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Impact of Teriparatide Acetate on Caspase-9 Gene Expression in Relation to Histological and Tunnel Assay of Apoptosis in Rat Kidneys and Livers

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ABSTRACT

The study aimed to evaluate the effects of Teriparatide on liver and kidney tissues in rats and to examine the relationship between these effects and the gene expression of caspase-9 focusing on the mechanism of apoptosis. The experiment included 20 male rats divided into a control group and a Teriparatide-treated group, and administered 10 µg/kg subcutaneously daily for 30 days. Histological analysis revealed significant pathological changes in the liver including portal vein congestion, increased fibrous tissue, bile duct hyperplasia, degeneration, hepatocellular necrosis and sinusoidal dilation. In the kidneys were observations of glomerular atrophy, Bowman's space dilation, hyaline casts in the tubular lumen, vacuolar degeneration and necrosis of the tubular epithelial cells, along with hemorrhage and inflammatory cell infiltration. TUNEL assay showed high levels of apoptosis in the liver and kidney tissues of the teriparatide-treated group. Regarding Caspase-9 gene expression, all positive samples indicated the presence of the gene at the 153 bp site, suggesting its activation in liver and kidney tissues. There was a substantial increase in Caspase-9 gene expression in the liver and kidneys compared to the control group. The study also showed that high doses of Teriparatide have severe side effects on the liver and kidney tissues of rats, where there was a clear activation of the intrinsic caspase-9 apoptotic activation pathway and with TUNEL analysis. The kidneys and liver were also impacted to a greater extent.

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INTRODUCTION

Teriparatide acetate is a synthetic version of a hormone composed of 34 amino acids at the beginning of its sequence **(Liu *et al.*, 2022).** This medication is effective in building bone strength **(Estell and Rosen, 2021)** and is commonly used to treat various forms of osteoporosis by promoting bone growth and reducing fracture risk **(Chotiyarnwong and McCloskey, 2020; Anam and Insogna, 2021).** However, the use of hyperparathyroidism has been found to have an extended number of effects on liver conditions on enzymes such as liver and ALT kidneys. These AST effects, which may include the inflammation elevation or damage of the liver **(Lei *et al.*, 2020).** In rare cases, jaundice may occur, causing yellowing of the skin and eyes due to bilirubin buildup **(Dilfuza *et al.*, 2022).**

As for the kidney, hypercalcemia can cause other kidney-related problems, for instance, kidney stones, or worsen **(Reddi, 2022).** In some cases, acute kidney failure may also manifest in individuals with preexisting kidney conditions. Additionally, bone metabolism can be disrupted by changes in bone resorption rates that exceed bone formation rates **(Wawrzyniak and Balawender, 2022).** Detecting the impacts of Teriparatide often involves monitoring caspase 9 levels, given its crucial role in the intrinsic apoptotic pathway **(Yip, 2023).**

Caspase 9, an enzyme in this pathway, activates in response to stress or medication-induced damage, especially affecting liver and kidney functions. Its activation triggers a cascade effect on caspases like Caspase 3, which are essential in carrying out apoptosis **(Wang *et al.*, 2021).** Concurrently, assays and gene expression analyses of Caspase 9 provide insights into apoptotic processes within tissues or cells **(Kari *et al.*, 2022).** The rationale for these tests lies in the TUNEL assay's capability to detect apoptotic cells by identifying exposed DNA fragment ends resulting from autolysis **(Moldovan *et al.*, 2023).** Monitoring Caspase 9 levels serves as evidence for activating the apoptotic pathway where Caspase 9 plays a central role. Using both assays, researchers can confirm the occurrence of apoptosis in cells **(Avrutsky and Troy, 2021).**

Hence, this study aimed to explore acetates effects on major organs such as kidneys and liver through histopathological examinations and investigate how apoptosis correlates with Caspase 9 activation. Studying Caspase 9 levels helps us delve into the process that triggers cell death, whereas the TUNEL test gives us an idea of how many cells are undergoing apoptosis. By combining these insights, we can better grasp the connection between enzyme activity and the outcomes of apoptosis indicated by the TUNEL assay.

MATERIALS AND METHODS

Materials

Drug

Teriparatide acetate, marketed under Forteo, is produced by Eli Lilly and Company, USA. The formulation used was a subcutaneous injection.

Animals

This study used male Swiss albino rats weighing between 220 and 250 grams and approximately 1.5 months old. The rats were obtained from the Veterinary Medicine College, Mosul University. The staff and veterinarian at the Animals House of the College of Veterinary Medicine, University of Mosul, Iraq, cared for the animals. The environmental conditions in the Animal House were maintained at 22±2°C, with a 12-hour light cycle and humidity of around 30%. Food and water were provided as needed.

Ethical Approval

Ethical approval was obtained from the University of Mosul, with a code from the College of Dentistry (UOM. Dent/A.L.). All procedures followed the ethical guidelines for animal research set by the institutional ethics committee.

Experimental Design

Twenty adult male rats were used and separated into two groups of 10 rats each. The study duration was 30 days, and the groups were as follows:

1. **Control Group:** Injected subcutaneously with 1 ml/kg of normal saline in the dorsal neck region once daily from day 1 to day 30.
2. **Treatment Group:** Receiving a daily sub-cutaneous injection of 10 µg/kg of Teriparatide in the dorsal neck region.

Sample Collection

All rats in the control and treatment groups were anesthetized using ether. Following anesthesia, the rats were dissected to remove the liver and kidneys, which were washed with regular water and then placed in containers filled with 10% formalin for histological and immunohistochemical studies.

Tissue Processing

The liver and kidney tissues were dehydrated in ethanol (70%, 80%, 90%, 100%) for one hour each. The tissue sections were cleared in two different xylene solutions (one hour each). After clearing, the tissue pieces were embedded in molten paraffin wax at 60°C for one hour each. The tissue sections were then placed into paraffin blocks, and 5-micron-thick sections were cut using a Reichert rotary microtome. The samples were placed on glass slides and treated with two xylene solutions to remove the paraffin, spending 5 minutes on each solution. They were then rehydrated using ethanol in decreasing concentrations (90%, 80%, and 70%), with each step lasting for two minutes. Lastly, the tissue slices were dyed with H&E before being observed under a microscope.

Micromorphometric measure

The histological images were assessed using USB 2.0 digital image camera (Omax ToupView 9.0-Megapexil China), with its picture processing software. The camera software was calibrated to all lenses of the microscope using a 0.01mm stage micrometer slide.

TUNEL Assay Procedure

Tissue Processing

Preserve tissues in formalin and encase them in paraffin. Slice the tissues into sections using a microtome (around 4-5 microns thick). Microtome from Reichert Rotary Microtome manufactured in Germany).

Duration of Sample Processing in Alcohol Before Paraffin Embedding

The samples were exposed to a graded series of ethanol conc (70%, 80%, 90%, 100%) for one hour at each concentration.

Paraffin. Revitalization

Eliminate paraffin through a sequence of xylene washes followed by ethanol solutions. Rehydrate tissues, with water rinses. Protein Digestion**:** Treat the tissues with a protein digestion solution (e.g., Proteinase K) to enhance DNA accessibility.

TdT Reaction

Apply a solution containing Terminal deoxynucleotidyl transferase (TdT) and labeled deoxythymidine triphosphate (dUTP) to add nucleotides to the exposed ends of fragmented DNA in apoptotic cells.

Signal Detection

Detect the incorporated nucleotides using fluorescent labels (e.g., fluorescein) or colorimetric substances (e.g., diaminobenzidine, DAB).

Microscopic Examination

Examine the slides under a microscope to identify and count apoptotic cells, which will appear dark brown stained. Microscope from Biosystems MiniAmpPlus Thermocycler (manufactured by Thermo Fisher Scientific, USA).

Methods for Quantifying and Analyzing Apoptotic Cells Using Image J

- Load the TUNEL-stained image into ImageJ.
- Use the **Threshold** function to adjust values and identify positive cells only.
- Apply the **Analyze Particles** tool to count positive cells, specifying appropriate particle size.

Caspase 9 Gene expression

Extraction of DNA from paraffin blocks

DNA recovery was performed using the Kit from Daejeon, ADD BIO INC DNA FFPE Tissue, Republic of Korea, following the recommended procedure (Abed and Dark, 2022; Untari *et al*., 2024). The number of tissue sections used for extraction is equal to that of all samples, 25 mg. DNA concentrations were measured using the Qubit device.

Primer design

Forword/ Reverse primer sequences 5’ AGTGAAGCTGGACCCATCTC 3’, 5’ ACATCATGAGCTCTGCCAGA 3’of Caspase 9 primers were designing in this study **(Fig. 1).**

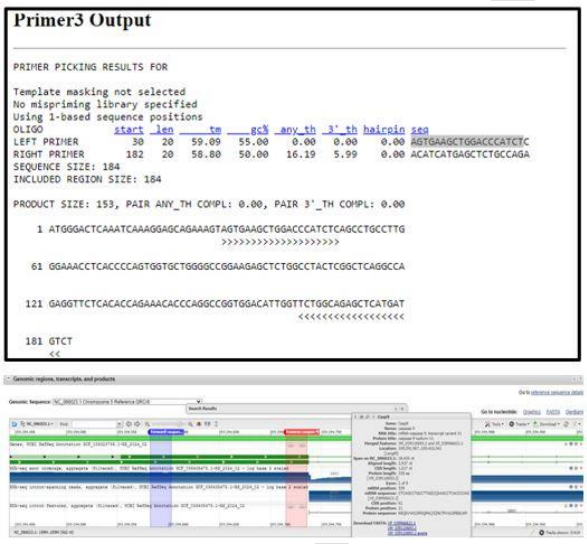

Fig. 1: Nucleotide sequence of target Caspase 9 primers design from the Gen Bank database (NCBI).

PCR analysis

The DNA samples were utilized as templates for PCR amplification, using primer pairs shown in the primer design section. In summary, the specimen was amplified using PCR Master Mix (Taq ADDBIO) by MiniAmpPlus Thermocycler (Applied Biosystems, by Thermo Fisher Scientific), then follow conditions: 95 °C for 5 minutes, then 35 cycles of 95 °C for 1 minute, 62 °C for 1 minute, then 72 °C for 1 min. This was accompanied by 5 minutes at 72°C for decisive extension, then held at 12°C. The DNA was isolated from tissues that were previously stored at a temperature of -20 degrees Celsius. Electrophoresis (Cleaver Scientific, England) was carried out on a 2% agarose gel containing gel-safe stain to separate charged DNA fragments according to their sizes.

Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR): qRT-PCR normalized by GAPDH targets the caspase 9 gene in the liver and kidney. **Livak and Schmittgen (2001)** applied the fold change approach for the relative expression of the target genes and GAPDH to the qPCR data.

Statistical analysis

The values were statistically tested by One-way ANOVA and then the significant differences between group were tested by T-test at the level of ($p \leq 0.05$).

RESULTS

Liver

The histological appearance of the liver section from the control revealed intact architecture of the hepatocytes, central vein, sinusoids, and Kupffer cells. The teriparatide acetate-treated group shows portal area with fibroplasia, hyperplasia of bile duct, and inflammatory cell infiltration. The teriparatide acetate-treated group showed portal area with congestion of the central vein, massive hydropic degeneration and necrosis of the hepatocyte, and expansion of the sinusoid **(Fig.2).** The sinusoidal dilation reflects a clear effect of the drug on liver architecture **(Table 1).**

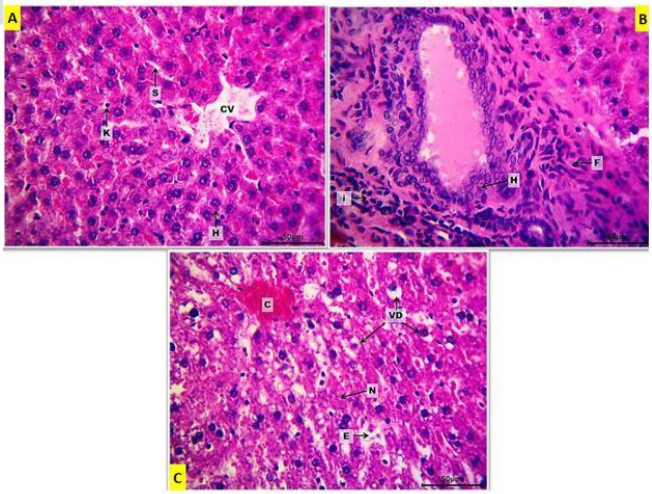

Fig. 2: liver of rat. [A]: Control shows intact architecture of the hepatocytes (H), Kupffer cell (K), sinusoids (S) and central vein (CV). [B] Teriparatide acetate group shows portal area with fibroplasia (F), hyperplasia of bile duct (H) and infiltration of inflammatory cells (i). [C]: Teriparatide acetate group shows portal area with congestion of central vein (C), massive vacuolar degeneration (VD) and necrosis (N) of the hepatocyte and expansion of the sinusoid (E). [A, B, C: 400X], H&E stain.

Table 1: Morphometric Measurements of Liver and Kidney Components

Parameter	Control Group (Mean ± SEM)	Treated Group (Mean ± SEM)	p-value
Sinusoidal Diameter (µm) in liver	8.3 ± 0.5	12.7 ± 0.6*	<0.01
Bowman's Space Width (µm) in kidney	10.2 ± 0.8	15.5 ± 0.7*	<0.05

*= refer to significant different from control group.

Kidney

The control group's histological sections of the kidney showed intact structure of glomeruli, proximal tubules, and distal tubules. The teriparatide acetate-treated group revealed glomeruli atrophy, hyaline cast in the lumen of renal tubules, Bowman's space dilatation, and degeneration and necrosis of epithelial cell lining renal tubules. The teriparatide acetate-treated group showed blood vessel congestion, hemorrhage, edema, and inflammatory cells **(Fig. 3).**

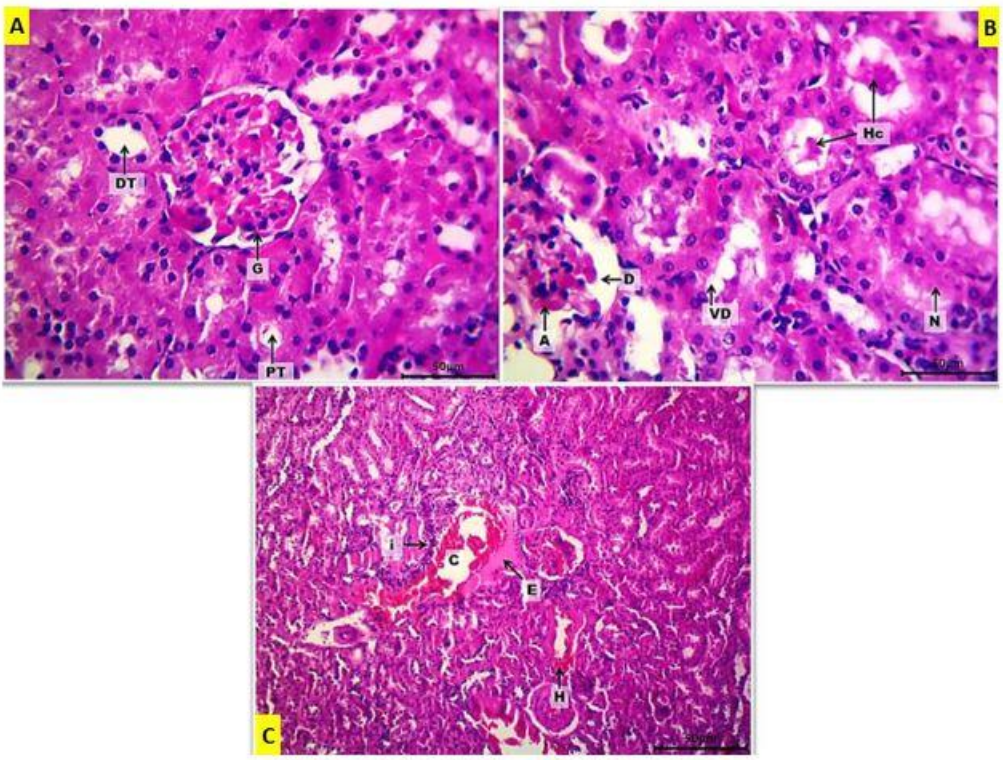

Fig. 3: Histological sections show control [A] with intact structure of distal tubule (DT), proximal tubule (PT) and glomeruli (G). [B]: Teriparatide acetate group shows glomeruli atrophy (A), hyaline cast in the lumen of renal tubules (Hc), Bowman's space dilatation (D), and degeneration (VD) and necrosis (N) of epithelial cell lining renal tubule. [C]: Teriparatide acetate treated group shows blood vessels congestion (C), hemorrhage (H), edema (E) inflammatory cells (i). [A, B, C, D & E: 400X]; [F: 100X], H&E stai

The treatment with the drug resulted in an increase in Bowman's space width which may indicate damage or structural changes in kidney function **(Table 1).**

TUNEL Assay Results

Histological sections of the TUNEL assay for apoptosis in liver of the control group (Without treating) showed negative reaction of apoptotic cells. Liver sections of Teriparatide treated group revealed highly positive stained apoptotic cells. Histological sections of the TUNEL assay in kidney of the control group demonstrated weak positive stained apoptotic cells. The kidney of Teriparatide treated group revealed moderate positive stained apoptotic cells **(Fig.4).**

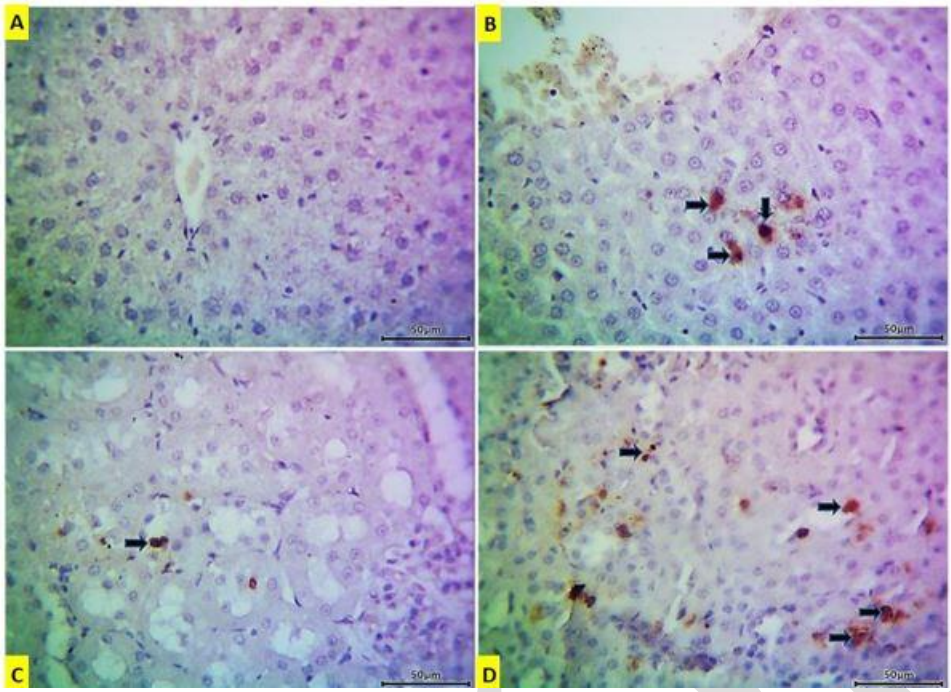

Fig. 4: Histological sections of the TUNEL assay for apoptosis in liver [A & B]. [A]: Control group (Without treating) shows negative reaction of apoptotic cells. [B]: Teriparatide treated group showing highly positive stained apoptotic cells (arrows). [C]: control group (Without treating) showing weak positive stained apoptotic cells (arrow). [D]: Teriparatide treated group showing moderate positive stained apoptotic cells (arrows). Hematoxylin stain, 400X.

Gene Expression Measurement: Caspase-9

The **Fig.5** represents an acrylamide gel showing the results of PCR to detect the presence of the Caspase-9 gene in formalin-fixed, paraffin-embedded (FFPE) liver and kidney tissues.

- **Lanes 1 to 8:** These lanes represent the tested samples of liver and kidney tissues.
- **Lane 9:** Represents the negative control sample.
- **Lane M:** Represents the DNA ladder (100 bp DNA ladder) from Addbio, Korea.

The bands observed at 153 bp indicate the presence of the Caspase-9 gene in the samples present in lanes 1 to 8. The absence of a band in lane 9 confirms the validity of the negative control, as no band is expected in a negative control sample.

- **Lanes 1 to 8:** All show bands at the 153 bp position, indicating that all the samples contain the Caspase-9 gene. This suggests that gene is activated in the FFPE liver and kidney tissues.
- **Lane 9:** The absence of a band confirms that the negative control sample was free from contamination or the presence of the Caspase-9 gene, thereby supporting the accuracy of the positive sample results.
- **Lane M:** Provides a reference for the band lengths, with the band at 153 bp indicating the target gene, Caspase-9.

In conclusion, the Caspase-9 gene in the tested samples in lanes 1 to 8 indicates potential apoptotic activity in the liver and kidney tissues. The result from lane 9 (the negative control) supports the reliability and accuracy of the test, confirming that the positive results in lanes 1 to 8 are indeed due to the presence of the gene and not due to contamination.

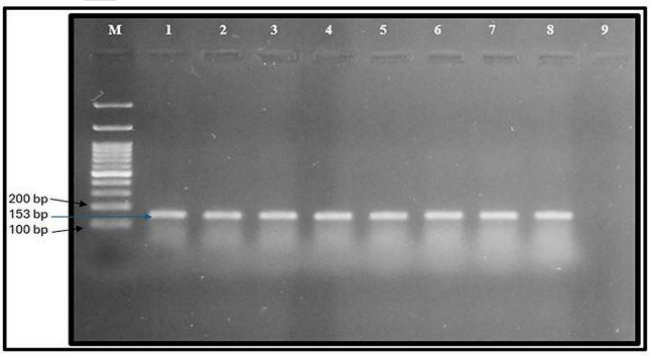

Fig. 5: Positive Caspase 9 gene located in lanes 1-8 (153 bp) (formalin-fixed, paraffin-embedded (FFPE) block of liver and kidney tissues), whereas negative control is located in lane 9. Lane M: 100 bp DNA ladder (Addbio company, Korea).

DISCUSSION

This study represents the first investigation focusing on the cellular and histopathological alteration induced by teriparatide in liver and kidney tissues. While the research done on patients has revealed that high doses of teriparatide are associated with liver and kidney problems, these studies only looked at clinical outcomes and failed to look at histological findings. The present findings also showed that teriparatide acetate was histologically characterized by alterations in hyperplasia due to congestion of the bile of the necrotic liver ducts, the liver, and sections of metabolism.

inflammation, portal may sinusoidal of infiltration areas, be dilatation. the by fibrosis, attributed the group inflammatory to actions administered cells, the of with extensive stimulation Teriparatide vacuolar of acetate changes, enzymes on as well as its impact on hepatic **(Sobh *et al.*, 2022a; Altememy *et al*., 2023; Aziz *et al*., 2023).**

Teriparatide acetate might stimulate or inhibit liver enzymes such as cytochrome P450 **(Sobh *et al*., 2022b).** This can alter the metabolism rate of the drug itself or other drugs, increasing the likelihood of accumulating toxic metabolites that may affect liver cells. Additionally, metabolic interactions can lead to increased production of reactive oxygen species (ROS), which can damage cell membranes, proteins, and DNA in liver cells, causing oxidative stress and cell death **(Clare *et al*., 2022).** The production of reactive oxygen species may be enhanced by inhibiting fatty acid oxidation in the mitochondria, altering both peripheral insulin resistance and lipid synthesis in hepatocytes, ultimately leading to hepatic histological changes **(Di Ciaula *et al.*, 2021).** Oxidative stress can stimulate an inflammatory response, increasing the production of cytokines such as TNF-α and IL-6, which can cause liver inflammation and damage **(Taherkhani *et al*., 2020; Alnuaimi and Alabdaly, 2023).** The teriparatide acetate-treated group showed atrophy of glomeruli, dilatation of Bowman's space, hyaline cast in the lumen of renal tubules, degeneration, and necrosis of epithelial cell lining renal tubules. The effects of teriparatide on the kidneys may alter the glomerular GFR and blood filtration **(Hsu *et al*., 2020).** The drug increases chances of kidney injury; thus, drugs impairing metabolites result in enhanced boosting of ROS and inflammation **(Eker, 2022; Alfathi *et al*., 2023).**

Metabolic interactions may also cause the accumulation of unfolded proteins in the endoplasmic reticulum, leading to endoplasmic stress and triggering signaling pathways that lead to apoptosis **(Ren *et al*., 2021).** Other researchers have reported that hypercalcemia can occur during teriparatide treatment, usually beginning within 4 to 6 hours after administration **(Miller *et al.*, 2021).** Hypercalcemia is a significant concern for physicians and clinicians, and once it occurs, the GFR decreases while sodium excretion and water depletion in the body increase, ultimately leading to metabolic alkalosis and kidney tissue damage **(Tuli *et al.*, 2020).** Apoptosis is a regulated process of programmed cell death, crucial for maintaining cellular homeostasis and eliminating damaged or unnecessary cells **(Santagostino *et al*., 2021).**

Apoptosis is activated via two main pathways: the intrinsic one, which is excited in response to internal stressors like DNA damage or nutrient deprivation, and the extrinsic one, which is initiated by external signals binding to death receptors on the cell surface, such as the Fas receptor **(Yue and López, 2020).** In the intrinsic pathway, Bcl-2 family proteins on the mitochondria are activated, leading to the release of proteins like cytochrome c from the mitochondria into the cytoplasm. Cytochrome C combines with Apaf 1 (protease activating factor 1) to create a complex called the apoptosome, which triggers Caspase 9 activation. Caspase 9 plays a role as an initiator in the pathway of programmed cell death. When activated within the apoptosome, Caspase 9 kickstarts the activation of executor caspases like Caspase 3 and Caspase 7 **(Khalil, 2021; Chimento *et al*., 2023).** These executor caspases break down the cells' proteins and facilitate DNA self-degradation, ultimately leading to planned cell death **(Ketelut-Carneiro and Fitzgerald, 2022).** This data provides evidence suggesting the initiation of the activation pathway in the apoptosis. The TUNEL assay helps to detect the pathway of caspase and understand the mechanism of the apoptosis in kidney tissue. The apoptosis process results in alteration in biochemical tissue, like the markers of medications negative side effects and the effect of toxins. By checking the levels of Caspase 9, we can assess the harm caused by Teriparatide, as higher Caspase 9 levels indicate an increase in cell death, implying tissue injury **(Wang *et al.*, 2023).**

Understanding how teriparatide impacts processes can help pinpoint the ways it triggers side effects. If Teriparatide induces stress in cells that activates the apoptosis pathway, measuring Caspase 9 can offer proof of this occurrence.

To sum up, our findings suggest that injecting teriparatide acetate results in its absorption and distribution to either the liver or kidneys, where it undergoes metabolism. The kidneys, in particular, play a crucial role in the distribution and metabolism of teriparatide, though not in its excretion. Caspase-9 measurement serves as a powerful tool to confirm the activation of apoptosis via the intrinsic pathway. Measuring enzymatic activity or protein levels can provide direct evidence of the initiation of apoptosis, which is valuable in studies examining the effects of drugs or treatments on cells and tissues.

CONCLUSION

The current study demonstrates that high doses of teriparatide have negative effects on liver and kidney tissues in rats. The use of the TUNEL assay revealed cells undergoing apoptosis by identifying the open DNA ends resulting from the self-degradation process, with kidney tissue being more affected. The measurement of Caspase-9 levels provided evidence of the activation of the intrinsic apoptotic pathway, with Caspase-9 being a key enzyme in this pathway.

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Conflict of Interest

The authors declare no conflict of interests.

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