5-2017

Inhibition of chemotherapeutic-induced apoptosis in esophageal squamous cancer cells by the keystone periodontal pathogen, Porphyromonas gingivalis.

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INHIBITION OF CHEMOTHERAPEUTIC-INDUCED APOPTOSIS IN ESOPHAGEAL SQUAMOUS CANCER CELLS BY THE KEYSTONE PERIODONTAL PATHOGEN, *PORPHYROMONAS GINGIVALIS*.

By

Atul Kumar Agrawal

A Thesis

Submitted to the Faculty of the

School of Dentistry of the University of Louisville

In Partial Fulfillment of the Requirement for the Degree of

Master of Science in Oral Biology

Department of Oral Health and Rehabilitation

University of Louisville, School of Dentistry

Louisville, KY

May 2017

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BDS

A Thesis approved on

April 21, 2017

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ACKNOWLEDGEMENTS

I owe many thanks to my mentor Dr. David A. Scott for his patience, support and encouragement throughout my master's degree. I would also like to thank my committee members Dr. Richard J. Lamont and Dr. Huizhi Wang for their feedback and assistance during this period.

A special thanks to Gwyneth Lamont, Zhen Gu and Diane Renaud for teaching me every single procedure with great patience. I would like to give thanks to Justin Hutcherson, Himabindu Gogeneni, Neelima Cherukumalli, Rajarshi Guha Niyogi and Shilpa Singh for their help in my project. I would also like to thank Celine Joyce, and Muhammad Mudassir for their support.

My sincere thanks to my parents Mr. Ashok Kumar Agrawal and Kumari Maya Saria for allowing me to realize my potential. Words cannot describe the love and encouragement they have provided to me over the years. Also a very special thanks to my brothers Arvind Agrawal and Ankit Agrawal for their emotional and financial support. Without their encouragement and support I would have never been able to achieve my dream. Lastly, thanks to all my friends Dheeraj, Ting, Krishna, Pramod, Praneet, Rajshekhar, Sree, Mahesh and all others for being there for me; I am grateful for you.

ABSTRACT

INHIBITION OF CHEMOTHERAPEUTIC-INDUCED APOPTOSIS IN ESOPHAGEAL SQUAMOUS CANCER CELLS BY THE KEYSTONE PERIODONTAL PATHOGEN, *PORPHYROMONAS GINGIVALIS.*

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Recent evidence has shown that *P. gingivalis*, a Gram-negative, anaerobic oral bacterium, is negatively associated with the presence and outcome of esophageal squamous cell carcinoma (ESCC). While potential underlying mechanisms are yet to be established, *P. gingivalis* infection is known to inhibit apoptosis in gingival epithelial cells. Therefore, we hypothesized that *P. gingivalis* may also inhibit of the induction of apoptosis in human ESCC cells exposed to the commonly employed anti-ESCC chemotherapeutic agent, cisplatin. The capacity of *P. gingivalis*, at variant multiplicities of infection, to suppress cisplatin-induced necrosis and apoptosis in EC9706 ESCC cells was established by lactate dehydrogenase (LDH) release assays, caspase-3-specific Western blots and activated caspase-3 ELISAs. LDH and activated caspase-3 levels were found to be significantly decreased by *P. gingivalis* for necrosis and apoptosis respectively, indicating that chemotherapeutic drug action in ESCC is rendered ineffective in presence of *P. gingivalis*. This study provides some of the first mechanistic insights

into how esophageal infection with *P. gingivalis* may be associated with ESCC-related mortality.

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CHAPTER 1: INTRODUCTION

1.1 Epidemiology of Esophageal Cancer

Esophageal cancer is cancer arising from the esophagus - the food pipe that runs between the throat and the stomach. Esophageal carcinoma affects more than 450,000 people worldwide and the prevalence is rapidly expanding (Pennathur, Gibson, Jobe, & Luketich, 2013). Currently, esophageal carcinoma is the eighth most common incident cancer in the world (Zhang, 2013). It is a growing health concern that is expected to increase in incidence over the next 10 years (Napier, Scheerer, & Misra, 2014). The American Cancer Society estimates that 16,940 new cases of esophageal cancer will be diagnosed and 15,690 people suffering from the disease will die in 2017 in the United States alone. Untreated esophageal carcinoma can cause difficulty in swallowing, chest pain, weight loss, vomiting, bleeding into esophagus and upper airway obstruction making it a significant problem. It is one of the least studied and deadliest cancers worldwide because of its extremely aggressive nature and poor survival rate (Zhang, 2013). The five year survival rate for the cancer that has spread to the organs or lymph nodes away from the tumor (distant) is as low as 4%, for the cancer that has spread to nearby surrounding lymph nodes or tissues (regional) is 21% and for localized is 40% (American Cancer Society 2016). Factors such as age, gender, heredity,

gene mutation, chemical exposure, diet, etc. have been reported as risk factors for esophageal cancer. Two major histological subtypes of esophageal cancer are known. Squamous cell carcinoma (ESCC) arises from squamous cells, the thin, flat cells lining the esophagus, most often found in the upper and middle part of the esophagus and is more prevalent among the developing nations. Adenocarcinoma (EAC), which arises from the glandular (secretory) cells in the lining of the esophagus that release mucus, forms in the lower part of the esophagus, near the stomach and is more prevalent in developed nations, including the United States (Gao et al., 2016).

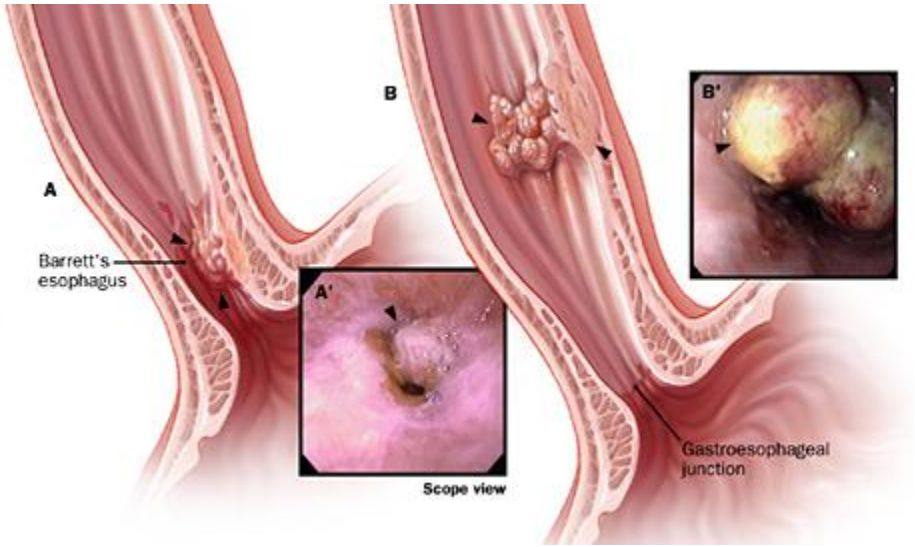

Fig 1. A, Adenocarcinoma; B, Squamous cell carcinoma of the esophagus; A', B', corresponding endoscopic views.
Illustration Copyright© 1998-2003 by The Johns Hopkins Health System Corporation and The Johns Hopkins University; used with permission from the Johns Hopkins Division of Gastroenterology and Hepatology (www.hopkinsmedicine.org/gi). Illustration created by Mike Linkinhoker.

1.2 Treatment of Esophageal Squamous Cell Carcinoma

Traditionally, surgery is considered the best treatment for esophageal squamous cell carcinoma in terms of long-term survival. Several factors, including the type and stage of cancer, possible side effects, and the patient's precedence and overall health help in choosing the treatment options and recommendations. Peculiarly for squamous cell cancer, chemotherapy and radiation therapy, a combination called chemoradiotherapy, are routinely recommended as the initial treatment to reduce the tumor in size, with surgery subsequently depending how well chemoradiotherapy worked.

In Japan, surgical resection was the mainstay of treatment for ESCC. In 2008, Japan Clinical Oncology Group (JCOG 9907) conducted a study comparing postoperative and preoperative chemotherapy with cisplatin and 5-fluorouracil (5-FU) in patients with clinical stage II or III ESCC. Preoperative chemotherapy was found to be superior to postoperative by inducing down staging and residual tumor reduction and improving overall survival of the patient without additional serious adverse events. Since the results of the Japan Clinical Oncology Group (JCOG) 9907 study were reported, neoadjuvant chemotherapy with cisplatin plus 5-fluorouracil followed by surgery has emerged as a new standard treatment (Higuchi et al., 2009).

Chemotherapy is a treatment, or combination of drugs, that circulates throughout the body to kill cancer cells wherever they may be. It is well

documented that most cytotoxic anticancer agents work by inducing apoptosis and the disruption of the apoptotic program can render the treatment ineffective (Lowe & Lin, 2000). Among the chemotherapeutic drugs, one of the most widely and clinically used drug is cisplatin. Cisplatin is a platinum coordination complex that is hydrolyzed intracellularly to produce a highly reactive moiety which causes cross linking of DNA. It cross-links DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible (Dasari & Tchounwou, 2014).

1.3 Apoptosis

Apoptosis is a process of programmed cell death that occurs in multicellular organisms. Different biochemical events lead to characteristic morphological cell changes such as blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation which ultimately lead to cell death (Ziegler & Groscurth, 2004).

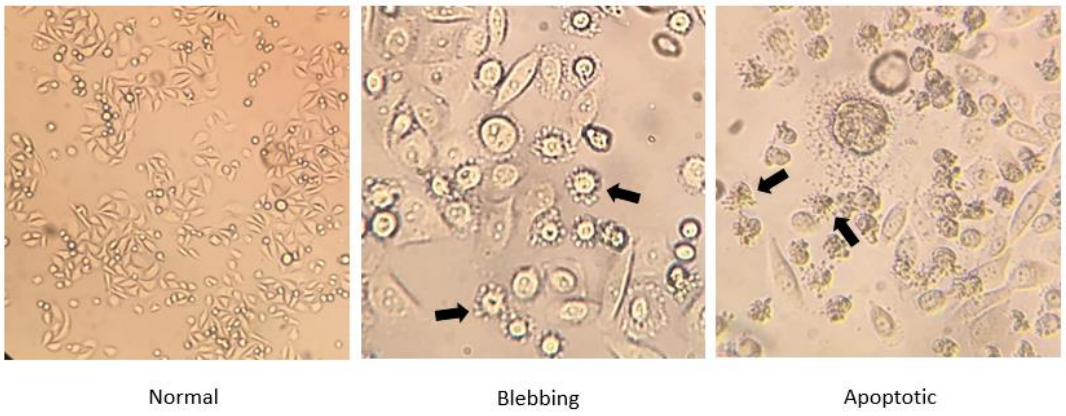

Fig 2. Morphological changes in cells in progression from normal to apoptotic.

Apoptosis can be initiated through one of two main understood pathways, intrinsic or extrinsic. In the *intrinsic pathway* the cell kills itself because it senses cell stress, while in the *extrinsic pathway* the cell kills itself because of signals from other cells. Both pathways initiate cell death by activating caspases, which are cell cycle-related proteases. The two pathways both activate initiator caspases, which then activate executioner caspases, which then kill the cell by degrading proteins indiscriminately. Most chemotherapeutic agents act by initiating the extrinsic apoptosis pathway in cancer cells. For example, 5-fluorouracil (5-FU) is converted in the body to the corresponding nucleotide, 5-fluoro-2-deoxy-uridine monophosphate (FdUMP), which inhibits thymidylate synthase and blocks the conversion of deoxyuridilic acid to deoxythymidylic acid interfering with the DNA replication or mis-incorporate into DNA or RNA ultimately causing cell apoptosis (Longley, Harkin, & Johnston, 2003). Similarly, cisplatin is hydrolyzed intracellularly to produce a highly reactive moiety which causes cross linking of DNA. It cross-links DNA in several different ways, interfering with cell division

by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible (Dasari & Tchounwou, 2014). Another chemotherapeutic agent, docetaxel, a member of the taxane drug class, binds to microtubules which stabilizes them and prevents depolymerization from calcium ions. Inhibition of the disassembly of the microtubules results in accumulation of microtubules inside the cell and initiation of apoptosis.

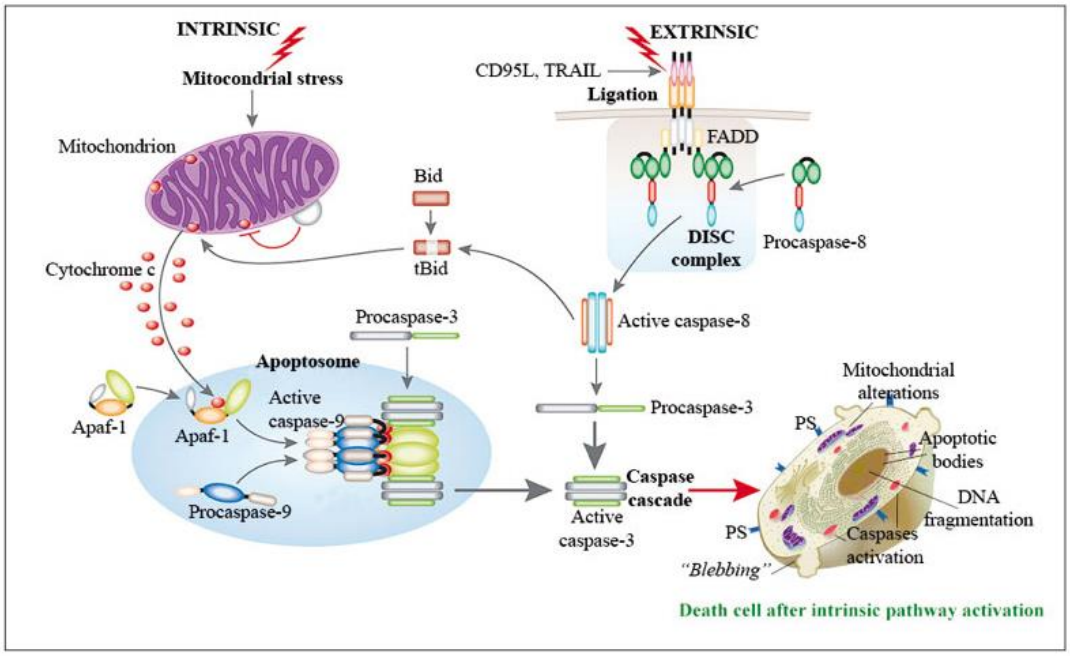

Figure 3. Apoptotic pathways: the extrinsic pathway involves so-called death receptors (CD95, TRAIL); the intrinsic one involves mitochondrial granules. Both pathways converge at caspase-3 activation, where classic biochemical and morphological changes in association with the apoptotic phenotype are originated.

1.3.1 Caspases including Caspase-3

Caspases (**c**ysteine-**asp**artic prote**ases)** are a family of proteolytic enzymes playing essential roles in programmed cell death (including apoptosis, pyroptosis and necroptosis) and inflammation. They are termed caspases because of their specific cysteine protease activity - a cysteine in its active site nucleophilically attacks and cleaves a target protein only at the C-terminal of an aspartic acid amino acid.

Apoptotic caspases are subcategorized as:

1. Initiator Caspases (Caspase- 2, Caspase- 8, Caspase-9, Caspase- 10)
2. Executioner Caspases (Caspase- 3, Caspase- 6 and Caspase- 7)

Once initiator caspases are activated, they produce a chain reaction, activating several other executioner caspases. Executioner caspases degrade over 600 cellular components in order to induce the morphological changes for apoptosis (Sollberger, Strittmatter, Garstkiewicz, Sand, & Beer, 2014).

1.3.2 Activation of caspases

Caspases are synthesized as inactive zymogens (pro-caspases) that are activated only in response to an appropriate stimulus. This post-translational level of control allows rapid and tight regulation of the enzyme.

Activation consists of dimerization and often oligomerization of pro-caspases, followed by cleavage into a small subunit and large subunit. The large and small subunit link with each other to form an active heterodimer caspase. The active enzyme often exists as a heterotetramer in the biological environment, where a pro-caspase dimer is cleaved together to form a heterotetramer (Shi, 2004).

Once appropriately dimerized, caspases cleave at inter-domain linker regions, forming a large and small subunit. This cleavage allows the active-site loops to take up a conformation favorable for enzymatic activity (Riedl & Shi, 2004). Initiator caspases cleave auto-proteolytically whereas executioner caspases are cleaved by initiator caspases. This chain of command allows an amplifying chain reaction or cascade for degrading cellular components, during controlled cell death.

Executioner caspase- 3

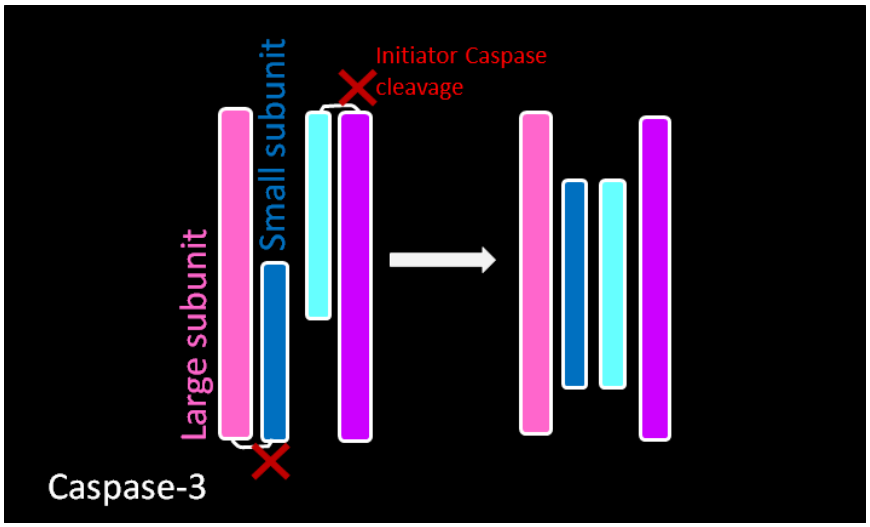

Fig 4. Capsase-3 activation: Executioner caspases can constitutively exist as homodimers. The red cuts represent regions where initiator caspases can cleave the executioner caspases. The resulting small and large subunit of each caspase-3 will associate, resulting in a heterotetramer (Lavrik et al., 2003).

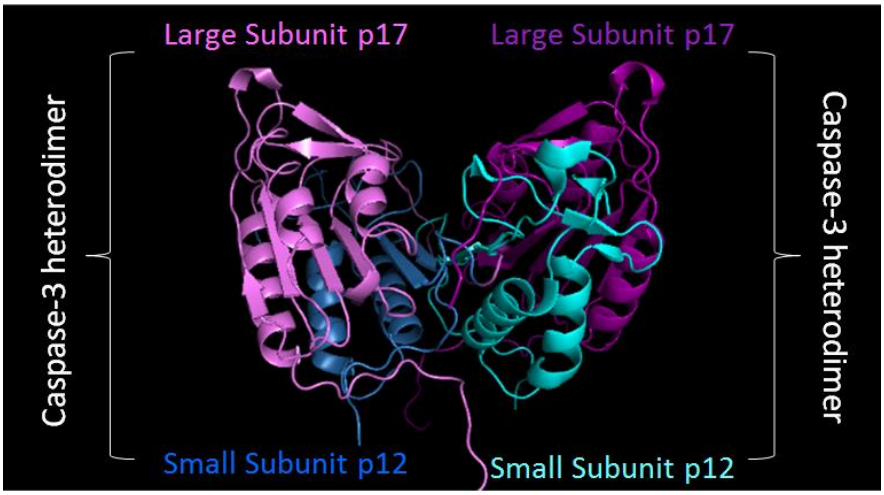

Fig 5. PDB image of Caspase- 3 (4QTX) in 'biological assembly'. Two shades of blue used to represent the two small subunits, while two shades of purple represent the two large subunits.

1.4 Necrosis

Necrosis is a form of cellular injury which results in premature cell death. It is caused by factors external or internal to the cell. In contrast to apoptosis, which follows defined signal transduction pathways, cellular death due to necrosis results in activation of various receptors, loss of cell membrane integrity and an

unrestrained release of products into the extracellular space (Proskuryakov, Konoplyannikov, & Gabai, 2003). Cellular changes that occur during this process are cell swelling followed by blebbing, nuclear shrinkage, chromatin condensation and finally dissolution of the nucleus into the cytoplasm (Kroemer et al., 2009). This process of cell death is almost always detrimental to the tissues and can be fatal because unlike apoptosis, cell membrane integrity is lost in necrosis and there is an uncontrolled release of cellular products into the extracellular space which initiates an inflammatory response in the adjacent tissues attracting leukocytes and phagocytes to eliminate the dead cells. The damaging substances released by the leukocytes keeps a check on the microbes as well as creates collateral damage to the surrounding tissues. Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many different cell types. It catalyzes the conversion of pyruvate, the final product of glycolysis, to lactate with conversion of NAD+ to NADH and lactate to pyruvate. Plasma membrane damage during necrosis releases LDH into the extracellular space. Thus we have employed caspase-3 in the study as a means for detection of apoptosis and LDH release as a means for detection of necrosis.

1.5 Bacteria and Cancer

After it has been found that *Helicobacter pylori* can cause gastric adenocarcinoma, numerous other relationships between specific bacteria and cancer have been documented (De Koster, Buset, Fernandes, & Deltenre, 1994; Kim, Ruiz, Carroll, & Moss, 2011). For example, *Salmonella typhi* with gall bladder

cancer (Randi, Franceschi, & La Vecchia, 2006), *Chlamydophila pneumoniae* with lung cancer (Littman, Jackson, & Vaughan, 2005), *Streptococcus bovis* and *Escherichia coli* with colon cancer (Boleij, van Gelder, Swinkels, & Tjalsma, 2011; Prorok-Hamon et al., 2014), *Porphyromonas gingivalis* with oral squamous cell carcinoma etc (Katz, Onate, Pauley, Bhattacharyya, & Cha, 2011).

Evidence for a potential role for microbes in the development of esophageal cancer has recently emerged. A study by Pei et al. concluded *Streptococcus*, *Prevotella* and *Veillonella* to be the most commonly detected microbial genera in esophageal biopsies (Pei et al., 2004; Pei et al., 2005). Another study by Yang et al. have categorized the microorganisms found in the esophagus into two subtypes: the *Streptococcus*-dominated type I microbiome, and the Gram-negative dominated type II microbiome. Type I is associated with normal esophagus whereas type II with Barrett's esophagus and esophagitis. 16S rRNA gene survey of the biopsy revealed these microbiome types located predominantly in these histological esophageal phenotypes (Yang et al., 2009). A significant association of esophageal squamous dysplasia, a precursor lesion of esophageal squamous cell carcinoma with the colonizers of the upper digestive tract microbiota has also been reported as these bacterial communities were characterized by 16S rRNA gene survey in biopsy samples taken from the dysplasia of the distal esophagus (Yang, Francois, & Pei, 2012). Very limited studies have been done on the esophageal microbiota (at the species level) in patients suffering from ESCC. Recent evidence suggests that the oral pathogen, *Porphyromonas*

gingivalis, is epidemiologically associated with ESCC as immunohistochemical detection of *P. gingivalis*, expression of *P. gingivalis* protease lysine-gingipain and *P. gingivalis*-specific 16S rDNA was significantly higher in esophageal cancerous and adjacent tissue as compared to the normal esophageal mucosa. In addition, Kaplan-Meier survival analysis showed that the higher expression of *P. gingivalis* whole cell antigens or lys-gingipain were both positively related with lower mean survival time of patients with ESCC. Hence the study indicates that the *P. gingivalis* infection could be a novel risk factor for ESCC.

1.6 *Porphyromonas gingivalis* and Esophageal Cancer

Porphyromonas gingivalis is a Gram-negative, proteolytic, asaccharolytic anaerobe. It is one of the members of the microbiota responsible for chronic and severe manifestations of the periodontal disease. According to the American Academy of Periodontology, periodontal disease is an inflammatory disease that affects the soft and hard structures that support the teeth. In its early stage, called gingivitis, the gums become swollen and red due to inflammation, which is the body's natural response to the presence of harmful bacteria. In the more serious form of periodontal disease called periodontitis, the gums pull away from the tooth and supporting gum tissues are destroyed. Bone can be lost, and the teeth may loosen or eventually fall out. *P. gingivalis* is a keystone oral pathogen, in mice, capable of invading the epithelial cells and manipulating the native immune system and cell cycle machinery of the host (Hajishengallis & Lamont, 2014;

Whitmore & Lamont, 2014). It has been shown that *P. gingivalis* localizes itself in the perinuclear region of the gingival epithelial cells (GECs) once invaded (Belton, Izutsu, Goodwin, Park, & Lamont, 1999) and is capable of inhibiting the apoptosis of the GECs (Nakhjiri et al., 2001). Several studies have established that periodontal diseases and tooth loss are related with oral cancer, gastric cancer and pancreatic cancer and may even relate to survival (Whitmore and Lamont 2014). *P. gingivalis* can induce the expression of the B7-H1 and B7-DC receptors in oral squamous cell carcinoma (OSCC) cells which contribute to chronic inflammation and supress effector T cells contributing to immune evasion by oral cancer. *P. gingivalis* infection also activates several pathways which induce MMP-9 expression and promotes carcinoma cell migration and invasion in the context of OSCC (Whitmore and Lamont 2014). In a prospective cohort study of over 400 cases and controls, >2 fold increase in risk of pancreatic cancer was observed among those with high levels of antibodies to *P. gingivalis* after adjusting for known risk factors (Michaud, 2013). Greater serum *P. gingivalis* IgG, independent of periodontal disease, inclined to be associated overall with increased orodigestive cancer mortality in a prospective cohort study of 12,605 subjects of the National Health and Nutrition Examination Survery III (NHANES III) survey (Ahn, Segers, & Hayes, 2012). In the context of oral squamous cell carcinoma, *P. gingivalis*-mediated immune avoidance, apoptosis impedance, carcinogen conversion, induction of MMP-9 and dysbiosis of the oral microbiota have all been posited as pro-tumorigenic mechanisms (Inaba et al., 2014; Whitmore & Lamont,

2014). A recent study by Gao et al. has shown that *P. gingivalis* is negatively associated with the presence and outcome of esophageal squamous cell carcinoma (ESCC) as described above. To date, however, the potential mechanisms by which the important oral pathogen, *Porphyromonas gingivalis*, may predispose to the development of esophageal cancer are entirely unknown.

HYPOTHESIS

Recent evidence has shown that *P. gingivalis*, a Gram-negative, anaerobic oral bacterium, is negatively associated with the presence and outcome of esophageal squamous cell carcinoma (ESCC). While potential underlying mechanisms are yet to be established, *P. gingivalis* infection is known to inhibit apoptosis in gingival epithelial cells. Therefore, we hypothesized that *P. gingivalis* may also inhibit of the induction of apoptosis in human ESCC cells exposed to commonly employed anti-ESCC chemotherapeutic agent, cisplatin.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials and Vendors

EC9706 cell line was gifted by Dr. Zhan Qimin (State Key Laboratory of Molecular Oncology, Chinese Academy of Medical Science, Beijing, China). (Rosewell Park Memorial Institute) RPMI-1640 (Gibco 11875-093) medium was purchased from Thermo Fisher Scientific (Rockford, IL). Sodium pyruvate 100mM, 2-Mercaptoethanol and Dimethyl Sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal Bovine Serum, penicillin (10,000 U/ml) and streptomycin (10,000 µg/ml) were purchased from Atlanta Biologicals (Flowery Branch, GA). HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 20mM was from Fisher Scientific (Fair Lawn, NJ), sodium bicarbonate 7.5% from Life Technologies Corporation (Grand Island, NY) and Plasmocin Prophylactic (2.5mg/ml – stock solution) was purchased from InvivoGen (San Diego, CA). Tissue culture treated flasks 25cm^2 and 75cm^2 were purchased from Corning Incorporated (Corning, NY.

Porphyromonas gingivalis ATCC 33277 (*Pg*) strain was originally obtained from the American Type Culture Collection. Gifu anaerobic medium (GAM), was purchased from Nissui Pharmaceutical (Tokyo, Japan).

Phosphate buffered saline (PBS) was from Thermo Fisher Scientific (Rockford, IL) Trypsin 0.25% was from Sigma Aldrich (St. Louis, MO). Cisplatin was purchased from EMD Millipore Corporation (Billerica, MA).

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reagent was purchased from Sigma Aldrich (St. Louis, MO), sodium dodecyl sulfate (SDS) – 10% (w/v) from Bio-Rad laboratories (Hercules, CA) and 0.5M hydrochloric acid (HCL) from Fisher Scientific (Fair Lawn, NJ).

Pierce LDH Cytotoxicity Assay Kit was purchased from Thermo Scientific (Rockford). Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit came from Thermo Fisher Scientific (Waltham, MA).

Caspase-3 antibody (#9662), β-Actin antibody (#8457 S), and anti-rabbit HRP-tagged and RIPA lysis buffer were purchased from Cell Signaling Technology (Beverly, MA). Phosphatase inhibitors 2 and 3, proteinase inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF)-serine protease inhibitor from Sigma Aldrich (St. Louis, MO). EDTA (0.5 M) was from Hoefer Inc. (San Francisco, CA). 28 ½ gauge insulin syringes were purchased from Beckton Dickinson & Company (Franklin Lakes, NJ). Pre-cast gels (NuPAGE™ 4-12% Bis-Tris Gel 1.5mm x 15well), MES SDS running buffer (20X) NP0006-1, transfer buffer 20X NP0002, See Blue plus and antioxidants were from Life Technologies (Carlsbad, CA). Cell scrapers 25cm were purchased from Sarstedt (Newton, NC). Magic Mark was purchased from Invitrogen (Carlsbad, CA). Tris buffered saline (TBS) – 10X and Tween 20 were

purchased from Fisher Scientific (Fair Lawn, NJ). Bovine serum albumin (BSA) came from Millipore Corporation (Kankakee, IL). Polyvinylidene difluoride (PVDF) membrane 0.2µm and filter papers purchased from Bio-Rad Laboratories (Hercules, CA). WestPico Chemiluminescent substrate and Pierce BCA Protein Assay kit were purchased from Thermo Scientific (Rockford, IL).

Human/Mouse Cleaved Caspase-3 (Asp 175) (DYC832-5) kit purchased from R&D Systems (Minneapolis, MN).

Methods

2.2 Selection of Cells – EC9706 is considered one of the more stable esophageal cancer cell lines and is widely used in research studies. It represents poorly-differentiated esophageal squamous cell carcinoma (ESCC). An alternative cell line, KYSE30, representing well-differentiated esophageal squamous cell carcinoma, was also considered for these studies. However, the stability of the KYSE series cell line is not well established, and the production of this series is no longer continuing. Considering these points, we proceeded further with the EC9706 cell line for our experiments.

2.3 Esophageal Cancer Cell Culture – Esophageal Cancer cell line - EC9706 was maintained as frozen stocks in 10% DMSO at -80^{0}C. Cells were cultured in RPMI-1640, supplemented with 10% Fetal Bovine Serum, sodium pyruvate 100mM, 2-mercaptoethanol, penicillin, streptomycin, HEPES buffer 20mM, sodium bicarbonate 7.5%. In addition, Plasmocin prophylactic was added at a

concentration of 5µg/ml to prevent mycoplasma. The cells were incubated at 37^0C, 5%CO_2. Culture was initially in 25cm^2 cell culture flasks until reaching 70-90% confluence. Subsequent passages were to 75cm^2 flasks.

2.4 *P. gingivalis* culture – *P. gingivalis* was maintained as frozen stock cultures and grown in GAM under anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2) at 37^0C in an anaerobic chamber. Bacterial cells were harvested at mid to late log phase (*P. gingivalis*- O.D at 600 nm =1.0, corresponding to 10^9 cells per ml).

2.5 Assessment of EC9706 cancer cell apoptosis and necrosis-

To enable us to assess the effects of *Pg* treatment on necrosis and apoptosis of EC9706 cells, it was first necessary to conduct several experiments to determine the effects of differing concentrations and length of exposure of cisplatin on % viability and proportion of cells in apoptosis. Once we had established optimal conditions for these parameters we were able to narrow down the range of concentration and time and test the effects of *Pg* on these factors.

2.5.1 Determination of viability of EC9706

Viability of EC9706 cells was assessed by colorimetric assay to determine extent of metabolic activity in cultures exposed to different dosages and exposure times of cisplatin compared to untreated cells. Water soluble MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) was purchased in powder form and diluted in PBS to 12mM solution and stored at 4^0C. 10,000 cells were seeded in each well of a 96 well plate and incubated overnight. After 8h,

specific wells were infected with *Pg* at a Multiplicity of Infection (MOI) of 10:1 and 100:1. RPMI-1640 media without antibiotics was used during addition of *Pg* and further. After 90 min of infection with *Pg*, the cells were washed with PBS three times and then exposed to cisplatin (0, 10, 100 and 500µM) for 12, 24, 36 and 48 h. After the desired incubation times the microplate was centrifuged at 350 g (x 1000) for 10 min and the supernatant was collected for LDH assay (see below). The cells attached to the bottom of the wells were used for MTT assay. 5ml of RPMI 1640 without phenol red was mixed with 500 µl of MTT reagent (12 mM) and 50 µl of this solution was added to each well containing the cells and incubated for 4 h. Next, 5ml of sodium dodecyl sulfate (SDS) was mixed with 100µl of 0.5M hydrochloric acid (HCL) and 50µl of this solution was added to each well and incubated for another 4 h. The plate was read at 570nm by a spectrophotometer. Viability was expressed as a function of OD/OD untreated cells.

The cytotoxic effects of cisplatin treatment under different conditions was further assessed by lactate dehydrogenase (LDH) release assay using the Pierce LDH Cytotoxicity Assay Kit.

Extracellular LDH in the media can be quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via NAD+ reduction to NADH. Purified diaphorase added to the mixture uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that can be quantified by absorption at 490nm. The level of formazan formation is directly proportional to

the amount of LDH released into the medium, which is indicative of cytotoxicity. We first determined the optimum cell number for the assay as per the manual. Serial dilution of cells (0-20,000 cells/100µl media) were plated in two sets of triplicate wells in a 96-well tissue culture plate. The plate was incubated overnight in an incubator at 37°C, 5% CO2. 10µL of sterile, ultrapure water was then added to one set of triplicates, and 10µL of lysis buffer (10X) to the other. The plate was mixed by gentle tapping. After a further 45 min incubation at 37°C, 5% CO_2, 50µL of each sample was transferred to a 96 well flat bottom plate and 50µL of reaction mixture (lyophilized substrate mix + 11.4ml of ultrapure water + 0.6ml of assay buffer) was added to each well. The plate was covered to protect from light, and incubated for 30 min at room temperature. 50µL of stop solution was added and the plate mixed by gentle tapping. Any bubbles present in the wells were broken by a syringe needle. The absorbance was measured at 490nm and 680nm. To determine LDH activity, the 680nm absorbance value (background signal from instrument) was subtracted from the 490nm absorbance.

Once optimum cell number had been determined, this assay was used to determine the cytotoxic effects of cisplatin. 10,000 cells were seeded per well and appropriate concentrations of cisplatin were added to triplicate cultures. LDH activity was determined in the manner described above. Supernatants collected from cell cultures used for the MTT assay were also analyzed in this manner.

2.6 Assessment of Cancer Cell Apoptosis

EC9706 cells were cultured in 12 well plates (cells/well). *Pg* (MOI 10:1; 100:1) and cisplatin (0, 10, 100, 500 μM) were added as described earlier. After incubation (0-48h) cells were harvested using 0.25% trypsin, trypsin neutralized with culture media, and washed in cold PBS. Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit was used to label cells following manufacturer's instructions.

1X annexin-binding buffer was prepared by diluting 5X buffer with deionized water. Propidium iodide (PI) 5μg/ml stock solution was diluted to a working concentration of 100 μg/ml in annexin-binding buffer.

The washed cells were re-centrifuged and re-suspended in annexin-binding buffer. 5μl Alexa Fluor 488 annexin V and 1 μl 100 μg/ml PI working solution was added to each 100 μl of cell suspension. The tubes were incubated at room temperature for 15 min in the dark. After the incubation, 400 μl annexin-binding buffer was added, mixed gently and kept on ice. The stained cells were analyzed as quickly as possible by flow cytometry.

Cleaved and uncleaved caspase-3 levels were detected by western blot. EC9076 cells (50,000/well) were seeded into 12 well plates and incubated at 37^0C, 5% CO_2. After 8h of incubation the cell culture media in the wells was replaced with fresh RPMI-1640 without antibiotics, and the cells were infected with *Pg* for 90 min at MOI of 100:1. The cells were then washed with PBS and exposed to Cisplatin 10μ M for different time periods (24-48h) as described above.

After incubation cultures were lysed in RIPA buffer (5 mM EDTA; SIGMA proteinase inhibitor cocktail, SIGMA phosphatase cocktails 2 and 3, and PMSF) taking care to collect both attached and floating cells. Supernatants containing detached cells were collected and centrifuged at 2.0 g (x 1000), 4^0C for 3 min, while remaining attached cells were washed in ice cold PBS. This PBS was used to re-suspend the cell pellets and centrifuge as before. Lysis buffer was added to attached cells in wells. Wells were scraped with a cell scraper and lysate collected and added to pelleted cells. Lysates were incubated on ice for 20 min and vortexed every 5 min. The DNA was sheared by passage through insulin syringe needle 7-8 x and the tubes centrifuged at max speed for 10 min to pellet debris. All steps were performed at 4^0C. The lysates were then aliquoted and frozen at -20^0C.

Protein levels were estimated using the Pierce BCA Protein Assay kit. Dilutions of each sample (50µl) were incubated in a 96 well plate with 50µl of the reagent mixture (Reagent A: Reagent B (50:1) and incubated at 37^0C for 30 min. A standard curve of serial 1/2 dilutions of BSA (1mg/ml) was prepared each time. The optical density was then read at 562nm in a spectrophotometer and estimated protein concentration calculated.

Culture extracts were separated by SDS-PAGE gel electrophoresis. Protein concentrations were normalized and 40 µg each sample denatured by 20 min incubation at 70^0C in 20 µl LB with reducing buffer. Samples were loaded onto pre-cast NuPAGE™ 4-12% Bis-Tris gels and run at 200V for 50 min in MES-SDS running

buffer with NuPAGE antioxidant in the upper chamber. To allow size differentiation, 5µl See Blue Plus + 5µl Magic Mark was loaded into the first well. The electrophoresis was stopped when the dye front reached the bottom of the gel.

Proteins were transferred to PVDF membrane (0.2µm) pre-soaked in 100% methanol for 2-5 min. The gel was removed from casing and the blot module loaded in the following order: 2-3 sponges/filter paper/gel/PVDF/filter paper/3 sponges. Sponges and filters were pre-soaked in ice cold transfer buffer containing 10% methanol and 1% antioxidant. The module was placed in the transfer unit and pre-cooled transfer buffer added to the assembly. The air bubbles were tapped out. Ice cold water was added to the tank. The whole assembly was placed on ice and connected to the power supply to run at 35V for 2h.

The blots were incubated with 5% powdered milk inTBS-Tween20 (0.1%) at room temperature for 1 hour. The blocking buffer was poured off and the membrane was washed 3 times for 5 min each with TBST.

The membrane was incubated in primary antibody (Caspase-3) solution (1:1000 dilution in TBST 5% powdered non-fat dry milk), overnight at 4^0C with gentle shaking. The blot was washed 3 times as before. Blots were then incubated in secondary anti-rabbit HRP-tagged antibody solution (1:2000 in 5% w/v nonfat dry milk) for 1 hour at room temperature. The blot was washed 3 times and then

incubated with WestPico Chemiluminescent substrate solution for 5min before imaging.

2.6.1 Quantification of Cleaved Caspase-3 in Culture Lysates

Human/Mouse Cleaved Caspase-3 (Asp 175) (DYC832-5) kit was used. Overnight cultures of EC9706 cells (one million) in 6 well plates were infected with *Pg* (MOI – 10:1) and incubated for 8 h. Triplicate groups were treated with cisplatin 10uM for 30, 36, 42, 48 and 54 hours together with control groups with no infection. Cell extracts were prepared in lysis buffer (1mM EDTA, 0.5% Triton X-100, 5mM NaF, 6M urea, proteinase inhibitor cocktail and phosphatase inhibitors) prepared according to manufacturer's instructions (R & D Human/Mouse Cleaved Caspase-3 (Asp 175) kit) Protein concentrations were estimated as described previously. Lysate concentrations were equalized with 6M urea lysis buffer, 6-fold dilutions were prepared to reduce urea concentration to 1M. Thereafter, further dilutions were made with 1M urea buffer.

Samples were plated in 96-well microplate coated overnight with 100µl 2ug/ml anti-total caspase 3 capture antibody. The plate had been blocked with 300µl of block buffer in each well for 1-2 hours. After a 2 hour incubation at room temperature, wells were washed 3 times and 100µl detection antibody (anti-cleaved caspase-3 (150ng/ml) added to each. After a further 2h incubation at room temperature, the aspiration/wash step was repeated. Streptavidin-HRP was

added to each well and incubated for 20 min at temperature, the plate being protected from direct light. Aspiration/wash step was repeated. 100µl of substrate solution was added to each well and incubated for 20 min at room temperature away from direct light. 50 µl of stop solution was added to each well and the optical density was determined immediately using microplate reader set to 450nm.

Statistical analysis (one way ANOVA) was done using Graph Pad Prism Software.

CHAPTER 3: RESULTS

3.1 Optimization of Cisplatin-induced Cancer Cell Death

First, we determined the optimal concentration and exposure duration of the chemotherapeutic drug, cisplatin, to induce maximal apoptosis with minimum effective dose in EC9706 cells, in order to facilitate the subsequent assessment of the effect of *P. gingivalis* on drug-induced programmed cell death in these ESC cells. Cell viability and necrosis in cisplatin-exposed EC9706 cells was also assessed.

Metabolic Activity of EC9706 cells is reduced at higher cisplatin concentrations

To examine the anti-tumor potential of cisplatin on esophageal squamous carcinoma cells (EC9706), cell viability analysis was performed using the MTT assay. EC9706 cells were incubated with a wide range of cisplatin concentrations (0, 10, 100 and 250μM) for 48 hours. As shown in the Fig 6. cisplatin induced growth inhibition in a dose-dependent manner in EC9706 cells. In addition, kinetic analysis revealed that cisplatin (10μM) induced a significant growth inhibition following 48h of incubation (Fig 7.).

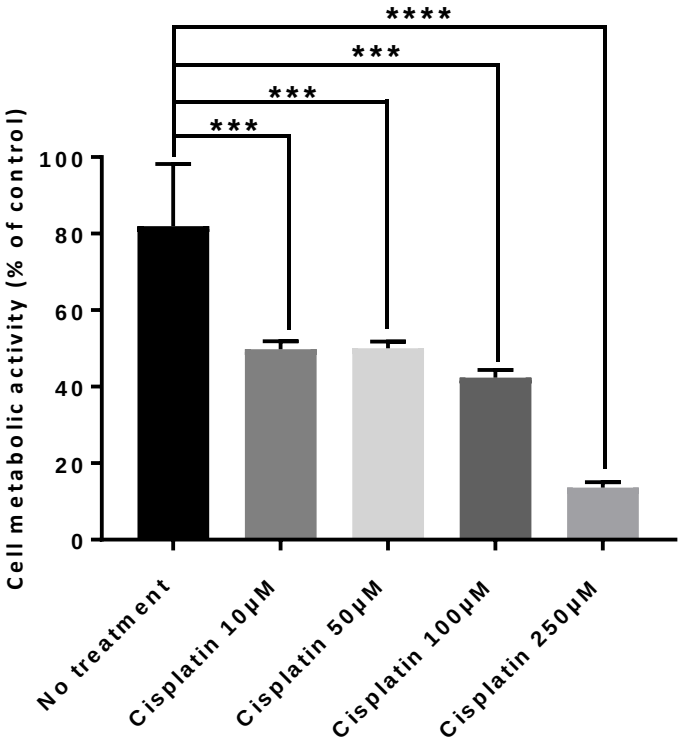

Fig 6. Cisplatin inhibits EC9706 cell growth in a dose-dependent manner.

EC9706 cells were treated with cisplatin (0, 10, 50, 100, 250μM) for 48h prior to assessing cell viability using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Data are presented as the mean ± standard error of three individual experiments. Values are calculated by subtracting OD 570nm - OD 570nm of media blank and expressed as a percentage of no treatment control.

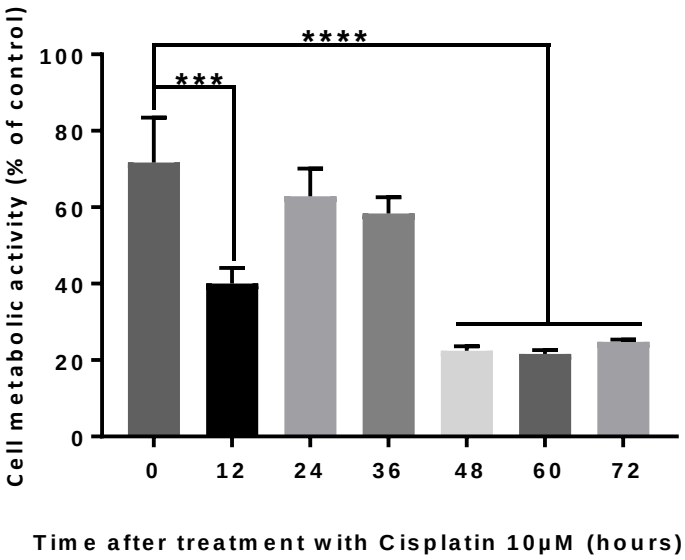

Fig 7. Cisplatin induces time-dependent cell growth inhibition in EC9706 cells.

The cells were treated with Cisplatin (10µM) for 0-72h. The cell viability was determined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Data are presented as the mean ± standard error of three individual experiments. Values are calculated by subtracting OD 570nm - OD 570nm of media blank and expressed as a percentage of no treatment control.

3.2 Analysis of type of cell death by cisplatin

Cisplatin-induced cell death was optimized by MTT as above. Since MTT gives us an idea about the relative metabolic activity of the cells and not the type of cell death, we had to analyze if cell death caused by cisplatin was due to apoptosis or necrosis. Several assays were performed to analyze the type of cell death.

Detection of Cleaved Caspase-3 but not Annexin V labeling of EC9706 cells could reliably indicate cisplatin-induced apoptosis– EC9706 cells were detached with trypsin, accutase, cell dissociation media and scraping. Flow cytometry was

performed but background levels of apoptosis were too high to determine the contribution by cisplatin.

Western blotting analysis and human active caspase-3 ELISA were performed to detect levels of cleaved caspase-3, an absolute biomarker of apoptosis, after different treatment regimes. These experiments indicated that cisplatin 10µM exposure for 42h caused maximum activated caspase-3 levels and the cells were apoptotic at this dose and duration of exposure (Fig 8. & Fig 9.)

Western Blot

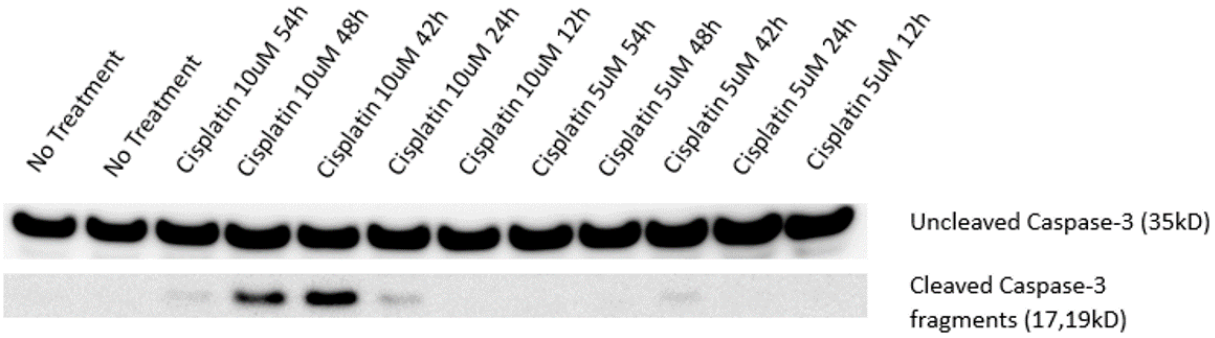

Fig 8. Cisplatin 10 µM induces maximum apoptosis at 42h of treatment.

Western blot of whole cell lysates of EC9706 cells treated with cisplatin (5, 10µM) for (12, 24, 42, 48, 54h). The cleaved and uncleaved caspase-3 levels were detected by caspase-3 primary antibody which showed cleaved caspase-3 peaked at 42h of treatment with cisplatin 10µM.

Human Active Caspase- 3 ELISA

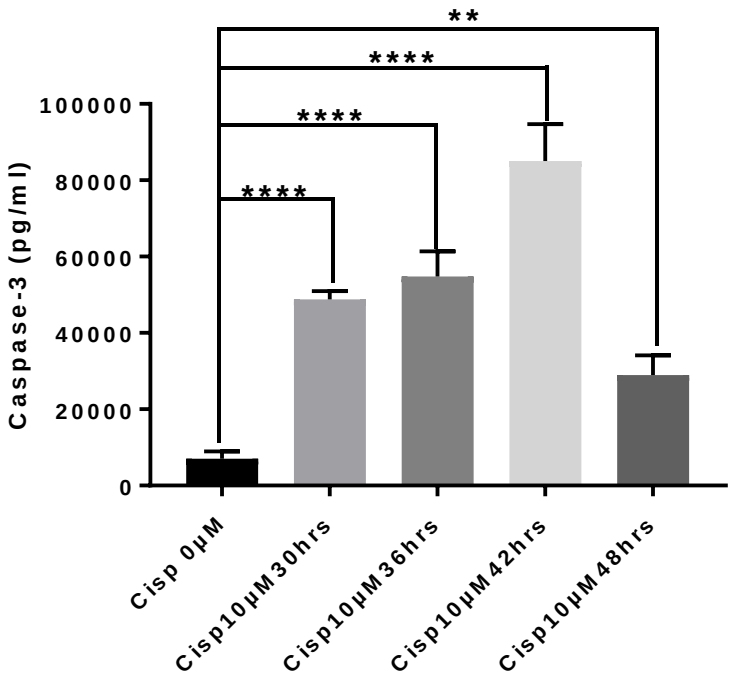

Fig 9. Cisplatin treatment at 10μM induces maximum cleaved caspase-3 from EC9706 cells at 42h of treatment.

EC9706 cells were treated with cisplatin 10μM for (30, 36, 42, 48h). Figure shows maximum cleaved caspase-3 levels at 42h of treatment with cisplatin 10μM. Data are presented as the mean ± standard error of three individual experiments. Cisp – cisplatin

3.3 Cisplatin Induces Necrosis in EC9706 cells in a dose-dependent manner

Cisplatin 10μM exposure for 42h caused maximum detectable cleaved caspase-3. (Fig. 9.) To confirm that the cells were not necrotic at that dose and duration of cisplatin, the LDH assay was performed. The goal of this experiment is to optimize

concentration and duration of treatment of cisplatin to induce maximum number of EC9706 cells in late apoptosis while minimizing necrosis.

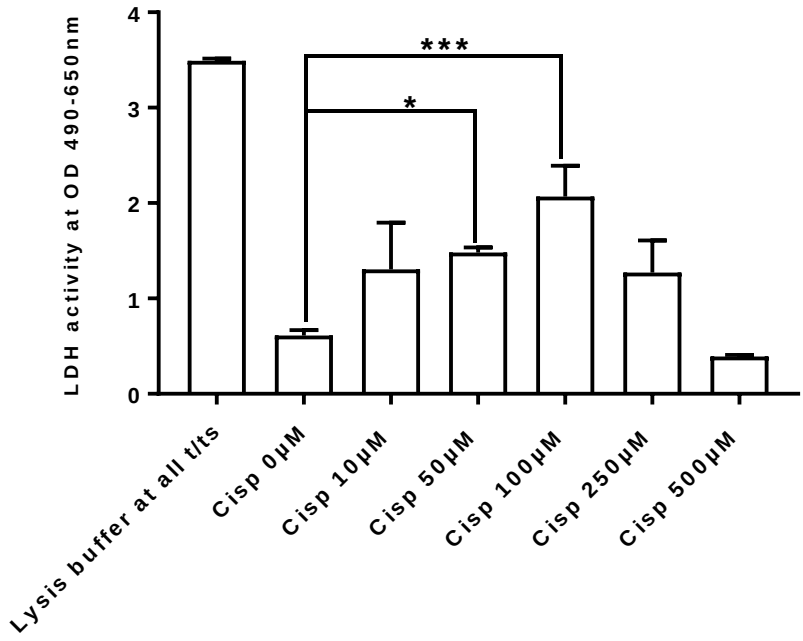

Fig 10. Cisplatin induces necrosis in EC9706 cells in a dose-dependent manner.

EC9706 Cell LDH activity treated with cisplatin (10, 50, 100, 250, 500μM) for 48h. Cisplatin 10μM shows the least LDH release which indicates minimal necrosis. Data are presented as the mean ± standard error of three individual experiments. Cisp - cisplatin

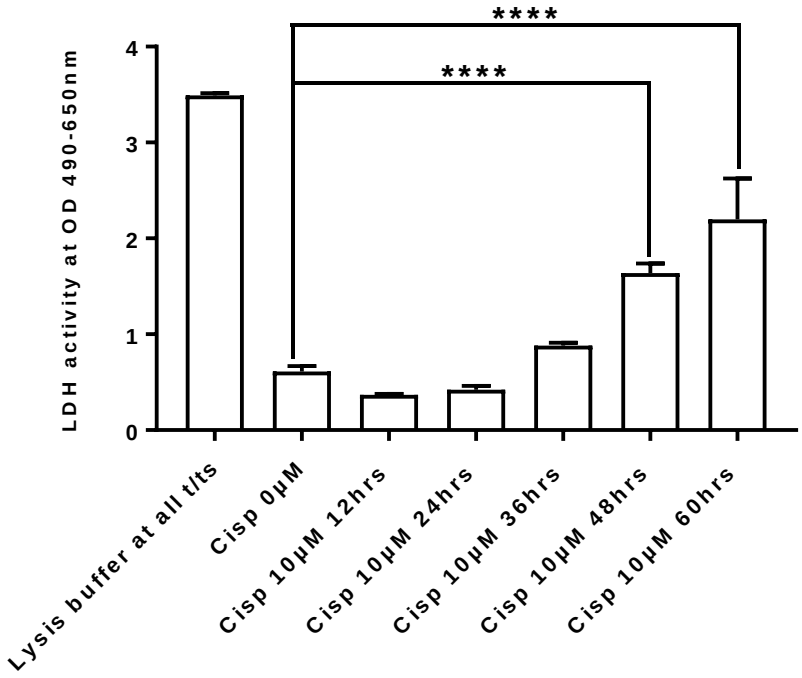

Fig 11. Cisplatin 10μM induces necrosis in a time-dependent manner.

Cisplatin induces time-dependent necrosis in EC9706 cells. The cells were treated with cisplatin (10μM) for different time periods (12-60h). Necrosis was determined using the lactate dehydrogenase (LDH) release activity assay. Data are representative of three individual experiments. Data are presented as the mean ± standard error of three individual experiments. Cisp - ciplatin

Based on the above experiments, we see that lower concentrations of chemotherapeutic agent, here cisplatin 10μM, can cause a progressive increase in necrotic and apoptotic cells. The slow death response, maximizing the duration of exposure to a modest but effective concentration of agent rather than maximizing the concentration present for a short duration can be an optimal strategy for initiating apoptosis of cancer cells. Use of the lowest effective dose will increase the potential to observe any potential inhibitory effect of *P. gingivalis* pre-treatment.

3.4 *P. gingivalis* Pre-treatment Inhibits Cisplatin induced Apoptosis and Necrosis

P. gingivalis was incorporated into our final experiments to see if this microbe can inhibit cisplatin induced apoptosis and necrosis. The cancer cells were infected with differing MOI of *P. gingivalis* (10:1 and 100:1) and the activated caspase-3 levels were detected by western blot (Fig 12, 13) and human active caspase- 3 ELISA (Fig 14, 15) whereas LDH levels were detected as described above (Fig 16, 17).

Western Blot–

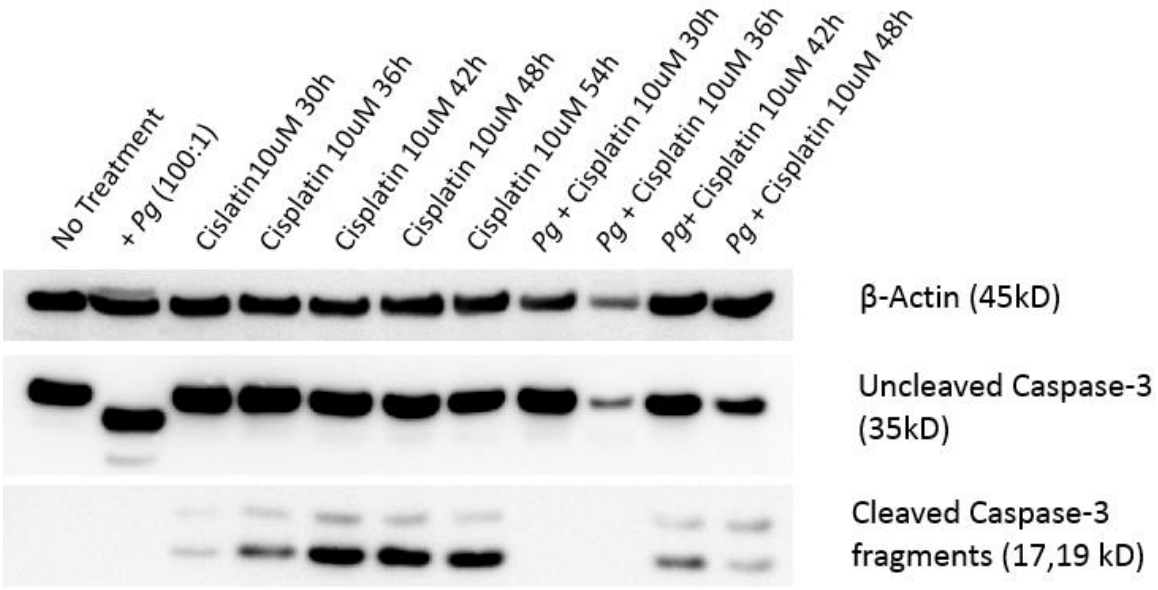

Fig 12. *P. gingivalis* inhibits levels of cleaved caspase-3 sub-units.

Western blot of whole cell lysates from *P. gingivalis*-infected (MOI – 100:1) and non-infected esophageal squamous cell carcinoma cells (EC9706). Blot was probed with antibodies to caspase-3 (#9662) as well as β-actin as loading control. *Pg – P. gingivalis*

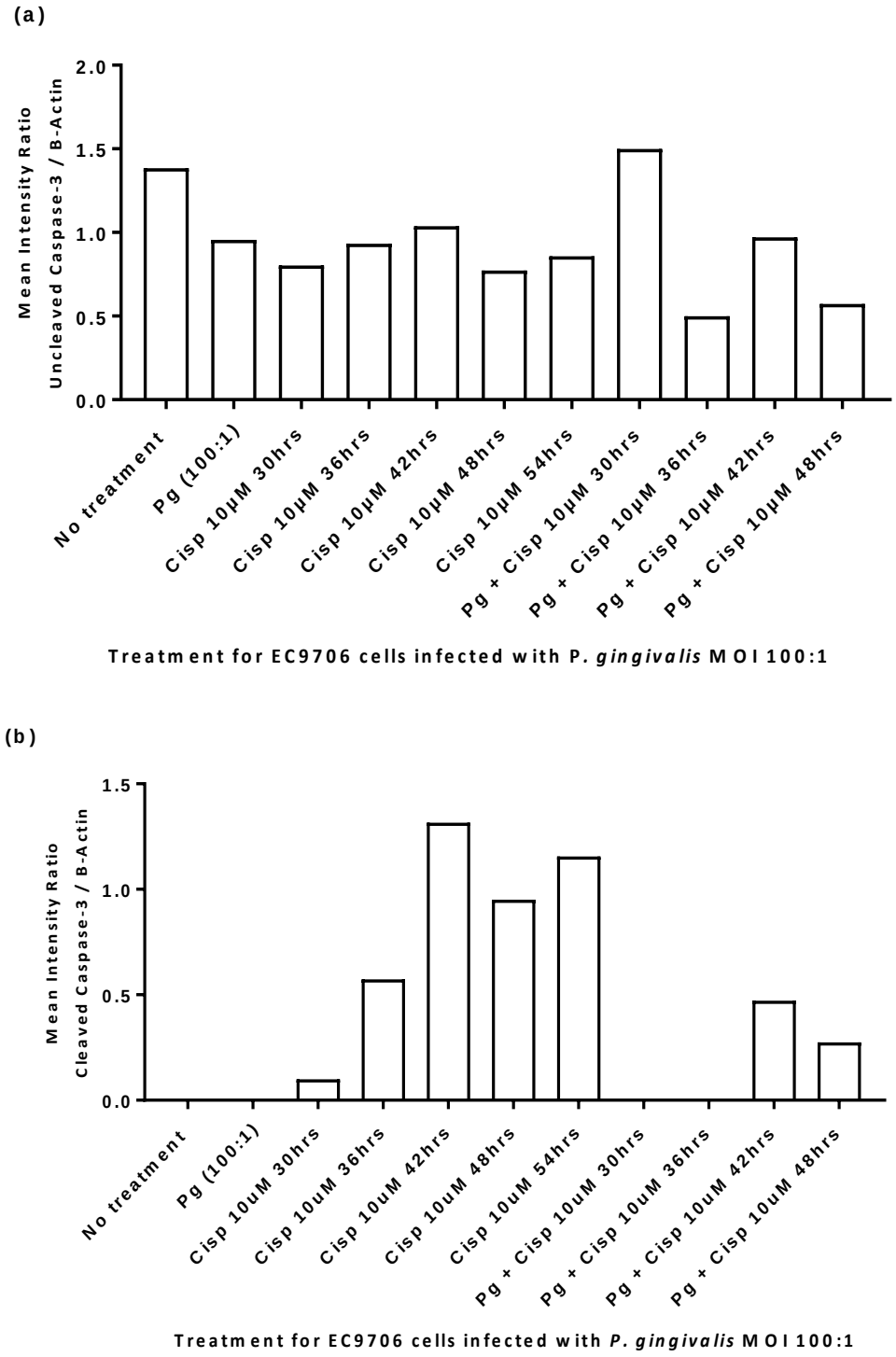

Fig 13. Densitometric quantification of caspase-3 levels of EC9706 cells treated with cisplatin 10µM for (30, 36, 42, 48h) with and without *P. gingivalis*. Densitometric quantification was performed by determination of the ratios of (a)

uncleaved caspase-3 (b) cleaved caspase-3 to total β-Actin signal using Image J software. *Pg - P. gingivalis*

Human Active Caspase- 3 ELISA

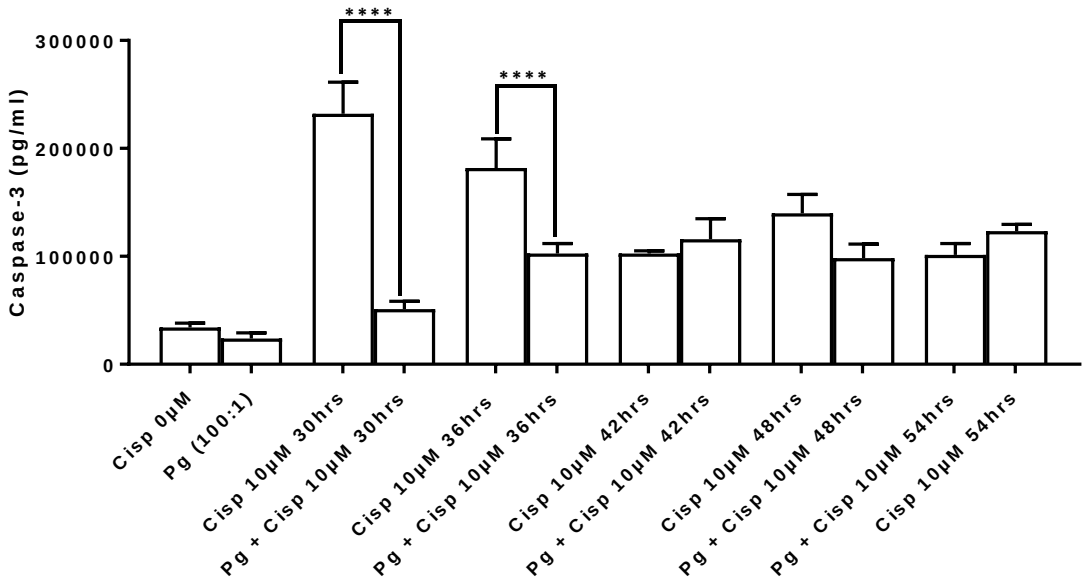

Fig 14. *P. gingivalis* inhibits apoptosis in EC9706 cell line.

ELISA of whole cell lysates (40µg protein) from *P. gingivalis* infected and non-infected EC9706 cells. The cells were infected with *P. gingivalis* at MOI (100:1) and incubated for24h prior to treatment with cisplatin 10µM. Level of apoptosis was determined using the human active caspase-3 immunoassay. Data are presented as the mean ± standard error of three individual experiments. (*Pg – P. gingivalis,* Cisp – cisplatin).

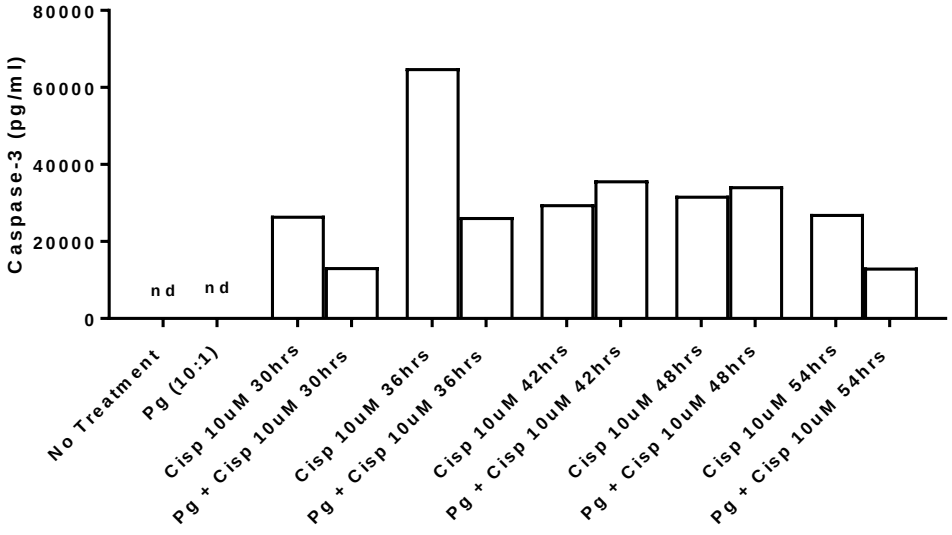

Fig 15. *P. gingivalis* inhibits apoptosis in EC9706 cell line.

ELISA of whole cell lysates (40µg protein) from *P. gingivalis* infected and non-infected EC9706 cells. The cells were infected with *P. gingivalis* at MOI (10:1) and incubated for24h prior to treatment with cisplatin 10µM. Level of apoptosis was determined using the human active caspase-3 immunoassay. (nd – not detectable, *Pg – P. gingivalis,* Cisp - cisplatin)

<u>Lactate Dehydrogenase Release Activity Assay</u>

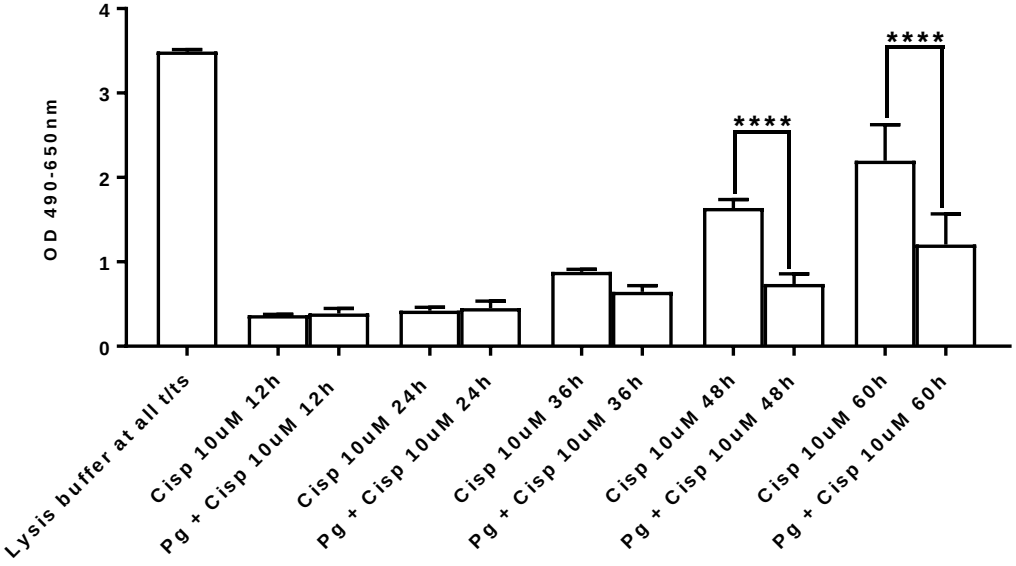

Fig 16. *P. gingivalis* inhibits cisplatin induced necrosis.

LDH activity assay showing decreased production of LDH when EC9706 cells were infected with *P. gingivalis* at MOI of 10:1 prior to treatment with cisplatin 10µM for 12, 24, 36, 48 and 60h. Data are presented as the mean ± standard error of three individual experiments. (*Pg – P. gingivalis,* Cisp – cisplatin).

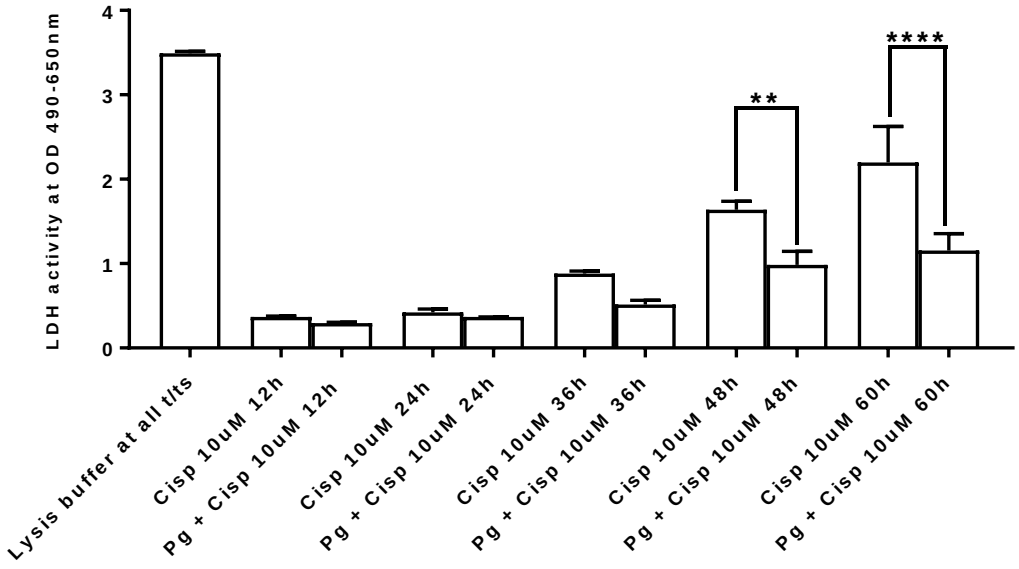

Fig 17. *P. gingivalis* inhibits cisplatin induced necrosis.

LDH activity assay showing decreased production of LDH when EC9706 cells were infected with *P. gingivalis* at MOI of 100:1 prior to treatment with cisplatin 10µM for 12, 24, 36, 48 and 60h. Data are presented as the mean ± standard error of three individual experiments. (*Pg – P. gingivalis,* Cisp – cisplatin).

CHAPTER 4: DISCUSSION

Esophageal squamous cell carcinoma is a major health problem worldwide and has several causative risk factors. It is a growing health concern that is expected to increase in incidence over the next 10 years (Napier et al., 2014). Like other parts of the gastrointestinal tract, the esophagus hosts a variety of bacteria (Gall et al., 2015). While environmental factors, host genetics and immune response have been implicated in the progression to esophageal carcinoma, studies investigating esophageal microbial communities suggest that bacteria may also play an important role in driving the inflammation that leads to disease (Kaakoush, Castano-Rodriguez, Man, & Mitchell, 2015). Association between microbes and cancer has been established in the past. *P. gingivalis* has been found in significantly higher levels in oral squamous cell carcinoma surfaces as compared to the healthy oral mucosa (Nagy, Sonkodi, Szoke, Nagy, & Newman, 1998). Similarly high levels of *P. gingivalis* antibodies have been detected in gingival carcinomas in comparison to the healthy gingival tissue (Katz et al., 2011). These findings showed a significant association between oral cancer and *P. gingivalis*. Evidence for a potential role for *P. gingivalis* in the development of esophageal cancer has recently emerged as the microbe and its antigens have been detected significantly higher in the esophageal cancerous and adjacent tissue in comparison

to the normal esophageal mucosa (Gao et al., 2016). Cisplatin, a well-known chemotherapeutic drug has been used for the treatment of numerous human cancers (Dasari & Tchounwou, 2014) including esophageal cancer. It is known to act by inducing apoptosis in the cancer cells. Based on the histological similarity between the oral squamous cells and the esophageal squamous cells, this thesis investigates if *P. gingivalis* can inhibit the apoptosis of the esophageal cancer cells caused by the chemotherapeutic drug, cisplatin. Studies have shown that *P. gingivalis* inhibits apoptosis in gingival epithelial cells (Nakhjiri et al., 2001). It has previously been established that *P. gingivalis* produces a nucleoside diphosphate kinase (NDK) that can promote epithelial cell survival by hydrolyzing extracellular ATP and preventing apoptosis (Choi et al., 2013). *P. gingivalis* can inhibit chemically induced apoptosis in gingival epithelial cells by upregulating JAK/Stat pathway and blocking activation of the effector caspase-3 (Mao et al., 2007). However the potential mechanisms by which the important oral pathogen may predispose to the development of esophageal cancer are entirely unknown. We show for the very first time, *P. gingivalis* is capable of inhibiting drug induced apoptosis as well as necrosis (atleast the LDH release) in the esophageal squamous cell carcinoma cell line EC0706. The best recognized biochemical hallmark of both early and late stages of apoptosis is the activation of cysteine proteases (caspases). Specifically detection of active caspase-3 in cells and tissue is an important method for apoptosis (Choudhary, Al-Harbi, & Almasan, 2015). Thus we employed detection of cleaved capsease-3 by western blot and enzyme linked

immunosorbent assay. Plasma membrane damage causes leakage of a soluble cytoplasmic enzyme, lactate dehydrogenase whose detection can be used as a convenient method for detection of necrosis. (Chan, Moriwaki, & De Rosa, 2013). The significance of measuring LDH is that the LDH release does not purely occur during necrosis but might be released by some cells undergoing apoptosis too. The apoptosis seen in the results might not be clean apoptosis and thus LDH release as well as cleaved caspase-3 were detected in the cancer cells after cisplatin 10μM exposure for 48h. Hence LDH assay was used for detection of necrosis. We found that Cisplatin induces apoptosis and necrosis of EC9706 cells in a dose and time dependent manner. But when the cancer cells are infected with *P. gingivalis* prior to the treatment with cisplatin, both apoptosis and necrosis is significantly reduced. *P. gingivalis* might also be causing cleavage of caspase-3 into subunits that are not active forms for apoptosis as seen by western blot. This finding may aid in the understanding of why chemotherapeutic drug action might be rendered ineffective in patients with esophageal squamous cell carcinoma and bad periodontal health. Improved oral hygiene and treatment of periodontitis may be useful in limiting the development or spread of cancer. With a clear need for better therapeutics and preventive strategies for esophageal cancer, *P. gingivalis* would serve as a good candidate for further exploration based on its capabilities shown herein. (Calandria, Irurzun, Barco, & Carrasco, 2004)

Future direction – Further exploration would be needed, If *P. gingivalis* leads to dysbiosis of the esophageal microbiota involved in the development of esophageal cancer. Also the mechanism of how *P. gingivalis* alters the cancer cell morphology and their response to chemotherapeutic agents need to be studied. Antibiotics targeted towards *P. gingivalis* which could combat this inhibition of apoptosis and progression of esophageal cancer could definitely be a good area of study as well.

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