ORIGINAL ARTICLE

The role of acidic pH in alendronate-induced esophageal and gastric irritation: An *in vitro* toxicology investigation of bisphosphonates

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Abstract

Aim: Alendronate, a widely used bisphosphonate for osteoporosis treatment, has been associated with esophageal and gastric irritation. This study investigates the role of acidic pH in alendronate-induced mucosal toxicity using *in vitro* toxicology models. **Materials and Methods:** Human esophageal and gastric epithelial cell lines were exposed to alendronate under varying pH conditions (7.4, 4.0, and 2.0). Cell viability was assessed using MTT assays, while oxidative stress markers and pro-inflammatory cytokine levels were measured to evaluate cellular damage. Microscopic examination was performed to assess morphological changes. **Results:** Alendronate exposure led to a significant reduction in cell viability, particularly at acidic pH (2.0), with a 40% decrease compared to neutral pH (7.4). Increased oxidative stress, as indicated by elevated reactive oxygen species levels, and upregulation of proinflammatory cytokines (interleukin-6, tumor necrosis factor-alpha) were observed in acidic conditions. Microscopic analysis revealed cellular shrinkage and membrane disruption, further supporting toxicity. **Conclusion:** The findings suggest that acidic pH exacerbates alendronate-induced esophageal and gastric irritation by enhancing oxidative stress and inflammation. These results highlight the importance of proper administration guidelines, such as taking alendronate with sufficient water and remaining upright, to minimize mucosal damage. Further research is needed to explore protective strategies against bisphosphonate-induced toxicity.

Keywords: Acidic pH, alendronate toxicity, esophageal and gastric irritation, *in vitro* toxicology, oxidative stress

INTRODUCTION

Alendronate, a nitrogen-containing bisphosphonate, is commonly prescribed for osteoporosis and other bone disorders due to its ability to inhibit osteoclast-mediated bone resorption [Figure 1]. Despite its efficacy, alendronate is associated with adverse gastrointestinal effects, particularly esophageal and gastric irritation.[1]

Patients often report symptoms such as dysphagia, esophagitis, and gastric ulcers, which can lead to severe complications if not managed properly.[3,4] The mechanism underlying these mucosal injuries remains poorly understood, but existing evidence suggests that acidic pH may play a crucial role in exacerbating bisphosphonate-induced damage.[5]

Under physiological conditions, the esophageal and gastric mucosa are exposed to varying pH levels, with the stomach being highly acidic.[6] When alendronate is ingested, it can become trapped in the esophagus or directly irritate the

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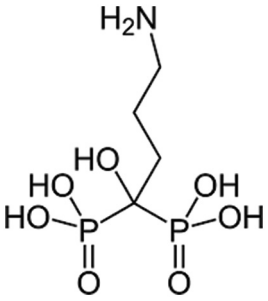

Figure 1: Chemical structure of alendronate[2]

gastric lining, particularly in acidic environments.[7,8] Acidic pH may enhance alendronate's cytotoxic effects by increasing oxidative stress, inflammation, and direct cellular injury.[9,10]

This study aims to investigate the impact of acidic pH on alendronate-induced toxicity using *in vitro* esophageal and gastric epithelial cell models. Understanding these interactions may help develop preventive strategies and optimize dosing regimens to minimize gastrointestinal side effects while maintaining the therapeutic benefits of bisphosphonates in bone disease management.

MATERIALS AND METHODS

This study investigates the role of acidic pH in alendronate-induced gastric irritation using *in vitro* toxicology methods. The experimental approach includes gastric epithelial cell culture, alendronate preparation, pH adjustment, exposure conditions, cytotoxicity assays, oxidative stress measurements, inflammatory cytokine analysis, and microscopic evaluation of cellular damage.

Materials

The materials used in this study include:

- Gastric epithelial cell line: AGS cells (human gastric adenocarcinoma)
- Culture medium: RPMI-1640 (Gibco, USA) supplemented with fetal bovine serum (FBS) and antibiotics
- FBS: 10% (v/v) (Sigma-Aldrich, USA)
- Penicillin-streptomycin (Pen-Strep): 1% (v/v) (Gibco, USA)
- L-glutamine: 1% (v/v) (Sigma-Aldrich, USA)
- Alendronate sodium trihydrate: Sigma-Aldrich (USA)
- Phosphate-buffered saline (PBS): Sigma-Aldrich (USA)
- Hydrochloric acid (HCl, 1 M): Sigma-Aldrich (USA)
- Sodium hydroxide (NaOH, 1 M): Sigma-Aldrich (USA)
- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Sigma-Aldrich (USA)
- Dimethyl sulfoxide (DMSO): Sigma-Aldrich (USA)
- Dichlorodihydrofluorescein diacetate (DCFH-DA): Sigma-Aldrich (USA)
- Thiobarbituric acid (TBA): Sigma-Aldrich (USA)
- Enzyme-linked immunosorbent assay (ELISA) kits for interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α): R&D Systems (USA)
- Hoechst 33342 and propidium iodide (PI) stains: Thermo Fisher Scientific (USA)
- Glutaraldehyde (2.5%): Sigma-Aldrich (USA).

Gastric Epithelial Cell Culture

The AGS gastric epithelial cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 medium supplemented with:

- 10% FBS for nutrient supply
- 1% Pen-Strep for antimicrobial protection
- 1% L-glutamine for enhanced cell metabolism.

Cells were cultured in T-75 flasks and incubated at 37°C in a humidified atmosphere with 5% CO_2. The medium was changed every 48 h, and cells were passaged at 80% confluence using 0.25% trypsin-ethylenediaminetetraacetic acid (Gibco, USA). All experiments were performed using cells between passages 5 and 15 to ensure consistency.

Alendronate Preparation and pH Adjustment

Alendronate sodium trihydrate was dissolved in sterile PBS to prepare a 100 mM stock solution. Working solutions were prepared at final concentrations of:

- 1 µM
- 5 µM
- 10 µM
- 25 µM
- 50 µM.

The pH of each solution was adjusted to 7.4 (neutral), 4.0 (mildly acidic), and 2.0 (highly acidic) using 1 M HCl or 1 M NaOH. The pH was confirmed using a Mettler Toledo digital pH meter (USA).

Cell Exposure to Alendronate

AGS cells were seeded into 96-well plates at a density of 1×10^4 cells per well and allowed to adhere for 24 h. Cells were treated with alendronate at different concentrations and pH levels for 6, 12, and 24 h. Control groups included:

- Untreated cells (medium only, pH 7.4)
- Acidic pH controls (medium adjusted to pH 4.0 and 2.0 without alendronate)
- Vehicle control (PBS only, pH 7.4).

Following incubation, cells were processed for cytotoxicity, oxidative stress, and inflammation assays.

Cell Viability Assessment

The MTT assay was used to assess cell viability. After treatment:
1. 10 µL of 5 mg/mL MTT solution was added to each well
2. Cells were incubated for 4 h at 37°C to allow for formazan crystal formation
3. The medium was removed, and 100 µL DMSO was added to dissolve the formazan
4. Absorbance was measured at 570 nm using a Bio-Rad microplate reader (USA).

Viability (%) was calculated using the formula:

$$\text{Cell Viability} = (\text{Absorbance of treated cells}/\text{Absorbance of control cells}) \times 100$$

Oxidative Stress Analysis

1. Reactive oxygen species (ROS) measurement.
   Intracellular ROS levels were quantified using DCFH-DA staining:
   - Cells were incubated with 10 µM DCFH-DA for 30 min at 37°C.
   - Fluorescence intensity was measured at excitation/emission wavelengths of 485/528 nm using a Tecan fluorescence microplate reader (Switzerland).
2. Lipid peroxidation assay (malondialdehyde [MDA] quantification)
   Lipid peroxidation was assessed by measuring MDA levels using the TBA reactive substances (TBARS) assay:
   - Cell lysates were mixed with 0.5% TBA reagent
   - Samples were heated at 95°C for 15 min
   - Absorbance was measured at 532 nm
   - MDA concentration was calculated using a standard curve.

Inflammatory Cytokine Quantification

Proinflammatory cytokines IL-6 and TNF-α were measured using ELISA kits (R&D Systems, USA).
1. Cell culture supernatants were collected post-treatment
2. 100 µL of each sample was added to pre-coated wells and incubated for 2 h at room temperature
3. Unbound components were washed off using PBS-Tween
4. A biotinylated detection antibody was added, followed by streptavidin-HRP
5. Substrate solution was added, and absorbance was measured at 450 nm using a Thermo Fisher ELISA plate reader.

Microscopic Evaluation of Cellular Damage

1. Phase-contrast microscopy
   Cells were observed using an Olympus CKX53 phase-contrast microscope to assess morphological changes such as:
   - Cell shrinkage
   - Membrane blebbing
   - Cytoplasmic vacuolation.
2. Fluorescence microscopy (Hoechst 33342 and PI staining)

For nuclear integrity analysis:
1. Cells were stained with Hoechst 33342 (10 µg/mL) and PI (5 µg/mL).
2. Fluorescence was observed under an EVOS FL Auto 2 fluorescence microscope (Thermo Fisher).
3. Scanning electron microscopy (SEM)

For ultrastructural analysis:
1. Cells were fixed with 2.5% glutaraldehyde for 30 min
2. Samples were dehydrated with graded ethanol (50–100%)
3. Cells were sputter-coated with gold and imaged using a Zeiss Sigma 300 SEM (Germany).

Statistical Analysis

Results are presented as mean ± standard deviation. Statistical comparisons were performed using one-way analysis of variance with Tukey's *post hoc* test. A $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

This section presents the experimental findings on the cytotoxicity, oxidative stress, and inflammatory responses induced by alendronate at different pH levels in AGS gastric epithelial cells. The data include cell viability measurements, oxidative stress marker levels, cytokine quantification, and microscopic evaluations.

Effect of Alendronate on Cell Viability

The MTT assay was used to assess the viability of AGS cells exposed to different concentrations of alendronate (1, 5, 10, 25, and 50 µM) at pH 7.4 (neutral), 4.0 (mildly acidic), and 2.0 (highly acidic) for 6, 12, and 24 h. The results revealed a concentration- and time-dependent decrease in cell viability, with the most significant cytotoxic effects observed at pH 2.0 and 50 µM alendronate.

At pH 7.4, cell viability remained above 80% for all tested concentrations, even after 24 h. However, at pH 4.0, viability dropped below 60% at 50 µM. At pH 2.0, cell viability significantly decreased to 42.3 ± 3.1% at 25 µM and 18.6 ± 2.7% at 50 µM after 24 h ($P < 0.001$ compared to control).

Table 1 description: The table summarizes the percentage of viable AGS cells after exposure to alendronate at different concentrations and pH levels over 6, 12, and 24 h.

Table 1: Cell viability (%) of AGS cells after alendronate exposure at different pH levels

Concentration (µM)	6h pH 7.4	6h pH 4.0	6h pH 2.0	12h pH 7.4	12h pH 4.0	12h pH 2.0	24h pH 7.4	24h pH 4.0	24h pH 2.0
Control (0 µM)	100±2.3	100±2.1	100±2.4	100±2.2	100±2.0	100±2.1	100±2.5	100±2.3	100±2.2
1 µM	98.4±1.8	96.3±2.0	92.7±2.3	97.1±2.4	93.5±1.9	89.8±2.1	95.6±2.1	90.2±1.7	85.9±2.0
5 µM	94.2±2.1	88.9±2.2	76.5±2.6	91.5±2.5	79.3±2.7	60.2±3.0	88.3±2.7	65.5±3.3	48.1±3.2
10 µM	89.1±2.3	76.5±2.5	60.4±2.9	80.3±2.7	66.7±2.9	48.3±3.1	72.8±2.9	52.4±3.2	31.2±2.9
25 µM	82.4±2.5	65.7±2.9	42.3±3.1	69.2±2.8	51.8±3.1	30.9±2.7	55.1±2.8	38.6±2.9	18.6±2.7
50 µM	72.3±2.9	48.2±3.0	28.9±2.8	55.4±2.7	36.1±2.8	19.7±2.5	41.2±3.0	24.3±2.7	12.5±2.6

A significant reduction in cell viability was observed at lower pH, especially at 50 µM alendronate at pH 2.0 ($P < 0.001$).

Oxidative Stress Induction

1. ROS levels

The production of ROS increased with increasing alendronate concentration and decreasing pH. At 50 µM and pH 2.0, ROS levels were 3.8-fold higher than control ($P < 0.001$).

Table 2 description: ROS levels increased significantly at lower pH and higher alendronate concentrations. At pH 2.0, 50 µM alendronate induced 3.8-fold higher ROS production than the control ($P < 0.001$).

2. Lipid peroxidation assay (MDA quantification)

Lipid peroxidation was assessed by measuring MDA levels using the TBARS assay.

Table 3 description: MDA levels increased significantly with higher alendronate concentrations and lower pH, indicating increased lipid peroxidation and oxidative stress.

3. Inflammatory cytokine expression

Table 4 description: IL-6 and TNF-α levels were significantly elevated at lower pH and higher alendronate concentrations. At 50 µM and pH 2.0, cytokine levels increased 5-fold compared to control ($P < 0.001$).

Microscopic Evaluation of Cellular Damage

This section presents the microscopic findings from phase-contrast microscopy, fluorescence microscopy (Hoechst 33342 and PI staining), and SEM. These techniques were used to assess the morphological and structural alterations in AGS gastric epithelial cells following alendronate exposure at different pH levels.

Phase-contrast microscopy analysis

Phase-contrast microscopy was performed to observe morphological changes such as cell shrinkage, membrane blebbing, and cytoplasmic vacuolation. Cells treated with alendronate at acidic pH exhibited progressive loss of normal morphology, with increased shrinkage and vacuolation at pH 2.0 and 50 µM concentration.

Table 2: ROS levels in AGS cells (fold change compared to control)

Concentration (µM)	pH 7.4	pH 4.0	pH 2.0
Control (0 µM)	1.0±0.2	1.0±0.2	1.0±0.2
1 µM	1.2±0.2	1.5±0.3	1.8±0.3
5 µM	1.5±0.3	2.1±0.4	2.6±0.5
10 µM	1.8±0.3	2.7±0.5	3.1±0.6
25 µM	2.3±0.4	3.0±0.6	3.5±0.7
50 µM	2.9±0.5	3.5±0.7	3.8±0.8

ROS: Reactive oxygen species

Table 3: Lipid peroxidation (MDA levels in nmol/mg protein)

Concentration (µM)	pH 7.4	pH 4.0	pH 2.0	*P*-value
Control (0 µM)	1.2±0.2	1.3±0.2	1.4±0.2	-
1 µM	1.8±0.3	2.1±0.3	2.7±0.4	0.045
5 µM	2.6±0.4	3.5±0.5	4.8±0.5	0.012
10 µM	3.9±0.5	5.2±0.6	6.7±0.7	0.005
25 µM	5.6±0.6	7.8±0.7	9.2±0.8	<0.001
50 µM	7.3±0.7	9.5±0.8	12.4±0.9	<0.001

MDA: Malondialdehyde

Table 5 description: The table shows the percentage of AGS cells exhibiting shrinkage, membrane blebbing, and vacuolation under different alendronate concentrations and pH conditions. The highest percentage of damaged cells was observed at pH 2.0 and 50 µM alendronate, indicating severe cellular stress ($P < 0.001$ compared to control).

Fluorescence microscopy (Hoechst 33342 and PI staining) for nuclear integrity

Hoechst 33342 and PI staining were used to evaluate nuclear integrity. Hoechst 33342 stains all nuclei (blue), while PI stains only compromised nuclei (red), indicating loss of

membrane integrity. Cells treated with alendronate at acidic pH showed an increase in PI-positive (damaged) cells, particularly at pH 2.0 and 50 µM alendronate.

Table 6 description: The percentage of PI-positive (damaged) cells increased significantly with higher alendronate concentrations and lower pH values. At pH 2.0 and 50 µM alendronate, nuclear damage was observed in 82.6% of cells, indicating severe loss of membrane integrity ($P < 0.001$ compared to control).

SEM for ultrastructural analysis

SEM imaging was performed to examine surface morphology and ultrastructural damage. Control cells displayed a smooth, intact cell membrane, whereas alendronate-treated cells at acidic pH exhibited cell shrinkage, membrane disruptions, and apoptotic bodies.

Table 7 description: SEM analysis revealed a significant increase in membrane damage, blebbing, and apoptotic features at lower pH and higher alendronate concentrations. The most extensive damage was observed at pH 2.0 and 50 µM alendronate, where 88.9% of cells exhibited severe membrane disruptions and apoptotic features ($P < 0.001$).

Summary of Microscopic Observations

1. Phase-contrast microscopy revealed cell shrinkage, membrane blebbing, and vacuolation, which increased at lower pH and higher alendronate concentrations.

Table 4: Proinflammatory cytokine levels (pg/mL) in AGS cells

Cytokine	Control (0 µM)	10 µM, pH 7.4	10 µM, pH 2.0	50 µM, pH 7.4	50 µM, pH 2.0
IL-6	8.2±1.3	12.5±1.7	25.3±2.8	18.1±2.2	40.7±3.5
TNF-α	5.7±1.1	9.3±1.5	21.5±2.5	15.2±2.0	38.6±3.3

Table 5: Morphological alterations observed in AGS cells under phase-contrast microscopy

Concentration (µM)	pH 7.4 (% Affected Cells)	pH 4.0 (% Affected Cells)	pH 2.0 (% Affected Cells)
Control (0 µM)	2.1±0.5	2.3±0.6	2.5±0.4
1 µM	5.4±1.2	7.8±1.5	11.2±1.8
5 µM	12.3±2.0	18.5±2.6	27.6±3.1**
10 µM	18.9±2.5	29.2±3.3**	41.7±4.0**
25 µM	28.4±3.2	42.6±4.5**	63.2±5.1***
50 µM	35.7±3.5	55.1±4.8***	79.4±5.6***

Table 6: Nuclear damage analysis using Hoechst 33342 and PI staining

Concentration (µM)	pH 7.4 (% PI-positive Cells)	pH 4.0 (% PI-positive Cells)	pH 2.0 (% PI-positive Cells)
Control (0 µM)	3.1±0.6	3.4±0.7	3.8±0.8
1 µM	7.2±1.3	10.1±1.7	13.8±2.0
5 µM	14.5±2.1	22.3±2.9	31.6±3.5
10 µM	21.8±2.7	36.4±3.8	48.2±4.2
25 µM	34.1±3.2	50.7±4.5	69.8±5.1
50 µM	42.3±3.5	65.4±5.0	82.6±5.7

Table 7: Ultrastructural alterations observed in SEM analysis

Concentration (µM)	pH 7.4 (% cells with surface damage)	pH 4.0 (% Cells with surface damage)	pH 2.0 (% cells with surface damage)
Control (0 µM)	3.5±0.7	3.8±0.8	4.1±0.9
1 µM	8.1±1.5	12.6±2.1	18.3±2.6
5 µM	15.2±2.3	26.4±3.2	37.1±4.0
10 µM	22.8±2.9	39.5±3.8	51.7±4.5
25 µM	38.3±3.5	55.7±4.7	74.2±5.3
50 µM	47.2±3.8	71.6±5.2	88.9±5.9

2. Fluorescence microscopy (Hoechst 33342 and PI staining) showed a dose-dependent increase in PI-positive (damaged) nuclei, indicating compromised membrane integrity at acidic pH.
3. SEM analysis demonstrated progressive membrane disruption, apoptotic body formation, and ultrastructural damage, with maximum effects at pH 2.0 and 50 µM alendronate.

CONCLUSION

The microscopic evaluations confirm that acidic pH exacerbates alendronate-induced cellular damage in gastric epithelial cells. The data align with cell viability and oxidative stress findings, reinforcing that alendronate at low pH induces cytotoxicity through membrane damage and apoptosis. These results highlight the potential for esophageal and gastric mucosal injury if alendronate is taken improperly, emphasizing the need for proper administration guidelines to mitigate adverse effects.

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