Journal of Environmental Pathology, Toxicology and Oncology, 36(2):131–150 (2017)

DNA Double-Strand Breaks Caused by Different Microorganisms: A Special Focus on *Helicobacter pylori*

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ABSTRACT: The association between inflammation and cancer has long been recognized. Several studies have found that different types of tumors develop at sites of chronic inflammation. It is stated that over 15%–20% of malignancies worldwide can be related to infections caused by viruses, bacteria, and schistosomes. Inflammatory conditions are characterized by overexpression of inducible nitric oxide synthase (iNOS) and overproduction of nitric oxide/reactive nitrogen species (ROSs/RNSs) in epithelial cells. Reactive oxygen species (ROSs) may also lead to cellular alterations and eventually to inflammation. A variety of chronic infectious diseases can generate steady-state levels of ROSs/RNSs within infected cells and possibly lead to different types of DNA lesions. Accumulation of DNA lesions may finally lead to mutations that may activate oncogenes or inactivate tumor suppressor genes. *Helicobacter pylori* has been shown to generate ROSs/RNSs, induce DNA damage, and lead to chronic inflammation in gastric epithelial cells. A limited number of studies have addressed the effects of *Helicobacter pylori* on DNA damage, particularly its impact on single-strand and double-strand DNA breaks. This bacterium is classified as a Group I carcinogen by the International Agency for Research on Cancer on the basis of numerous animal and epidemiological studies. Chronic *Helicobacter pylori* infection can lead to increased risk of gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma. This review addresses the DNA-damaging and double-strand break–inducing effects of different microorganisms and their toxins, specifically focusing on *Helicobacter pylori.*

KEY WORDS: DNA damage, double-strand DNA break, gastric cancer, *Helicobacter pylori*, inflammation, reactive oxygen species

ABBREVIATIONS: DSB, double strand break; **HTGTS**, high-throughput genome-wide translocation sequencing; **PCR**, polymerase chain reaction; **SSB**, single-strand break

I. INTRODUCTION

Inflammation is the natural self-defense mechanism of the immune system in response to consistent challenges such as injury, toxins and microorganisms, low-level radiation (X-rays, γ-irritation, UVA/B), drugs, and a variety of chemicals.[1] It is estimated that pathogen-induced genomic damage contributes to tumorigenesis and that 15%–20% of all human cancers are linked to pathogenic agents.[2] Several studies have found that many types of bacteria produce toxins and/or cause chronic infections that may both destroy the cell cycle and result in altered cell growth.[3]

Pathogens affect host cell genomes directly via the genotoxins and oncoproteins they secrete. These proteins may induce alterations in cellular DNA and cause the impairment of DNA repair mechanisms. Genetic stability is also affected indirectly as a consequence of pathogen replication and induced inflammation.[4] The inflammation-cancer link can result from extrinsic and intrinsic pathways.[5–7] The extrinsic pathway is maintained by inflammatory signals—infiltrating leukocytes, phagocytes (e.g., monocytes, macrophages, eosinophils, neutrophils), cytokines, chemokines (e.g., tumor necrosis factor-α, or TNF-α, and interleukin-1β, or IL-1β), growth factors, lipid messengers, matrix-degrading

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enzymes, key transcription factors (e.g., nuclear factor kappa light-chain enhancer of activated B cells, or NF-κB, and signal transducer and activator of transcription 3, or STAT3), and autoimmune factors. All of these establish inflammatory conditions that can increase cancer risk. The chronic inflammation associated with infections (caused by nearly all pathogens: hepatitis B and C viruses, *Helicobacter pylori*, and the like), mechanical stress, radiation, and chemical insults trigger the secretion of these inflammatory signals and favor initiation and progression of malignant tumors.[8]

On the other hand, the intrinsic pathway is mainly affected by genetic alterations that may lead to inflammation and finally to cancer. Genetic events leading to neoplastic transformation promote the formation of an inflammatory environment.[5,9] In general, the intrinsic pathway involves the activation of oncogenes and/or the inactivation of tumor suppressors. Such genetic changes may trigger the inflammatory cascade and lead to tumor progression. The epidermal growth factor receptor (EGFR) family of proteins exhibits tyrosine kinase activity and is suggested to play a role in human cancers. The activation of the Ras family of oncogenes, the most frequently mutated dominant oncogenes in human cancer, can lead to high expression and production of inflammatory mediators.[10] Myc, another well-studied oncogene, encodes a transcription factor that is overexpressed in many human tumors, causing autonomous cell proliferation, and leads to remodeling of the extracellular microenvironment with inflammatory cells and mediators. The myc-activated genetic program induces the CC chemokines, which in turn cause the recruitment of mast cells. Mast cells provide new vessel formation (angiogenesis) that leads to sustainment and growth of tumors.[11]

Phagocytes become activated when they are exposed to an inflammatory stimulus and produce large numbers of ROSs and reactive nitrogen species (RNSs). ROSs, which are produced during inflammation and/or infection include superoxide ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), hypochlorous acid (HClO), singlet oxygen ($^1O_2$), and hydroxyl radical (•OH). Under normal conditions, ROSs are protective against pathogens; however, they may also cause tissue damage and contribute to the development or progression of various diseases, specifically cancer.[12,13] In addition to ROSs, chronic inflammation upregulates inducible nitric oxide synthase (iNOS) and enhances the production of nitric oxide radical (•NO). RNSs include peroxynitrite ($ONOO^-$), nitrogen dioxide radical ($\bullet NO_2$), and other oxides of nitrogen.[14,15]

ROSs and RNSs can cause DNA base alterations, DNA strand breaks, tumor suppressor gene depletion, and increased expression of proto-oncogenes. These changes induce malignant transformations in the affected cells.[9,10] •NO can rapidly auto-oxidize to yield a variety of intermediates (e.g., dinitrogen trioxide, or N_2O_3) in the presence of molecular oxygen; these potent nitrosating agents can produce carcinogenic and mutagenic nitrosamines when they react with secondary/tertiary amines.[15] Increased formation of nitrosamines has been reported to occur in inflammation and infection.[16] In addition, studies have indicated that $ONOO^-$ exposure usually leads to greater DNA damage than exposure to an equivalent dose of •NO. $ONOO^-$ can also react directly with the sugar moiety of DNA and cause sugar fragmentation that generates a DNA strand break. Moreover, it has been stated that after $ONOO^-$ exposure, the DNA damage spectrum can be very complex.[16]

Proteins and lipids are the main targets of oxidative and nitrosative attacks. RNSs and ROSs may also react with proteins to modify amino acid residues by oxidation, nitrosation, and/or nitration. Alterations in protein structure and functions can contribute to carcinogenesis as well.[17,18] Such alterations, called epigenetic changes, are functionally relevant changes to the genome that do not involve a change in the nucleotide sequence. Examples of mechanisms that produce such changes are DNA methylation, DNA acetylation, and histone modification (methylation/acetylation), each of which alters how genes are expressed without altering the underlying DNA sequence. These common epigenetics have a critical role in cancer progression.[19] Many studies have shown that nitrated protein levels are elevated in inflamed tissues, including the

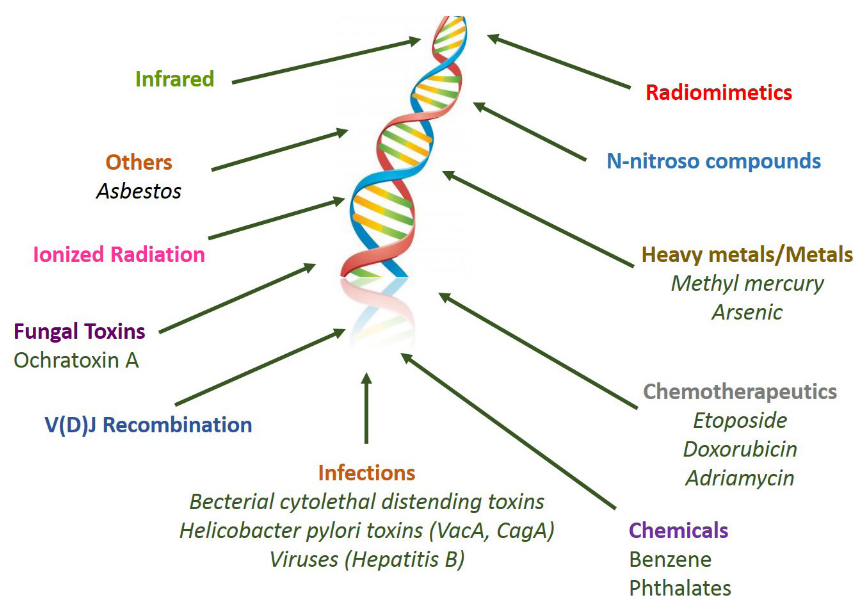

FIG. 1: Causes of DNA double-strand breaks

gastric mucosa of patients with *Helicobacter pylori*—induced gastritis.[20–25]

Oxidative modification of lipids can lead to formation of genotoxic lipid peroxidation intermediates, which can react with protein and DNA and ultimately lead to mutagenesis and cancer. Additionally, protein oxidation may arise independently when high cellular RNS or ROS levels are achieved. If oxidative modifications in DNA polymerase or DNA repair enzymes are present, this phenomenon may lead to low DNA repair capacity and high incidence of genotoxicity, which can further lead to mutations and sequential cancer formation.[18]

This review addresses the oxidative and nitrosative DNA-damaging and DNA double-strand break (DSB)–inducing effects of different pathogens and their toxins. We focus on *Helicobacter pylori* because approximately 50% of the world's population has been estimated to be infected by this bacterium.

II. DNA DOUBLE-STRAND BREAKS

The DNA double-helix structure consists of two strands that comprise heterocyclic DNA bases in humans. DNA is subject to many endogenous and exogenous insults that lead to impairments in its replication, chromosome segregation, and repair. Different types of damage can be observed, the most dangerous of which is DSB, which can be induced by radiation, UV, infrared, radiomimetics, certain chemotherapeutics (e.g., adriamycin, etoposide, doxorubicin), natural antibiotics (e.g., calicheamicin, esperamicin, dynemicin A, neocardinostatin), heavy metals (e.g., arsenic, methyl mercury), asbestos, environmental chemicals (e.g., benzene, phthalates), fungal toxins (e.g., ochratoxin A), microorganisms (as mentioned subsequently), variable (V) diversity (D) and joining (J), or V(D)J, recombination, and high cellular ROS and/or RNS levels.[26–32] Causes of DSB are summarized in Fig. 1.

In the last two decades, several methods have been tested to detect DSBs. Because some detect all kinds of DNA breaks, not particularly DSBs, scientists are in need of more accurate ways to detect DSB specifically. Current detection methods are summarized in Fig. 2 and discussed in the following paragraphs.

Various DSB repair inhibitors that permit estimation of the frequency of spontaneous

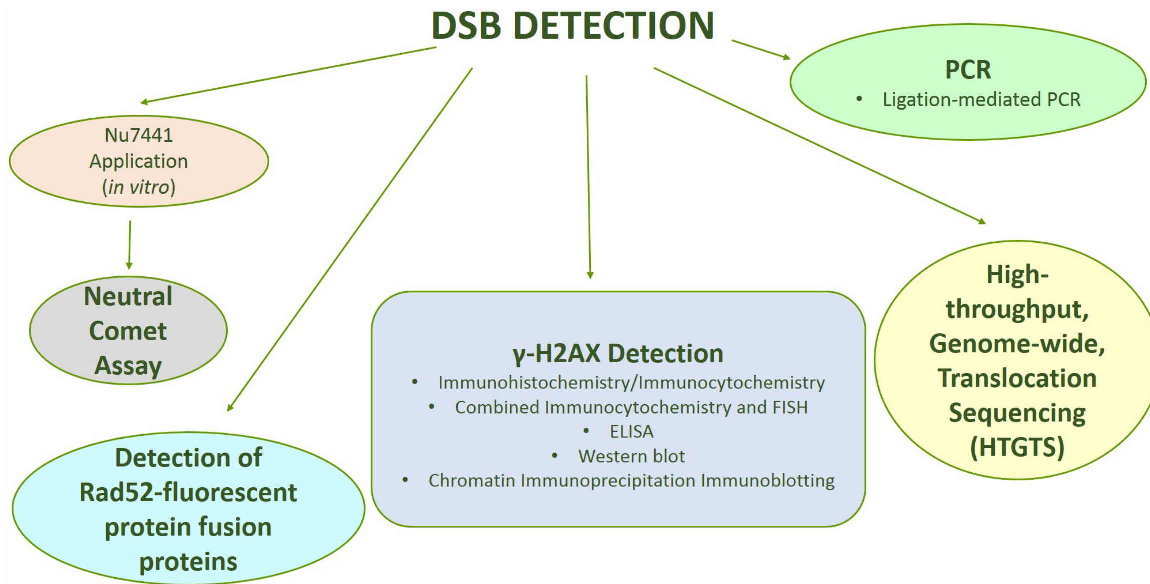

FIG. 2: Detection of DNA double-strand breaks

DSBs are commercially available—for example, 8-(4-dibenzothienyl)-2-(4-morpholinyl)-4H-1-benzopyran-4-one and Nu 7441. DSBs can be measured in cells in which DSB repair is prevented.[33,34] After the application of Nu7441, for example, neutral single-cell gel electrophoresis (Comet) assay can be applied to detect the unrepaired DSBs. However, Comet assay can also be used directly. Because DSBs measured by Comet assay are now being associated with other types of DNA damage, the results may overestimate the incidence of DSBs.[34]

DSBs can be detected by monitoring the formation of damage-induced foci, either by indirect immunofluorescent staining or by the fusing of fluorescent proteins to proteins that are recruited to the sites of DNA damage as part of the damage response. The H2A histone family member X (H2AX), a variant of the H2A protein family, is a component of the histone octomer in nucleosomes. An endogenous or exogenous insult to DNA is always followed by the phosphorylation of H2AX on serine 139. The kinases of the PI3 family—ataxia telangiectasia mutated protein (ATR) and DNA-dependent protein kinases (DNA-PKcs)—are responsible for this phosphorylation, especially the ataxia telangiectasia mutated protein (ATM). In vertebrate cells, H2AX phosphorylation produces gamma-H2AX (γ-H2AX), which is often used as a DSB indicator.[35–37] Phosphorylation can happen accidentally, during replication fork collapse or in response to different chemical (several chemotherapeutics), physical (i.e., ionizing radiation), and biological agents, and it can occur during controlled physiological processes, such as V(D)J recombination. γ-H2AX is associated DNA damage other than DSBs, and so results from experiments with it may exaggerate DSB incidence. However, it is still considered a good DSB biomarker.[38]

The binding of other key DNA repair proteins—53BP1, replication protein A (RPA), and Rad51 foci—serves as a DSB indicator as well.[39–41] In budding yeast, the most frequently used live-cell marker of DSB damage is the recruitment of Rad52-YFP into damage-induced foci. Even multiple DSBs may result in a single Rad52 focus as they aggregate into a repair center.[42] However, recent literature has proposed that Rad52-fluorescent protein fusion proteins have a remarkable ability to aggregate that allows them to strongly mark independent DSBs.[40,42,43]

High-throughput genome-wide translocation

sequencing (HTGTS) robustly detects DSBs generated by engineered nucleases across the human genome, based on their translocation to other endogenous or ectopic DSBs.[44] Polymerase chain reactions (PCRs) represent another technique now being used to detect DNA breaks. Ligation-mediated PCR is a relatively new technique suggested to be both sensitive and fast.[45]

The DNA repair ability of a cell is vital to the integrity of its genome and thus to the normal functionality of the organism. DSBs must be repaired to preserve chromosomal integrity and protect genetic stability. All organisms have DNA repair mechanisms to prevent their genetic material from different environmental factors, including pathogens.[46,47] In mammalian cells, single-strand breaks (SSBs) are the most common lesions and SSB repair is a rapid and efficient process that removes SSBs within minutes by a global pathway that is accelerated by the SSB sensor protein poly [ADP-ribose] polymerase 1 (PARP1) and the molecular scaffold protein X-ray repair cross-complementing protein 1 (XRCC1).[48] SSB repair defects can cause chronic diseases (e.g. diabetes, high blood pressure), accelerated aging, and an increased risk of cancer.

DSBs are repaired in a more complicated and less efficient way[49,50] involving two important pathways. DNA nonhomologous end joining (NHEJ) is the major DSB rejoining process and occurs in all cell cycle stages; it is the first choice of mechanisms, repairing approximately 80% of X-ray-induced DSBs with rapid kinetics. Homologous recombination (HR) can additionally function to repair irradiation-induced two-ended DSBs in the G2 phase. HR predominantly uses a sister chromatid as a template for repair; thus it functions only in the late S/G2 phase.

The accumulation of DSBs in tissues depends on the dose and type of DSB-casing agent as well as the organism subjected to these agents. For instance, in human fibroblasts, γ-H2AX formation is first detected 20 seconds after irradiation, with half-maximal and maximal formations reached 1 minute and 10 minutes after irradiation, respectively.[51] DSB repair is an essential cellular process required to maintain genomic integrity in the face of potentially lethal genetic damage. Failure to repair a DSB can trigger cell death, whereas misrepairs can cause the generation of chromosomal translocations leading to the development or progression of cancer.[52]

III. ROLE OF BACTERIAL TOXINS AND BYPRODUCTS IN HOST CELL DNA DSBS AND ONCOGENESIS

In the twentieth century, understanding the role of chronic bacterial inflammation in cancer development gained extreme importance. Several bacterial infections were discovered to lead to oncogenesis.[3,53] Bacteria-caused alterations in hosts such as inflammation, antigen-attacked lympho-proliferation, and induction of stress-related hormones can cause epithelial cell proliferation. Bacteria can also promote carcinogenesis by their direct effect on cell transformation or by their toxic and carcinogenic metabolites.[53] Some bacteria can generate a wide range of toxic products that interact with host cellular signaling components or directly attack the host genome.[54]

A. Cytolethal Distending Toxins

Pathogens that manipulate host cell function are usually able to generate bacterial products that mimic host proteins. These bacterial products are either direct homologues or structural mimics.[55] Cytolethal distending toxins (CDTs) are a family of bacterial protein toxins produced by various Gram-negative bacteria, including enteropathogenic *Escherichia coli*, *Campylobacter species*, enterohepatic *Helicobacter* species, *Shigella* species, and *Haemophilus ducreyi*. They act directly in the nuclei of their target cells and their major effect is the induction of DNA strand breaks. Additionally, these toxins may activate DNA damage checkpoint responses, which can then lead to cell cycle arrest or apoptosis in intoxicated cells.[56,57]

Studies have shown that CDTs cause sensitive eukaryotic cells to become blocked in the G2 (or the earlier M) phase via an incompletely charac-

terized mechanism.[58] A CDT is a triple halotoxin formed from CdtA, CdtB, and CdtC.[59] CdtB is the active subunit of the CDT halotoxin and has structural homology with the phosphodiesterase family of enzymes that include mammalian DNAase1. Because of its nuclease activity, CdtB causes limited DNA damage, triggers the DNA-damage response, induces DSBs, and then may cause cell cycle arrest and apoptosis.[55,60] Several studies have demonstrated that CDT-type bacteria are active on different types of mammalian cells. For instance, *Campylobacter jejuni*'s CDT is active on Caco-2 cells (a human colon carcinoma cell line), *Haemophilus ducreyi*'s CDT is toxic to HaCat cells (human keratinocyte cell line) and Don cells (hamster lung fibroblasts), *Actinobacillus actinomycetemcomitans*' CDT is toxic to CD41 and CD81 human T cells and has immunosuppressive potential, and finally *Escherichia coli*'s CDT is toxic to HeLa cells (a human cervical cancer cell line).[58,61]

B. N-Nitroso Compounds

Human exposure to endogenously formed N-nitroso compounds is suggested to be a highly important causative factor for carcinogenesis, particularly for cancers of the gastrointestinal tract. N-nitroso compounds can be formed via two different pathways:

- Through direct chemical reaction between secondary amino compounds and nitrite. This process is strongly pH dependent and does not proceed rapidly at neutral pH even in the presence of chemical catalysts.[62]
- Through direct bacterial catalysis of N-nitrosation. N-nitrosation catalysis is induced by bacterial enzyme systems and proceeds much more rapidly at neutral pH than through direct chemical reaction. *In vitro* studies have shown that organisms that commonly cause chronic infections, such as *Escherichia coli*, *Proteus morganii*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae*, have the ability to reduce nitrate to nitrite and use nitrite to nitrosate secondary amines.[62]

Bacteria can also produce N-nitrosamines as part of their metabolism. For example, *Escherichia coli* produces reductases, which can catalyze the conversion of nitrates into nitrites and allow the formation of N-nitrosamines. N-nitroso compounds from bacteria may lead to bladder cancer after chronic urinary tract infections.[53] Stickler et al.[63] reported that several bacterial species (including *Escherichia coli*, *Klebsiella oxytoca*, *Providencia stuartii*, and *Proteus mirabilis)* commonly and persistently colonize in the urinary tracts of paraplegic patients and may lead to high levels of nitrate, nitrite, nitrosamines, and volatile N-nitroso compounds in their urine. This accumulation may further lead to tumors in the urinary bladder in these patients.

IV. BACTERIA THAT MAY CAUSE DNA DSBS

A. *Escherichia coli*

Escherichia coli, a commensal Gram-negative bacillus found in the large intestine of humans and animals, is a common cause of human infection. Certain *Escherichia coli* species have a set of genes that specify the biosynthesis of different virulence proteins. Some of these proteins prolong bacterial survival while some are responsible for the toxic effects of this bacterium on host organisms.[64] For instance, *Escherichia coli* expresses cyclomodulins, which are bacterial effectors that modulate the eukaryotic cell cycle. These proteins target the host cell cycle and influence whether an infected cell will survive or die.[65]

CDTs are considered the prototype of inhibitory cyclomodulins. Expressed by *Escherichia coli*, they are heterotripartite toxins consisting of three protein subunits, CdtA, CdtB, and CdtC. The proposed role of CdtA and CdtC is the transport of CdtB to the target cell and its attachment to the cell surface. The active subunit, CdtB, exhibits DNase activity in the target cell, causing DNA damage and cell cycle arrest. All three subunits are needed to cause DNA damage in the host.[66] Some microorganisms (e.g. *Helicobacter pylori*, *Bartonella spp.*, *Lawsonia intracellularis*, and *Citro-*

bacter rodentium) also express *Escherichia coli*–like cyclomodulins that induce cell proliferation in the host.[66] Using Comet assay, Nougayre`de et al. showed that *Escherichia coli* infection leads to DSBs in infected HeLa cells, which exhibit nuclear γH2AX within 4 hours of infection along with morphological changes.

B. *Chlamydia trachomatis*

Chlamydia trachomatis is an ovoid-shaped non-motile, Gram-negative, and non-spore-forming bacterium. The most common cause of sexually transmitted diseases (STDs) in the United States, it can cause chlamydia, trachoma, lymphogranuloma venereum, nongonococcal urethritis, cervicitis, salpingitis, pelvic inflammatory disease, and pneumonia. The infection caused by *Chlamydia trachomatis* is associated with the development of cervical and ovarian carcinoma.[67] The underlying mechanisms for *Chlamydia*'s carcinogenicity are poorly described. Chumduri and collegues[68] showed that it induces DSBs, leading to the induction of γ-H2AX. Additionally, its infection promotes cellular viability by limiting DNA damage signaling to and complications in host cell chromatins, enforcing the survival of damaged host cells.

C. *Pseudomonas aeruginosa*

Gram-negative *Pseudomonas aeruginosa* is an opportunistic pathogen, frequently associated with devastating nosocomial infections in cystic fibrosis or immunosuppressed patients (such as AIDS patients), who have undergone a surgical procedure or suffered severe burn wounds. It induces several types of DNA alterations, such as SSBs, DSBs, and oxidative DNA damage, and thus it activates several pathways to repair these lesions.[69] Infection of immune or epithelial cells by *Pseudomonas aeruginosa* has been shown to trigger the phosphorylation of histone H2AX to form γH2AX.[70]

The ExoS bacterial toxin produced by *Pseudomonas aeruginosa* is identified as the major causative factor for gene deletion, mutagenesis, and γH2AX induction. Elsen et al.[71] observed a strong induction of H2AX phosphorylation in lung epithelial cells after *Pseudomonas aeruginosa* infection, which they found to be genotoxic to host cells, to generate multiple types of DNA damage, particularly DSBs, and to initiate several DNA repair pathways. ExoS toxin does not have nuclease activity, so its underlying mechanism to produce DSBs in the host cell is still unknown. However, it has been suggested that ExoS bacterial toxin might cause ROS-related oxidative DNA damage.[69]

D. *Haemophilus ducreyi*

Haemophilus ducreyi, a rigorous Gram-negative bacterium, is the causative agent of chancroid. Its chromosomal gene cluster encodes CDT (called HdCDT). Li et al.[72] demonstrated that HdCDT induces DSBs in HeLa cells. Additionally, Frisan et al.[73] showed that treatment of HeLa cells with CdtAC or CdtB alone did not induce breaks but that the combination of all three CDT components caused DNA damage in intact HeLa cells. The researchers used pulsed field gel electrophoresis (PFGE) to separate high-molecular-weight DNA and show that HdCDT intoxication induces DSBs in a time-dependent manner.

V. STILL A NIGHTMARE FOR THE DEVELOPING WORLD: *HELICOBACTER PYLORI*

Helicobacter pylori is a helix-shaped Gram-negative bacteria that is both microaerophilic and neutralophilic. It usually colonizes in the upper gastrointestinal tract[74] and chronically infects the human gastric mucosa.[75] *Helicobacter pylori* colonizes in 50% of the world's population, causing chronic infection that leads to gastritis and peptic ulcer, and later to gastric cancer and gastric MALT.[76,77] Toxicity mechanisms of *Helicobacter pylori* are summarized in Fig. 3.

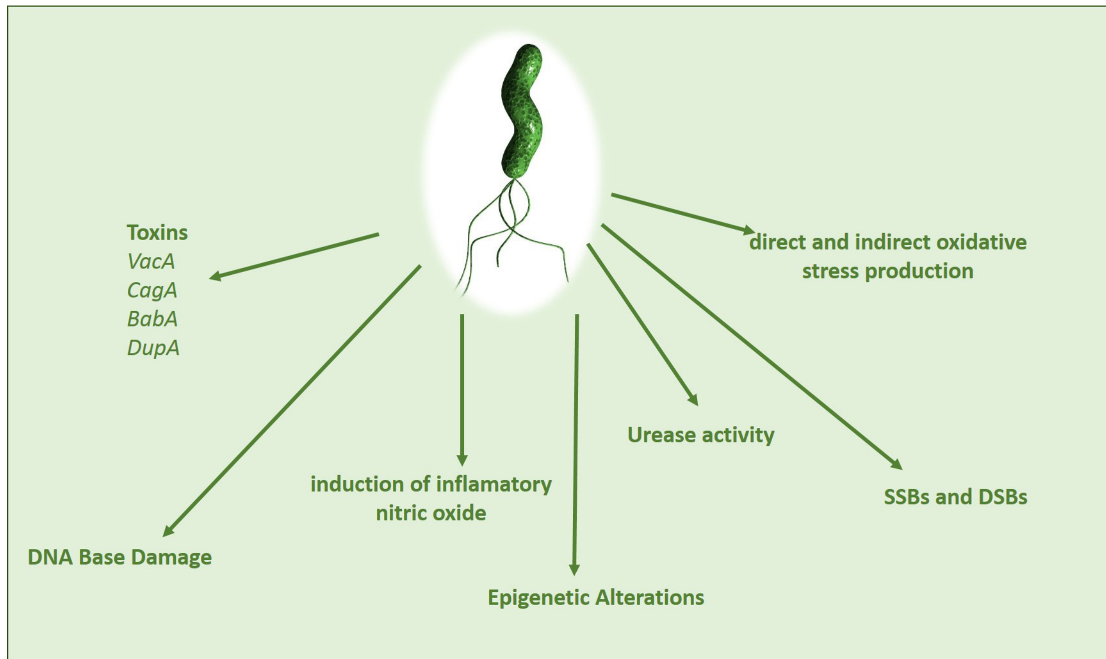

FIG. 3: Toxicity mechanisms of *Helicobacter pylori*

A. Role of *Helicobacter pylori*'s Virulence Factors in Gastric Inflammation and Carcinogenesis

Helicobacter pylori has four major virulence factors: the cytotoxin-associated gene product A (CagA), the vacuolating toxin (VagA), the duodenal ulcer–promoting toxin (DupA), and the adhesion protein (BabA).[78,79]

1. CagA

The CagA protein, one of the major pathogenic factors of *Helicobacter pylori*, has an approximate 125–140 kDa of molecular mass and is encoded by the CagA pathogenicity island (cagPAI). It is translocated into host epithelial cells through injection, via the Type IV secretion system (T4SS). T4SS is also encoded by cagPAI,[78,80] which is linked with several important gastric pathologies.[81]

CagA adheres to host epithelial cells and localizes to the inner surface of the plasma membrane. It undergoes tyrosine phosphorylation by kinases of the Src family.[80] *Helicobacter pylori* disrupts the host epithelial barrier as a result of CagA-related events.[82] Various studies have revealed that CagA disrupts the functions of intercellular junctions.[83] This disruption allows the activation of EGFR and thereby leads to epithelial cell motility. In addition, CagA has inductive effects on cytoskeletal reorganization, immune response, and apoptosis.[84] The CagA gene is not directly responsible for cell damage; however, it has been shown to affect the cell cycle and postpone prophase and metaphase, causing orientation default of the mitotic spindle, aberrant division axis, and finally genomic instability.[85] CagA contributes to inflammatory processes and induces epithelial layer disruption, which leads to the generation of gastric cancer and MALT lymphoma.[83] Serological studies have shown that CagA seropositivity is linked to the fast progression of gastric cancer.[86,87]

2. VacA

The VacA toxin is released by toxigenic *Helicobacter pylori* strains. It is encoded by a chromosomal gene known as vacA.[88] Several studies have

shown that VacA disrupts cell proliferation and migration[86] and is responsible for formation of large intracellular vacuoles in gastric epithelial cells; it also disrupts the tight connections of epithelial cells and induces gastric inflammation by blocking T lymphocyte activation and proliferation.[89]

Epidemiological studies have indicated that active variants of VacA are associated with increased secretion of proinflammatory cytokines, such as interleukin-8 (IL-8), interleukin-10 (IL-10), TNF-α, macrophage inflammatory protein 1α (MIP-1α), and interleukin-13 (IL-13). Moreover, it is suggested that VacA causes β-catenin release, modulation of apoptosis, and alterations in cell cycle phases.[90] Winter et al.[91] recently reported that *Helicobacter pylori* strains that have more active VacA toxin than other strains are more likely to induce metaplasia in mice.

3. BabA

BabA is an outer membrane protein that mediates adherence to the ABO/Lewis b antigen in gastric epithelial cells. It is expressed by 40%–95% of *Helicobacter pylori* strains, although its expression varies in different regions of the world.[90]

4. DupA

DupA is encoded by the duodenal ulcer gene A and associated with duodenal ulcer and gastric cancer. It induces high production of IL-8 and interleukin-12 (IL-12). Recent studies have shown that 42% of DupA-positive *Helicobacter pylori*–infected patients are diagnosed with duodenal ulcer and 9% are diagnosed with gastric cancer irrespective of nationality.[92]

B. Carcinogenicity of *Helicobacter Pylori*

Helicobacter pylori has been classified as a Group I carcinogen (carcinogenic to humans) by the International Agency for Research on Cancer (IARC).[93] Gastric cancer is the third most common cause of cancer-related deaths worldwide, and IARC estimates that 78% of all cases are linked with *Helicobacter pylori* infections.[93] Many studies have also shown that *Helicobacter pylori* is an important risk factor in the development of noncardia gastric cancer. Although *Helicobacter pylori* infections and, as a consequence, gastric cancers, are decreasing in the developed world, they remain a major threat to human populations in developing countries.[94]

The molecular mechanisms of *Helicobacter pylori*–associated gastric carcinogenesis are as yet undefined.[75] Studies show that the bacterium can produce genomic instability both directly and via epigenetic pathways.[95] In addition, its infection can induce direct changes in the nuclear and mitochondrial DNA of the host, such as oxidative damage, methylation, decreased expression of mismatch repair genes, increased chromosomal instability, microsatellite instability, and mutations.[96] Chronic inflammation also causes increased cell proliferation and prominent acid inhibition due to bacterium urease activity, which makes it a major risk factor for gastric carcinoma.[97]

1. Urease Activity

Helicobacter pylori uses its urease activity for survival and pathogenesis. Through hydrolysis of urea, bacterial urease generates NH_3 and CO_2 and causes a counterbalance in gastric acidity, leading to neutralization of gastric acid.[98,99] It is suggested that *Helicobacter pylori* uses L-arginase as a substrate for converting urea to NH_3[98] in a process that generates a neutral microenvironment in the gastric lumen, leading to enhancement of bacterial motility.[99,100] Also, it has been reported that urease activity can extinguish a host's •NO defense.[101] On the other hand, increased NH_3 can catalyze the production of monochloramine and other NH_3-derivated compounds, which have cytotoxic effects in host cells.[102] Monochloramine, an oxidant emerging by from the reaction of HClO and NH_3, causes significantly more damage in cultured mucosal cells when compared to cells exposed to HClO or H_2O_2 at physiological concentrations.[102]

2. Induction of Inflammatory Nitric Oxide Synthase

•NO has important roles in the host's defense and is responsible for endogenous production of N-nitroso compounds.[98,103] DNA-damaging metabolites such as nitrosating agent N_2O_3 or oxidizing agents such as $ONOO^-$ can be generated by the interaction of •NO and oxygen radicals. This reaction is one of the most important events in the promotion of carcinogenesis.[104]

Recent studies have focused on the relationship between *Helicobacter pylori* CagA-positive strains with host iNOS expression. Using real-time PCR (RT-PCR), Rieder et al.[104] investigated the association between chronic *Helicobacter pylori* infection and iNOS expression in samples obtained from gastric carcinoma patients as well as in antral biopsies from patients with *Helicobacter pylori*–associated gastritis. They reported that a significant increase in iNOS mRNA signaling was present only in one-third of the biopsies from *Helicobacter pylori*—associated gastritis patients. Additionally, CagA-positive strains were shown to have more activity in inducing iNOS expression than CagA-negative strains. The researchers concluded that CagA-positive *Helicobacter pylori* strains are associated with the expression and activity of iNOS and might contribute to the development of intestinal metaplasia after the development of gastric cancer.

3. Direct and Indirect Oxidative Stress Production

After infection of the host with *Helicobacter pylori*, excessive ROSs/RNSs can be produced that can cause histological damages in human gastric mucosa.[105] Additionally, *Helicobacter pylori* by itself generates ROSs.[99,106] It has been reported that it can release $O_2•^-$ and •OH, both of which cause gastric epithelial cell damage.[80,89] Studies have shown that •NO reacts with $O_2•^-$ (which is endogenously formed in *Helicobacter pylori*) and in this way can inhibit the antibacterial effects of •NO, which is synthesized by inflammatory cells.[107] iNOS can produce much higher concentrations of •NO than the other ROSs—endothelial (eNOS) and neuronal (nNOS) oxidase synthases.[104] Its expressions have been found to be significantly higher in *Helicobacter pylori*–induced gastritis patients, further contributing to carcinogenesis.[108] Along with DNA damage, *Helicobacter pylori*–induced oxidative stress induces epithelial cell apoptosis and later cell proliferation through the stimulation of inflammatory mediators.[109]

Helicobacter pylori infection induces active inflammation with neutrophilic infiltration. It also provokes chronic inflammation by infiltration of lymphocytes, macrophages, and plasma cells in stroma sides.[110] The activation of monocytes and neutrophils causes these cells to produce ROSs (e.g., $O_2•^-$, H_2O_2, and •OH). Enhanced ROS levels cause increased oxidative DNA damage in the gastric mucosa of *Helicobacter pylori*–infected patients.[111] The long-standing chronic inflammation of gastric mucosa caused by *Helicobacter pylori* infection can lead to potentially mutagenic and carcinogenic gene modifications that destroy the natural capability of highly proliferating epithelial gastric gland stem cells to repair DNA damage.[112] For this reason, oxidative stress caused by *Helicobacter pylori* is believed to be one of the major factors in predisposition to gastric cancer.[17]

4. DNA Base Damage

The cellular consequences of DNA oxidation by ROSs can lead to the formation of oxidized bases, abasic sites, oxidized deoxyribose sugars, SSBs, and DSBs. Additionally, ROSs can decrease tumor suppressor gene expression and increase protooncogene expression.[113,114]

In nuclear and mitochondrial DNA, the most common base damage caused by physical, chemical, or biological agents is 7,8-hydroxy-2′-deoxyguanosine (8-OHdG), which is the oxidized form of deoxyguanosine. Oxidized bases are one of the predominant forms of free radical–induced oxidative lesions and have been widely used as biomarkers of oxidative stress and carcinogenesis because they cause abasic sites, SSBs, and DSBs.[97,115]

Oxidation of deoxyguanosine contributes to carcinogenesis in two ways: modulation of gene expression and induction of mutations. The formation of 8-OHdG can cause a G-T transversion that promotes carcinogenesis. Studies have shown that urinary 8-OHdG is a good biomarker of various cancers and degenerative diseases.[115]

Using alkaline Comet assay, Arabski et al.[116] measured the DNA damage of gastric mucosa cells in 22 *Helicobacter pylori*–infected patients. They found this damage to be significantly higher in patients versus controls, thus demonstrating that *Helicobacter pylori* infection can be a potent source of free radicals that cause oxidative DNA damage resulting in the formation of 8-OHdG or 8-oxodG. The researchers also suggested that oxidative DNA damage can be introduced by both inflammatory cells and direct bacterial products such as urease. A study by Nishibayashi et al.[117] showed that 8-OHdG levels in the gastric mucosa cells of *Helicobacter pylori*–positive patients are significantly higher than in *Helicobacter pylori*–negative patients, suggesting that *Helicobacter pylori* can be a major risk factor for gastric carcinoma. In addition, using immunohistochemical techniques, Ma et al.[118] demonstrated that *Helicobacter pylori* infection induces 8-nitroguanine formation and accumulation of proliferating cell nuclear antigen (PCNA), a prognostic factor for gastric cancer, in gastric gland cells. Also, they showed that levels of 8-nitroguanine, 8-oxodG, and PCNA are significantly higher in patients with *Helicobacter pylori* infection than in patients without it.

5. SSBs and DSBs

Helicobacter pylori infection can directly trigger both SSBs and DSBs in gastric inflammation and carcinogenesis. Clinical and epidemiological studies have demonstrated that gastric carcinogenesis is strongly related to mucosal damage induced by *Helicobacter pylori* inflammation and to high ROS production. Both intraepithelial neutrophil infiltration and various *Helicobacter pylori* toxins (which contribute to mucosal damage and ROS/NOS production) cause genetic instability in *Helicobacter pylori*–induced inflammation.[119] The most dangerous DNA damage, DSBs, can cause remarkable genetic instability due to chromosomal aberrations. Over the last decades, studies have shown have shown that, like many bacteria, *Helicobacter pylori* induces DSBs and SSBs.[120]

Helicobacter pylori can introduce DNA breaks in a host genome. The formation of γ-H2AX can be a consequence of DSB formation in the presence or absence of ROSs and has been detected in infected gastric mucosa epithelial cells.[75] Sentani et al.[121] showed that expression of the γ-H2XA protein correlates with the degree of gastric malignancy. In addition, its expression in neoplastic gastric mucosa epithelial cells is significantly higher than in nonneoplastic gastric mucosa cells. Xie et al.[122] studied 302 patients who had undergone gastroduodenoscopy because of chronic gastritis, intestinal dysplasia, and gastric carcinogenesis. Immunohistochemical analysis and Western blotting revealed γ-H2AX in the nuclei of epithelial cells and its overexpression in gastric carcinoma cells, correlating with the pathological features of carcinoma such as tumor location, differentiation, lymph node metastasis (TNM) stage, and metastasis.

Yabuki et al.[119] examined cell proliferation and DNA damage in 35 *Helicobacter pylori*–infected Japanese subjects who had undergone total and partial gastrectomy due to intestinal adenocarcinoma, evaluating both SSBs and DSBs by in situ end labeling (transfer of biotinylated nucleotide to the 3’-OH end) in the gastric mucosa. The investigators showed that irreversible DNA fragmentation, SSBs, and DSBs were present and that they had led to apoptosis. They also reported that intraepithelial neutrophil infiltration significantly contributed to mucosal damage and carcinogenesis in *Helicobacter pylori* inflammation.

Studies have demonstrated that cagPAI-encoded T4SS has a critical role in *Helicobacter pylori*–induced DNA damage. Hartung et al.[123] reported that it induces DSB, induces nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, and induces IL-8 production.[123] NK-kB and IL-8 are critical in inflammation and carcinogenesis. CagA interacts with several host proteins

to activate β-catenin, NF-κB, Ras, and ERK in downstream pathways.[124]

ATM is involved in the DSB-induced cell cycle arrest that might be expected following *Helicobacter pylori*–induced DNA damage.[95] Koeppel et al.[120] demonstrated the effects of *Helicobacter pylori* on human primary gastric cells, human gastric cells, human gastric adenocarcinoma (AGS) cells, and gastric tubular adenocarcinoma (MKN74) cells. Following infection of gastric AGS and MKN74 cells, the researchers observed an accumulation of γH2AX as early as 6 hours postinfection which reached levels comparable to those induced by 10 Gy IR after 18 hours postinfection. DSB was observed to accumulate in AGS cells during the first hours, reaching a steady state between 6 and 18 hours postinfection, most likely due to a balance between repair and damage. Accumulation in MKN74 cells was slower compared to AGS cells, although DSB accumulation after 18 hours postinfection was comparable to IR-induced damage.

On the other hand, Comet assay showed that AGS cells had significant DNA damage at 6 hours postinfection whereas MKN74 cells had marked DNA damage at 18 hours. CagA-positive strains caused a more significant decrease in NBS1 expression and some specific action on MRE11 phosphorylation in AGS cells. Additionally, the researchers demonstrated that *Helicobacter pylori* causes G1/S arrest because the p53-binding protein (53BP1) was localized in the separated foci during the G1 phase of the cell cycle.[120]

Toller et al.[75] showed that *Helicobacter pylori* infection directly contributes to genomic disintegration of host cells and introduces DSBs in primary and transformed murine and human epithelial and mesenchymal cells. After infecting AGS cells for 6 hours, the researchers observed DNA fragmentation and DSB induction in a time- and dose-dependent manner. The DNA disruptions in the host nuclear DNA required direct contact with live bacteria (bacterial adhesion); however, it was suggested that these disruptions were independent of the *Helicobacter pylori* virulence determinants, such as VacA and Cag PAI, as well as ROS production. The DNA discontinuities triggered a damage-signaling and repair response involving the sequential ATM-dependent recruitment of repair factors—53BP1, mediator of DNA damage checkpoint protein 1 (MDC1), and H2AX phosphorylation. Although most breaks were repaired efficiently at the termination of infection, prolonged active infection was suggested to cause the saturation of cellular repair capabilities, which in turn can lead to different pathological conditions, particularly cancer.[75]

In two different studies, Hanada et al.,[95,125] using microarray screening, examined *Helicobacter pylori*–infected human gastric biopsy specimens to identify the genes responsible for DNA damage response to DSBs. The researchers confirmed the presence of DSBs by observing γ-H2AX in *Helicobacter pylori*–infected human gastric epithelium. They also showed that activated ATM was present as well. The researchers examined the effect of Cag-PAI on *Helicobacter pylori*–induced chromosome instabilities. Their results indicated that infection activates the ATM-dependent DNA damage response and that infections with both Cag-PAI-positive and Cag-PAI-negative strains are associated with the development of gastric cancer; in fact, the presence of Cag-PAI approximately doubles the risk.[95,125]

Anikeenok et al.[126] used Comet assey to examine the genotoxic potential of two strains of *Helicobacter pylori*—P12 (wild type) and PAI-deficient mutant deltaPAI—on AGS and HeLa cells at 20–500 multiplicities of infections (MOIs). They showed that infection of AGS and HeLa cells with both strains for 6 hours and infection of AGS with both strains for 12 hours did not induce DNA damage. However, a significant dose-dependent increase in tail moment of the AGS cells after infection with deltaPAI for 24 h was detected whereas the genotoxic effects of P12 (wild type) under the same conditions were not.[126]

As explained previously, DNA oxidation can lead to several types of damage, one of the most common being oxidized bases. Base excision repair (BER) is the major repair mechanism for oxidized bases, such as 8-oxodG. Studies have shown

that cells undergoing *Helicobacter pylori*–induced chronic inflammation possess high levels of ROSs/RNSs and cytokines, which can directly lead to tumors in BER-deficient cells.[113,114] Kidane et al.[97] suggested that *Helicobacter pylori* infection induces oxidative and small base damage and later enhances the number of abasic sites, leading to the accumulation of BER intermediates in host cells. These intermediates can increase DSBs. The researchers infected a GES-1 cell line derived from nontumorigenic human gastric epithelial cells for 12 hours. After incubation, γH2AX positive cells were found distributed in all phases of the cell cycle, particularly G1. In addition, AP sites were generated at a high frequency in genomic DNA during infection and were further processed into DBSs, leading to genomic instability in 8-oxoguanine DNA glycosylase (OGG1) –proficient *Helicobacter pylori*–infected cells during all cell cycle phases.[97]

In summary, studies support that in the human stomach *Helicobacter pylori* infection can introduce DSBs with γ-H2AX formation as a consequence with and without the presence of ROS induction.

6. *Epigenetic Alterations*

Studies have shown that *Helicobacter pylori* can produce genomic instability directly or via epigenetic pathways.[95] Infection can cause epigenetic alterations such as methylation of DNA, miRNA-dependent posttranscriptional silencing, and histone modifications.[95,125]

Methylation and acetylation pattern alterations in tumor suppressor genes are of particular importance in *Helicobacter pylori*–induced carcinogenesis.[125,127] It is not completely clear how *Helicobacter pylori* inflammation stimulates DNA methylation. Investigation of methylation of CpG islands of multiple genes such as cyclooxygenase 2 (COX-2), E-cadherin, p16, and runX3 in precancerous lesions and carcinogenic cells has revealed that aberrant CpG island methylation accumulates and leads to gastric carcinogenesis.[127] Also, the runX3 tumor suppressor gene's promoter regions were found to be methylated in *Helicobacter pylori*–induced gastric cancer patients. Katayama et al.[128] conducted a study on the human gastric epithelial cell line MKN45 infected with *Helicobacter pylori* strains, and reported that the infection caused runX3 gene methylation and loss of runX3 expression. On the other hand, they reported that promoter methylation of the CDH1 gene (which expresses E-cadherin) was more frequent in the gastric mucosa of infected patients than in controls.[129] *Helicobacter pylori* induces promoter region methylation of the E-cadherin gene, which, as Huang et al.[130] demonstrated, is generated by IL-1β activation due to direct interaction between *Helicobacter pylori* and gastric cells.

By determining the methylation levels of 8 marker CpG islands, Maekita et al.[131] observed that infected patients had much higher methylation levels in their gastric mucosa (5.4- to 303-fold) than did control subjects without infection ($p < 0.0001$). In addition to the 8 marker CpG islands, which are associated with protein-coding genes, CpG islands of microRNA genes were found to be methylated in these patients as well. Also, the methylation levels of the marker CpG islands in the gastric mucosa were shown to correlate with gastric cancer risk. Patients with gastric cancers had 2.2- to 32-fold higher methylation levels than did healthy individuals, and patients with multiple gastric cancers had significantly higher methylation levels than did those with a single gastric cancer.[132,133] This correlation vigorously endorses the general concept that the accumulation of aberrant methylation in the gastric mucosa produces an epigenetic field for cancer—that is, a field defect.[132–135]

Studies in animal models support the hypothesis that *Helicobacter pylori* infection also induces gastric carcinogenesis with epigenetic changes such as DNA methylation and histone modifications due to high ROS production. Obst et al.[111] showed that infection promotes DNA methylation via inflammation in Mongolian gerbils.

VI. DISCUSSION

Epidemiological studies support the notion that

chronic infections are linked to an increased risk of cancer. Several chronic bacterial inflammations have been suggested to be the cause of tumor development. During infections, pathogens and host cytokines recruit and activate inflammatory cells, including neutrophils and macrophages/monocytes,[91] which produce ROSs that cause DNA damage. Damage to DNA leads to substitutions, deletions, or translocations; SSBs or/and DSBs; and ultimately mutations. These mutations alter both gene patterns and gene modifications, and such alterations are potentially mutagenic or carcinogenic.[94]Additionally, bacterial toxins can interfere with the regulation of cell cycle progression and apoptosis, which may contribute to the formation of the mutator phenotype.[113] Any imbalance between DNA damage and host DNA repair mechanisms has a harmful impact on the integrity of the host genome. Recent evidence supports that bacterial infections can impair host cell DNA repair mechanisms.[114]

Escherichia coli, *Chlamydia trachomatis*, *Pseudomonas aeroginosa*, and *Haemophilus ducreyi* all lead to infection in humans. Their toxins are suggested to cause DNA damage leading to tissue damage or carcinogenesis in the target organs. *Helicobacter pylori* is one of the main causes of gastric and duodenal cancers in the developing world. Many strains of this bacterium lead to DNA damage in host cells. Two possible cancer mechanisms in *Helicobacter pylori* infection are being investigated.

The first mechanism involves the enhanced production of ROSs/RNS. After oxidative/nitrosative stress, the bacterium increases the rate of host cell mutation. Although it is also suggested to cause DNA base lesions, the predominant mechanism of DNA damage after infection seems to be DSBs, which are also the most dangerous. In patients with low DSB repair capacity, *Helicobacter pylori* can lead to serious infection that may result in cancers of the stomach or duodenum. Moreover, in people with high oxidative stress (i.e. chronic infections, chronic diseases such as diabetes), DNA repair mechanisms can be impaired. Studies have shown that *Helicobacter pylori* infection alters the host genome and reduces the synthesis of DNA mismatch repair and BER proteins. It is also believed to alter levels of the proapoptotic regulator p53.[49] These effects may lead to higher levels of unrepaired DNA damage, resulting in carcinogenesis.

The second mechanism under investigation is the perigenetic pathway, which involves enhancement of the transformed host cell phenotype by alteration of cell proteins such as adhesion proteins.[103] It is indisputable that *Helicobacter pylori* causes inflammation along with high levels of TNF-α and/or interleukins, particularly interleukin 6 (IL-6). The perigenetic mechanism suggests that inflammation-associated signaling molecules, such as TNF-α, alter gastric epithelial cell adhesion and cause the dispersal and relocation of mutated epithelial cells without the need for additional mutations in tumor suppressor genes such as those that code for cell adhesion proteins.[136] However, *Helicobacter pylori* may express other proteins that are responsible for its effects on the host genome. The effects of the outer-membrane proteins OipA (HopH) and SabA (HopP), expressed by *Helicobacter pylori*, are still under investigation.

Although it is difficult to isolate *Helicobacter pylori* toxins, research should be conducted on them one by one to determine whether they cause DNA damage, particularly DSBs.[137] Once all genes that contribute to the virulence of *Helicobacter pylori* have been identified, it may become easier to develop novel therapeutic drugs or vaccines to treat and prevent this infection.

VII. CONCLUSIONS

A variety of DNA lesions can be generated either spontaneously or as a result of exposure to exogenous DNA-damaging chemical, physical, and biological agents. Among these lesions, DSBs are particularly detrimental and should be repaired to maintain genomic integrity and stability. Insufficient repair can cause genomic rearrangements (deletions, translocations, and fusions) in the DNA. If not repaired at all, DSBs can lead to cell death.[26–32]

Among all causes of DSBs in the host genome, exposure to microorganisms is usually neglected. Although DSBs can be repaired efficiently in healthy organisms, prolonged bacterial infections can lead to full depletion of the repair capabilities of host cells, leading to ineffective and mutagenic DSB repair. Studies show that infection with different types of bacteria, particularly *Helicobacter pylori*, can cause DNA damage (specifically DSBs) and impairment of the host's DNA repair capacity.[95] These effects may lead to genetic instability, frequent chromosomal aberrations, and finally promotion of gastric and/or duodenal cancers. Because humans are constantly exposed to large numbers of microorganisms, the role of various bacterial toxins, isolated or combined (from one microorganism or from different microorganisms), should be elucidated by mechanistic studies so treatments of bacterial infections can be developed. Antioxidants should also be considered as preventive supplementations or as co-treatment options in *Helicobacter pylori* infections, as they reduce intracellular oxidative stress—one of the main causative factors in *Helicobacter pylori*–mediated cancers.[14] In conclusion, comprehensive studies are needed to evaluate the DNA damage–causing effects of common microorganisms with the goal of developing new strategies to overcome them.

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