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Review Article

Damage to the Upper Gastrointestinal Tract - The Role of Pepsin

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Abstract

Discovered in 1836, pepsin is now widely considered to be the most damaging, aggressive enzyme of the gastrointestinal contents. Produced via the precursor pepsinogen, pepsin makes up a family of isoenzymes allowing activity to range from pH 2 - 7.5 resulting in many complications. Pepsin is a digestive enzyme present in all vertebrates; however minor structural differences occur between species. Salivary pepsin has recently been recognised as a diagnostic marker for reflux and has been demonstrated to cause harm to cells via endocytosis, weakening protective proteins and damaging golgi apparatus and mitochondria.

This review explores contemporary literature comprising of books, articles and conference presentations reporting on all roles of pepsin and the damage it can cause to the upper gastrointestinal tract. Some controversy is exhibited between Fruton, Bhown and Righetti regarding pepsin's isoelectric point. Similarly, Bulmer and Tack display differences in their findings on how pepsin is most damaging to the upper gastrointestinal tract.

Keywords: *Pepsin; Digestive Enzymes; GERD; Diagnostic Biomarker; Reflux Disease; Endocytosis*

Introduction

Reflux disease is a growing condition responsible for causing damage to the upper gastrointestinal tract. Defined as Gastro-esophageal reflux disease (GERD), Extra-esophageal reflux disease (EER) and Laryngopharyngeal reflux (LPR), reflux is often incorrectly referred to as heartburn [1], a symptom associated with GERD. The growing awareness of reflux has led to many advances - it is now characterised as a prevalent disorder in gastroenterology [2], yet there are gaps in the current knowledge.

Defined as "reflux that causes troublesome symptoms, mucosal injury in the oesophagus, or both of these" [3], GERD is apparent when damage to the lower oesophageal sphincter occurs, allowing gastric contents to leave the stomach (Figure 1) [3]. Contents of gastric juice are expelled into the esophagus causing impairment to tissues and problematic, painful symptoms including heartburn and regurgitation which if left untreated may lead to esophageal adenocarcinoma or laryngeal cancer [4,5].

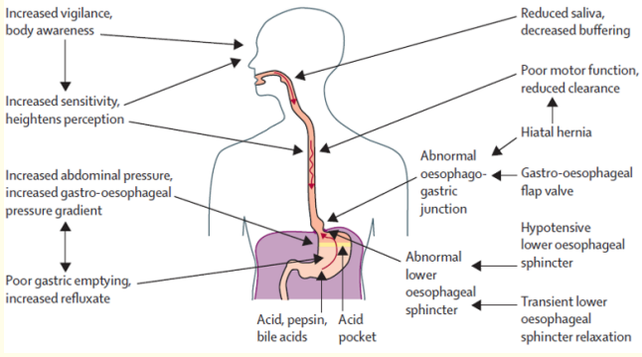

Figure 1: *A schematic of GERD's physiological process, displaying its connections with impairment.*

With the expanding knowledge on reflux disease, a recent association was found between pepsin and the disease [6], leading to the awareness of EER and LPR. Known as "the retrograde movement of gastric contents into the larynx and pharynx" LPR is often recognised as silent reflux [7] due to the lack of the more classical symptoms such as heartburn and symptoms being respiratory-like.

Purpose of the Study

The purpose of this review is to evaluate the current literature on pepsin from its discovery and structure to the comparison of concentration between adults and neonates. The review also aims to link the damaging effects of pepsin on the gastrointestinal tract and how these effects lead to reflux disease.

The history of pepsin

Discovery of pepsin

Discovered by Theodor Schwann in 1836 [8], pepsin is now considered the most widely associated aggressive proteolytic enzyme in the gastric contents [9]. During the discovery, Schwann was studying the role of digestion and obtained a filtrate that he noted affected digestion - Schwann later named this filtrate pepsin. Realising the effects of pepsin were different to those of HCl, Schwann later established its physiological role [10] and contribution to protein metabolism [11].

Berzelius suggested Schwann's pepsin should be isolated to examine the effects with greater precision; however, this caused scientists, including Blondlot, to believe Schwann's pepsin was a false discovery due to many failed attempts of isolation [8]. Northrop's contributions on pig pepsin in 1930 allowed Herriott to study the conversion of pepsinogen to pepsin in 1938, embedding Schwann's discovery [10].

Pepsin production within the body

Pepsin is present in the gastric contents of all vertebrates [10] and is secreted and synthesised as the precursor pepsinogen [12] by the chief cells located in the gastric mucosa [13]. Pepsinogen is inactive at neutral pH but in the presence of stomach acid, converts into the active, damaging form known as pepsin [14,15]. Pepsin is considered the key enzyme of the digestive system [16].

The structure of pepsin

In humans and the chemistry around pepsin

By using X-ray crystallography, pepsin has been characterised as a large concaved molecule consisting of two regions composing of amino acids, one containing an N terminal, the other a C terminal [4]. Both Huang and Tang and Fujinaga., *et al.* confirm porcine and human pepsins have extremely similar structures [17,18] with only minor differences occurring in their amino acid sequences. However, recorded research is sparse on the structure of human pepsin. Huang and Tang reveal at amino acid 8, glutamine is present in the human structure whereas in porcine pepsin it is lysine. Similarly, at amino acid 18, human pepsin contains phenylalanine [19] contrasting to the porcine structure containing a molecule of tyrosine (Figure 2) [17].

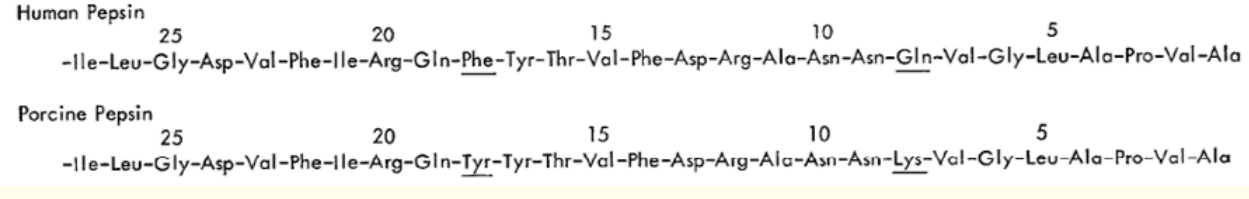

Figure 2: *Comparisons between human and porcine pepsin amino acid sequences [17].*

Following on from previous research, Fujinaga., *et al*. split human pepsin into three domains before carring out X-ray crystallography to determine the structure. The first section contained the backbone of the active site - a six stranded antiparrallel β-sheet. The remaining two divisions were each region, made up of additional β-sheets orthoganally packed together. Differences between the regions occurred through the number of β-sheets present: the C terminal region comprised of two whereas the N terminal consisted of three. The crystallography pattern broadened the knowledge on human pepsin, generating a computerised structure (Figure 3) [18] and additional key information presented in table 1 [18].

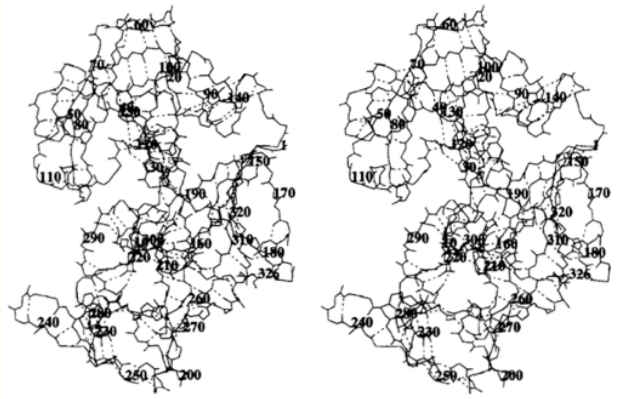

Figure 3: *A computer generated image of human pepsin [18].*

Parameter	Human Pepsin
Number of protein atoms	2438
Number of H_2O molecules	102
Disulphide bond separation (Å)	2.0
Energy of hydrogen bonds in the main backbone chain (kcal mol^{-1})	-2.1
Chirality of torsion angles (°)	33.9
Helix torsion angles (°)	-65.1, -37.1

Table 1: *Key information obtained from performing x-ray crystallography on human pepsin [18].*

Furthermore, research has proven the optimal pH for pepsin digestion is 2, despite showing maximum activity at pH 3.2 and 4.2. The enzyme continues to be active up to pH 6.5 but is irreversibly denatured at pH 8 or higher due to depletion of the enzyme's secondary structure. However, pepsin remains stable at pH values as high as 7.5 which can cause further implications to patients that suffer from GERD and EER [19-21].

The structure of animal pepsins and their amino acid structures

Pepsin has been studied extensively in a vast range of animals including; pigs, chickens and cold-water fish, displaying pepsin is present in two main isoforms - pepsin A and pepsin C [22].

Studies into porcine pepsin revealed there is a lot of controversy in the published literature regarding its isoelectric point. Fruton [10] and Bhown [23] both report porcine pepsin to have an isoelectric point, pI, of 1, much lower than human pepsin which is calculated to

have a pI in the region of 2 - 3 [10]. In contrast, Righetti's work (also conducted in 1990) displays porcine pepsin to have a value between 2.76 - 2.90, correlating with Righetti and Caravaggio's results showing the pI value of porcine pepsin to be 2.86 [24]. Furthermore, porcine pepsin contains four basic amino acid residues [25] containing arginine and lysine which should increase the isoelectric point of porcine pepsin above the values recorded for human pepsin agreeing with Righetti's findings. However, as pI is a measure of the overall balance of the whole protein, despite porcine pepsin having more basic amino acid residues, it could in fact have a greater number of acidic residues than human pepsin causing Fruton and Bhown's research to appear more accurate. The lack of knowledge on isoelectric points is further emphasised by the study of chicken pepsin. Chicken pepsin contains a cysteine residue, resulting in the molecule being strongly basic and an isoelectric point of approximately 4 to be recorded [10].

Karlsen and colleagues performed extensive research on pepsin in cold-water cod to note whether the structure of pepsin differed to that in mammals. They demonstrated pepsin in cod has a greater number of water molecules (161) than human pepsin (102) and the structure contained 324 amino acids mainly consisting of glycine, lysine, arginine, histidine and glutamine. The arrangement of this amino acid sequence causes the pepsin structure to have high conformational freedom with the aromatic region of histidine interacting through hydrogen bonding to other amino acid residues providing stability to the enzyme.

Karlsen also found cold-water fish have a higher optimum pH value and lower Arrhenius activation energy compared to mammals [22].

Pepsin isoforms

Pepsin is a range of 8 isoforms rather than being one individual molecule [4]. Gastric juice in humans contains pepsin isoforms, known as pepsin 1, 3a, 3b, 3c - each isoform has different characteristics, sensitivities and optimal pH values [19,26]. Pepsin 3b has been proven to be the most common state - this isoform contributes 70% to 80% of human gastric juice [20]. The nomenclature for pepsin isoenzymes is due to their ability to mobilise in electrophoresis with isoenzyme 1 displaying the most movement [19,27].

Pepsin in neonates

When is pepsin first produced in the body?

Reflux is particularly predominant in neonates [13] suggesting pepsin is present within the human body directly after birth. This is emphasised in the literature by Farhath, Elabiad and Zhang who report pepsin to be present in samples taken from neonates up to three days old [28,29]. Samples taken directly after birth, before feeding, display lower pepsin concentrations than those taken later in the neonate's life, specifically compared to postprandial samples taken. Further studies into pepsin secretion have been carried out on premature neonates displaying pepsin activity is much lower compared to that of overdue neonates [30]. Due to the limited research available, an explanation for this is not known, however one possible hypothesis is overdue babies have had longer to develop and establish mechanisms, therefore having naturally secreted a higher concentration of pepsin causing greater activity to occur.

The study performed by Metheny and colleagues reported the fasting concentrations of pepsin to be 349.1 μg ml^{-1} in adults and 76.11 μg ml^{-1} in neonates [30], much lower than the normalised value for adults, known to be 0.91 mg ml^{-1} [31]. As there is limited information available regarding pepsin concentrations in neonates, it is difficult to directly compare pepsin concentrations of neonates and adults in its normalised form.

Gastroesophageal reflux disease

GERD is a condition becoming more frequent affecting up to 60% of the adult population [32]. From as early as 1903 when Coffin hypothesised the cause of rhinorrhoea and hoarseness was due to gas being refluxed from the stomach [33] continuous research has been conducted to clarify this hypothesis - to date many studies have been performed.

Gastric refluxate

Gastric refluxate is responsible for damage occurring during a reflux event consequently resulting in GERD. The contents of refluxate include; pancreatic enzymes, pepsin, bile acids and HCl [34,35]. Expanding knowledge has found pepsin to be the element of refluxate responsible for inducing deterioration on tissues despite controversy in findings [9]. The techniques available for detecting pepsin in clinical samples are listed in table 2. Many of these techniques are time consuming and require technical knowledge. The most recent addition to the available tests is Peptest™, a rapid lateral flow test which is now commercially available worldwide.

Immunohistochemistry
Enzymatic Assay
Western blot
ELISA
Lateral Flow Technology (Peptest™)

Table 2: *Techniques available for pepsin detection in gastric refluxate.*

Pepsin diagnosis

Studies indicate pepsin is widely used in the diagnosis of reflux due to its proven high sensitivities and specificities as well as its ability to detect non-acidic reflux unlike invasive techniques [16,36].

Pepsin as a biomarker for reflux disease

Pepsin is synthesised by the chief cells situated in the stomach and is present in all refluxate, allowing the enzyme to be an established biomarker for reflux disease [37]. If a reflux event has occurred, pepsin will be present outside of the stomach by the movement of gastric contents. Using pepsin as a biomarker for reflux disease has proven to be superior to that of invasive testing. This is due to its sensitivity, specificity and ability to not require the placement of an invasive catheter [16] causing many researchers to claim detecting the presence of pepsin to be the new 'gold-standard' for diagnosing GERD [38].

Peptest

Kim and colleagues hypothesised pepsin detection in saliva may lead to a non-invasive, patient preferred sensitive method for diagnosing GERD [39]. Dettmar followed this hypothesis and launched Peptest (RD Biomed Limited, Hull) - the latest diagnostic method for reflux. The technique is a simple, non-invasive, 'office-based' lateral flow device, producing rapid results without any discomfort for the patient [39]. The test is based on a pepsin-immunoassay within a lateral flow device [33,35] designed on two monoclonal antibodies specific to human pepsin - one detects pepsin and the other captures the enzyme. The detection antibody is labelled with blue latex beads and forms a sandwich ELISA with the capture antibody to generate a 'T' line. Any excess detection antibody migrates to the 'C' line proving the test has worked, as displayed in figures 4 [40] and 5 [41]. The intensity of the 'T' line can be measured and converted into pepsin concentration, providing accurate diagnostic results for GERD (Figure 6).

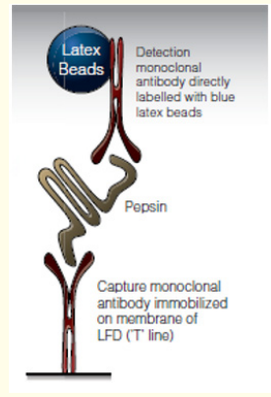

Figure 4: *A simplified visual representation of how two unique monoclonal antibodies work together in Peptest [40].*

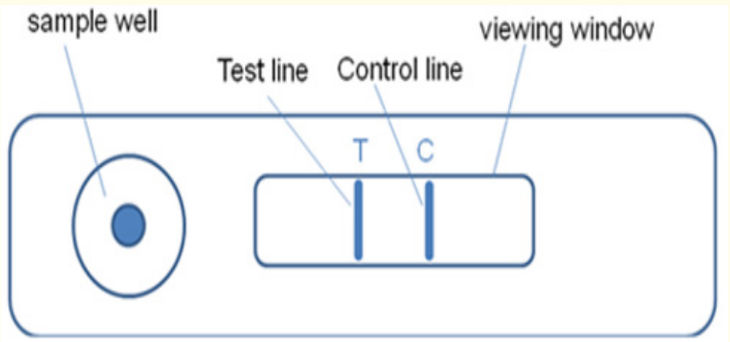

Figure 5: *A labelled annotation of Peptest displaying a positive pepsin result [41].*

Figure 6: *Peptest Cube reader designed to generate a pepsin concentration in ng ml^{-1}.*

The monoclonal antibodies are responsible for the rapid detection of pepsin, providing the test with sensitivity and specificity values of 88% and 87% respectively [12].

It is believed that this is the most favourable diagnostic technique for the patient due to its non-invasive nature - the recommendation is for the patient to collect three saliva samples before sending them to the laboratory for analysis. In the laboratory, the samples are analysed and processed allowing results to be issued to the patient within the same day. This expressive turn-around from sample collection to receiving results reiterates the quick speed and convenience of Peptest, increasing its recognition and allowing the device to be available worldwide [12]. The availability of Peptest has made pepsin detection in clinical samples far easier and this is reflected in the number of gastrointestinal and respiratory diseases now identified as strongly associated with pepsin as listed in table 3.

Gastro-esophageal reflux disease (GERD)
Extra Esophageal Reflux (EER)
Laryngopharyngeal Reflux (LPR)
Chronic cough
Asthma
Sinusitis
Cystic fibrosis (CF)
Lung allograph rejection
Otitis media with effusion
Non-allergic rhinitis
Chronic Pharyngitis
Chronic obstructive pulmonary disease (COPD)
Idiopathic Pulmonary Fibrosis (IPF)

Table 3: *Pepsin detection in association with gastrointestinal and respiratory diseases.*

Invasive diagnostic tests for pepsin and reflux disease

The majority of GERD diagnostic techniques are invasive, expensive, time consuming and not preferred by the patient due to their discomfort, however the invasive 24-hour pH monitoring technique is currently the 'gold-standard' for diagnosing GERD [42]. Despite its prominence in diagnosing GERD, 24-hour pH monitoring has low sensitivity ranging between 50 - 80% [38]. This low sensitivity value is due to 24-hour pH monitoring not being capable of detecting weak, mildly acidic reflux events which occur at pH values less than 4 [43,44] with Hayat claiming the monitoring misdiagnosed 1/3 of all reflux cases [45]. This was in contrast to Peptest - a device suited to diagnose all reflux events [46].

Furthermore, Samuels and Johnston reported 24-hour pH monitoring has an extremely low reproducibility rating of 55% [16], allowing Farhath and colleagues' research on reflux in neonates and Fox provided an explanation for this low reproducibility. For example, the majority of neonates produce minimal stomach acid resulting in an unreliable pH to be detected. Furthermore, in premature neonates the pH of the stomach is often greater than 4 for up to 90% of the time [13,28]. In addition, Fox stated that symptoms of reflux altered daily, implying different reflux results would be recorded if 24-hour pH monitoring was repeated monthly compared to the results recorded if the monitoring time was extended to 3-4 days confirming the techniques lacked reproducibility [47].

Shields., *et al.* believed another development of 24-hour pH monitoring - pH monitoring combined with multichannel intraluminal impedance (pH-MII) - was set to be classified as the new 'gold-standard' for diagnosing GERD and EER due to the advanced knowledge and understanding it provided [48]. This additional understanding included the formation of a relationship between the reflux event and the symptom association as well as changes in oesophageal pH [49]. The technique works through MII's ability to detect changes in resistance on alternating electrical currents. This means the technique does not rely on pH change, allowing this diagnostic method to be more accurate and dependable through increasing the sensitivity to detect GERD [44,50]. This novel way of diagnosing GERD and EE offered many additional advantages compared to conventional 24-hour pH monitoring, including its ability to detect non-acidic reflux events occurring at pH values greater than 4 (due to the presence of multichannel intraluminal impedance) concurrently to detecting acid reflux events [50], increasing the technique's sensitivity [51]. However, pH-MII is not a widely available technique for diagnosing GERD [50].

Upper gastrointestinal endoscopy (UGE) is reported to be the most disputed diagnostic technique; Aviv claims endoscopic examinations are adequate to diagnose reflux disease if patients have reported suffering from reflux associated symptoms [52]. Contrastingly, Kim states UGE was not a satisfactory method for diagnosing GERD as the technique cannot distinguish between pathological / physiological reflux [39].

Figure 7 illustrates an algorithm of when Peptest™ (pepsin detection) could be typically used in clinical practice to determine the presence of reflux disease and a suggested treatment regime.

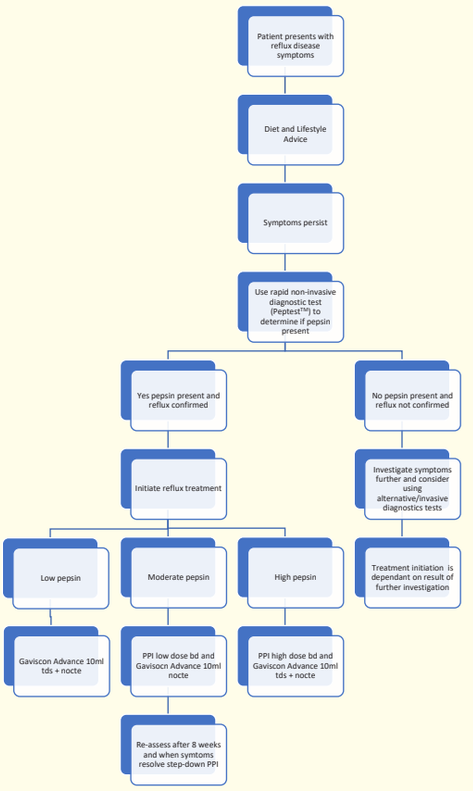

Figure 7: *Algorithm for using pepsin diagnostic test to determine if reflux present.*

Cell biology of pepsin endocytosis

Via endocytosis, dormant pepsin is taken up by receptor cells [53] situated on the mucosal epithelial. During this process, pepsin enters the golgi apparatus triggering a sequence of chemical reactions to occur, resulting in damaged cells [33]. Pearson and Parikh as well as Luebke report altered gene expression in stress, and damage to the mitochondria to be present after pepsin endocytosis [21,53].

How pepsin damages the cells?

Pepsin is the active form of pepsinogen, responsible for causing damage in reflux disease. This is due to gastric juice damaging mucosal defences [33] allowing pepsin to bind to laryngeal cells [54], where it can remain dormant until re-acidification occurs either from hydrochloric acid in the gastric juice, from consuming acidic drinks including fruit juice [44] or via endocytosis where it is then reactivated [4].

As pepsin can remain dormant up to pH 8, Bulmer and colleagues displayed pepsin alone is the main component of gastric juice to cause injury. As pepsin is active over a wide range of pH values [19], they visually noted damage when compared to stomach acid alone [55]. However, controversy in the literature was displayed by Tack who believed the combined effects of pepsin and acid were responsible for causing major damage to the oesophagus [56] due to the link between reflux disease and pepsin only being found recently.

Damaging effects of pepsin in mammals

Pepsin is known as a major aggressive enzyme, due to the vast amount of damage it may cause to tissues and organs [57] if secreted out of the stomach. Proven to cause a plethora of damaging effects in all mammals, pepsin has been widely documented in the literature. The most serious, life threatening effect pepsin possess in humans is its ability to contribute to laryngeal cancer as well as Barrett's oesophagus - a condition where the lining of the oesophagus replicates the lining of the intestine [58]. Furthermore, at low pH, pepsin is more prevalent in damaging tissues in the lungs and in preterm neonates, which can progress into lung disease [28].

Gotley and colleagues state pepsin is responsible for causing destruction to the oesophagus [59] with Wood and colleagues confirming when anatomical walls are damaged, pepsin is able to enter the oesophagus causing laryngeal damage [34], potentially resulting in cancer. Additionally, research has displayed pepsin to weaken protective proteins in the larynx through destroying the golgi apparatus and mitochondria [60,61].

Research performed on canines, felines, rabbits and pigs has provided further insight into the damaging effects of pepsin, proving it is the major aggressive enzyme in refluxate. The results displayed an increase in oesophageal damage regardless of pH. Furthermore, the rabbit model confirmed oesophageal damage due to pepsin destroying the proton barrier allowing a higher proportion of pepsin to adhere to the laryngeal cells, ready for re-acidification or endocytosis. Similarly, studies conducted on pig and canine models displayed destruction to the larynx at weakly acidic pH, contrasting to the damaging effects of stomach acid alone [42].

In mammalian tissue, pepsin has also been shown to be involved in neuroendocrine tumours. The incidence of type 1 gastric neuroendocrine tumours (T1-GNETs) has significantly increased and are associated with chronic atrophic gastritis (CAG) where the stomach becomes unable to produce sufficient amounts of pepsinogen and pepsin due to gastric chief cell injury. T1-GNETs tend to display a nearly benign behaviour and a low risk of progression or metastasis. Further studies are needed to identify appropriate treatment regimes for these tumours [62]. Studies have also shown that pepsin in LPR has the potential to contribute to the development of laryngopharyngeal carcinogenesis. Pepsin plays its role through promoting IL-8 signalling enhancing cell proliferation and metastasis [63].

Survival of pepsin in the cells

Dormant pepsin is recorded to survive in the cells for more than 24 hours at pH 7. However, activity loss is experienced if pepsin is dormant for a longer period - re-acidified pepsin only possesses 80% of its original activity [34]. Conversely, pepsin absorbed through the endocytosis mechanism has a much shorter survival time in the cells where it is only detectable for 6 hours [46] before it is excreted via kidney filtration [31].

Conclusion

Damage to the upper gastrointestinal tract affect a great number of people, with pepsin being recognised as a major causative factor. GERD and more recently the awareness of EER and LPR are becoming more prevalent affecting patient's quality of life. Despite invasive diagnostic techniques being the current gold-standard, the development of a rapid non-invasive test; Peptest, has led to the detection of salivary pepsin in reflux disease and is becoming the new gold-standard for detecting reflux disease.

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