ORIGINAL ARTICLE

Nephrotoxicopathology Properties of Gold and Iron Oxide Nanoparticles With Perchloric Acid & SiPEG as Radiographic Contrast Media

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ABSTRACT

Introduction: Exponential research and development of nanotechnology has lead to its implementation in medical line such as radiographic imaging. In current practice, iodine is clinically used as a contrast media in radiographic analyses. However, contraindication of iodine to kidney in clinical practice warrants for a better contrast enhancer with lower toxicity. Gold nanoparticles (GNPs) and Iron Oxide nanoparticles (IONPs) have been proposed as potential iodine's substitute due to their novel biocompatibility. **Methods:** In accordance with technology-driven toxicity impact, an animal modeling study has been conducted to assess the nephrotoxicopathology of GNPs and IONPs with Perchloric Acid and SiPEG by biochemical study, in-depth tissue examination by histopathology, apoptosis, and ultrastructural observation, and molecular analysis by Comet Assay. **Results:** Renal function test (RFT) revealed significant alteration in iodine group compared to nanoparticles and negative control group ($p<0.05$). Reactive oxygen species (ROS) generation and lipid peroxidation (MDA) levels demonstrated significant reduction in both nanoparticles' groups compared to iodine ($p<0.05$), suggesting for lower oxidative stress induction. Morphological aberration demonstrated by histology and ultrastructural evaluation (TEM) showed a distortion in kidney tissues and nucleus' structure of iodine-administered group as compared to control and nanoparticles' group. Apoptosis detection by TUNEL assay for GNPs and IONPs group also revealed a significant reduction in apoptotic cells compared to iodine group. Comet assay revealed significant reduction in DNA damaging effect of GNPs and IONPs group compared to iodine group. **Conclusion:** The present study may postulate that GNPs and IONPs show better contrast enhancer properties with lesser toxic properties than iodine

Keywords: Gold nanoparticles, Iron oxide nanoparticles, Nanotechnology, Toxicity, Pathology

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INTRODUCTION

Multi-application of nanotechnology has led to a great demand in diversified disciplines and could provides great benefits for society in general as well as financial gains (1). Nanotechnology has penetrated deep into human life in diverse areas, significant impact on medical and health sciences (2). A variety of NPs products are being developed, including a possibility in becoming contrast enhancer for radiographic imaging. Parveen et al, (3) discussed the important of NPs that can be exploited as a marker in diagnostic purposes and medical imaging. In current radiographic imaging, iodine contrast medium has been widely used. However, the limitations of iodine such as high toxicity to iodine-intolerance patients have been documented (4). This warrants for a finding of new and safer contrast medium, where the advancement of nanotechnology has drawn researcher's attention for NPs as a new contrast media. Contrast medium properties of NPs should be utilized for advance use in imaging of certain diseases in vivo. Due to their diversified utilization, metal NPs such as gold nanoparticles (GNPs) and iron oxide nanoparticles (IONPs) with functionalized surface are becoming focus owing to its uniqueness which could triggers the various applications in biological and medicine (5).

Although the bulk gold is considered as inert and biocompatible, Jenkins et al, (6) has articulated the increasing concern regarding toxicity of nano-sized GNPs which is of utmost importance in medical imaging and also for diagnostic protocols. Based on

their promising application in laboratory and the physiochemical stability, GNPs were expected to be safe (7). Jeerage et al, (8) mentioned that GNPs are potential candidate for biomedical application due to their stability and easy to be fabricated. Previously, gold has demonstrated a strong ability as a contrast agent for x-ray (9) but there is lack in scientific reports of GNPs toxicity following its use as a contrast medium. Apart from GNPs, there is also a drain interest in developing iron oxide nanoparticles (IONP) as a contrast medium, due to a need in developing another new contrast agent with less cost.

Thorough and critical studies have been conducted on IONPs which demonstrated that several types are becoming major interest due to its convincing biocompatibility (10). Peng et al, (11) stressed that IONPs could be regarded as biodegradable with lower to no appreciable toxicity. Moreover, the poly-ethylene glycol (PEG) that could be used to coat the IONPs would be additional advantage in reducing the opsonization of IONPs. Brullot et al, (12) discussed that silane-PEG (SiPEG) on the other hand might acts as stabilizer thus making IONPs to be inert and highly biocompatible. However, some studies reported that IONPs could induce unfavourable events in certain conditions (13). Concerning to application of metallic NPs in biomedical, the unpredictability of its toxicity criteria warrants for systematic risk analysis and assessment.

Fundamental research will furnish the basis of NPs risk assessment by the expanded of data from previous research models (14). The unique physical and chemical properties of NPs including its size, surface area, shape, and solubility may enhance the biological reactivity thus attracts for commercialization. However, it may also enhance the toxicity of materials that are inert in bulk form to become toxic in nanoscale-form. Thus, it is essential to further study on the potential toxicity carried by metallic NPs (3). However, certain nanotoxicology studies have documented conflicting findings pertaining the minor modification and differences in physicochemical features of NPs, and several studies have reported that these outcomes were due to its size, surface charge, shape, and coating materials (15). Nanotoxicity studies postulate that metallic NPs could lead to undesirable effects on health, but the exact mechanism remains ambiguous. The interactivity of metallic NPs with human body system has gained great interests for collaborative transdisciplinary research in material science and biology (7). Therefore, the various toxicological aspects for NPs has aggressively started to be assessed due to its potential nanotoxicity which remains controversial (15). Kwon et al, (15) also urged that physicochemical properties of NPs could be studied by characterizing various parameters which may indicate biocompatibility of NPs.

Kidney is a vital organ playing key role in eliminating toxic substances from body. In this particular case, regardless the routes of exposure, it is proven that NPs are able to circulate in blood vessel and gain access to renal, hence highlighting the importance of assessing nephrotoxicity (1). Besides, renal also plays a major role in xenobiotics elimination from biological systems, hence leads to a hypothesis that absorbed NPs in systemic circulation could be excreted by kidney (16). Thus, current atmosphere has lead to a nanotoxicity study in kidney following NPs administration and to safeguard that nanotechnology development in medicine is beneficial, a diversified nanotoxicity assessments need to be documented (17).

MATERIALS AND METHODS

Animal use and study design

Animal modelling study was managed following the guidelines of Universiti Teknologi MARA Committee on Animal Research and Ethics (UiTM CARE). Twenty healthy four weeks old Wistar rats obtained from Laboratory Animal Facility Management (LAFAM) were acclimatized for 14 days with normal pellet diet. Animals were categorized into control group (Cx), iodine administered group (Ix), Gold Nanoparticles administered group (GNPx), and Iron Oxide Nanoparticles administered group (IONPx). Animal in Ix, GNPx and IONPx received intravenous administration of iodine, GNPs and IONPs for 0.5 ml of 300ug/ml solution. Animals underwent diethyl ether anaesthesia after 24 hours for blood sampling via retro-orbital before euthanized by cervical dislocation and kidney tissues were collected.

Kidney biochemistry analyses

Whole blood sample in plain tubes were centrifuged for 15 minutes at 3000rpm to obtain the serum. Serum for kidney biochemistry analyses were sent to University Veterinary Hospital, Universiti Putra Malaysia Serdang for determination of serum creatinine, urea and lactate dehydrogenase (LDH).

Reactive oxygen species (ROS) production assay

Fifty milligrams of kidney tissues were resuspended in one mL PBS and was homogenized on ice. Homogenates were centrifuged for five minutes at 3000 rpm to obtain the supernatant. ROS level was determined by Oxiselect™ ROS Assay kit.

Lipid peroxidation end product (MDA) assay

Hundred milligrams of kidney tissues were resuspended in one ml PBS containing 1X butylated hydroxytoluene (BHT) and homogenized on ice. Homogenates were spun at 3000rpm for five minutes to obtain the supernatant and its MDA level was determined by Oxiselect™ TBARS Assay kit.

Histology observation

Fresh kidney tissues were fixed in 10 % prior to tissue grossing and processing. Tissues were embedded in paraffin wax prior sectioning with rotary microtome. Sectioned tissue were fixed on microscope glass

slide and stained with Haematoxyling & Eosin stain for macroscopic observation. Histology scoring was conducted under veterinary pathologist's supervision.

Transmission electron microscope (TEM) evaluation

Fresh kidney tissues were slit into 1 mm x 1 mm under 4 % glutaraldehyde for fixation, and washed with buffer sodium cacodylate. Tissues were fixed with 1 % osmium tetroxide in 0.1M cacodylate buffer prior dehydration with ethanol. Tissues were then rinsed in propylene oxide before embedding in mould from resin. Tissue's sections underwent trimming and ultrathin sectioning before being contrasted with uranyl acetate and lead citrate. Finally the tissues were observed by TEM (FEI TECHNAI G2).

Apoptosis TUNEL detection

The evaluation of apoptosis-induced toxicity after NPs and iodine administration was conducted by Apoptag® Peroxidase *In Situ* Apoptosis Detection from Millipore. This method applied the labelling of cells undergo apoptosis by modifying its DNA fragments utilizing terminal deoxynucleotidyl transferase (TdT). This staining method was performed by *in situ* DNA strand breaks which were detected by TUNEL assay after visualization by microscope.

Comet assay evaluation for DNA damage

Detection of DNA single and double strand break was conducted by using Comet Assay Kit (3-Well Slides) from OxiSelect™ Cell Biolabs, Inc. Tissues were minced in ice cold PBS before being centrifuged and supernatant was discarded. Cells were combined with agarose before placed onto slides. After the gel became solidified, slides underwent lysis solution prior to electrophoresis at 35 V for 30 min. Comet slides were placed into distilled water, followed by alcohol and air dried. Slides were stained with ethidium bromide prior to onservation by confocal laser microscope (Leica Company) and software analysis by CASPlab comet assay analysis.

Statistical analysis

Statistical analyses of the obtained data were conducted using IBM SPSS version 21.0. Analysis of variance (ANOVA) was performed and followed by *post hoc* Tukey test. Statistical significance was determined at 0.05 probability level ($p < 0.05$).

RESULTS

General examination

Animals were observed for any physical and pathological changes following iodine, GNPs and IONPs administration. No sign of mortality and significant changes was noted. Gross examination of kidneys yielded no significant pathological changes.

Kidney biochemistry

Renal function test (RFT) consisted of serum creatinine, urea and lactate dehydrogenase (LDH) levels were conducted to evaluate the renal status of all experimental groups (Fig 1(a) and 1(b)). Serum creatinine level in Fig 1(a) demonstrated a significant elevation in Ix, GNPx, and IONPx in comparison to Cx, ($p < 0.05$) respectively. Levels of serum creatinine in Ix also showed to be significantly elevated compared to both NPs-administered groups, ($p < 0.05$). No appreciable differences among GNPx and IONPx was noted. Serum urea levels in Ix and GNPx shown significant increment as compared to Cx ($p < 0.05$), meanwhile no remarkable dissimilarity was noted in IONPx. Referring to Fig 1(b), LDH level for Ix shown to be significantly elevated when juxtaposed to Cx, GNPx, and IONPx, ($p < 0.05$)

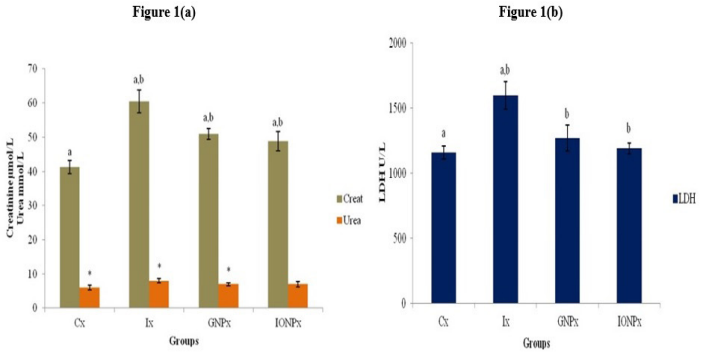

Fig. 1(a) – Kidney biochemistry values
The bar chart shows the levels of Creatinine and Urea in Cx, Ix, GNPx and IONPx. Values were expressed as mean ± S.D (n = 5), (*p<0.05).*
[a]Indicates significant differences when compared to Cx, (*p<0.05*)
[b]Indicates significant differences when compared to Ix, (*p<0.05*)
Fig. 1(b) – LDH levels
The bar chart shows the levels of LDH in Cx, Ix, GNPx and IONPx groups. Values were expressed as mean ± S.D (n = 5), (*p<0.05*).
[a]Indicates significant differences when compared to Cx, (*p<0.05*)
[b]Indicates significant differences when compared to Ix, (*p<0.05*)

respectively.

ROS production assay

Fig 2 depicts ROS production values in kidney tissues of iodine, GNPs and IONPs-administered animals. Significant elevation of ROS production level was recorded in Ix as compared to Cx, GNPx, and IONPx, ($p < 0.05$) respectively. Level in GNPx on the other hand has shown significant elevation compared to Cx ($p < 0.05$), meanwhile no notable differences were observed among Cx and IONPx.

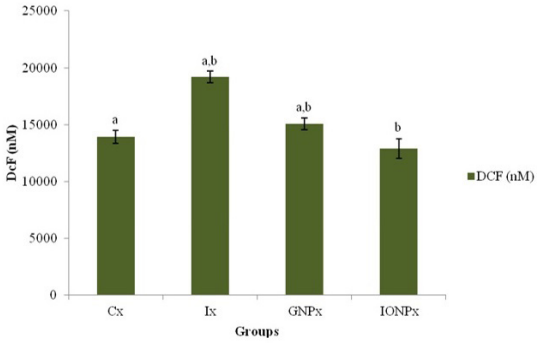

Fig. 2 – The levels of ROS production
Bar chart shows the levels of ROS production in kidney tissues for Cx, Ix, GNPx and IONPx. Values were expressed as mean ± S.D (n = 5), (*p<0.05*).
[a]Indicates significant differences when compared to Cx, (*p<0.05*)
[b]Indicates significant differences when compared to Ix, (*p<0.05*)

Lipid peroxidation product level, MDA Assay

Determination of MDA in kidney tissues could reflects the lipid peroxidation occurrence because it is the main product for oxidation. Fig 3 showed significant increment of MDA level in Ix as compared to Cx, GNPx, and IONPx, ($p < 0.05$) respectively. MDA level for GNPx was depleted compared to Cx, however the outcomes were not significant.

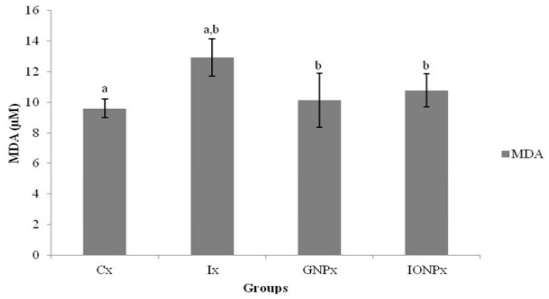

Fig. 3 – Kidney MDA levels
The bar chart shows the leves of MDA level in kidney's tissue of Cx, Ix, GNPx and IONPx.Values were expressed as mean ± S.D (n = 5), ($p<0.05$)
[a] Indicates significant differences when compared to Cx, ($p<0.05$)
[b] Indicates significant differences when compared to Ix, ($p<0.05$)

Histology observation

Pathologic changes of kidney tissues were evaluated by histology and the lesion assessment was performed under Veterinary Pathologist guidance. Fig 4 (A-D) depicted the kidney tissues histology. Kidney histopathology of Ix (B) also revealed the features of more compact glomerulus (⟵) with broader Bowman's space (◄) compared to Cx (A), GNPx (C) and IONPx (D).

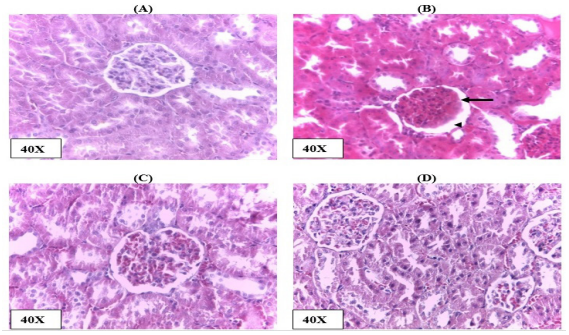

Fig 4 – Kidney histology
Photomicrograph of kidney tissues in histology H&E stain (A) Cx (B) Ix (C) GNPx (D) IONPx. Tissues in Cx, GNPx and IONPx showed no significant pathological changes, while in Ix the glomerulus was noted more compact and tightly packed (⟵), and the Bowman's space (◄) was broader which suggestive for mild glomerulopathy

TEM ultrastructural analysis

The ultrastructural observation of the kidney's nucleus was shown in Fig 5 (A, B, C and D). Ultrastructure of nucleus membrane (⟵) of Ix demonstrated an irregularity and aberrant in shape as compared to Cx (A), GNPx (C) and IONPx (D). Thickness of nucleus membrane has also noted in Ix (B), while the nucleus for GNPx and IONPx did not show any pathological changes compared to Ix.

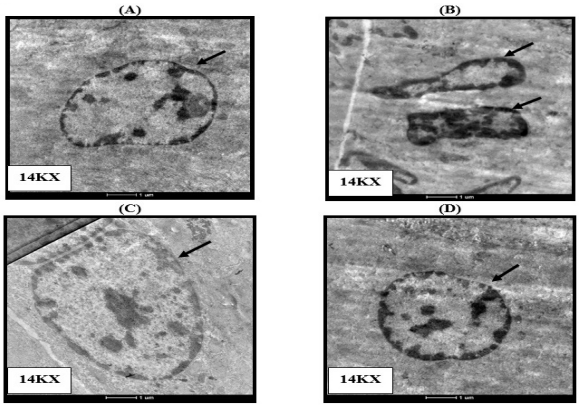

Fig. 5 – Ultrastructure evaluation
Ultrastructure of nucleus in kidney tissues (arrow pointed figure). (A) Cx nucleus (B) Ix nucleus (C) GNPx nucleus and (D) IONPx nucleus. Cx, GNPx and IONPx nucleus show smooth regular membrane while there is irregular shape of Ix nucleus. Denser layer of nuclear membrane has also been noted in Ix with remarkable reduce in size.

Apoptosis TUNEL detection

Apoptosis TUNEL assay marker for kidney tissues in Fig 6 (A, B, C, D) revealed the abundant of apoptotic cells in Ix (B) compared to Cx (A), GNPx (C) and IONPx (D). Prominent of brownish cells reflect the apoptosis occurrence and there was fewer apoptotic cells in Cx, GNPx and IONPx.

Table I - Comet assay analysis

Comet assay analysis by measuring Olive Tail Moment (OTM) (Tail DNA % x Tail Moment Length, performed by CASPlab Comet Assay Analysis Software (Mean ± SD).

Groups	Kidney Tissues
Cx	1.253 ± 0.048 [a]
Ix	4.042 ± 0.394 [a, b]
GNPx	2.252 ± 0.642 [b]
IONPx	1.386 ± 0.752 [b]

[a]Indicates significant differences compared to Cx
[b]Indicates significant differences compared to Ix

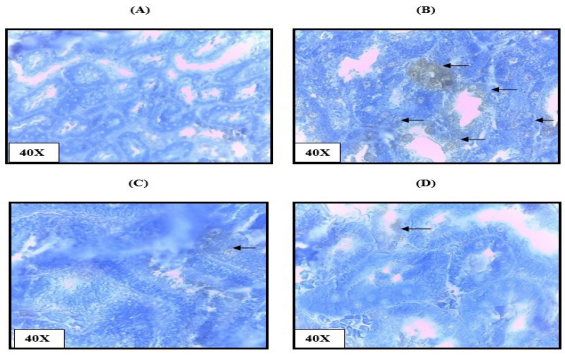

Fig. 6 – Apoptosis determination
TUNEL staining assay demonstrating apoptosis occurrence in kidney tissues. (A) Cx tissues (B) Ix tissues (C) GNPx tissues and (D) IONPx tissues. Apoptotic appearance was denoted by brown appearances. Cx, GNPx and IONPx depicted few to no apoptotic appearance while Ix showed abundance of apoptotic occurrence.

Comet assay evaluation for DNA damage study

Comet assay DNA damaging effect evaluation reported that olive tail moment (OTM) in kidney tissues of Ix increased significantly compared to Cx ($p < 0.05$),

suggesting progressive damage in Ix kidney tissues. Results in Table 1 proposed that DNA damage in Ix was prominent in Ix compared to Cx, GNPx and IONPx.

DISCUSSION

A complete and systematic evaluation of nanotoxicity has become a major concern to researchers as it could provoke the biological response and elicit the damage to the body. One of major key factor in understanding the biological impact of NPs is to address their in vivo nephrotoxicity. Kidney tissues showed absence of any significant pathological changes and the gross examination of animals after euthanasia revealed no abnormalities. This was supported by previous studies from Guo et al, (18) and Zhang et al, (19) that reveals no alteration in general examination of NPs treated animals. Physical examination was followed by a series of reliable toxicity assessment in term of hematological and biochemical evaluation, oxidative stress induction, microscopic evaluation of tissues morphology and DNA damage enhancement.

Renal function test (RFT) consist of serum creatinine, urea and Lactate dehydrogenase (LDH) are mainly conducted to assess kidney function is routinely performed in clinical pathology evaluation for standard in vivo toxicity study (18, 20). In current study, level of serum creatinine in Ix, GNPx and IONPx were significantly increased compared to Cx. This could suggest that administration of iodine and NPs may trigger renal functions impairment. However, there are also significant reductions in levels of serum creatinine for GNPx and IONPx compared to Ix. Based on the results obtained, it may clarify that administration of iodine and NPs could trigger renal distortion, however, administration of NPs signifies a less propensity to induce nephrotoxicity as compared to iodine. Level of urea in serum demonstrated a significant elevation in NPs groups (Ix and GNPx) as compared to Cx, while no notable elevation was observed in IONPx. These results further suggest that administration of iodine and GNPs could trigger alteration in urea levels, with the level in GNPx was lesser than in Ix while no significant changes in urea level were observed in rat administered with IONPs.

Concerning to serum creatinine and urea levels, iodine has shown plausibility to induce nephrotoxicity compared to GNPs and IONPs. It was in line with Guo et al, (18) who stressed that any significant alteration in serum biochemistry for RFT involving serum creatinine and urea are markers for kidney impairments. Moreover, level of LDH in Ix was significantly increased as compared to Cx, GNPx, and IONPx. Elevation of LDH in response to cellular damage or necrosis could suggest that iodine-administration may lead to renal impairment, while NPs-administration showed lesser tendency (18).

Mohammad et al, (21) proposed the ability of excessive ROS generation which may distort redox balance status and induce oxidative stress that can lead to various pathological disorders. The results for kidney tissues' ROS level revealed that iodine administration showed to induce excess ROS in kidney tissues compared to Cx and NPs-treated groups. In exception, it was noted that level of ROS generation in IONPx showed no significant increased compared to Cx. These ROS results in kidney provide evidence that both iodine and GNPs could lead to excess ROS generation, however, the odds of GNPs to reduce ROS generation compared to iodine is plausible. On the other hand, result also suggests that IONPs administration in biological system of rats did not triggers any noteworthy increment in ROS generation from Cx and thus, the propensity to induce oxidative stress to the kidney following intravenous administration could be avoidable. Previous study emphasis that toxicity of NPs is highly associated with production of ROS and induction of oxidative stress. Roy et al, (2) and Fu et al, (17) highlighted that the ability of NPs to promotes ROS generation are the main critical determinant of toxicity. Excessive generation of ROS could distort redox balance in cells and induce oxidative damage by interacting with cellular constituents such as protein and lipids (22). Measuring MDA levels may reflect the occurrence of lipid peroxidation (LPO) mechanism. Kidney tissues of Ix recorded a notable elevation in MDA level when compared to Cx, GNPx, and IONPx, while there are no appreciable differences were observed in Cx and both NPs-administered groups. This indicates that GNPs and IONPs showed better characteristics, which could minimally induce LPO to both tissues as compared to iodine. Current findings were in positive relation with ROS production which recorded that ROS levels in both NPs groups were significantly reduced than in iodine group lower than in Ix. The discoveries of present study was supported by previous scientific works by Ebabe-Elle et al, (23) which stressed that the administration of NPs would elicit remarkable alteration in MDA levels (24). The outcomes in MDA levels in this study were in agreement with ROS production findings which postulate that NPs did not lead to significant nanotoxicity.

Histology evaluation on kidney tissues have demonstrated that in iodine administered group, the presence of features suggestive for glomerulopathy has been observed which characterized by compact glomerulus and enlargement of Bowman's space. This histology evaluation was in agreement with findings in RFT that denoted presences of renal biochemistry alterations which are related to the pathophysiology of Ix toxicity. Chen et al, (25) urged that nanotoxicity study in term of histopathology would revealed degenerative and inflammation in liver tissues, while in kidney tissues it would alter the renal glomerulus. Coccini et al, (26) and Cho et al, (27) emphasized that histology analysis could suggest early occurrence of pathological changes and a remarkable evaluation in scrutinizing the toxicity of NPs. Histology outcomes in current research is congruent with previous study by Guo et al, (18) which reported that NPs might only causes slight degeneration in hepatocytes of administered animals and did not lead

to significant toxicity.

TEM analyses could be performed to evaluate the toxicity effect from NPs exposure by evaluating cellular uptake, intracellular localization and organelles pathology which could reflect the structural damage induced by iodine and NPs. Photomicrograph from TEM evaluation showed no significant pathological changes in ultrastructural image of kidney's nucleus from both GNPs and IONPs-administrated groups compared to Cx. The results for kidney's ultrastructural nucleus evaluation revealed that Ix nucleus showed an irregularity and bizarre in shape as compared to nucleus in Cx, GNPx and IONPx. The thickness of nucleus membrane also noted in Ix while there is no pathological change in Cx and NPs-administered kidney tissues. These findings were in agreement with other parameter results such as RFT, ROS generation and MDA levels which showed no deterioration of kidney tissues in NPs-administered animals. Li et al, (28) mentioned that NPs such as IONPs might evoke the toxicity effect in significantly higher dosage and with repeated administration, while Brullot et al, (12) asserted that IONPs which coated with substances such as SiPEG is biocompatible. These results suggest that NPs demonstrate lower toxic properties compared to iodine.

Apoptosis detection by TUNEL assay has been adapted in toxicology for detecting toxic effect of particular substances including iodine and NPs. Apoptosis staining in kidney tissues have revealed the occurrence of apoptosis in iodine and both NPs administrated rats. Interestingly, tissues from Ix showed abundant of apoptotic cells which characterized by the presence of brownish to dark coloured components while no similar coloured cells were observed in Cx. On the other hand kidney tissues from GNPx and IONPx only depicted a few apoptotic cells which suggest for normal cell turnover compared to Ix. This study outcome could suggest that iodine administration has lead to DNA fragmentation and apoptosis, while GNPs and IONPs administration showed less predisposition in inducing apoptosis in kidney tissues. In addition, the occurrence of apoptosis could be related with significant increased in ROS and MDA levels in Ix compared to GNPx and IONPx. Sarkar et al, (5) previously mentioned that NPs may penetrate cellular membrane and induce cell death or apoptosis and mainly related to depends on NPs dosage, duration of exposure and cellular system.

A quantities of previous literatures speculate the NPs interaction with biological molecules could induce DNA strand break and damage (2, 5). Roy et al, (2) mentioned that NPs could lead to DNA damage by inducing apoptosis and micronucleus formation. In assessing DNA damage following NPs introduction in vivo, comet assay were used an established protocol to examine genotoxicity. It is performed by analyzing unrepaired DNA strands breaks and lesion using electrophoresis methods and characterized by Olive Tail Moment (OVT) which reflect the amount of DNA breakage (29). According to the present study, the OTM in Ix has shown a significant increment when comparison was made to control and NPs groups. This phenomenon may provide evidence that administration of NPs did not induce appreciable toxicity effects. Current results were supported with published work by Gerber et al, (30) whom reported that NPs did not trigger any significant DNA damaging effect as observed by comet assay evaluation. The current findings demonstrated that NPs injection did not induce significant DNA damage and the findings for comet assay are supported by other parameter findings including biochemistry, ROS and MDA level, histology and ultrastructural evaluation which revealed no alteration in biological environment of rats following GNPS and IONPs administration.

CONCLUSION

Present study has postulated that GNPs and IONPs showed lesser tendencies to induce nephrotoxicity as compared to iodine, which may be applied as a safer contrast enhancer in radiographic imaging.

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