Section A-Research paper
ISSN 2063-5346

Hepatotoxicity Assessment of Biosynthesized Silver Nanoparticle from *Citrus maxima* Fruit in Rodent Model

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ABSTRACT

Biosynthesized nanoparticles are gaining attention because of biologically active plant secondary metabolites that help in green synthesis and their unique biological applications. This study reports an ecofriendly, reliable, and cost-effective synthesis of silver nanoparticles using the fruit extract of *Citrus maxima* (*C. maxima*). The biosynthesized silver nanoparticles (AgNPs) were characterized using UV–visible spectroscopy and transmission electron microscope. To determine in vivo cytotoxicity of biosynthesized AgNPs and to compare the effects of different AgNP doses. On rats, we conducted an *in vivo*. The rats were given three dosages of AgNPs (5mg, 7mg, and 10 mg), and three-month-old rats were placed into five groups, each with six rats. A liver function test (ALT, AST, Bilirubin, Alkaline phosphate) and histological parameters were performed as preventive measures. The toxic effect of AgNPs on rats was dose- and time-dependent, where no side effects or lethality was observed in the tested animals over the study period. Our data showed that AgNPs at low dosages (5 and 7 mg/kg) were acceptable for biomedical usage and had no negative effect. In contrast, higher doses (10mg/kg) may be cytotoxic or lethal. Our study shows that high doses of AgNPs can be lethal to eukaryotic cell-based systems. In addition, we have also discovered that azithromycin was more hazardous than AgNPs. This further suggests the necessity of comprehensive in vivo studies to determine the extent of nanotoxicity.

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Keywords: Silver nanoparticles, Antimicrobial, Hepatotoxicity, Oxidative stress, *Citrus maxima*, Azithromycin

INTRODUCTION

Nanotechnology is a developing field of research that has produced a wide range of metal nanoparticles. These nanoparticles are incredibly small, ranging from 1 nm to 100 nm in size, and have a huge surface area, resulting in a high Metals and metal oxides have received much attention in recent years due to their safety for humans and animals and their ability to survive harsh weather conditions [1].

Silver, gold, copper, CuO, TiO_2, and ZnO are inorganic nanoparticles that are more stable and can endure high pressure and temperature and have extreme antibacterial activities [2,3]. As a result, nanoparticle research has sparked tremendous interest, particularly in the use of silver nanoparticles in the biomedical sector, where many goods, such as lotions, bandage materials, and wrapping materials, are currently on the market [4].

AgNPs are the most common metal nanoparticles employed as antibacterial agents in various ailments [5]. AgNPs are effective in the diagnosis of various diseases because of their antioxidant properties [6]. Chemical approaches have been used to synthesize AgNPs, but there are several limitations, including being time-consuming, expensive, and damaging to the environment. Green nanoparticle synthesis, on the other hand, is substantially less expensive and safer for the environment [7]. Plant-oriented AgNPs application in medicine and pharmaceutical interference may become the way to go owing to the availability, nontoxicity and variety of metabolites that help to bring down Ag ions in biological system [8].

Citrus maxima, also called pomelo, is expectoration, nausea, and throat rashes [9]. Citrus species are dispersed globally, high in minerals, vitamins, carotenoids, flavonoids, pectins, fibers and proven to have antimicrobial, anti-inflammatory, antioxidant, antidiabetic and antihepatotoxic [10]. It is also known that *C. maxima* peel has blood pressure reduction, antiallergic, antioxidant and antitumor properties [11]. From Over the past few years, *C. maxima* has been extensively used as a healthy food for its active substances, including essential oils, polysaccharides, and flavonoids [12].

In a previous study, we synthesized AgNPs with the help of pulp, peel and seed of C. maxima fruit and also compared the number of nanoparticles produced from respective extract. Further we analyzed the bactericidal property of formulated NPs. AgNPs formed through fruit extracts exhibit significant antibacterial properties against gram-positive, gram-negative strain, i.e. *S. aureus, E. coli.* [13]. This study was designed in continuation with our previous study to evaluate cytotoxic effect of AgNPs, synthesized through *C. maxima* and provide comparative data of AgNP biodistribution and commercial antibiotic medicine (Azithromycin) *in vivo*.

MATERIALS AND METHODS

Preparation of silver nanoparticles (AgNPs)- Our previous research study described the fabrication and characterization of silver nanoparticles [13]. Briefly, the AgNPs were synthesized by adding 5 mM silver nitrate in 8 ml of *Citrus maxima* fruit extract and defined by UV-visible spectroscopy, transmission electron microscopy and Fourier transform infrared (CDRI, Lucknow, Uttar Pradesh, India). The size range of the AgNPs used in this study was between 52.36 and 12.38 nm.

Experimental animals- Thirty Wistar rats (males; 3 months; 170-180 gm) were obtained from the Indian Veterinary Research Institute (IVRI), Bareilly, U.P. India. Animals were housed in a clean environment under standardized temperature (22°C-24°C) and 12 hours light/dark cycles and received standard food and water. This research was carried out after acceptance of the Institutional Animal Ethics Committee (IAEC) at SRMSCET, Bareilly Uttar Pradesh, India (Protocol approval number 2021/715/CPCSEA) and animal handling were done as per guidance given for the use of animal in scientific research prepared by Indian National Scientific Academy (INSA).

Animal grouping and treatment- The entire study took 14 days, the rats were sorted into five observational groups, each consisting of six animals (Table 1). Group 1 was the control group given distilled water whereas group 2 was given azithromycin (AZM) dissolved in distilled water. Groups 3, 4, and 5 were given AgNPs doses of 5 mg/kg, 7 mg/kg and 10 mg/kg, separately as prescribed[11]. All treatments were given orally through a cannula on daily basis for

14 days. On day 14, post-treatment, animals were anesthetized and sacrificed per the CPCSEA guidelines, and liver tissues were dissected for histopathology.

Table 1. Group distribution for study of cytotoxicity effect of nanoparticles

Group	**Type**	**Number of rat**	**Biochemical examination (AST, ALT, Bilirubin and serum alkaline phosphate)**		**Histopathological testing**
			After 7 Days	After 14 days	After 14 days
Group 1	Control	6	6	6	1
Group 2	Azithromycine	6	6	6	1
Group 3	Peel NPs (5mg/kg)	6	6	6	1
Group 4	Peel NPs (7mg/kg)	6	6	6	1
Group 5	Peel NPs (10mg/kg)	6	6	6	1

Clinical signs and body weight measurement- Clinical symptoms of animals were carefully evaluated before and after medication, including appearance, activity, hair, probable trauma, and death, as per the standard methods.

Biochemical examination- Blood samples were collected of all animal groups (1-5) at day 0, day 7 and day 14, respectively, through the major veins of the abdominal cavity for serum aminotransferases (SGPT/SGOT; Angstrom Biotech Pvt. Ltd., Rajasthan, India), alkaline phosphate (Meril Diagnostics Pvt. Ltd., Gujrat, India) and bilirubin (Meril Diagnostics Pvt. Ltd., Gujrat, India) analyses using commercial diagnostic kits as per the manufacturer's instructions.

Histopathological examination- The liver tissues of animals (n = 5) were collected on day 14. After deep ether anesthesia, the liver tissues dissected on the ventral were fixed, dehydrated, paraffin-embedded before slicing, and staining for histological examination. Briefly, tissues were quickly washed in saline and homogenates were ready by using Tris hydrochloric acid buffer followed by centrifugation at 3000 rpm for 15 min. Tissues were fixed instantly in phosphate-buffered formal saline, washed properly, desiccated in ethanol and fixed firmly in paraffin wax.

Tissues were finely sliced, stained with eosin, hematoxylin and the mounted specimens were examined under a light microscope.

Statistical analysis- The mean and standard deviation were used to present all of the data. The data were statistically analyzed using one-way ANOVA. The Dunnett test was applied as a post hoc test. At $p<0.05$, mean values were judged statistically significant.

Ethical approval- Institutional Animal Ethics Committee (Protocol approval no. 2021/715/CPCSEA) of SRMS College of Engineering & Technology, Bareilly Uttar Pradesh, India.

Results

Biophysical characterization of AgNPs- The synthesized AgNPs were brownish in colour. The maximum UV absorption of AgNPs was 430 nm, and the TEM-determined size ranged between 12.38 nm to 53.36 nm (Fig. 1).

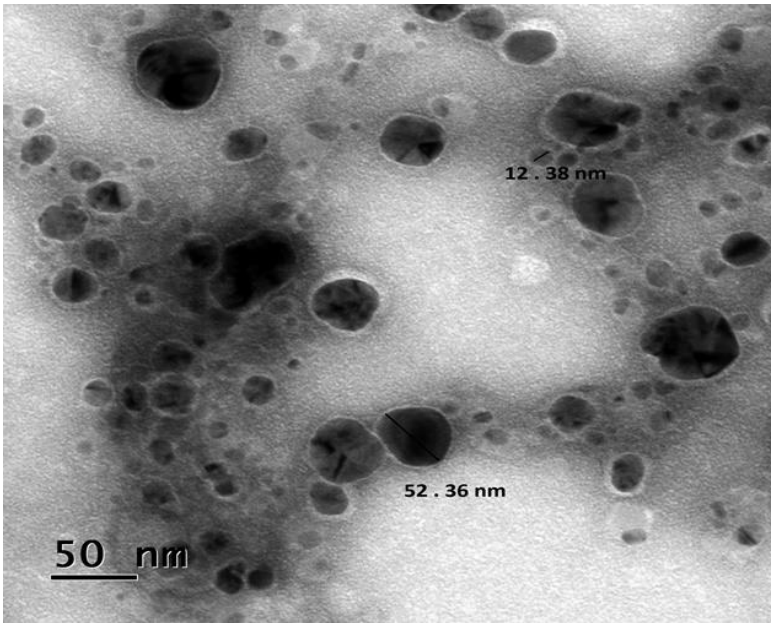

Fig. 1: Transmission electron micrograph (TEM) image of the biosynthesized silver nanoparticles (AgNPs) from *C. maxima*. The scale bar corresponds to 50 nm.

Biochemical examination- To test the cytotoxicity of AgNPs, rats were given doses of 5, 7 and 10 mg/kg for group 3, 4 and 5 respectively. During this procedure, no lethargy, other adverse signs or lethality was observed in the animals.

The ALT levels of group 1 (control group) were 48.28±4.56, 48.30±4.60, 49.66±5.63 on day 0, day 7 and day 14, respectively. In group 2, animals exposed with azithromycin drug, ALT levels were 48.62±3.35, 74.60±1.98, 98.02±1.64, on day 0, day 7 and day 14, receptively ($p > 0.05$). In group 3, ALT levels were 52.09±7.38, 57.29±3.45, 56.18±3.61 on day 0, day 7 and day 14 of treatment. The p value was more than 0.05 and data were found statistically none significant. In group 4, ALT levels were 54.09±6.43, 60.58±1.89, 60.26±2.02 on day 0, day 7 and day 14 orderly ($p<0.05$). In group 5, ALT levels were 56.17±4.66, 62.91±7.08, 71.10±6.72 on day 0, day 7 and day 14 ($p<0.05$), (Fig. 2).

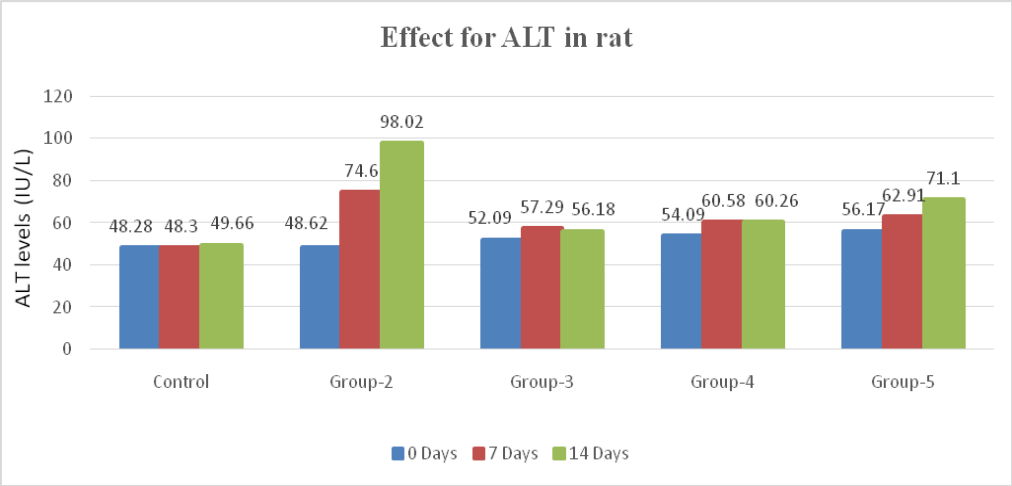

Fig. 2: Impact of exposure of drugs on Alanine aminotransferase (ALT) levels. Group 1 was the control group, and they were given distilled water on a daily basis for 14 days, group 2 was given azithromycin (AZM), groups 3, 4, and 5 were given 5 mg/kg, 7 mg/kg, and 10 mg/kg AgNPs, respectively. In group 3 and 4, ALT levels were increased differentially on day 0, day 7 and day 14 of treatment. Data were found statistically none significant ($p>0.05$). In group 5, ALT levels were increased in time and dose dependent manner. Data was found statistically significant ($p< 0.05$).

A time dependent increased in AST levels were estimated in Group-2 rats following the treatment of azithromycin (Fig. 3). On day 7, AST level was 133.0±7.02; on day 14, its level was

215.3±3.46. The data was statistically significant ($p<0.05$). In group 3, AST level were 98.40±1.17 and 98.27±1.05 on day 7 and day 14, respectively. Data was statistically significant ($p<0.05$). The level of AST in group 3 remains low as compared to group 2. In group 4, on day 7 and day 14, AST level were 91.26±1.52 and 92.60±1.71 respectively. In group 5, AST level were 117.4±6.76 and 178.1±7.83 on day 7 and day 14 of exposure.

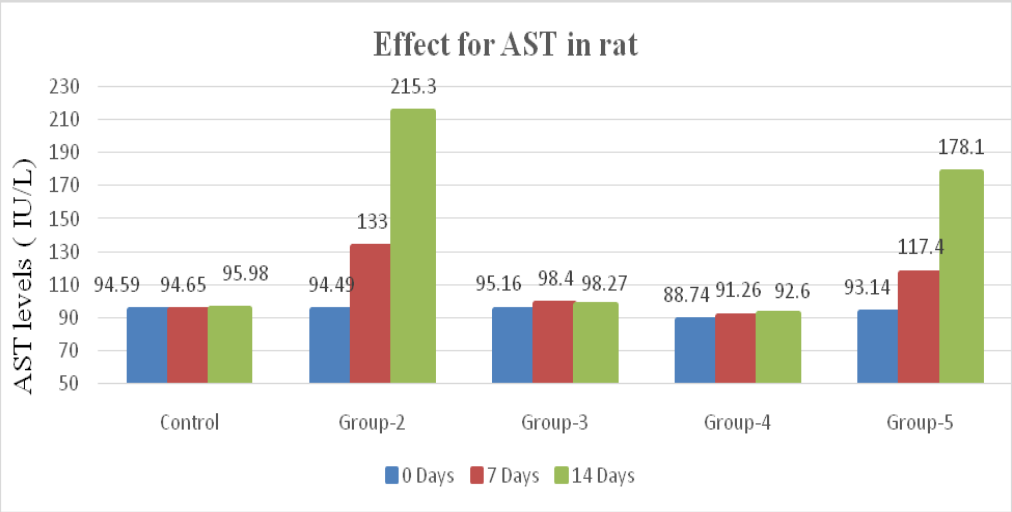

Figure 3. Impact of exposure of drugs on Aspartate aminotransferase (AST) levels. AST levels increased on increasing periods of time in Group-2 rats following treatment of azithromycin. Group 3, 4, 5 rats were given 5 mg/kg, 7 mg/kg, and 10 mg/kg AgNPs. The levels of AST were increased differentially in time and dose dependent manner. Data was statistically significant ($p<0.05$).

In control group, bilirubin levels were 0.565±0.17, 0.566±0.17, 0.606±0.15 on day 0, day 7 and day 14, respectively. In group 2, bilirubin levels were 0.648±0.16, 0.87±0.03, and 1.375±0.01 on day 0, day 7 and day 14. In group 3, bilirubin levels were 0.55±0.08, 0.621±0.04, 0.615±0.04 on day 0, day 7 and day 14. Data were found statistically none significant ($p>0.05$). In group 4, bilirubin levels were 0.581±0.04, 0.66±0.02, 0.66±0.02 and in group 5, levels were 0.593±0.07, 0.851±0.04, 1.166±0.03 on day 0, day 7 and day 14 ($p<0.05$).

In control group rats, alkaline phosphatase levels were 169.7±15.4, 169.9±15.4, 170.1±13.7 on day 0, day 7 and day 14, respectively. In group 3, alkaline phosphatase levels were 165.5±8.77, 170.3±1.93, 170.6±2.52 on day 0, day 7 and day 14 orderly ($p>0.05$). In group 4, alkaline phosphatase levels were 160.7±13.2, 168.0±2.46, 168.3±2.73 on day 0, day 7 and day 14

($p<0.05$). In group 5, alkaline phosphatase levels were 180.4±9.87, 194.5±7.89, 270.9±16.9 on day 0, day 7 and day 14 orderly ($p<0.05$).

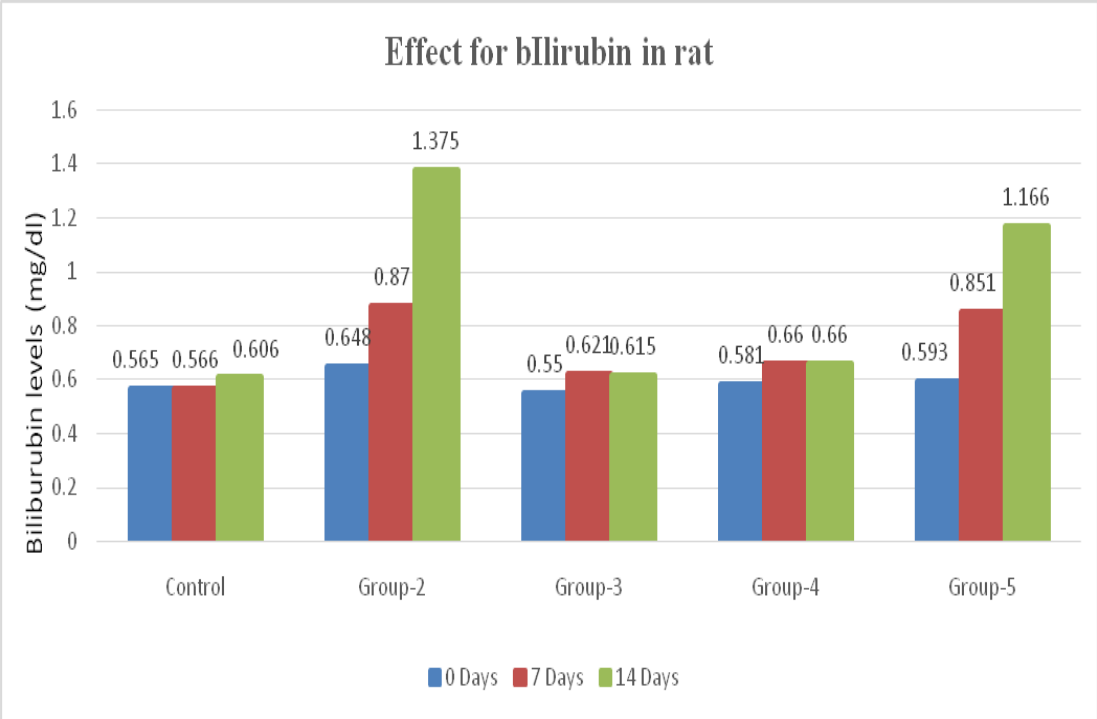

Fig. 4: Impact of exposure of drugs on bilirubin levels. In group 3 rats were treated with biosynthesized AgNPs (5 mg/kg), bilirubin levels were found statistically none significant ($p>0.05$). In group 4 and 5, bilirubin levels were increases as duration and doses increases and data was found statistically significant ($p<0.05$).

Liver histopathological evaluation- The histological photomicrographs of the liver tissue are shown in Fig. 5. No histopathological alteration was observed in the liver of control animals, group 3 and 4. In AZM treated group significant inflammation near centro-veinular region compared to postal region was observed with single cell necrosis and appearance of acidophilic bodies. In all of the NPs treatment groups, there were no significant changes in histopathological liver tissues were observed at 5 and 7 mg/kg, according to the findings of this study.

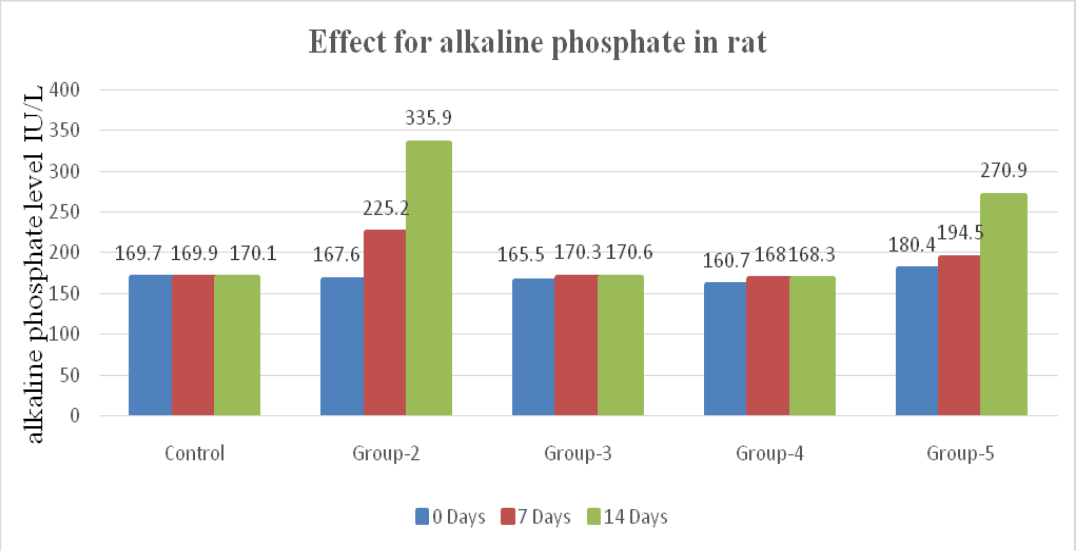

Fig. 5: Impact of exposure of drugs on alkaline phosphatase levels. In group 3, alkaline phosphatase levels were none significant ($p>0.05$). In group 4 and 5, alkaline phosphatase levels were increased from day 0 to day 14 and data was found statistically significant ($p<0.05$).

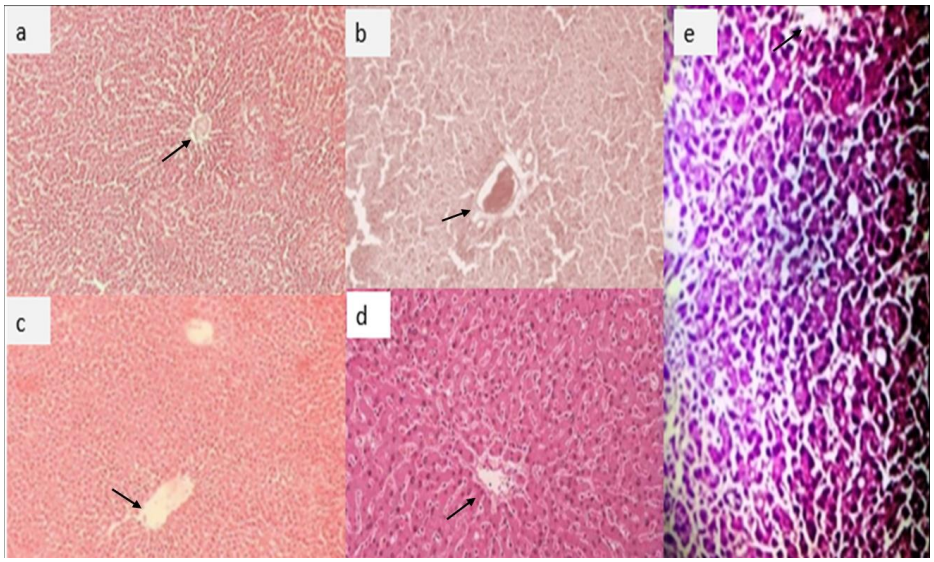

Fig. 6. Histological examination of rat liver tissue treatment after 14 days: Indicative image of rat liver tissue. A-control, B-treated with Azithromycin, C, D, and E- treated with AgNPs (5mg/kg, 7mg/kg, 10mg/kg).

Discussion

Development in the field of nanotechnology progressively replaced antibiotics with nanoparticles. However, excessively high doses of nanoparticles have negative effects[14]. The in

vivo toxicity of nanoparticle-containing medical devices is receiving a lot of interest right now, and further research is needed for the safety evaluation and risk management of nanoparticles. An analysis by Ramadi *et al*., stated that systemic application of AgNPs was observed to cause liver toxicity and NLRP3-dependent inflammation. These nanoparticles raised the levels of AST, ALT and LDH as well. Moreover, about 24 hours after exposure to AgNPs, a dose-dependent increase was noticed in peritoneal neutrophils and up-regulation in the expression of several pro-inflammatory gene mediators, comprising tumor necrosis factor-alpha and interleukin B. The result of the liver tissue pathology displayed hepatic necrosis and an increase in the sinusoidal kupffer cells and granulomatosis 24 h after the exposure of AgNPs [15].

After systemic delivery, nanoparticles are typically small enough to enter even extremely minute capillaries throughout the body, making them the most effective method of tissue distribution. Although, nanoparticles can alter the physiology of any cell in an animal's body since they can pass through biological membranes [16,17]. AgNPs are gaining popularity due to their antibacterial properties. These nanomaterials can be ingested by humans in various ways, and accumulate in vital organs causing biological harm [18].

The effects of AgNPs in rats at various dosages were analyzed in this study. Various biochemical markers and enzymatic assays have been employed for tracking AgNPs-associated clinical symptoms. Treatment with AgNPs for 14 days had no negative impacts on growth during the research period, as no significant variations in body weight were identified between the AgNPs-treated and control rats (data not shown). Furthermore, no abnormal clinical symptoms and way of behaving were identified in both the control and treated groups. It is well established that when cells are damaged, the AST and ALT might seep into the bloodstream, causing an increase in activity [18,19].

Compared to the control group, AgNPs administration dramatically boosted all enzymes in the rats' liver in a dose-dependent way. When we compared the enzyme levels of the normal group with the treated groups of rats, we found that the enzyme level of the azithromycin treated group was raised from the normal level in 14 days, whereas the enzyme level of the AgNPs treated group 3 and 4 (dose: 5 and 7 mg/kg) did not change, but the 10 mg/kg dose of AgNPs increased the enzyme level but less than the azithromycin-treated group

We got similar results for the levels of bilirubin and alkaline phosphatase in rats. Our findings showed 'no-observed adverse effect level limit for AgNPs in rats' as 10.0 mg/kg body weight. Furthermore, at high doses of these nanoparticles (10 mg/kg), an increase in liver enzyme concentrations and a minimal amount of toxicity was observed.

The histopathological examination of liver of group 5 rats revealed degenerative changes in hepatocytes characterized by granular cytoplasm compared to control groups. These degradative changes were found to be dose dependent and indicated that AgNPs dose of 5 mg/kg and 7 mg/kg were non-toxic. The hepatotoxicity at doses of 10 mg/kg might be explained by the fact that the liver primarily metabolizes most of the xenobiotic.

Conclusion

We have demonstrated that the high doses of AgNPs could be fatal to eukaryotic cell-based systems. In addition, we have also discovered that azithromycin was more hazardous than AgNPs.

Taken together, this further suggested the necessity of in-depth *in vivo* studies to determine the extent of nanotoxicity.

CONTRIBUTION OF AUTHORS

Research concept- Prof. Tanzeel Ahmed, Prof. Lalit Singh

Research design- Prof. Tanzeel Ahmed, Prof. Lalit Singh

Supervision- Prof. Tanzeel Ahmed, Prof. Lalit Singh

Materials- Priyanka Mishra

Data collection- Priyanka Mishra

Data analysis and Interpretation- Priyanka Mishra, Prof. Tanzeel Ahmed, Prof. Lalit Singh

Literature search- Priyanka Mishra

Writing article- Priyanka Mishra, Prof. Tanzeel Ahmed

Critical review- Prof. Tanzeel Ahmed

Article editing- Prof. Tanzeel Ahmed

Final approval- Prof. Tanzeel Ahmed

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