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Alendronate effect in esophagus, stomach and liver: An animal based pathological study

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Summary. Bisphosphonates are commonly used in clinical practice. Their effectiveness is indisputable, however their adverse effects, especially in the GI tract, are still controversial. In our report, we demonstrate pathological findings of the effect of systematic alendronate administration in esophagus, stomach and the liver of an *in vivo* animal model of 15 Wistar rats. Light microscopy with immunohistochemistry and electron microscopy were used. Microscopic findings of inflammation of the stomach and mild hepatic dysfunction were observed. Conclusively, alendronate can potentially affect gastric mucosa and liver function on this animal experimental model.

Key words: Alendronate, Esophagus, Stomach, Liver, Immunohistochemistry, Electron microscopy

Introduction

Bisphosphonates (BP) are commonly used in clinical practice (Jeal et al., 1997; Lanza et al., 2000; Sener et al., 2005; Cremers and Papadopoulos, 2011; Costa et al., 2013). They are divided into nitrogen containing bisphosphonates (alendronate, pamidronate, ibandronic acid, risedronate, zoledronate, olpandronate, neridronate) and non-nitrogen containing bisphosphonates (Lanza et al., 2000; Cremers and Papadopoulos, 2011). They act as osteoclast selective inhibitors by reducing osteoclasts' effect in site of increased bone remodeling and therefore increase bones' density and forestall osteoporotic bone fractures (Lanza et al., 1998).

Bisphosphonates are absorbed by the gastrointestinal (GI) tract and rapidly distributed from the plasma (6 h) (Jeal et al., 1997; Costa et al., 2013), achieving maximum concentration in thyroid gland, bone marrow, cartilage, kidney, aorta and spleen (Cremers and Papadopoulos, 2011) Oral bioavailability is extremely small (0.9-1,8%) (Lieverse, 1998) and relative to gastric pH, food consumption, and calcium and magnesium levels in the gastric fluid (Lieverse, 1998). Regarding metabolism, only etidronate and clodronate are metabolized, being however unknown how these metabolites are excreted and what's their bioavailability (Cremers and Papadopoulos, 2011). Most of them are slowly eliminated by the kidneys and excreted unchanged into urine and in a small percentage into bile (Cremers and Papadopoulos, 2011).

Although their clinical effectiveness is undisputable, BPs have been associated with many adverse effects, mainly gastrointestinal (GI), such as epigastric pain, dyspepsia, nausea and vomiting. These were presumed to the result of upper GI irritation (Bauer et al., 2000; Lanza et al., 2000; Carrere et al., 2002; Sener et al., 2005; Papapetrou, 2009; Costa et al., 2013). De Groen et al. presented 3 case reports in which he associated the use of alendronate with esophageal and gastric ulceration, consistent with drug's direct toxic effect to the mucosa with direct recovery when the responsible drug(alendronate) was stopped (De Groen et al., 1996). However, there is still a controversy regarding the significance of GI adverse effects between BP and

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placebo users (Bauer et al., 2000; Cryer and Bauel, 2002). Furthermore, endoscopy studies were not able to establish a correlation between esophageal mucosal lesions and BP administration and had been controversial regarding their gastric effects (Lanza et al.,1998; Cryer and Bauer, 2002). Pathophysiology is still unclear (Costa et al., 2013). Although esophagitis was correlated with the retention of the alendronate capsule in the esophagus, later *in vivo* studies have correlated that effect with daily exposure of alendronate in lower gastric pH conditions (De Groen et al., 1996; Cryer and Bauer, 2002).

Gastric mucosal microcirculation plays a fundamental role in protection against ulceration (Sørbye and Svanes, 1994; Ceranowicz et al., 2009). Mucosal microcirculation was also found to be regulated by gastrointestinal hormones like ghrelin, which had proven to have a protective effect on experimental rat models of gastric mucosal damage, by increasing mucosal blood flow and mucosal cell proliferation (Ceranowitz et al., 2009, 2015). In previous studies alendronate was found to increase the susceptibility of rat gastric mucosa to damage and to delay gastric ulcer healing (Elliott et al., 1998; Kanatsu et al., 2004). Elliott et al had shown that alendronate did not affect gastric acid production, prostaglandin synthesis or mucosal blood flow (Elliot et al., 1998). However, further studies had shown that alendronate caused exfoliation of epithelial cells, significant increase in gastric mucosal blood flow and slight hemorrhagic damage (Kanatsu et al., 2004). Other studies have claimed that alendronate disrupts phospholipid protective effect within adherent mucosal gel (Lichtenberger et al., 2000). Its effect in microcirculation remains still uncertain.

The aim of this study was to evaluate the extent of the esophageal, gastric and hepatic cell damage after alendronate administration, based on an *in vivo* mice model and using, optical and electron microscopy assistance.

Materials and methods

Fifteen female Wistar rats, 12-month old, weighing approximately 500 g, were used. Rats were housed in stainless steel cages, with one rat per cage, 12h light-dark cycle and relative humidity and temperature control. This study was previously approved by the Bioethics Committee of the Medical School of the Aristotle University of Thessaloniki (Approval date:5-11-2015/Project Identification Code:165).

The experimental group consisted of 10 randomly allocated animals. Five of them were used as controls. Alendronate (Fosamax, Merck) was administered orally to animals at a dose of 0.05 mg/kg body weight/week dissolved in 3cc normal saline for a period of 13 weeks. The drug was administered thirty minutes prior to breakfast. Human dosing protocol was used. The duration of the study was 13 weeks and after euthanasia, the liver, stomach and esophagus of the animals was extracted, and specimens were processed for electron and optical microscopy examination.

Light microscopy-immunohistochemistry

Tissue sections from both groups were fixed in 10% of formalin, embedded in paraffin and cut into thin sections of 3-4 μm. First, deparaffinization was performed in xylene. Afterwards, specimens were immersed in absolute alcohol with degressive densities 100%, 96% and 70% v/v consecutively. Finally, they were rinsed out with distilled water. Antigen retrieval was performed by incubation at 650°C. Following this, specimens were first rinsed with PBS buffer, then incubated in H_2O_2 for 5 min to quench endogenous peroxidase activity and finally rinsed again with PBS buffer. Thereafter, specimens were covered with a solution of the primary tonic monoclonal antibody against MPO (myeloperoxidase). Eventually, they were washed using WAS solution.

For the detection of immunohistochemical staining, specimens were firstly immersed in Post-Primary solution. After being washed, they were immersed in polymer solution and then in chromogen-diaminobenzidine (DAB) solution. Then, they were stained with Hematoxylin- Eosin. Specimens were rinsed out in tap water and dehydrated with escalating densities of ethanol solution and xylene (70, 96 and 100% v/v consecutively). Finally, they were covered with tape, placed in glass plates and immersed in Canada balsam. Previously reported immunohistochemical staining procedure was repeated twice. Specimens were examined using an optical microscope (Zeiss) and photographs were taken using a camera (Contax), attached to the microscope. Staining was evaluated by two independent reviewers.

Transmission electron microscopy

Esophagus, gastric and liver tissue samples were sectioned into pieces with size below 1 cm^3. Afterwards, they were placed into glutaraldehyde 3% for 2 hours, then into osmium tetra oxide (OsO_4) 1% for 1 hour. The staining process was performed with uranyl acetate 1% for 16 hours. Then, samples were dehydrated with increased ethanol concentrations. Afterwards, sample tissues were embedded into Epon resin and ultra-thin sections were cut (600-900 Å). Finally, sections were stained with Reynolds's stain. Samples were observed with a Transmission Electron Microscopy (JEOL 1011).

Results

Esophagus

Light microscopy

No pathological changes were observed. Neither epithelial hyperplasia nor intraepithelial inflammatory

infiltration were present. The muscularis mucosa, the submucosa and the muscularis propria were normal (Fig. 1).

Electron microscopy

No pathological changes were observed. However, in four samples a partial disorganization of the muscular fibrils was found.

Stomach

Light microscopy

Out of fifteen cases, nine showed areas of squamous metaplasia within the glandular epithelium. The metaplastic squamous epithelium was hyperplastic compared with the non-glandular epithelium with basal cell hyperplasia and elongated rete ridges. Hyperkeratosis was also observed (Fig. 2). The subepithelial stroma was fibrotic, accompanied by inflammatory infiltration, composed mainly by eosinophils. Mild intraepithelial infiltration with lymphocytes was also present (Fig. 3).

The immunohistochemical stain for Myeloperoxidase (MPO) stained the eosinophils and neutrophils of the stroma (Fig. 4).

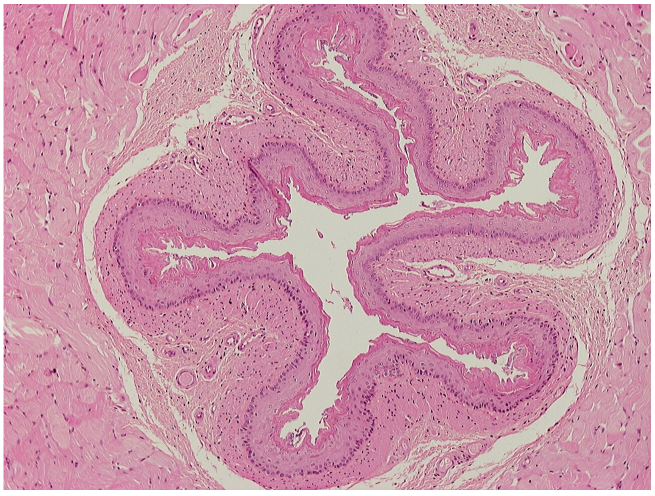

Fig. 1. Medium power view of the esophagus shows no pathological changes. H&E. x 100.

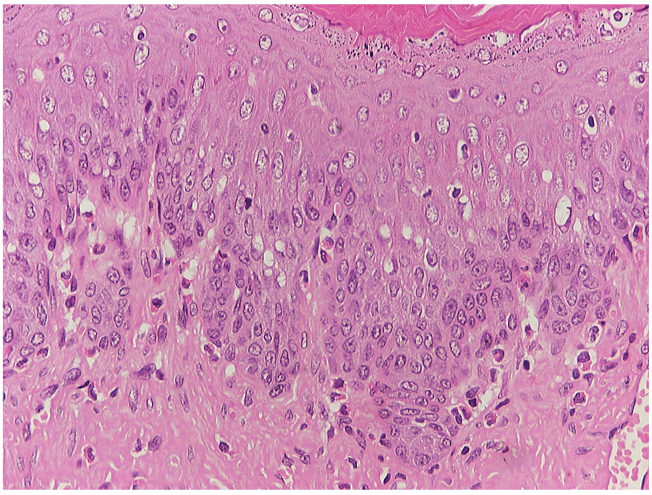

Fig. 3. High power view shows a site of squamous metaplasia of the glandular epithelium of the stomach with inflammatory infiltration of the subepithelial stroma, composed mainly of eosinophils. Intraepithelial lymphocytes are also present. H&E. x 400.

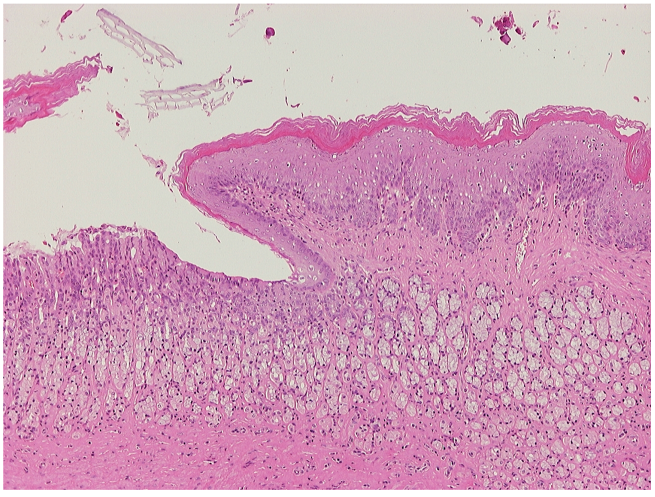

Fig. 2. Medium power view shows a site of squamous metaplasia of the glandular epithelium of the stomach with hyperplasia, hyperkeratosis and elongated rete ridges. H&E. x 100.

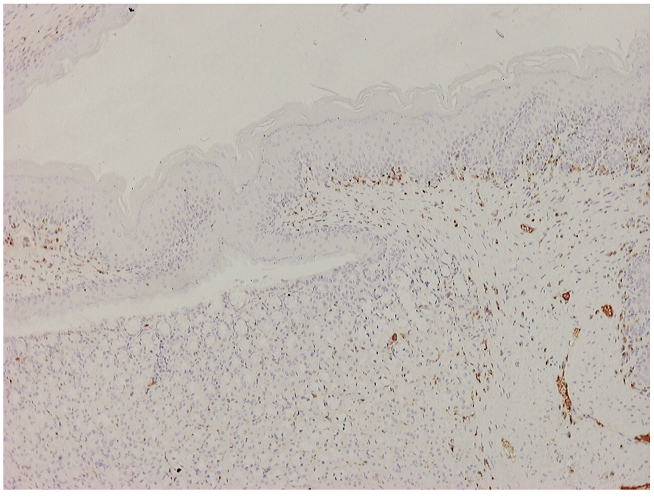

Fig. 4. Increased number of eosinophils and neutrophils at a site of squamous metaplasia of the glandular epithelium of the stomach. Immunoperoxidase for MPO with hematoxylin counterstain. x 100.

Electron microscopy

Gastric glands and their cells were normal in their majority, with minor histological changes in three of them. These changes consisted of vacuole like formations within gastric cells along with rupture of their nuclear membrane, pyknosis of the nuclei and degeneration of mucosal cells.

Liver

Light microscopy

No pathological changes were observed (Fig. 5). All three elements of the portal triad (veins, arteries and intrahepatic ducts) were normal (Fig. 6). Neither inflammatory infiltration, nor bile ducts reaction was observed. The hepatic trabeculae were of normal thickness (1-2 cells thick) and the hepatic sinusoids were not dilated. Fibrosis was not present.

Electron microscopy

Evidence of mild dysfunction of the hepatic cells was found in all sections and there was an extensive depletion of the glycogen in the hepatocytes. Dilated sinusoids and increased number of vacuoles in the cytoplasm were also seen. Moreover, the microvilli of hepatocytes on Disse's space was absent in multiple foci in comparison with control. Collagen fibers were detected in the extracellular matrix.

Discussion

Numerous histopathological studies had investigated BPs' side effects on upper GI tract in the past, most of them based on light microscopy (De Groen et al., 1996; Elliot et al., 1998; Lichtenberger et al., 2000; Dobrucali et al., 2002; Kanatsu et al., 2004). To our knowledge, there were no other experimental studies on rats, regarding systematic BP administration effect on liver, esophagus and stomach using both electron and light microscopy.

What is more, our study group was treated with the usual human dose of alendronate. Alendronate damage to the gastric and esophagus mucosa was known to be related to the concentration and dosage of the drugs used (De Groen et al., 1996; Wallace et al., 1999). Other researchers used different concentrations (Elliot et al., 1998) not always according to the human model (Blank et al., 1997; Wallace et al., 1999; Kanatsu et al., 2004; Sener et al., 2005). Furthermore, alendronate was not always administered orally (Elliot et al., 1998; Wallace et al., 1999; Kanatsu et al., 2004). Of course, different methodology in drug dosage and route of administration led to various findings which differ from study to study.

Microcirculation in gastric mucosa, regulated by ghrelin was found to be of great importance for its protection and healing and its changes can induce inflammation and ulceration (Ceranowitz et al., 2009; Ceranowitz et al., 2015). Most common findings in previous histopathological studies, regarding alendronate effect in gastric mucosa, were epithelial damage and exfoliation (Elliot et al., 1998; Lichtenberger et al., 2000; Kanatsu et al., 2004; Sener et al., 2005), hemorrhage (Kanatsu et al., 2004), inflammatory infiltration and antral or corpus mucosal necrosis (Blank et al., 1997; Wallace et al., 1999; Lichtenberger et al., 2000; Sener et al., 2005), degeneration of mucosal cells (Sener et al.,2005), loss of surface and glandular epithelium and collapse of gastric glands (Blank et al., 1997), disorganization of gastric glands in deep zones (Lichtenberger et al., 2000; Sener et al., 2005), hypocellular mucosa (Blank et al., 1997), pyknosis of remaining nuclei (Blank et al., 1997), increased mucus

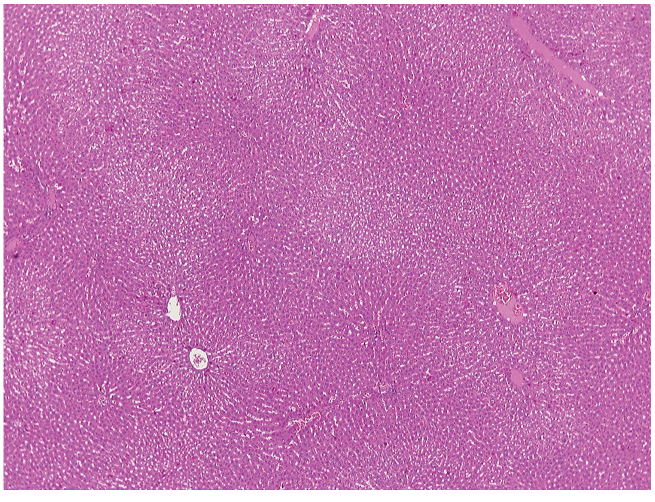

Fig. 5. Low power view of the normal liver. H&E. x 40.

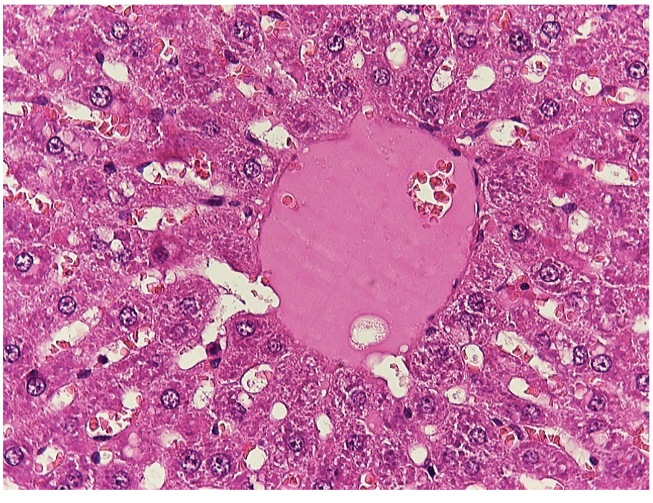

Fig. 6. High power view of the normal liver shows a central vein. H&E. x 400.

on the epithelium (Blank et al., 1997; Elliot et al., 1998) and severe dilatation of gastric pits (Sener et al., 2005). Our results are mostly in accordance with those previously reported. Myeloperoxidase (MPO), produced by degranulation of neutrophils, had proven to be a useful indicator of oxidative stress and inflammation and was found to be present in peptic ulcers as well (Khan et al., 2018). In accordance with previous reports, we have also detected MPO along with neutrophil infiltration in gastric mucosa post alendronate administration.

Regarding esophagus, no pathological changes were observed with both electron and light microscopy similar with previous research, which did not report major changes in the esophagus (Dobrucali et al., 2002).

A few case reports have correlated alendronate use with hepatic dysfunction (Lichtenberger et al., 2000; Pérez et al., 2001; Yanik et al., 2007). Besides alendronate, other bps had also been associated with hepatic dysfunction or lesion (Wallace et al.,1999). According to one case report, alendronate was the cause of severe hepatitis in a 77- year old woman. Hepatitis was resolved after voiding the responsible drug (Lieverse, 1998). Furthermore, alendronate may affect liver function, by inhibiting hepatic synthesis of cholesterol (Halabe et al., 2000). Our study agreed with previous reports and electron microscopy had shown evidence of mild hepatic damage (extensive depletion of the glycogen, increase number of vacuoles, absence of microvilli in peri-sinusoidal space). Hepatic stellate cells (HSCs), also known as perisinusoidal cells are the major cell type involved in liver fibrosis (Panebianco et al., 2017). Liver fibrosis is the formation of scar tissue in response to liver damage. When liver is damaged, these cells form collagen scar tissue, which can lead to cirrhosis. Therefore, the presence of collagen in the extracellular matrix, which was also noticed in our electron microscopy study, may indicate hepatic damage (Panebianco et al., 2017). Conclusively, alendronate can potentially cause mild hepatic damage (Ni et al., 2013; Xu et al., 2014; Korulczuk et al., 2016; Panebianco et al., 2017).

Our study demonstrated an *in vivo* animal model of possible upper GI toxicity of alendronate administration. However, there were limitations, such as the small number of animals used, the absence of an adequate number of controls, lack of multiple sections and of course immunochemistry. Further research is needed, in order to enlighten the full spectrum of BP side effects to human tissue.

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