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Nano-Curcumin Mitigates Against Hydroxyapatite Nanoparticle-Induced DNA Damage and KIM-1, LCN-2 Gene Responses in Kidney Rats

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ABSTRACT

Hydroxyapatite nanoparticles are regarded as an inorganic constituent found in natural bone and are primarily employed in tissue engineering because of their exceptional biocompatibility and bioactivity. Nevertheless, it remains uncertain whether they are safe for medical applications owing to their petite nano-scale particle size. From the previous studies, there was not enough data on nephrotoxicity and molecular changes induced by (high or low HAP-NPs) and the protection role of nano-antioxidants (CurNPs) in minimizing their toxicity. Therefore, the present study aimed to investigate the effect of high and low doses of HAP-NPs on the kidney system in male rats. At the molecular level, these results presented that the gene expression level, high HAP-NPs alone caused a significant increase in both kidney Injury Molecule-1 (KIM-1) and Lipocalin-2 (LCN-2) genes also, caused a significant increase in oxidative DNA damage (8-OHdG). The presence of Curcumin nanoparticles minimized the toxicity of high or low HAP-NPs due to their antioxidant properties and scavenging abilities against active free radicals.

Keywords: Hydroxyapatite; nanoparticles*;* Curcumin nanoparticles; oxidative DNA damage.

1- Introduction

Hydroxyapatite (HAp) with the chemical formula Ca10(PO4)6(OH)2 holds significant importance in the realm of nanotechnology. The heightened interest in HAP nanoparticles can be attributed to their structural and compositional resemblance to the mineralized framework found in natural bone, enamel, and dentin. In the medical field, there is a growing utilization of synthetic HAp nanoparticles as a bioresorbable platform for precisely controlled drug release in the management of conditions like cancer, osteoporosis, and osteomyelitis **(5).**

The increased applications of HAP nanoparticles have prompted heightened concerns regarding their potential harm to both humans and the environment. However, the existing body of knowledge on the toxicity of HAP nanoparticles concerning human health is notably limited, with only a few findings from fundamental studies having been disseminated. Some of these studies have provided compelling evidence indicating that the cytotoxicity associated with HAP nanoparticles may be linked, in part, to their capacity to induce cellular oxidative stress by generating (ROS) **(26)**.

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The precise molecular mechanisms underlying apoptosis induced by nano-Hydroxyapatite (nano-HAP) are still not fully elucidated, and research on nano-HAP production of apoptosis in cancer cells is limited. However, a notable study by **(4)** reported that nano-HAP had a pronounced effect in diminishing cell capability and prompting apoptosis in SGC-7901 cells. This apoptotic response was discernible through hypodiploid DNA content, alterations in cell morphology, and DNA fragmentation.

Numerous protein products have undergone investigation as potential new biomarkers for the early stages of acute renal failure. These include kidney injury molecule-1 (KIM-1), and gelatinase-associated lipocalin (NGAL), among others. KIM-1, a type I transmembrane glycoprotein, is typically absent in healthy kidneys. However, heightened levels of this protein have been observed, particularly on the apical membrane of proximal tubule cells, following instances of ischemic and nephrotoxic injuries **(20,23).** However, it was used two markers such as KIM -1 and NGAL in this study.

It's important to highlight that KIM-1, NGAL, and NAG are urinary biomarkers that serve as indicators of tubular damage and acute kidney injury (AKI). These markers tend to manifest changes earlier and exhibit greater sensitivity compared to plasma creatinine levels in cases of acute renal failure **(19).** Notably, urinary KIM-1 and NGAL are of particular interest because they are crossed by tubular epithelial cells in reaction to injury, and they have the potential to identify individuals at risk of developing chronic kidney disease (CKD) when progressive interstitial fibrosis and tubular atrophy start to develop **(6).**

Research conducted by **(30)** underscored the dose-dependent effects of HAp nanoparticles on provoking oxidative damage, genotoxicity, and cytotoxicity in human blood cells. These findings highlight the necessity for a thorough understanding of the potential risks associated with HAp nanoparticles.

The integration of antioxidants into nanoparticle formulations capitalizes on their innate ability to promote the removal of free radicals. This synergy between material science and nano-biotechnology is set to markedly increase their efficiency in mitigating oxidative damage caused by free radicals in biological systems. Consequently, the adoption of antioxidant nanoparticles is poised to outperform conventional antioxidant therapies, promising an improved quality of life and an extended lifespan for individuals. This breakthrough holds the potential to revolutionize approaches to combat oxidative stress and its associated health challenges **(15).**

Nanocomposites are new tools for the development of nano antioxidants. The integration of nanoscience with biomedicine to develop nano-antioxidant therapy has witnessed significant progress, which has advanced the pharmaceutical and biotechnology industries. It is one of the solutions to address the challenges associated with traditional antioxidant compounds. What is even more interesting is that nano-antioxidants have strong radical scavenging and quenching capabilities compared to natural antioxidants. Nano antioxidants enhance the pharmacokinetics of natural antioxidant compounds by shielding them from rapid degradation under stressful conditions, accomplished through either nanoencapsulation or nano-delivery techniques **(13).**

Nanocurcumin plays a dual role: It liquefies particles and forms heterogeneous nuclei. Agglomeration is prevented by the constant dispersion and high collision potential of the particles driven by ultrasound energy, thus achieving the highest possible charge. Nanocormine appears to be an effective radical scavenger with antioxidant effects and potential inhibition of oxidative stress. It can also protect the liver of mice from changes in liver function **(31)**. Nanocormin has many biological functions, such as antitumor, anti-inflammatory, antioxidant, anti-amyloid, antimicrobial, and antifibrotic effects **(1,30).**

The HANPs cause increasing toxicity despite their various uses. Therefore, it has become necessary to know what are the mechanisms and measures through which it can be prevented. This study investigated the renal toxicity caused by HANPs in male rats and the protective role of curcumin nanoparticles (CurNPs).

2- Material and Method

2-1- Chemical materials

Hydroxyapatite nanoparticles (HAPNPs)and Curcumin nanoparticles (CurNPs) Nanopowder (Yellowish Brown) >30 nm particle size was purchased from (Nanotech Egypt for Photo Electronics, sales@nanotecheg.com). The dose of hydroxyapatite nanoparticles was high (400 mg/kg B/W) and low (200 mg/kg B/ W) according to **(9).** The CurNP dose was 15 mg/kg body weight and was determined according to **(29).**

2.2. Characterization of Hydroxyapatite and Curcumin

High-resolution transmission electron microscopy (HR-TEM) was used to examine the morphology and characterization of hydroxyapatite and curcumin nanoparticle samples.

2.3. Transmission Electron Microscope Analysis.

TEM analysis was performed for both samples using a JEOL JEM-2100 high-resolution transmission electron microscope with an acceleration voltage of 200 kV.

2.4. Experimental Design.

Sixty adult male albino rats with an average weight of approximately (150±100 g) were used in this study. These rats were divided into six groups. group 1, as control; group 2, was orally administered with a dose (15 mg/kg) of (CurNPs); group 3, was injected intraperitoneally with a low dose (200 mg/kg) of (Ld HAP-NPs); group 4, was injected intraperitoneally with a high dose (400 mg/kg BW/day) of (Hd HAP-NPs); group 5, was injected intraperitoneally with (Ld HAP-NPs) with (CurNPs); group 6, was injected intraperitoneally with (Hd HAP-NPs) with (CurNPs) for successive 30 days.

2.5. Collection of Samples

Following the conclusion of the experiment, rats in each group were humanely euthanized while under ether anesthesia. Blood samples were then obtained and placed in heparin-coated tubes to aid in serum separation. The separation was accomplished by subjecting the samples to centrifugation at 860 ×g for 20 minutes. The resultant plasma samples were meticulously stored at -80°C for later use in subsequent biochemical analyses.

Kidney samples were also obtained, their weights were recorded, and they were rinsed with a cold saline solution (0.9%) to remove any residual connective tissues and adhering fats. These kidney samples were then divided into three distinct sections, each designated for specific analyses:

The first section was reserved for a DNA fragmentation assay. The second part of the kidney samples was allocated for gene expression analysis. The last portion of the kidney samples was minced and homogenized to create a 10% (w/v) homogenate.

2.6. Measured Parameters

2.6.1. Assay of Analysis of (Kim-1) and Lipocalin-2 Gene Expression Using RT-PCR.

The quantitative assessment of Kim-1 and lipocalin-2 expression in renal tissue was carried out as described by **(21).** This involved the use of (qRT-PCR). Initially, total RNA was extracted from the kidney. Subsequently, the isolated RNA was subjected to reverse transcription with the aid of inverse transcriptase enzyme to synthesize harmonizing DNA (cDNA). Following this, specific primers were utilized for amplification, and real-time PCR was employed for detection purposes.

A segment of Kim-1 was amplified with the use of a forward primer (F: 5'-CGCAGAGAAACCCGACTAAG-3') and a reverse primer (R: 5'-CAAAGCTCAGAGAGCCCATC-3'). Similarly, the gene Lipocalin-2 was amplified with a primer pair consisting of a forward primer (F: 5'-TCTGGGCCTCAAGGATAACAAC-3') and a reverse primer (R: 5'-AGACAGGTGGGACCTGAACCA-3').

Table (1): PCR Protocols for Kim-1 and lipocalin-2

	Kim-1	Lipocalin-2
Initial Activation	95 ^{0}C for 10 min	
Cycles	40	40
Denaturation	95^{0}C for 15 se	95^{0}C for 15 se
Annealing	55^{0}C for 15 se	55^{0}C for 15 se
Extension	60 ^{0}C for 15 se	60 ^{0}C for 15 se
Final Extension	60^{0}C for 10 min	
Melting analysis		

2.6.2. Assay of 8-OHdG in kidney

To quantify 8-OHdG, an enzyme-linked immunosorbent assay (ELISA) kit provided by Abcam (ab201734, Cambridge, UK) was employed following the manufacturer's guidelines.

2.7. Statistical Analysis.

The data is represented as mean ± standard error (SE). Statistical analysis was conducted using the general linear model (GLM) developed by SAS Institute, Inc. To assess the significance of variations between specific treatments, a Duncan multiple-range test was employed. Statistically significant differences in values are identified when the p-value is less than 0.05.

3. Results

3.1. Characterization of (hydroxyapatite and curcumin) nanoparticles

3.1.1. Transmission Electron Microscope Analysis (TEM)

The prepared TEM images of the HAP-NPs and Cur-NPs are presented in Figures 1 and 2, respectively, providing additional verification of the samples' structure and morphology. The analysis conducted via transmission electron microscopy affirms that HAP NPs exhibit a needle-like crystal morphology, with dimensions measuring approximately 200 ± 20 nm in length and 50 ± 10 nm in diameter, as depicted in Figure 1.

3.2. Gene expression analysis of Kim-1 and LCN2 of rats

The group treated with curcumin alone showed a significantly lower expression level of Kim-1 in comparison to the control group. In contrast, those treated with HAP-NPs (low or high doses) alone showed significant induction of renal expression of Kim-1 to be (3.5, 5.2) fold-control values. Groups treated with Cur-NPs with low or high HAP-NPs significantly reduced the expression of Kim-1 (**Table 2**).

Regarding the renal expression of lipocalin-2, treatment with Cur-NPs alone exhibited no significant effect compared control group. Treatment with low or high HAP-NPs alone significantly induced the expression of lipocalin-2 to be (1.8, 3.63 fold control values) However, the presence of Cur-NPs with low or high HAP-NPs significantly repression the expression of lipocalin-2 to values(1.3, 2.01 fold control values) as shown in **(Table 2)**.

3.3. Renal tissue content of oxidative DNA damage

The results of the 8-OHdG in the kidney tissue are in Table (3). Treated with Cur-NP alone had no significant effects on 8-OHdG levels relative to the control group. In turn, showed the treated group with two doses of HAP-NPs alone a significant increase in renal 8-OHdG levels compared to control values. In contrast, treatment with Cur-NP with HAP-NP significantly increased renal 8-OHdG levels in the kidneys. However, the OHdG content remains well above the normal value.

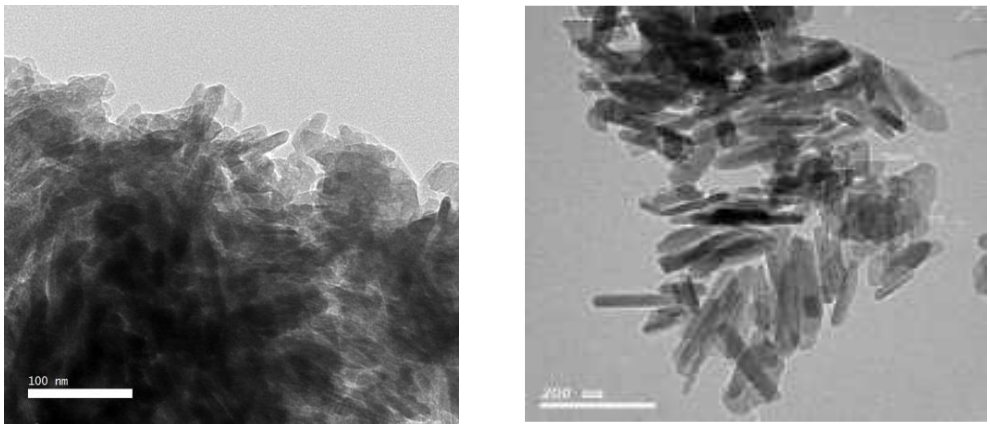

Figure (1). TEM images of HA NPs in adhesive pretending the appearance of the obtained n-HAP needle-like shape crystals at different scale bars 200 ± 20 nm (L), 50 ± 10 nm (D).

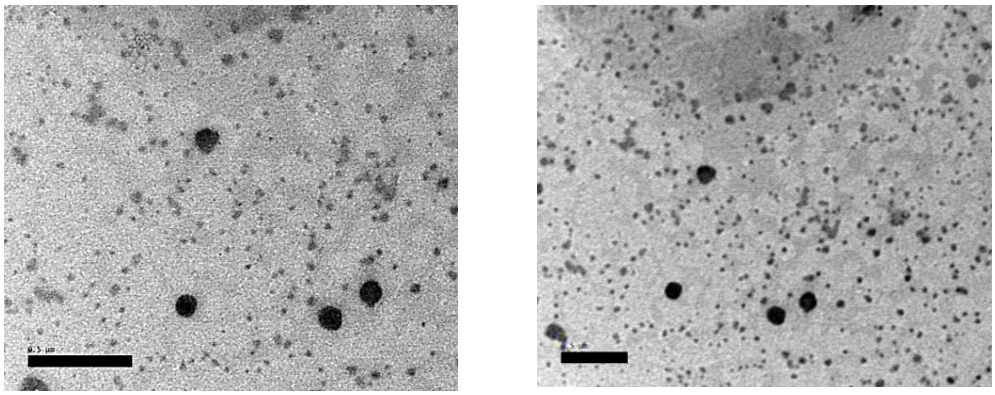

Figure (2). TEM images of curNPs were performed on JEOL JEM-2100 high-resolution transmission electron microscope

Table (2): Mean values ± SE of kidney expression of Kim-1 and lipocalin-2.

Experimental Groups	Kim-1 (Fold change)	Lipocalin-2 (Fold change)
Control	1.01 ± 0.07^{a}	1.03 ± 0.16^{ab}
Cur-NPs	0.67 ± 0.11^{a}	0.75 ± 0.06^{a}
Ld (HAP-NPs)	3.51 ± 0.14^{d}	1.83 ± 0.16^{c}
Hd (HAP-NPs)	5.24 ± 0.23^{e}	3.63 ± 0.19^{d}
(Ld HAP+Cur) NPs	1.93 ± 0.20^{b}	1.30 ± 0.09^{b}
(Hd HAP+Cur)NPs	2.75 ± 0.23^{c}	2.01 ± 0.16^{c}

Table (3): Mean values ± SE of kidney content of 8-OH-2-deoxygunaine.

Experimental Groups	8-OHdG (pg/µg DNA)
Control	3.93 ± 0.09^{ab}
Cur-NPs	4.30 ± 0.49^{a}
Ld (HAP-NPs)	6.83 ± 0.35^{cd}
Hd (HAP-NPs)	12.13 ± 0.99^{e}
(Ld HAP+Cur) NPs	6.07 ± 0.44^{bc}
(Hd HAP+Cur)NPs	8.47 ± 0.41^{d}

5. Discussion

The kidney plays a vital role in removing metabolic waste byproducts and foreign substances from the bloodstream. While performing this function, the kidney can be exposed to potential harm. The mechanisms for eliminating foreign substances involve glomerular filtration and tubular secretion. Substances with a size smaller than 50 to 65 kDa can freely pass through the glomerulus **(22).**

Toxicity specific to the kidneys is primarily linked to their elevation of blood influx rates, which results in the transfer of elevated concentrations of foreign substances in renal tissue. Consequently, the proximal tubule epithelium faces a greater susceptibility to renal toxicity. These cells express multiple carriers that facilitate the functional uptake and deposit of metabolites or harmful substances within the cell. Moreover, proximal tubular epithelial cells can convert non-toxic compounds into reactive intermediates, which can lead to cellular damage to macromolecules. These cells are highly metabolically active **(16).**

Over the past 25 years, hydroxyapatite has emerged as a crucial material among calcium phosphate ceramics, especially in the context of clinical

treatments for musculoskeletal system disorders. Its notable feature lies in its capacity to enhance bone growth, primarily attributed to its osteoconductive properties. Hydroxyapatite exhibits a favorable and functional bioactive behavior, where its interaction with the body's physiological environment leads to the formation of a layer that closely resembles bone apatite in terms of composition, referred to as bone-like apatite. These distinct attributes have spurred the utilization of hydroxyapatite nanoparticles (HANPs) across a wide range of biomedical applications, including bone enhancements, dental materials, tissue engineering, drug delivery, and bioactive coatings on metallic implants **(11).**

The HANPs are regarded as disquiet despite the considerable scientific interest and the capability they hold for numerous applications. So, the understanding of its mechanism (s) of toxicity is of great importance to suggest a suitable protective intervention. The present study aimed to investigate HAP-NPs-induced renal toxicity in male rats. Also, they investigate the protective role of CurNPs in alleviating this toxicity. Hence, HAP-NPs-induced nephrotoxicity was demonstrated at multiple levels. The HAP-NPs caused acute alteration in the Kim-1 and Lipocalin-2 gene expression of one of the most prominent and earliest observed effects in animals' kidneys treated with HAP-NPs.

As indicated in **Table (3)** in comparison to other biomarkers used to identify proximal tubular toxicity, urinary Kim-1 and lipocalin-2/NGAL demonstrate the highest levels of sensitivity and specificity for detecting renal damage. This is supported by studies demonstrating a significant increase in their mRNA expression following instances of acute kidney injury **(33).** Moreover, various studies have consistently revealed changes in lipocalin-2 levels in the kidney, serum, or urine, both in animal models susceptible to chemically induced tubular damage and in patients with acute or chronic kidney disease **(18,27).**

However, these responses are consistent with a possible function of Kim-1. As a putative epithelial cell adhesion molecule, Kim-1 may play an important role in the proliferation and regeneration of epithelial cells, thereby contributing to the restoration of renal function and morphological integrity. Changes in renal Kim-1 expression appear to correspond to histopathological changes observed after HAP-NP treatment. It has also been suggested that Lipocalin-2/NGAL plays a role in the regeneration process **(12,32).**

Emerging evidence proposed that LCN2 is a pro-inflammatory cytokine associated with obesity-related complications e.g. heart failure and insulin resistance, and cardiomyocyte apoptosis induction at least in part via induction of the intrinsic mitochondrial pathway, elevation in intracellular iron levels, translocation and activation of Bax **(24).** **Liu et al. (14)** found that the diffusion exposure effect of HAP-NPs resulted from the activation of apoptotic cells, though the precise molecular process by which HAP-NPs induce apoptosis remains uncertain. HAP has demonstrated clear abilities in laboratory settings to induce apoptosis and inhibit cell growth. Apoptosis is a regulated, predictable process of programmed cell death. HA NPs have been observed to trigger apoptosis in various cell types, **(28).**

Oxidative stress is linked to the oxidation of proteins and lipids, which can ultimately result in a significant disruption of mitochondrial function. Any modification in the permeability of the mitochondrial membrane is recognized as an initial occurrence in the process of apoptosis **(25**). To assess DNA oxidation, the quantification of 8-OH-dG adducts was conducted. The process involved digesting DNA by subjecting it to incubation with DNAase I, endonuclease, and alkaline phosphatase **(7)**. The total of 8-OH-dG was determined by high-activation fluid chromatography with electrochemical reveal according to established methods described previously **(2).**

At the molecular level, exposure to HAP-NPs induced oxidative stress in kidney tissue, resulting in a significant increase in an oxidized DNA marker

known as 8-hydroxy-deoxyguanine (8-OHdG) Table (4). ROS react with nitrogenous bases and deoxyribose in DNA and cause oxidative reactions. ROS react with nitrogenous bases and deoxyribose in DNA and cause oxidative reactions. This leads to mutations, apoptosis, carcinogenesis, necrosis, and genetic diseases. By fragmenting DNA through the destruction of nucleosomes, changes in its functional properties can then lead to errors that lead to mutations **(3).**

The limitations associated with curcumin can be overcome by creating curcumin nanoparticles, which can enhance its circulation time, permeability, and resistance to metabolic processes. Chemicals that have been modified using nanotechnology have demonstrated their effectiveness in delivering drugs and precisely targeting the intended tissues **(17, 10).** This study gives clear evidence through the results of the ability of CurNP to reduce damage to the level of antioxidants in the kidneys by increasing its activity and regulating the number of free radicals, as well as improving the value of 8-OH-dG, an indicator of oxidative DNA damage, as well as regulating the significant increase in the expression level of kidney damage-related genes Kim-1 and LCN2 induced by PAH-NPs toxicity.

Conflicts of Interest:

The authors affirm that there are no conflicts of interest.

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