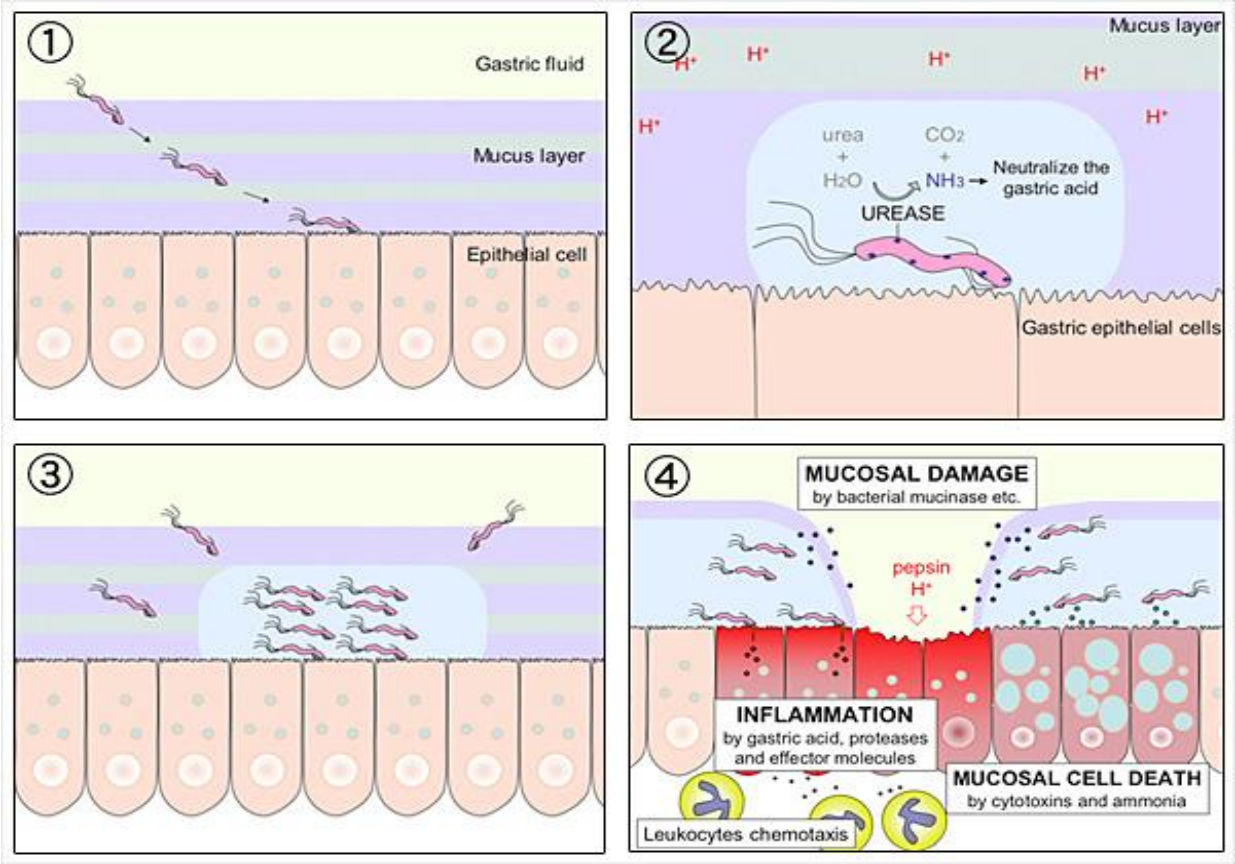


Figure 2.2: Schematic diagram of bacteria and host cell Interaction.

Source: (https://www.researchgate.net)

2.4 Role of VacA in Establishing the Disease

H. pylori strains secrete VacA, a 95-kDa protein that induces vacuolization in epithelial cells in vitro (Leunk *et al*., 1988). The VacA protein plays an important role in the pathogenesis of both peptic ulceration and gastric cancer (Cover and Blaser, 1992). VacA cytotoxin which is responsible for bringing about the inflammatory reaction and epithelial damage and induces cytoplasmic vacuolation both in-vivo and in-vitro. It was reported that VacA significantly contribute to murine gastric colonization by *H. pylori*.

The activities of VacA include membrane channel formation, disruption of endosomal and lysosomal activity, effects on integrin receptor-induced cell signaling, interference with cytoskeleton-dependent cell functions, induction of apoptosis, and immune modulation (Kuipers *et al.,* 2000). Although vacuolization is readily observed in vitro, it

does not seem to occur in vivo. VacA affects several cell types including T-cells and epithelial cells; it is likely that different host cell receptors are involved in cell binding (Augusto *et al.,* 2007). VacA is reported to induce pro-inflammatory effects and produces cytokines, including IL-1b, IL-6, IL-8 and TNF-α. Due to the sequence heterogeneity within the *vacA* gene (Palframan *et al.,* 2012) at the signal region (s) and the middle region (m). The s region of the gene, encoding signal peptide, occurs as either an s1 or s2 type, whereas the m region, which contains the p58 cell binding domain, exists as an m1 or m2 type (Atherton *et al.,* 1995). Vacuolating activity is high in s1/m1 genotypes, intermediate in s1/m2 genotypes, and absent in s2/m2 genotypes (Van Doorn *et al.,* 1998). In line with this, *vacA* s1/m1 genotypes are more frequently associated with peptic ulceration and gastric carcinoma (Cover and Blanke, 2005). Due to the rapid growth of the bacterium, the VacA expression levels differ over time which seems to be constantly adapting its genetic makeup to aid persistent infection (Papini *et al.,* 1998). Helps in membrane channel formation and vacuolation. VacA regulates inflammatory processes and induces the production of inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-10, and IL-13.

After entering the stomach *H. pylori* upregulates the immune response which is characterized by the expression of varied pro-inflammatory cytokines, including IL-1b, IL-6,IL-8, and TNF-α. *H. pylori* can evade host defence, immunity and gastric mucosa, via virulence and colonization factors such as urease,flagellin (provides motility), adhesins, VacA induced T-cell suppression (IIver *et al.,* 1998; Athmann *et al.,*2000). The activation of inflammatory reaction then leads to the recruitment of macrophages,lymphocytes, and neutrophils, to the gastric tissue. Macrophages are able to engulf *H. pylori,* but the bacterium develops some mechanism to evade killing upon phagocytosis. *H. pylori,* inside the macrophages, actively delay the phagosome formation and actin polymerization. *H. pylori* containing phagosomes undergo wide clustering and fusion resulting in the formation of “megasomes” having various bacteria, which might be a cause of resistance to intracellular killing (Lina *et al.,* 2014).

2.5 Host cell response against *H. pylori* Infection

Upon entry of pathogenic bacteria, neutrophils in the body immediately engulf the bacteria (phagocytosis) to form phagosomes, in which neutrophils kill the invaginated pathogens by ROS produced by Nox catalysis on the phagosome (Ding *et al., 2007).* ROS produced by non-phagocytic cells are byproducts of energy metabolic processes in the mitochondrial electron transport chain, whereas in phagocytic cells ROS is an actively produced product, not a byproduct. *H.pylori* infected person produces more ROS and RNS. Sources of ROS/RNS include neutrophils, vascular endothelial cells, gastric mucosal cells .During phagocytosis, gp91phox, the catalytic subunit of the phagocytic Nox, becomes activated and receives an electron from cytoplasmic NADPH (Lina *et al.,* 2014).

Nicotinamide adenine dinucleotide phosphate (NADPH oxidase [NOX]) on the cell membrane catalyzes the ROS production to kill bacteria and an electron transfer takes place from NADPH in cells to oxygen inside and outside cells, and the oxygen molecules that receive an electron become superoxide radicals (O^{2-}), which is rapidly converted to hydrogen peroxides (H_2O_2) by superoxide dismutase catalysis (SOD), and hydroxyl radicals (•OH) (Athmann *et al.,* 2000). In neutrophils, myeloperoxidase also results in the formation of the more toxic hypochlorous acid (HOCl) from H_2O_2 in the presence of chloride ions. The host neutrophils and epithelial cells also express a critical enzyme, the inducible nitric oxide synthase (iNOS), which produces NO. NO reacts with metals and O^{2-} to produce peroxynitrite, a strong oxidant (Butcher *et al.,* 2017).The excessive ROS production induces oxidative stress to the gastric mucosa, and may damage cellular components, including polyunsaturated fatty acids (lipid peroxidation), proteins(protein carbonylation), and DNA(DNA fragmentation) (Ding *et al., 2007).*

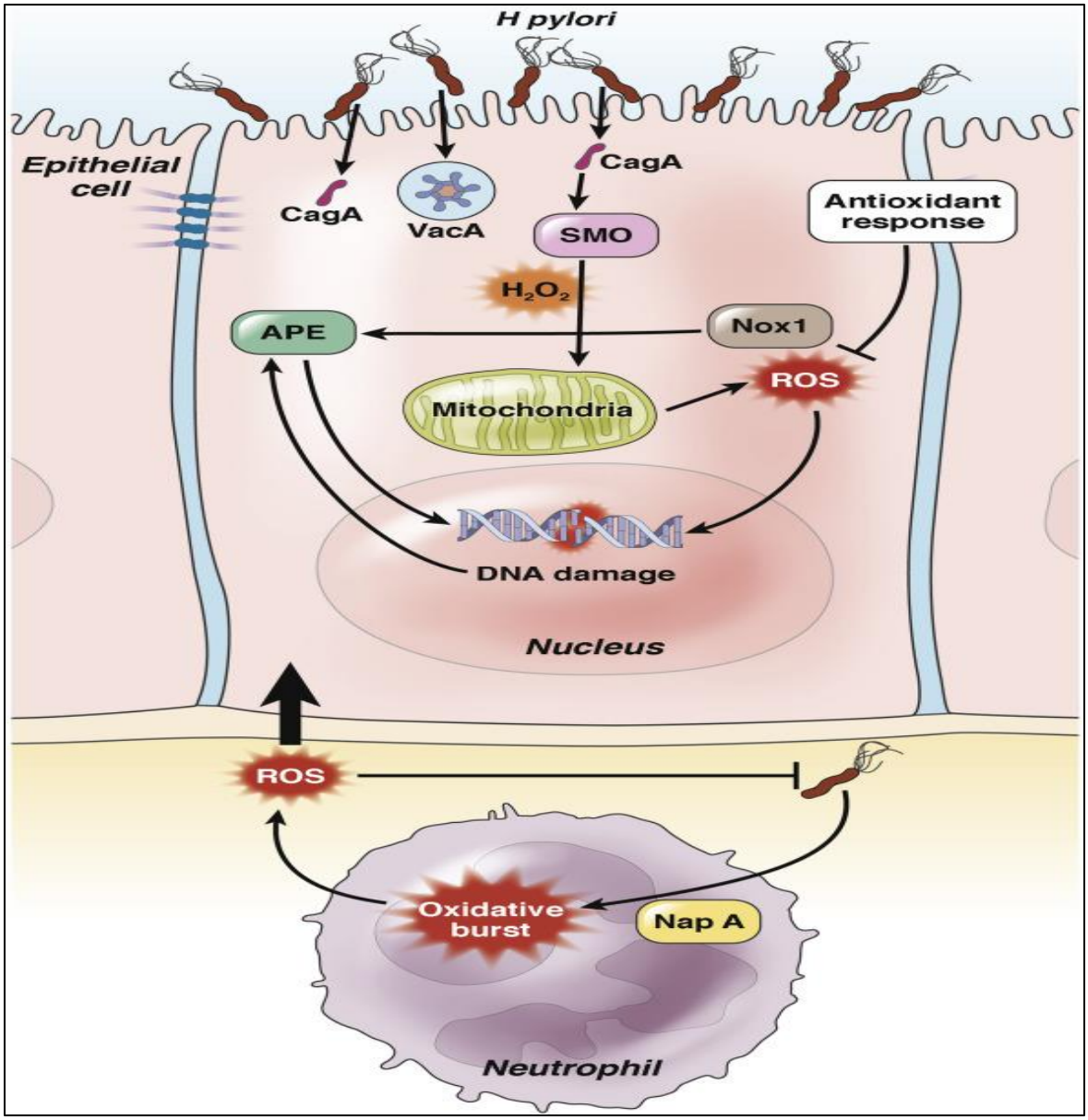


Figure 2.3: Role of Oxidative stress during *H. pylori* infection.

Source: Butcher, Lindsay D, 2017

2.6 Host Defence Mechanism

Upon infection, *H. pylori* causes strong inflammation in gastric mucosa. The presence of *H. pylori* results in the influx of phagocytic cells in an effort to clear the infection (Butcher *et al.,* 2017). Macrophages and neutrophils phagocytize the bacteria in an attempt to kill the organism with ROS/RNS. The host neutrophils and epithelial cells also express a critical enzyme, the inducible nitric oxide synthase (iNOS), which produces NO. Increased iNOS induces NO production using L-arginine as a substrate in these cells, where the main source of NO production is macrophages (Algood and Cover, 2006). NO production by macrophages is a normal host immune response against *H. pylori*, and this NO can kill *H. pylori* in in-vitro experiments. NO reacts with metals and O2- to produce peroxynitrite, a strong oxidant (Papini *et al.,* 1998). *H. pylori* infection results in the formation of ROS and RNS by increasing the immune cell expression of iNOS.

Patients infected with *H. pylori* have increased levels of ROS along with increased levels of NO-derived metabolites, indicating the activation of iNOS (Bliss *et al.,* 1998). In vivo studies with iNOS-deficient mice show decreased gastric cancer incidence after infection with *H. pylori* compared with wild-type mice (Mitchell *et al.,* 2007). Virulence factor that may increase the chance for the development of gastric cancer is VacA. VacA is capable of inducing an influx of Ca^{2+} and the generation of ROS that results in the activation of nuclear factor-κβ, thereby increasing proinflammatory immune response. *H. pylori* has the ability to protect itself from oxidative bursts with the aid of virulence factors urease, enzyme catalase and neutrophil activating factor A (NapA). Urease and NapA recruit neutrophils to the site of infection and induce the oxidative burst from the neutrophils once they arrive (IIver *et al.,* 1998). Contributing to the survival of *H. pylori* while creating a chronic inflammatory state, the neutrophils are less likely to undergo apoptosis, and *H. pylori* located in the lumen is protected from the oxy-radicals released by NapA and catalase. *H. pylori* is also able to protect itself from the host immune response by inducing apoptosis of macrophages. In vitro macrophages stimulated by the lipopolysaccharide of *H. pylori* produce polyamine, which suppress their iNOS and induces apoptosis (Yamasaki *et al.,* 2006).

In present work we aim to evaluate the levels of oxidative and nitrosative stress in human gastric and human monocytic cell line. A clear understanding of how *H. pylori* damages the human gastric cells and effects the host immune cells to avoid its elimination by the host immune response is an essential step to understand its pathogenesis and can give some critical leads for the development of novel diagnosis markers and therapeutic approaches for the eradication of *H. pylori*.

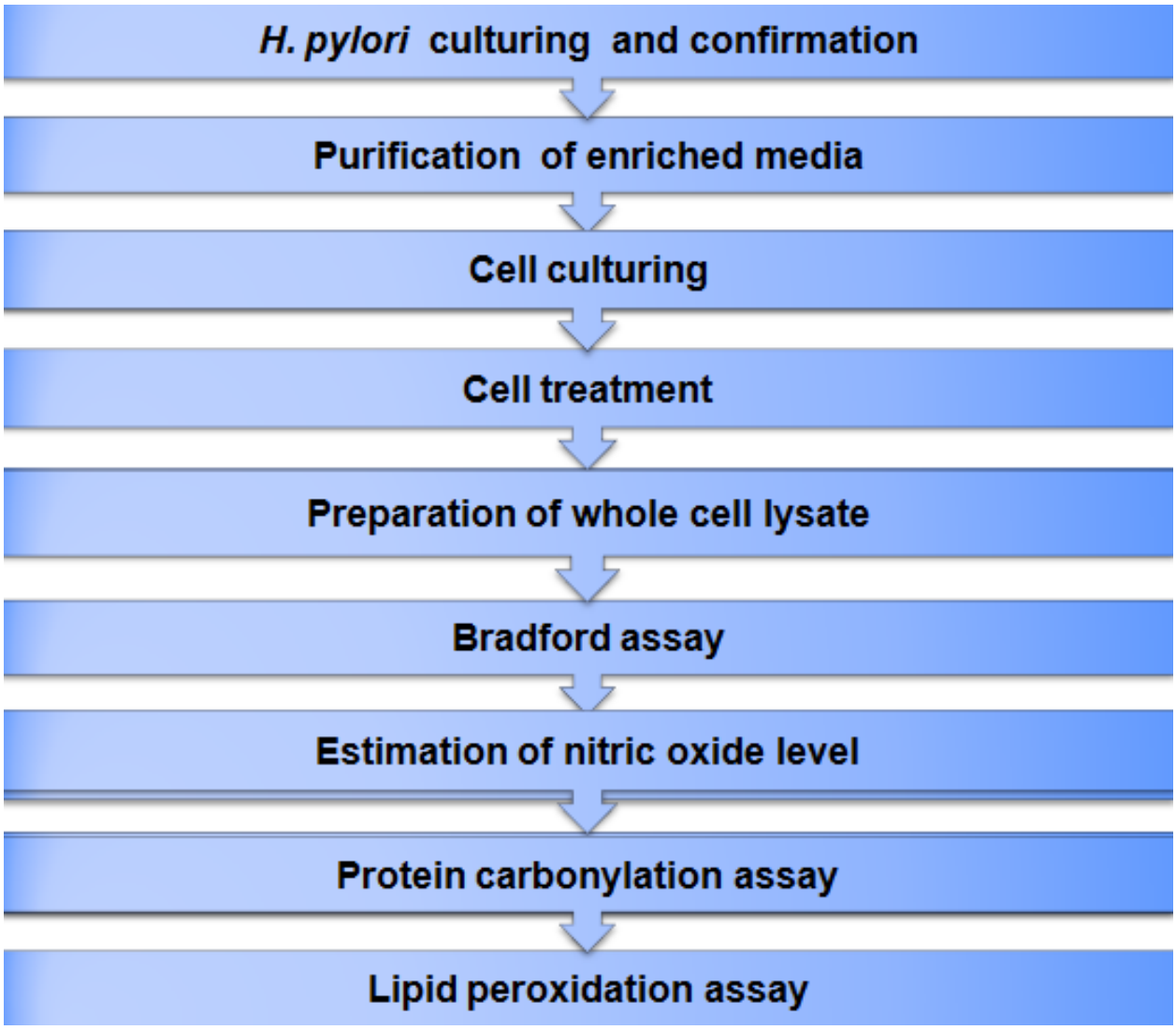
CHAPTER- 3

MATERIALS AND METHODS

MATERIALS AND METHODS

All experiments were done by following biosafety level 2 (BSL-2) protocols and Insitutional Biosafety Committee (IBC) approval was procured (CUPB/IBSC/2017/03).

3.1 Experimental Design for the Present Work



3.2 *H. pylori* culturing

H. pylori was cultured in micro-aerophilic conditions at 37^{o}C in Brain heart infusion broth (BHI) supplemented with 10% serum and other supplements such as β-cyclodextrins or

IsoVitaleX, or by using activated charcoal(Clover and Blaser, 1992). Antibiotics such as trimethoprim, vancomycin, amphotericin B and cefsoludin were added to the medium. For agar plates the Brucella Agar supplemented with blood or serum (2 to 10%) was used (Kusters *et al.,* 2006).

3.3 Confirmatory tests for *H. pylori*: Single colony of *H.pylori* was collected for confirmation tests and biochemical tests for confirmation were performed**:**

3.3 a) Gram Staining

Principle:

The structure of the cell wall of organism determines whether the organism is gram positive or negative. When stained with a primary stain and fixed by a mordant, some bacteria are able to retain the primary stain by resisting decolorization while others get decolorized by a decolorizer. Gram positive bacteria are those which retain the primary stain and those bacteria which get decolorized and then get counterstained are called Gram negative.

Procedure:

A free glass slide was taken. A loopful of culture was transferred by a sterile loop and smear was made at the center and dried. It was fixed by passing the slide 3-4 times through the flame quickly with the smear side facing up. Crystal violet stain, Gram's iodine solution, decolorizing agent was applied on it and washed under running tap water. The slide was counterstained with safranin and observed under microscope at 100X.

3.3 b) Catalase test

Principle:

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. Due to presence of enzyme catalase, when a small inoculum is introduced into hydrogen peroxide, rapid elaboration of oxygen bubbles occurs. Bacteria protect themselves from the lethal effect of Hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism.

Procedure:

Small amount of colony growth in the surface of a clean, dry glass slide was transferred. A drop of 3% H_2O_2 in the glass slide was placed and observed for the release of oxygen bubble.

3.3 c) Oxidase test

Principle:

The oxidase test is used to identify bacteria that produce cytochrome c oxidase, an enzyme of the bacterial electron transport chain. In the presence of cytochrome c oxidase it oxidizes the reagent tetramethyl-p-phenylenediamine to indophenols purple color end product. When the enzyme is not present, the reagent remains reduced and is colorless.

Procedure:

A filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride.It was moistened with sterile distilled water. The colony to be tested was picked with platinum loop and was applied on the filter paper. It was observed for a color change to deep blue or purple within 10-30 seconds.

3.3 d) Urease test

Principle:

Urea is a diamide of carbonic acid. It is hydrolyzed with the release of ammonia and carbon dioxide. Many organisms have an urease enzyme which is able to split urea in the presence of water to release ammonia and carbon dioxide. The ammonia combines with carbon dioxide and water to form ammonium carbonate which turns the medium alkaline, turning the indicator phenol red from its original orange yellow color to bright pink.

Procedure:

The broth medium was inoculated with a loopful of a pure culture of the test organism

Test tube was incubated the test tube at 35 °C in air for 18 to 24 hours, and colour change was observed.

3.4 Purification of Enriched Media

The broth culture of *H.pylori* after 48 hours was pelleted by centrifugation at 7000Xg for twenty minutes at 4^{o}C and supernatant was saturated up to 50% by adding ammonium sulphate. Precipitated proteins was pelleted by centrifugation at 7000Xg for twenty minutes at 4^{o}C and was dissolved in 1X PBS (pH 7.7) (González-Rivera *et al.,* 2016). The excess ammonium sulphate salt was removed by dialyzing the supernatant in dialysis tube .Protein quantification was performed by Bradford assay (Bradford, 1976). SDS-PAGE was performed to observe the quality of the protein. The protein was filter sterilized using syringe filter of 0.45µM and aliquots of the *H.pylori* enriched media (HPEM) was stored in -20˚C for future experiments.

3.5 Cell culturing

3.5.1 Culturing of THP-1 cells

The suspension of THP-1 human macrophage cell line was cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L $NaHCO_3$, 4.5 g/l glucose, 10 mM HEPES and 1.0 mM sodium pyruvate, 0.05 mM of 2-mercaptoethanol and 10% fetal bovine serum at 37^{o}C in the presence of 5% CO_2 for twenty four hours. The cells were treated with 10 ng/ml PMA for 24hrs which induces terminal differentiation of THP-1 cells into macrophages which then shows adherence.

3.5.2 Culturing of AGS cells

Human gastric AGS cells were maintained at 37˚C in a 5% CO2 humified atmosphere. Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), penicillin, 100mg/l streptomycin cells were cultured to 60-80 % confluence and were used for further experiments.

3.6 Cell viability assay (MTT ASSAY)

Principle:

This is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cell and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product which is largely impermeable to cell membranes thus resulting in its accumulation within healthy cells. Then, the cells are solubilized with an organic solvent and released, solubilized formazan reagent is determined spectrophotometrically. The number of surviving cells is directly proportional to the level of formazan product created .Since reduction of MTT can only occur in metabolically active cells the level of activity is measure of the viability of the cells.

Procedure:

1 x 10^5 THP-1 cells were seeded in a 96-well plate and were treated with different concentrations of HPEM (1.5 μg/ml, 3 μg/ml, 5 μg/ml, 7 μg/ml and 10 μg/ml) or buffer and incubated for 24 h. MTT (5mg/ml) was added to each well and incubated for 3 h. After incubation, formazan crystals were dissolved by adding equal volume of acidified propanol (prepared by adding 0.1 N HCl in isopropanol). After the crystals were completely dissolved, absorbance was measured at 570 nm.

Viable cell% = Treated cells*100/ Untreated cells

3.7 Preparation of Whole Cell Lysate

After treatment of the cells with HPEM(5 μg/ml) for 24 h, the culture supernatants were discarded.The cells were trypsinised and lysed using RIPA buffer (150Mm sodium chloride, 50 Mm tris HCl ,pH 7.4,1Mm phenylmethylsulfonyl fluoride, 1% sodium deoxycholic acid ,0.1% sodium dodecylsulfate). Cell debris was removed by the centrifugation. Protein concentration was measured with Bradford assay and was stored at 4˚C for future experiments.

3.8 Bradford assay

Principle:

Estimation of protein concentration is an essential method for quantifying the protein in protein study. Bradford assay is the preferred assay than the Lowry method because this assay is simpler, faster and more sensitive than the Lowry method. The basic principle relies on the binding of Coomassie Brilliant Blue to proteins. Bradford's reagent was formed by dissolving 50 mg of Coomassie Brilliant Blue in 50 ml of methanol and 85% phosphoric acid was added. The solution was filtered to remove precipitates and stored in dark bottle at 4˚C.

Procedure:

The standard curve was prepared using serial dilution of BSA. The protein sample was added to 1X PBS to make final volume 80 µl and to this 20 µl of Bradford reagent was added to make final volume 100 µl. After 10-15 min of incubation, the blue colour formed was measured at 595 nm wavelength with microplate reader.

3.9 Estimation of nitric oxide level (Griess Assay)

Principle:

Nitrite is detected and analyzed by formation of a red pink colour upon treatment of a NO_2^- containing sample with the Griess reagent. (Ding, Minohara *et al.* 2007). When sulphanilic acid is added the nitrites form a diazonium salt. When the azo dye agent (N-alpha-naphthyl-ethylenediamine) is formed a pink colour develops. This diamine is used in place of the simpler and cheaper alpha-naphthylamine because this is a potent carcinogen and moreover the diamine forms a more polar and hence a much more soluble dye in acidic aqueous medium.

Nitrate + Sulfanilamide + NEDD ⟶ Diazo coloured product

Procedure:

The NO production will be detected by using Griess reagent. The culture supernatants from the control and HPEM treated AGS and THP-1 cells was incubated with 0.2% naphthylethylenediamine dihydrochloride, and 2% sulphanilamide in 5% phosphoric

acid in 1:1 ratio. The optical density was measured at 543 nm. The nitrate content of each sample was evaluated from standard curve obtained with sodium nitrate and expressed in µm (Dhiman *et al.,* 2013).

3.10. Western Blotting for iNOS

Cell lysate (50 µg/µl) protein sample from control and treated groups were taken in a microcentrifuge tube. Protein sample was boiled for 10 min at 95˚C and then centrifuged for 2-3sec to remove any precipitation. The supernatant containing the protein was then separated on 10 % SDS-PAGE. The separated protein samples were transferred from gel to nitrocellulose membrane at 4˚C. The membrane was blocked in 5% non-fat dry milk (NFDM). The membrane was washed three times in 1X PBST for 15min. The membrane was incubated overnight at 4˚C with rabbit Anti-iNOS (1:1000) 5% NFDM in PBST and then membrane was washed again three times (5 min each) in 1X in PBST for 15 min. The membrane was incubated with HRP-conjugated at anti-rabbit secondary antibody (1:5000) for 2 hr and then membrane was washed in 1X PBST for 15min (Dhiman *et al.,* 2012). Enhanced chemiluminiscence (ECL) reagent was used as a chemiluminescent substrate to detect the signal followed by imaging and data analysis using Bio-Rad ChemiDocTM imaging system.

The same nitrocellulose membrane was stripped and treated with mouse Anti- β-actin (1:1000) 5% NFDM in PBST and then membrane was washed again three times (5min each) in 1X in PBST for 15 min. The membrane was incubated with HRP-conjugated at anti-mouse secondary antibody (1:5000) for 2 hr and then membrane was washed in 1X PBST for 15min (Dhiman *et al.,* 2012). Enhanced chemiluminiscence (ECL) reagent was used as a chemiluminescent substrate to detect the signal followed by imaging and data analysis using Bio-Rad ChemiDocTM imaging system.

3.11 Oxidative Stress Assays

3.11 a) Protein Carbonylation Assay

Principle:

Protein carbonyl assay is a very convenient technique to quantify and detect the oxidative modification of proteins. 2, 4-dinitrophenylhydrazine (DNPH) reacts with protein carbonyls to produce hydrazones (Dalle-Donne *et al.,* 2003). Hydrazones can be detected spectrophotometrically at an absorbance 370nm or by fluorescence. Western blotting techniques are widely used for sensitive and specific detection of the protein 2, 4-dinitrophenyl hydrazone moiety.

Procedure:

AGS and THP-1 cell lysate (50-100 μg) were treated with 20% TCA (w/v) to precipitate the proteins and centrifuge at 6000 rpm for 3-5 min. Supernatant was removed. 500 ul of 10 mM DNPH was added to the pellet. The mixture was kept in dark place for one and half hour for derivatization with DNPH. The protein was again precipitated with 500 μl of 10 % TCA by centrifugation for 3 min at 6000 rpm. Supernatant was discarded and the pellet was then washed with ethanol/ethyl acetate (1:1) for three times using 6000rpm/3min.The pellet was then dissolved in 6M guanidium HCl (500 μl) and centrifuged at 6000 rpm/ 3min. The supernatant was separated and its reading was taken at 360-385 nm.

Protein Carbonyls = O.D X Total sample dilution factor/21000 x Protein (mg) X Sample volume

3.11 b) Western Blotting for Protein Carbonylation

Principle:

The basic principle involves the formation of carbonyl group (CO) on protein side chains (arginine, lysine, proline, and threonine) upon oxidation. The hydrazones that are formed by the reaction of protein carbonyl and DNPH was analyzed by western blotting (Dalle-Donne *et al.,* 2003).

Procedure:

Western blotting was done to detect the protein oxidative modification in AGS and THP-1 cell lines. Protein sample from control and treated groups were taken in a microcentrifuge tube and derivatized with DNPH and 12 % SDS. In control set protein sample from all groups was taken and only 12% SDS was added and then both derivatized and non-derivatized samples were kept at room temperature 37˚C for 30 min followed by vortexing for 5 min each. After 30 min freshly prepared neutralization buffer was added to stop the reaction and samples were kept at 4˚C. Both derivatized and non-derivatized samples were separated on 10 % SDS-PAGE. The separated protein samples were transferred from gel to nitrocellulose membrane at 4˚C. The membrane was blocked with 1% BSA prepared in PBST for 2 hrs. The membrane was washed three times in 1X PBST for 15min. The membrane was incubated overnight at 4˚C with rabbit Anti-DNP (1:1000) 1% BSA in PBST and then membrane was washed again three times (5min each) in 1X in PBST for 15 min. The membrane was incubated with HRP-conjugated oat anti-rabbit secondary antibody (1:5000) for 2 hr and then membrane was washed in 1X PBST for 15min (Dhiman *et al.,* 2012). Enhanced chemiluminiscence (ECL) reagent was used as a chemiluminescent substrate to detect the signal followed by imaging and data analysis using Bio-Rad ChemiDocTM imaging system.

3.11 c) Lipid Peroxidation Assay

Principle:

Lipid peroxidation is a major mechanism of cellular injury in many biological systems of plant and animal origin. In this mechanism unsaturated lipids are oxidized to form radical species as well as harmful toxic by-products. Polyunsaturated fatty acids are susceptible to this type of damage and can react to form lipid peroxides. Lipid peroxides are themselves unstable, and after additional decomposition to form complex compounds including reactive carbonyl compounds. Polyunsaturated fatty acids peroxides further react to form malondialdehyde (MDA). It is the most widely reported analyses for the purpose of estimating oxidative stress effects in lipids or as a convenient biomarker for lipid peroxidation (Lykkesfeldt, 2007).

Procedure:

MDA, an end product of unsaturated fatty acid peroxidation, can react with TBA to form coloured complex called TBARS. TBA reactivity was assayed by the method of (Erdincler *et al.,* 1997). TCA was dissolved in hot distilled water and TBA was dissolved in 25ml HCL.100µg of sample was taken in a vial and 200µl of TBA was added to it. The mixture of sample and stock solution was heated for 30min in a boiling water bath and pressure was released at every 3-4min. After cooling centrifuged at 1000 rpm for 10 min. Absorbance of sample was taken at 530 nm at multiplate reader.

TBARS = Total volume X O.D/0.152 X Volume X Protein in mg

3.12 Statistical Analysis

Results are expressed as the means ± standard errors of the means (SEM). Data were compared by Student's *t* test (unpaired unless otherwise noted) or analysis of variance, and results were considered significant if *P* values were less than 0.05.

CHAPTER- 4

RESULTS

Results

4.1 *H. pylori* culture

H. pylori was cultured on BHI and blood agar. Grey translucent colonies were observed after three days (Figure 4.1).

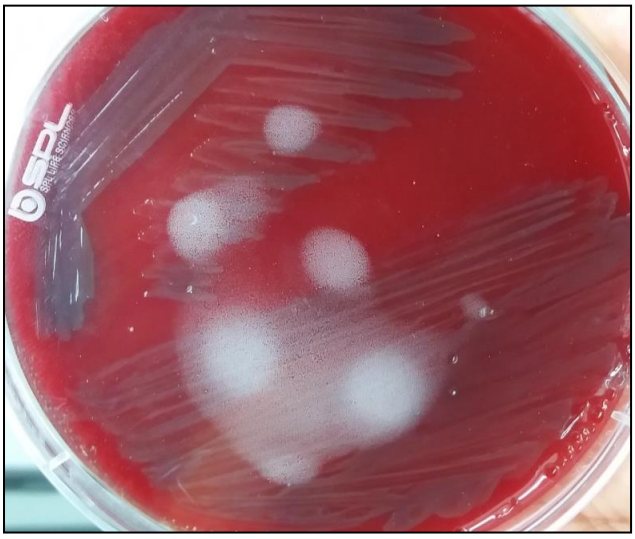


Figure 4.1: *H. pylori* culture on blood agar.

4.2 Confirmation tests

Biochemical Tests	Results
a) Gram Staining	Gram -ve
b) Oxidase Test	+ve
c) Urease Test	+ve
d) Catalase Test	+ve

Table4.1 : Biochemical tests for *H. pylori* confirmation.

Gram staining: Gram's staining differentiates between Gram-positive and Gram-negative bacteria on the basis of their cell wall. The strain was shown to be Gram-negative as they appeared pink in color and rod and helical in shape (Figure 4.2)

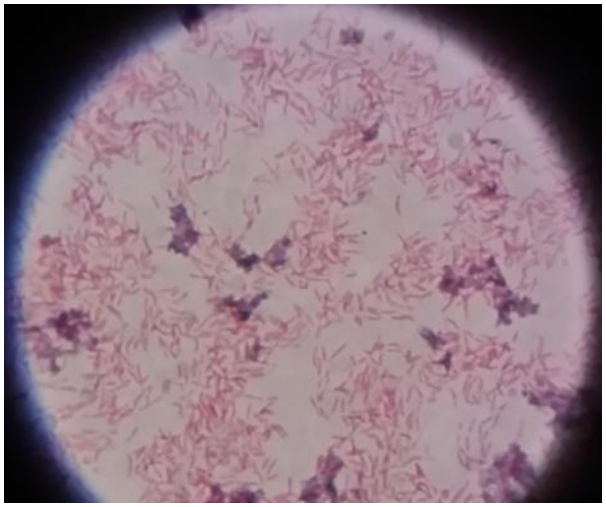


Figure 4.2: Gram staining – Helical shaped *H. pylori* was observed.

Oxidase test: The bacteria having the ability to produce cytochrome c oxidase are called the oxidase positive bacteria. The bacterial culture produced purple color when streaked on a paper soaked with Gordon Mc Leod reagent (Figure 4.3).

Figure 4.3: Oxidase test showing the purple color formation

Catalase test: Catalase is an enzyme which degrades hydrogen peroxide to oxygen and water. The *H. pylori* culture when inoculated with hydrogen peroxide, the release of gas bubbles indicates the presence of catalase enzyme hence the bacteria is catalase positive (Figure 4.4).

Figure 4.4: Catalase test showing the release of oxygen

Urease test: Urease test is a charactestic test for the detection of *H. pylori.* Urease enzyme degrades urea into ammonia, water and carbon dioxide creating an alkaline environment, this alkaline pH changes the color phenol red into dark pink under. The bright pink color was observed when the strain was incubated in urea broth 6 hours thus, showing urease test (Figure 4.5).

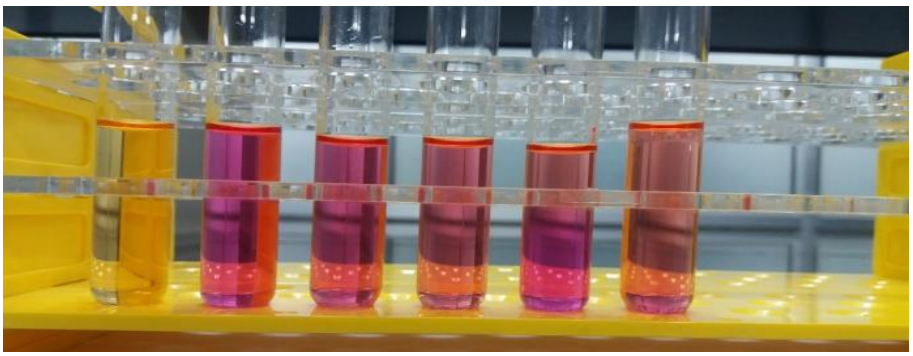


Figure 4.5: Urease test showing the phenol red from turning bright pink from its original orange yellow color

4.3 Protein Profile of *H. pylori* extracellular enriched media (HPEM)

The *H. pylori* extracellular enriched media after dialysis was separated on a 10%SDS-PAGE to analyse its protein pattern.

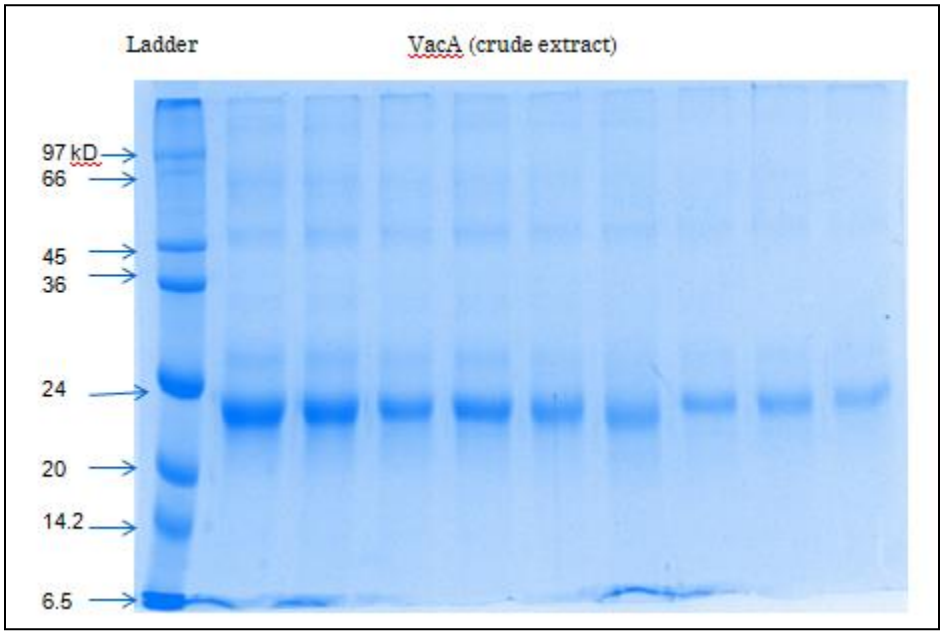


Figure 4.6: SDS PAGE for *H. pylori* extracellular crude extract.

4.4. MTT ASSAY

MTT assay for cell viability was used to quantify the cytotoxic response of HPEM on THP-1 and AGS cells.THP-1 and AGS cells were exposed to various concentrations of (0-20µg) *H. pylori* enriched media for 24 h.

THP-1 cells - At 5µg treatment 30% of the THP-1 cells were getting killed (Figure 4.7).

AGS cells- The AGS cells when treated with various concentrations of (0-20µg) *H. pylori* enriched media for 24hrs showed very interesting results where the cells were getting proliferated and the viability was increased by 20% at 2 & 5µg of HPEM treatment (Figure 4.8).

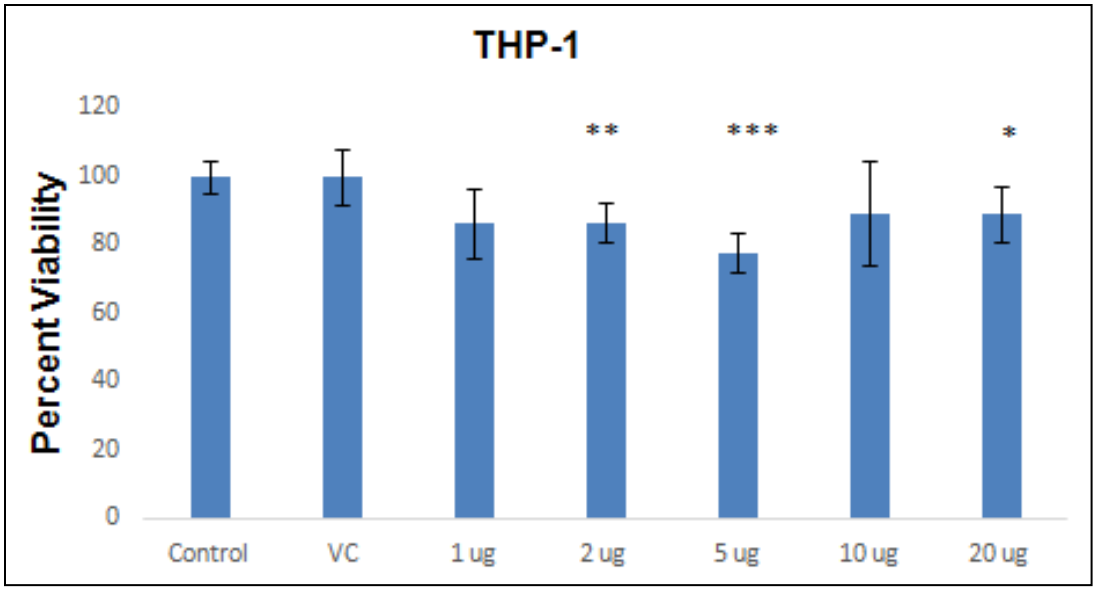


Figure 4.7: Percent viability of THP-1 cells when treated with *H. pylori* enriched media. The results were expressed as percent cell viability. Student t-test was performed to evaluate the significance of the results, the data were considered as statistically significant at p*≤ 0.05 when HPEM-treated cells were compared with untreated controls. The results are presented as the mean ± standard deviation (n=5).

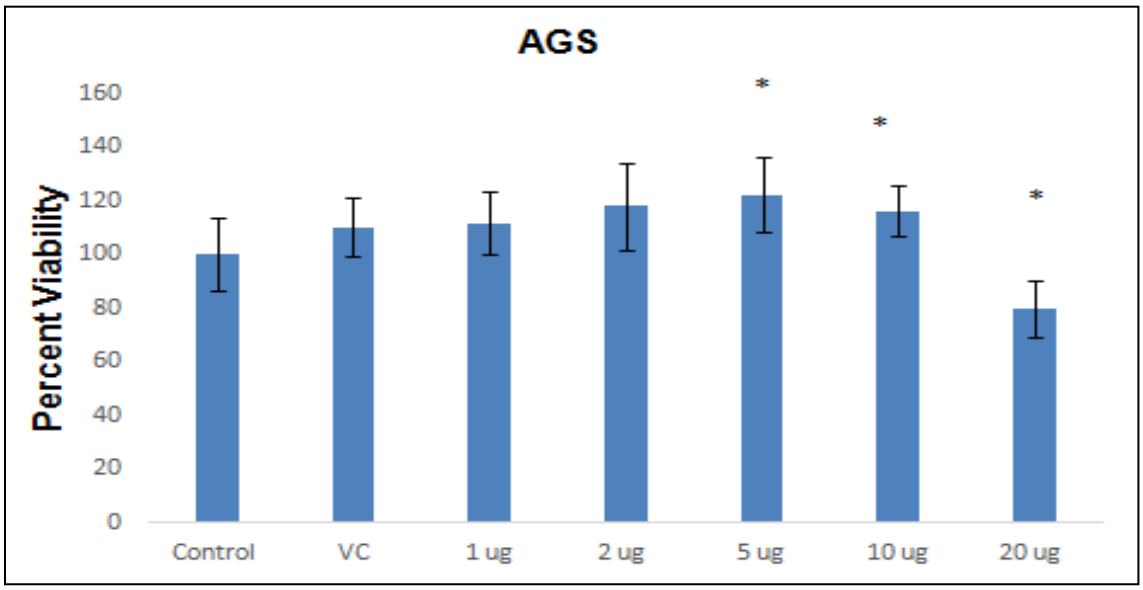


Figure 4.8: Percent viability of AGS cells when treated with *H. pylori* enriched media. The results were expressed as percent cell viability. Student t-test was performed to evaluate the significance of the results, the data were considered as statistically significant at p*≤ 0.05 when HPEM-treated cells were compared with untreated controls. The results are presented as the mean ± standard deviation (n=5).

4.4 Estimation of protein concentration in the samples by Bradford assay

The total amount of protein (concentration) present in whole cell lysates of THP-1 and AGS cells was calculated by Bradford method using BSA as a standard (Fig 4.9). This protein from the cell lysate (Table 4.2) was further used for future experiments

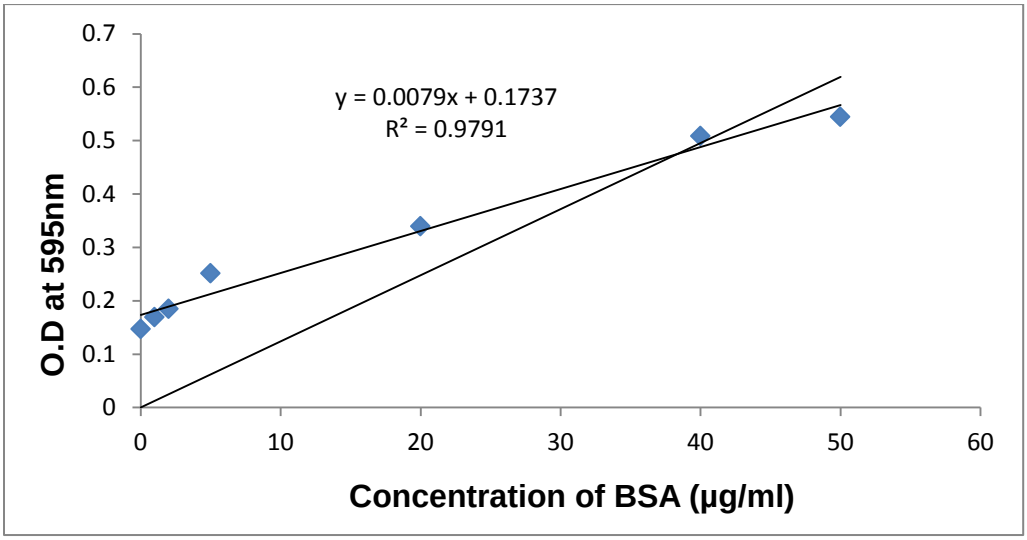


Figure 4.9: The standard curve of known concentration of BSA for the estimation of protein concentration by Bradford method.

Samples	Final Protein concentration(ug/ul)
THP-1 control	1.561111
THP -1control	2.064583
THP-1treated	2.144444
THP-1 treated	2.245139
THP-1 treated	2.325
AGS control	2.2625
AGS control	1.620139
AGS treated	2.422222
AGS treated	2.252083
AGS treated	2.463889

Table 4.2: Protein quantification by Bradford Method. Total Protein concentration in THP-1 and AGS cell lysate was estimated by Bradford assay.

4.5 Estimation of nitric oxide level in THP-1 and AGS cell lysate

Nitric Oxide (NO) can be spectrophotometrically assayed by measuring the accumulation of its stable degradation products, nitrate and nitrite. Griess reagent is used to detect nitrite in sample which forms a diazo product which is pink in colour and reading was taken at 543nm.The concentration of extracellular NO was determined by using Griess assay.

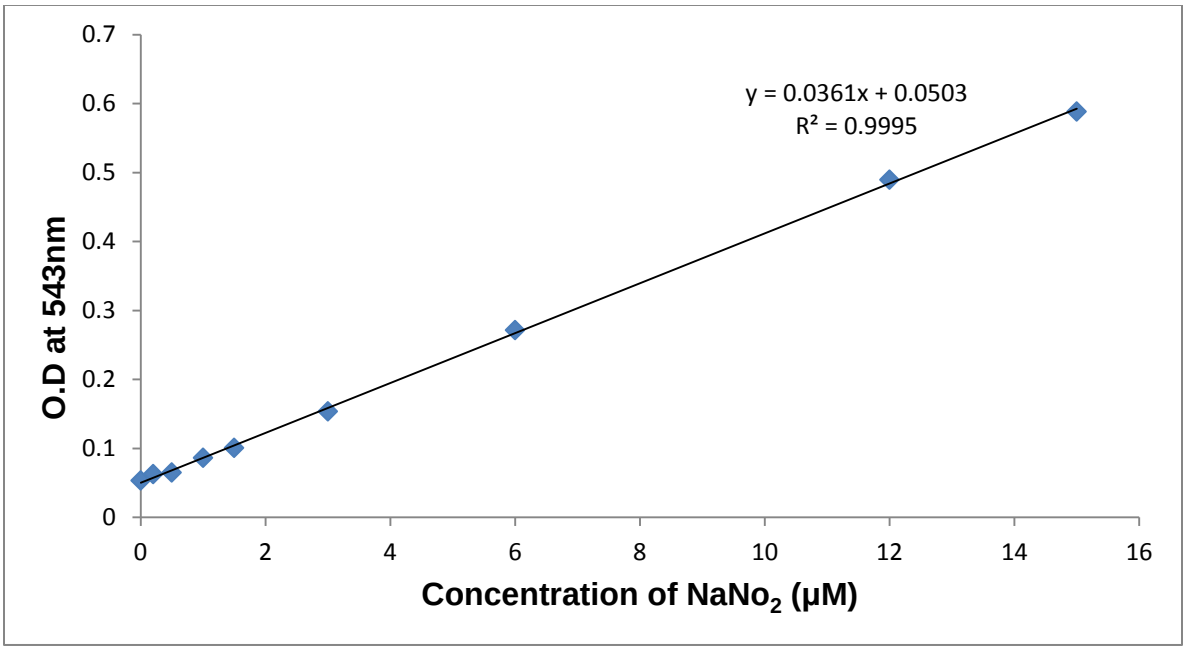


Figure 4.10: The standard curve of $NaNO_2$ for Griess assay.

4.5.1 Extracellular NO levels in culture supernatant

The NO level was detected in treated THP-1 and AGS cell culture supernatants. The levels of NO in AGS cells showed a significant increase of 3 folds when compared to the untreated controls. The THP-1 cells did not show any significant change in the NO level.

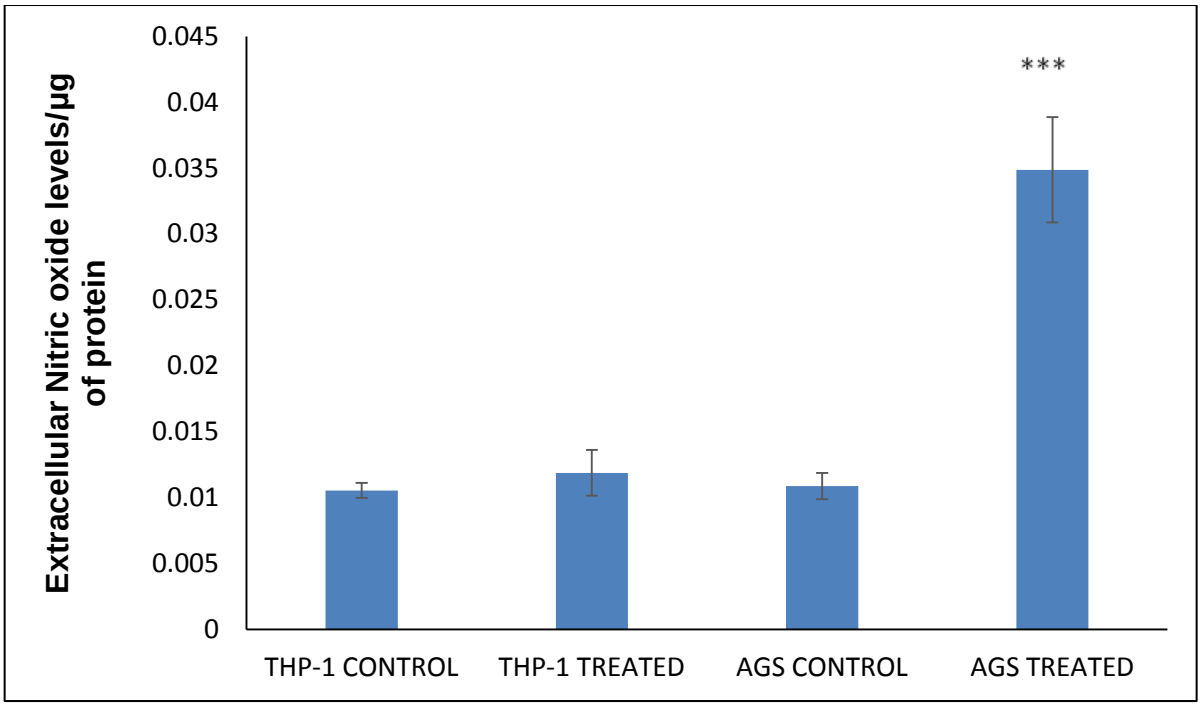
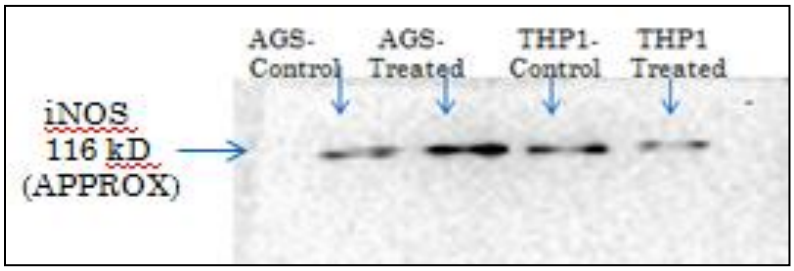


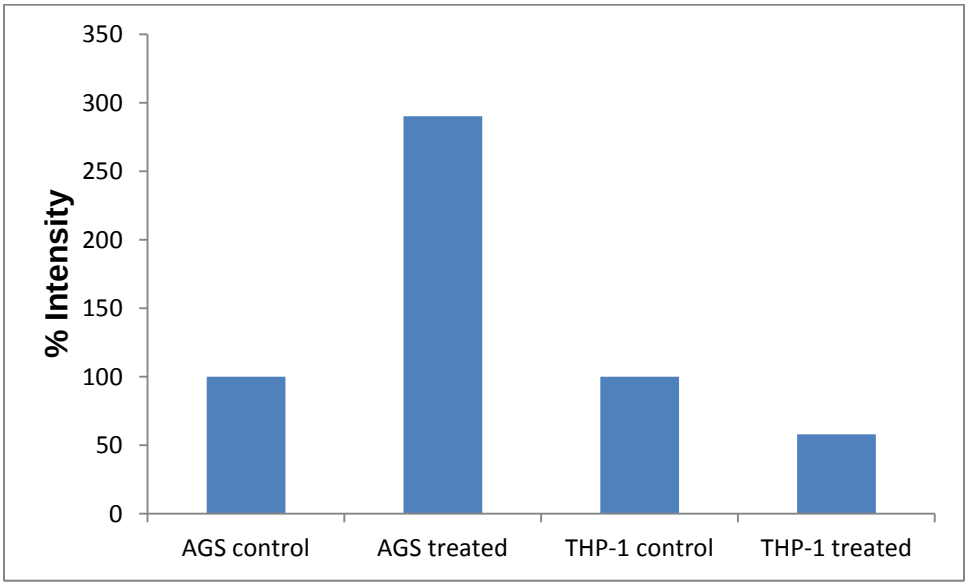
Figure 4.11: Detection of extracellular NO levels in THP-1 and AGS culture supernatants. Extracellular NO was detected using Griess assay in treated THP-1 and AGS culture supernatants. Student t-test was performed and there was significant changes observed in treated AGS culture supernatant.

4.5.2 iNOS Detection in AGS and THP-1 cell lysate by Western Blotting

iNOS is a key factor for determining the intracellular NO production in AGS and THP-1 cells due to oxidative stress induced by *H. pylori* enriched media as it is the main source of intracellular NO generation. Western blotting was performed for iNOS expression in the cell lysate of THP-1 and AGS cells treated with HPEM. The Western blot results confirmed the spectrophotometry results of NO levels. The levels of iNOS in AGS cell lysate increased significantly in HPEM treated samples where no change was seen in THP-1 cells.



A



B

Figure 4.12: A) Western Blot image showing expression of iNOS in AGS and THP-1 cell treated with *H. pylori* enriched media. B) Densitometry of the Western blot showing expression of iNOS in AGS and THP-1 cell treated with *H. pylori* enriched media.

4.6 Protein Carbonylation Assay (Protein oxidation)

Protein carbonyl content is widely used as a marker for oxidative stress and a measure of oxidative damage. Method used to determine the carbonyl utilize the reaction of carbonyl groups with 2, 4-dinitrophenylhydrazine (DNPH) to form protein bound 2, 4-dinitrophenylhydrazones which can be quantified spectrophotometrically. The Protein carbonyl content in both the cell lysate from THP-1 and AGS cells lines showed a significant increase suggested protein getting severely damaged/oxidized after HPEM treatment.

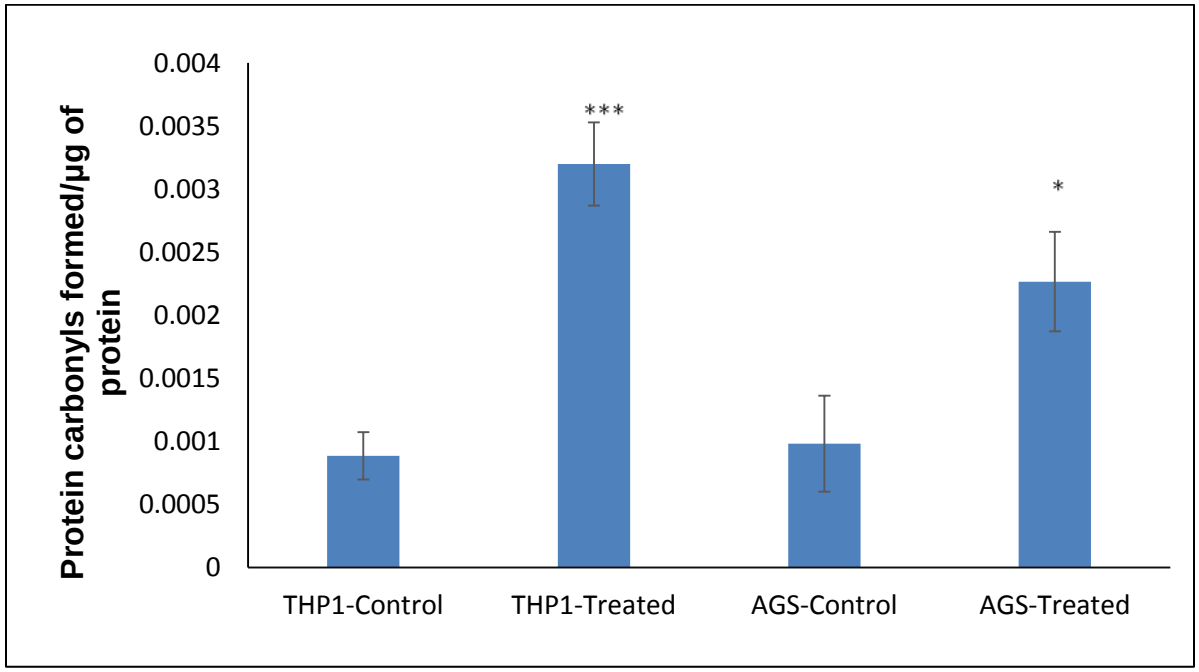


Figure 4.13: Level of protein carbonyls was estimated in THP-1 and AGS cells when treated with *H. pylori* enriched media. Student t-test was performed to evaluate the significance of the results, the data were considered as statistically significant at p*≤ 0.05 when HPEM-treated cells were compared with untreated controls. The results are presented as the mean ± standard deviation (n=5).

4.7 Lipid Peroxidation Assay

Malondialdehyde (MDA) an end product of unsaturated fatty acid peroxidation, can react with thiobarbituric acid (TBA) to form coloured complex called thiobarbituric acid reactive substance (TBARS). The absorbance of a sample is determined at 535 nm. TBARS level gradually increases in cells treated with *H. pylori* enriched media. The level of lipid peroxidation was significantly higher in cell lysate from the both cell lines that is THP-1 as well as AGS cells when compared with untreated control cells.

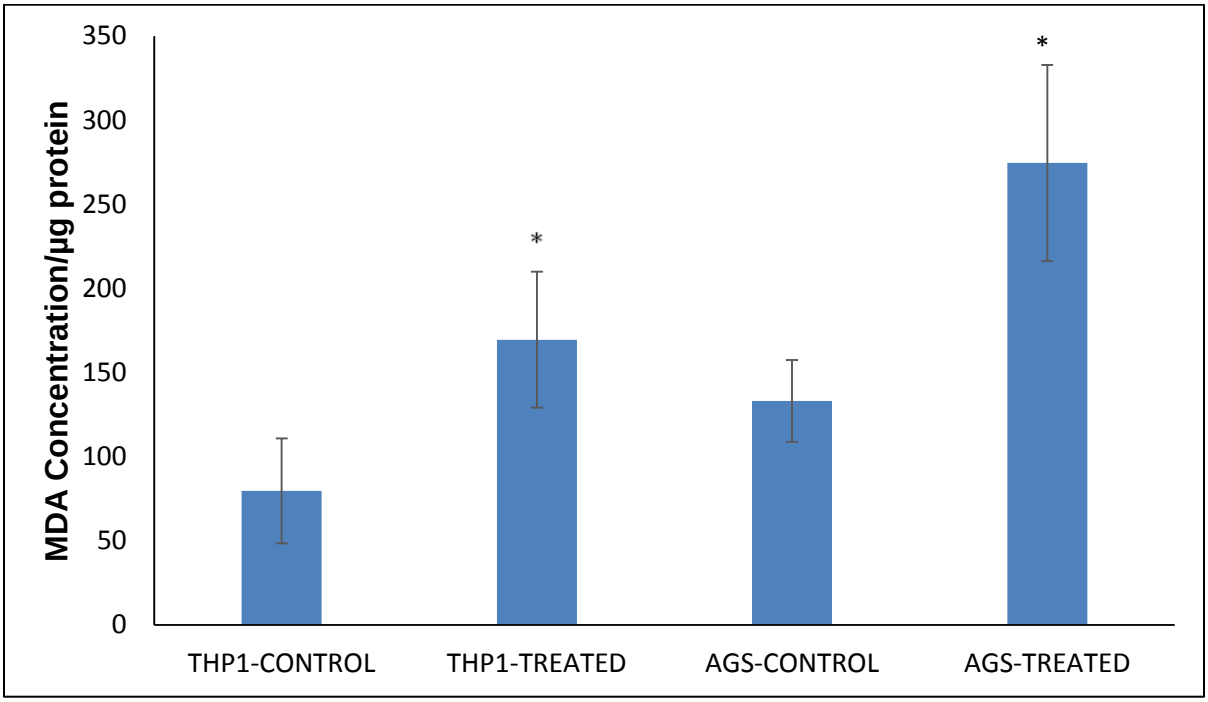


Figure 4.14: Effect of *H. pylori* enriched media on TBARS levels in THP-1 and AGS cell lysate. Treated cells showed a highly significant TBARS level in comparison to control cells. Student t-test was performed to evaluate the significance of the results, the data were considered as statistically significant at p*≤ 0.05 when HPEM-treated cells were compared with untreated controls. The results are presented as the mean ± standard deviation (n=5).

CHAPTER -5

DISCUSSION

Discussion

Oxidative stress contributes in both pathological and physiological pathways (Schieber and Chandel, 2014).Recent studies have shown an important role for ROS in tumor development (Ishikawa *et al.,* 2008).ROS and RNS are produced by mechanism of oxidative stress and various inflammatory cytokines due to persistent inflammation and in turn they activate various oxidant generating enzyme like inducible nitric oxide synthase (iNOS). Both ROS and RNS lead to oxidative damages of biomolecules like DNA, RNA, proteins, lipids and finally alter the normal mechanism of signaling pathways which lead to the carcinogenesis.

In this study we investigated the *H. pylori* enriched media induced oxidative stress and altered responses generated in THP-1 and AGS cells. The role of *H. pylori* enriched media protein (HPEM) in the induction of inflammation is well characterized and it contains intrinsic NADPH oxidase as reported previously (Butcher, *et al.,* 2017). NADPH oxidase rapidly increases the level of ROS in gastric cell line. The most common form of ROS is superoxide, H_2O_2 and OH° and these can react with other macromolecules such as proteins, lipids and DNA. The objective of this study was to see the cytotoxicity of *H. pylori* enriched media, for which MTT test was employed. The response of THP-1 and AGS cells after various concentrations of enriched media was investigated and the concentrations where 20-30% change was happening was for future experiments to induce oxidative stress.

High concentrations of NO are likely to have toxic effects resulting from the formation of nitrites, No further rests with superoxide radical and forms highly toxic peroxynitrite radical which due to its very short half life is highly reactive. Our finding suggests that the oxidative stress induced by enriched media showed high level of NO in AGS cell culture supernatants as compared to its control but very little to no change was seen in THP-1 cells. There were significant changes observed in treated AGS culture supernatant. In the previous report it was reported that $NO^{\bullet}$ level significantly increased in tissues in patients with colon tumors (Haklar *et al.,* 2001). $NO^{\bullet}$ levels was higher in gastric cancer than in the control group (Ebubekir Bakan *et al.,* 2002).

Further, we investigated that whether *H. pylori* enriched media can induce oxidative stress in THP-1 and AGS cells. Oxidative stress is the result of an imbalance between free radicals and an array of antioxidant defence systems of the cell which either directly scavenges ROS or produce compounds which serve this purpose. Proteins are major targets for RNS / ROS because they are mainly responsible for most functional processes within the cells. It has been estimated that proteins can scavenge the majority (50-75%) of reactive species (ROS/RNS) generated. Exposure of proteins to ROS /RNS may alter every level of protein structure from primary to quaternary, causing major physical changes in protein structure. Oxidative damage to proteins is induced either directly by ROS / RNS or indirectly by reaction of secondary by products of oxidative stress and can occur via different mechanisms. Spectrophotometric DNPH assay for the determination of carbonyl content in purified proteins is very sensitive (Dalle-Donne *et al.,* 2003). The protein carbonyls assay results showed that oxidative stress induced by exposure to *H. pylori* enriched media showed significant change in protein carbonyl levels in comparison to untreated control cells. Previous finding have reported increased protein carbonyl level in gastric cancer (Yongsheng Ma *et al.,* 2013). Cancer patients have higher levels of protein carbonyls (D.Chang *et al.,* 2008).

Next, we measured lipid peroxidation by TBARS assay in *H. pylori* enriched media treated cell lysate from THP-1 and AGS cells. We observed that the exposure of THP-1 and AGS cells to enriched media showed very high and significant increase in TBARS levels. The treatment of *H. pylori* enriched media increased the TBARS levels significantly by 2 folds in THP-1 and AGS cells. In some previous studies it is shown that the level of plasma MDA (lipid peroxidation marker) was significantly higher in patients with gastric cancer compared with the healthy control group (Ebubekir Bakan *et al.,* 2002). Increased serum MDA levels were reported in patients with gastric cancer (Khanzode *et al.,* 2003). Arivazhagan *et al.,* 1997 reported increased erythrocyte MDA levels in patients with gastric cancer.

Overall in the present study we observed increased oxidative stress detected in terms of TBARS and protein carbonyls when the cells treated with HPEM. The THP-1 cells which are human monocytic cell line are getting killed by the HPEM cells and also

the NO level in these cells is not getting changed after HPEM treatment. The detailed mechanism needs further investigation but present study has provided some preliminary and important data for further studies on HPEM induced oxidative stress in two important human cells cell lines.

Summary and Conclusion

In this study, the effects of oxidative stress on THP-1 and AGS cell line were studied. The response of THP-1 and AGS cells to *H. pylori* enriched media (HPEM) exposure was investigated. ROS once generated and accumulated can damage biological molecules or macromolecules. It can affect lipids and protein structure and functions. Hydroxyl radical is the most potent and can damage cells oxidation of protein, lipids, fatty acids and nucleic acids.

Human immune THP-1 cells and human gastric AGS cells were treated with various concentration of HPEM. The treated THP-1 and AGS cells showed increased accumulation of oxidative stress hence damaging the membrane lipids and resulted in lipid peroxidation. The protein oxidation determined in term of protein carbonyls was also increased upon he HPEM treatment. NO is a free radical which once formed can lead to many deleterious effects. Our results showed that:

- The NO levels in treated THP-1 did not alter significantly but in AGS cells it was significantly higher increase of 3 folds when compared to the untreated controls.
- Level of protein carbonyls were considered as statistically significant at $p^* \leq 0.05$ when HPEM-treated cells were compared with untreated controls. In treated THP-1 cells it showed a significant increase of 3 folds and in AGS cells it showed a significant increase of 2 folds when compared to the untreated controls.
- The level of lipid peroxidation was significantly higher in cell lysate from the both cell lines that is THP-1 as well as AGS cells when compared with untreated control cells.

From this work it can be concluded that HPEM can significantly increase ROS and RNS formation which result in increased oxidative stress.

References

Algood, H. M. S., & Cover, T. L. (2006). *Helicobacter pylori* persistence: an overview of interactions between H. pylori and host immune defenses. *Clinical Microbiology Reviews*, **19(**4), 597-613.

Amieva, M., & Peek, R. M. (2016). Pathobiology of *Helicobacter pylori*–induced gastric cancer. *Gastroenterology*, **150**(1), 64-78.

Arivazhagan, S., Kavitha, K., & Nagini, S. (1997). Erythrocyte lipid peroxidation and antioxidants in gastric cancer patients. *Cell Biochemistry and Function: Cellular biochemistry and its modulation by active agents or disease*, **15**(1), 15-18.

Atherton, J. C., Cao, P., Peek, R. M., Tummuru, M. K., Blaser, M. J., & Cover, T. L. (1995). Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori* association of specific vacA types with cytotoxin production and peptic ulceration. *Journal of Biological Chemistry*, **270**(30), 17771-17777.

Athmann, C., Zeng, N., Kang, T., Marcus, E. A., Scott, D. R., Rektorschek, M., ... & Sachs, G. (2000). Local pH elevation mediated by the intrabacterial urease of *Helicobacter pylori* cocultured with gastric cells. *The Journal of Clinical Investigation*, **106**(3), 339-347.

Augusto, A. C., Miguel, F., Mendonça, S., Pedrazzoli Jr, J., & Gurgueira, S. A. (2007). Oxidative stress expression status associated to *Helicobacter pylori* virulence in gastric diseases. *Clinical Biochemistry*, **40**(9-10), 615-622.

Bakan, E., Taysi, S., Polat, M. F., Dalga, S., Umudum, Z., Bakan, N., & Gumus, M. (2002). Nitric oxide levels and lipid peroxidation in plasma of patients with gastric cancer. *Japanese Journal of Clinical Oncology*, **32**(5), 162-166.

Björkholm, B., Zhukhovitsky, V., Löfman, C., Hultén, K., Enroth, H., Block, M., Rigo, R., Falk, P. and Engstrand, L. (2000). *Helicobacter pylori* entry into human gastric epithelial cells: a potential determinant of virulence, persistence, and treatment failures 1. *Helicobacter,* **5**(3), 148-154

Bliss, C. M., Golenbock, D. T., Keates, S., Linevsky, J. K., & Kelly, C. P. (1998). *Helicobacter pylori* lipopolysaccharide binds to CD14 and stimulates release of interleukin-8, epithelial neutrophil-activating peptide 78, and monocyte chemotactic protein 1 by human monocytes. *Infection and Immunity*, **66**(11), 5357-5363.

Bonis, M., Ecobichon, C., Guadagnini, S., Prévost, M. C., & Boneca, I. G. (2010). A M23B family metallopeptidase of *Helicobacter pylori* required for cell shape, pole formation and virulence. *Molecular Microbiology*, **78**(4), 809-819.

Bussière, F. I., Chaturvedi, R., Cheng, Y., Gobert, A. P., Asim, M., Blumberg, D. R., ... & Wilson, K. T. (2005). Spermine causes loss of innate immune response to *Helicobacter pylori* by inhibition of inducible nitric-oxide synthase translation. *Journal of Biological Chemistry*, **280**(4), 2409-2412.

Butcher, L. D., den Hartog, G., Ernst, P. B., & Crowe, S. E. (2017). Oxidative stress resulting from *Helicobacter pylori* infection contributes to gastric carcinogenesis. *Cellular and Molecular Gastroenterology and Hepatology*, ***3*(**3), 316.

Celli, J. P., Turner, B. S., Afdhal, N. H., Keates, S., Ghiran, I., Kelly, C. P., ... & Bansil, R. (2009). *Helicobacter pylori* moves through mucus by reducing mucin viscoelasticity. *Proceedings of the National Academy of Sciences*, **106**(34), 14321-14326.

Chang, D., Wang, F. A. N., Zhao, Y. S., & Pan, H. Z. (2008). Evaluation of oxidative stress in colorectal cancer patients. *Biomedical and Environmental Sciences*, **21**(4), 286-289.

Cover, T. L., & Blanke, S. R. (2005). *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. *Nature Reviews Microbiology*, **3**(4), 320.

Cover, T. L., & Blaser, M. J. (1992). Purification and characterization of the vacuolating toxin from *Helicobacter pylori. Journal of Biological Chemistry*, **267**(15), 10570-10575.

Dhiman, M., & Garg, N. J. (2011). NADPH oxidase inhibition ameliorates Trypanosoma cruzi-induced myocarditis during Chagas disease. *The Journal of Pathology*, **225**(4), 583-596.

Dhiman, M., Estrada-Franco, J. G., Pando, J. M., Ramirez-Aguilar, F. J., Spratt, H., Vazquez-Corzo, S., ... & Garg, N. J. (2009). Increased myeloperoxidase activity and protein nitration are indicators of inflammation in patients with Chagas' disease. *Clinical and Vaccine Immunology*, **16**(5), 660-666.

Dhiman, M., Wan, X., Popov, V. L., Vargas, G., & Garg, N. J. (2013). MnSODtg mice control myocardial inflammatory and oxidative stress and remodeling responses elicited in chronic Chagas disease. *Journal of the American Heart Association*, **2**(5), e000302.

Dhiman, M., Zago, M. P., Nunez, S., Amoroso, A., Rementeria, H., Dousset, P., ... & Garg, N. J. (2012). Cardiac-oxidized antigens are targets of immune recognition by antibodies and potential molecular determinants in Chagas disease pathogenesis. *PloS one*, **7**(1), e28449.

Ding, S. Z., Minohara, Y., Fan, X. J., Wang, J., Reyes, V. E., Patel, J., ... & Crowe, S. E. (2007). *Helicobacter pylori* infection induces oxidative stress and programmed cell death in human gastric epithelial cells. *Infection and Immunity*, **75**(8), 4030-403

Dundon, W. G., de Bernard, M., & Montecucco, C. (2001). Virulence factors of *Helicobacter pylori. International Journal of Medical Microbiology*, **290**(8), 647-658.

Erdinçler, D. S., Seven, A., Inci, F., Beğer, T., & Candan, G. (1997). Lipid peroxidation and antioxidant status in experimental animals: effects of aging and hypercholesterolemic diet. *Clinica Chimica Acta*, **265**(1), 77-84.

Gong, M., Ling, S. S. M., Lui, S. Y., Yeoh, K. G., & Ho, B. (2010). *Helicobacter pylori* γ-glutamyl transpeptidase is a pathogenic factor in the development of peptic ulcer disease. *Gastroenterology*, **139**(2), 564-573.

Same ref repeated González-Rivera, C., Gangwer, K. A., McClain, M. S., Eli, I. M., Chambers, M. G., Ohi, M. D., ... & Cover, T. L. (2010). Reconstitution of *Helicobacter pylori* VacA toxin from purified components. *Biochemistry*, **49**(27), 5743-5752.

Haklar, G., Sayin-Özveri, E., Yüksel, M., Aktan, A. Ö., & Yalçin, A. S. (2001). Different kinds of reactive oxygen and nitrogen species were detected in colon and breast tumors. *Cancer letters*, **165**(2), 219-224.

Handa, O., Naito, Y., & Yoshikawa, T. (2007). CagA protein of *Helicobacter pylori*: a hijacker of gastric epithelial cell signaling. *Biochemical Pharmacology*, **73**(11), 1697-1702.

Handa, O., Naito, Y., & Yoshikawa, T. (2010). *Helicobacter pylori*: a ROS-inducing bacterial species in the stomach. *Inflammation Research*, **59**(12), 997-1003.

Ilver, D., Arnqvist, A., Ögren, J., Frick, I. M., Kersulyte, D., Incecik, E. T., ... & Borén, T. (1998). *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science*, **279**(5349), 373-377.

Ishikawa, K., Takenaga, K., Akimoto, M., Koshikawa, N., Yamaguchi, A., Imanishi, H., ... & Hayashi, J. I. (2008). ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science*, **320**(5876), 661-664.

Khanzode, S. S., Khanzode, S. D., & Dakhale, G. N. (2003). Serum and plasma concentration of oxidant and antioxidants in patients of Helicobacter pylori gastritis and its correlation with gastric cancer. *Cancer Letters*, **195**(1), 27-31.

Kim, J. M., Kim, J. S., Lee, J. Y., Kim, Y. J., Youn, H. J., Kim, I. Y., ... & Song, I. S. (2007). Vacuolating cytotoxin in *Helicobacter pylori* water-soluble proteins upregulates chemokine expression in human eosinophils via Ca2+ influx, mitochondrial reactive oxygen intermediates, and NF-κB activation. *Infection and Immunity*, **75**(7), 3373-3381.

Kuipers, E. J., Israel, D. A., Kusters, J. G., Gerrits, M. M., Weel, J., Van Der Ende, A., ... & Blaser, M. J. (2000). Quasispecies development of *Helicobacter pylori* observed in

paired isolates obtained years apart from the same host. *The Journal of InfectiousDiseases*, **181**(1), 273-282.

Kusters, J. G., van Vliet, A. H., & Kuipers, E. J. (2006). Pathogenesis of Helicobacter *pylori* infection. *Clinical Microbiology Reviews*, **19**(3), 449-490.

Leunk, R. D., Johnson, P. T., David, B. C., Kraft, W. G., & Morgan, D. R. (1988). Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori. Journal of Medical Microbiology*, **26**(2), 93-99.

Lina, T. T., Alzahrani, S., Gonzalez, J., Pinchuk, I. V., Beswick, E. J., & Reyes, V. E. (2014). Immune evasion strategies used by *Helicobacter pylori*. *World Journal of Gastroenterology: WJG*, **20**(36), 12753.

Lykkesfeldt, J. (2007). Malondialdehyde as biomarker of oxidative damage to lipids caused by smoking. *Clinica Chimica acta*, **380**(1-2), 50-58.

Ma, Y., Zhang, L., Rong, S., Qu, H., Zhang, Y., Chang, D., … Wang, W. (2013). Relation between Gastric Cancer and Protein Oxidation, DNA Damage, and Lipid Peroxidation. *Oxidative Medicine and Cellular Longevity*, *2013*, 543760. http://doi.org/10.1155/2013/543760

Mitchell, P., Germain, C., Fiori, P. L., Khamri, W., Foster, G. R., Ghosh, S., ... & Lombardi, G. (2007). Chronic exposure to *Helicobacter pylori* impairs dendritic cell function and inhibits Th1 development. *Infection and Immunity*, **75**(2), 810-819.

O'Hara, A. M., Bhattacharyya, A., Bai, J., Mifflin, R. C., Ernst, P. B., Mitra, S., & Crowe, S. E. (2009). Tumor necrosis factor (TNF)-α-induced IL-8 expression in gastric epithelial cells: role of reactive oxygen species and AP endonuclease-1/redox factor (Ref)-1. *Cytokine*, **46**(3), 359-369.

Palframan, S. L., Kwok, T., & Gabriel, K. (2012). Vacuolating cytotoxin A (VacA), a key toxin for *Helicobacter pylori* pathogenesis. *Frontiers in Cellular and Infection Microbiology*, **2**, **92**.

Papini, E., Satin, B., Norais, N., de Bernard, M., Telford, J. L., Rappuoli, R., & Montecucco, C. (1998). Selective increase of the permeability of polarized epithelial cell monolayers by *Helicobacter pylori* vacuolating toxin. *The Journal of Clinical Investigation*, **102**(4), 813-820.

Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. *Current Biology*, **24**(10), R453-R462.

Shahi, H., Reiisi, S., Bahreini, R., Bagheri, N., Salimzadeh, L., & Shirzad, H. (2015). Association between *Helicobacter pylori* cagA, babA2 virulence factors and gastric mucosal interleukin-33 mRNA expression and clinical outcomes in dyspeptic patients. *International Journal of Molecular and Cellular Medicine*, **4**(4), 227.

Tang, B., Li, N., Gu, J., Zhuang, Y., Li, Q., Wang, H. G., ... & Chen, L. (2012). Compromised autophagy by MIR30B benefits the intracellular survival of *Helicobacter pylori*. *Autophagy*, **8**(7), 1045-1057.

Tsugawa, H., Suzuki, H., Saya, H., Hatakeyama, M., Hirayama, T., Hirata, K., ... & *Hibi, T. (2012). Reactive oxygen species-induced autophagic degradation of Helicobacter pylori* CagA is specifically suppressed in cancer stem-like cells. *Cell Host & Microbe*, **12**(6), 764-777.

Unemo, M., Aspholm-Hurtig, M., Ilver, D., Bergström, J., Borén, T., Danielsson, D., & Teneberg, S. (2005). The sialic acid binding SabA adhesin of *Helicobacter pylori* is essential for nonopsonic activation of human neutrophils. *Journal of Biological Chemistry*, **280**(15), 15390-15397.

van Doorn, L. J., Figueiredo, C., Sanna, R., Pena, S., Midolo, P., Ng, E. K., ... & Quint, W. G. (1998). Expanding allelic diversity of *Helicobacter pylori* vacA. *Journal of Clinical Microbiology*, **36**(9), 2597-2603.

Wang, G., Hong, Y., Olczak, A., Maier, S. E., & Maier, R. J. (2006). Dual roles of *Helicobacter pylori* NapA in inducing and combating oxidative stress. *Infection and Immunity*, **74**(12), 6839-6846

Yamaoka, Y., Ojo, O., Fujimoto, S., Odenbreit, S., Haas, R., Gutierrez, O., ... & Graham, D. Y. (2006). *Helicobacter pylori* outer membrane proteins and gastroduodenal disease. *Gut*, **55**(6), 775-781.

Yamasaki, E., Wada, A., Kumatori, A., Nakagawa, I., Funao, J., Nakayama, M., ... & Hirayama, T. (2006). *Helicobacter pylori* vacuolating cytotoxin induces activation of the proapoptotic proteins Bax and Bak, leading to cytochrome c release and cell death, independent of vacuolation. *Journal of Biological Chemistry*, **281**(16), 11250-11259.

Yue, P., XuN, L. I., & QIAo, L. (2015). Role of *Helicobacter pylori* in gastric cancer: advances and controversies. *Discovery Medicine*, **20**(111), 1-9.

URKUND

Urkund Analysis Result

Analysed Document:	Pallavi Samal Urkund_May 28.docx (D39309507)
Submitted:	5/28/2018 9:11:00 AM
Submitted By:	monisha.dhiman@gmail.com
Significance:	2 %

Sources included in the report:

https://www.dovepress.com/inhibition-of-mitoticnbspaurora-kinase-a-by-alisertib-induces-apoptosi-peer-reviewed-fulltext-article-DDDT
https://www.dovepress.com/danusertib-a-potent-pan-aurora-kinase-and-abl-kinase-inhibitor-induces-peer-reviewed-fulltext-article-DDDT
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4298344/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3928159/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1952011/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3728185/
http://www.jbc.org/content/early/2004/11/23/jbc.M412820200.full.pdf

Instances where selected sources appear:

9

URKUND

Urkund Analysis Result

Analysed Document:	Pallavi Samal Urkund_May 28.docx (D39309507)
Submitted:	5/28/2018 9:11:00 AM
Submitted By:	monisha.dhiman@gmail.com
Significance:	2 %

Sources included in the report:

https://www.dovepress.com/inhibition-of-mitoticnbspaurora-kinase-a-by-alisertib-induces-apoptosi-peer-reviewed-fulltext-article-DDDT
https://www.dovepress.com/danusertib-a-potent-pan-aurora-kinase-and-abl-kinase-inhibitor-induces-peer-reviewed-fulltext-article-DDDT
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4298344/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3928159/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1952011/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3728185/
http://www.jbc.org/content/early/2004/11/23/jbc.M412820200.full.pdf

Instances where selected sources appear:

9