Volume 22, No 1 | International Journal of Radiation Research, January 2024

Comparative bio-effect of gadolinium with alternative contrast medium of magnesium chloride and zinc oxide nanoparticles on single-strand DNA following exposure to magnetic resonance imaging

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► ***Original article***

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Received: February 2023
Final revised: July 2023
Accepted: August 2023

Int. J. Radiat. Res., January 2024; 22(1): 131-137

DOI: 10.52547/ijrr.21.1.19

Keywords: *Magnetic resonance imaging, gadolinium, magnesium chloride, zinc oxide nanoparticles, DNA single-strand, alkaline comet assay.*

ABSTRACT

Background: Gadolinium (Gd) is a widely used MRI contrast agent that improves visibility and aids in accurate diagnosis. However, conflicting reports exist regarding its genotoxic effects. This study investigates the bio-effects of Gd and alternative contrast media, magnesium chloride ($MgCl_2$), and zinc oxide nanoparticles (ZnO NPs) on DNA single-strand breaks. ***Materials and Methods:*** In this in-vitro comparative experiment, 12 adult New Zealand rabbits (males aged between 4.5 to 5.5 months, weighing 2.5 to 3.0 kg) were utilized. The rabbits received intravenous injections of different contrast agents, namely Gd (n=3), $MgCl_2$ (n=3), ZnO NPs (n=3), and a control group (n=3). Following this, all the rabbits were subjected to MRI at 1.5 Tesla (T) with an RF of 64 MHz for 20 minutes. The alkaline comet assay assessed the presence of single-strand breaks (SSB). ***Results:*** The results of the study revealed a statistically significant increase in DNA SSB in both the Gd ($p<0.010$) and ZnO NPs ($p=0.006$) treated groups compared to the normal control group. However, the alternative $MgCl_2$ treatment did not elicit a statistically significant effect on the DNA single-strand compared to the control group ($p=0.277$). ***Conclusion:*** The contrast medium Gd and alternative ZnO NPs were demonstrated to cause significant DNA single-strand breaks, with the Gd causing more damage than ZnO NPs. However, the alternative contrast $MgCl_2$ was safer with no effect on DNA single-strand. This suggests that $MgCl_2$ is more suitable as an alternative contrast media in MRI scanning.

INTRODUCTION

Contrast agents are frequently used in magnetic resonance imaging (MRI) scans to enhance the visibility of internal body structures [1]. However, conflicting reports exist regarding the potential genotoxic effects of MRI scans conducted with the commonly used contrast medium like gadolinium (Gd). Once the DNA is damaged, it will lead to genetic mutations, which can gradually lead to cancer. Moreover, reports suggest that MRI scans ranging from 1.5 to 7.0-T do not elicit genotoxic effects [2-4]. However, these studies often examined only one contrast medium (such as Dotarem, Gd) or focused on a few genes. Hence, it is crucial to conduct investigations and compare the effects of alternative contrast agents, which involve a wider range of genes associated with repair and apoptosis. This approach aims to provide a comprehensive understanding of their impact.

The contrast agents are believed to shorten the relaxation times of nuclei of human cells [5]. Additionally, recent findings indicate that small quantities of Gd contrast agents can be retained in the tissues, predominantly in the bones, with a minimal amount found in the brain [6]. Additionally, the European Medicines Agency (EMA) previously suggested suspending the marketing of four linear Gd contrast agents based on significant evidence indicating the accumulation of Gd in the brain [7]. Some reported a higher risk of nephrogenic fibrosis [6] and DNA single-strand breaks (SSB) [8, 9]. Other studies showed increased DNA SSB detected through γH_2AX, as well as a higher incidence of micronuclei and enhanced DNA damage, particularly assessed through alkaline single-cell gel electrophoresis [4, 10, 11].

However, replacing the contrast medium Gd with substituted elements, such as $MgCl_2$ and ZnO NPs, could produce an improved bio-effect and be an

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[DOI: 10.61186/ijrr.22.1.131]

alternative contrast medium. Through cyclic voltammetry technology, researchers have found that Mg^{+2} functions as an antioxidant in the blood medium [12]. $MgCl_2$ is a good choice for an alternative contrast medium in MRI. It has been demonstrated to produce high-resolution MR images, making it usable as a T1 contrast agent $MgCl_2$, simultaneously being an alternative to gadolinium [6, 8, 9, 13]. Mg is present in over 300 human enzymes, making it one of the five most abundant elements in the human body [14]. The electrochemical study of redox of Gd contrast in the blood medium with the cyclic voltammetry technique demonstrated high oxidation rates [12].

Nanotechnology is concerned with creating and using materials that are up to 100 nanometers in size. Metal oxide nanoparticles, which are being developed, have promising and wide-ranging applications in the biomedical field [15]. A recent study demonstrated that utilizing ZnO NPs as an alternative contrast medium in computed tomography imaging resulted in high-resolution imaging of rabbit organs, including the heart, kidneys, and liver [13, 15]. In another study, ZnO NPs were ideal for increasing contrast in Optical Coherence Tomography (OCT) imaging [16]. Additionally, ZnO NPs have advantageous electrochemical characteristics, particularly in blood medium [13], in contrast to Gd compounds that cause oxidation [17]. In biomedical applications, ZnO NPs have drawn increased interest. In a mouse atopic dermatitis model study, Ilves *et al.* (2014) ZnO NPs showed anti-inflammatory effects by significantly reducing local skin inflammation [18]. At the same time, Wang *et al.* (2008) found that mice exposed to ZnO NPs orally experienced liver damage [19].

The comet assay is a valuable method extensively utilized in genotoxicity research and biomonitoring to detect single- and double-strand DNA breaks [20]. This is the first study to compare the bio-effects of Gd with alternative contrast to ZnO NPs and $MgCl_2$ in MRI using the alkaline comet assay. This study used the alkaline comet assay to investigate the bio-effects of contrast Gd compared to the alternative contrast $MgCl_2$ and ZnO NPs on DNA single-strand.

MATERIAL AND METHODS

Study design and animals

In this *in-vivo* experimental non-randomized controlled study, white New Zealand rabbits (n=12, males) were selected from the Serum and Vaccine Institute in Alammriah Baghdad, Iraq, as presented in Table 1. The rabbits were divided into four main groups: negative control (n=3), contrast media gadolinium, Gd (Magnevist) (n=3), alternative contrast magnesium chloride, $MgCl_2$ (n=3) and alternative contrast zinc oxide nanoparticles, ZnO NPs (n=3) groups. The rabbits were exposed to a 1.5T (RF- 64 MHz) MRI for 20 minutes. To assess the DNA damage bio-effects, we employed the alkaline comet Aasay to study the DNA single-strand breaks. We measured three parameters, namely the mean % DNA in the tail, tail length (TL), and tail moment (TM), to quantify the extent of DNA damage. The primary focus of this study was on DNA single-strand breaks observed in the lymphocytes of rabbits treated with a contrast medium. The choice of rabbits as the experimental model was based on their phylogenetic closeness to humans, supported by DNA sequence similarities, which suggests their relevance in studying similar biological responses.

Preparation of alternative contrast $MgCl_2$

The hydrated magnesium chloride ($MgCl_2.6H_2O$) crystals (AR, Technician-SDFCL, Mumbai, India) were used to prepare alternative contrast of the $MgCl_2$ solution. We collected 10.15 gm of crystal, dried it in the oven for one minute to remove the moisture, and dissolved it in 10 ml of deionized water to produce a 0.5M solution of $MgCl_2$. Preparation was carried out in the laboratory of the Department of Radiation Techniques, College of Health and Medical Technology, Baghdad.

Preparation of alternative contrast ZnO NPs

Pure nanoparticles of zinc oxide (ZnO) 40 nm powder (Company-MK Nano, Malaysia), ranging in colour from white to light yellow, were used. Prepared 0.16 gm was dispersed in 10 ml of deionized water to get a 0.1 M ZnO NPs solution. Preparation was carried out in the laboratory of the Department of Radiation Techniques, College of Health and Medical Technology, Baghdad.

Characterization ZnO NPs

Standard techniques were employed to characterize the physicochemical properties of 40 nm ZnO NPs. The visualization of size and surface morphology shape was achieved through field emission scanning electron microscopy (FESEM) (NOVA Nano SEM-450, Thermo Fisher Scientific, USA) operation at 10 KV. To perform FESEM, a drop of ZnO NPs solution was placed on a sample holder silicon wafer and deposited on a conductive glass substrate. Meanwhile, imaging and analysis of the morphology of the surface, the chemical content of the sample, atomic structures, and compositional data can be collected by FESEM utilizing the Energy-Dispersive X-ray (EDX) detector (Oxford, UK).

Experimental procedure

The rabbits (n=9) were injected with 1 ml anesthesia dose of Ketamine (10 mg/kg) + Xylazine (3 mg/kg) (Both from Alfas, Holland) through the muscles. After the anesthesia for 5 -7 minutes, the rabbits were injected through the ear vein puncture with contrast Gd (n=3) (Magnevist 469 mg,

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[DOI: 10.61186/ijrr.22.1.131]

Bayer-Berlin, Germany), $MgCl_2$ solution 0.5 M (n=3) and ZnO NPs solution 0.1M (n=3), at a dose of 1.1/2 ml. The rabbits were put on the MRI couch and exposed for 20 minutes to an MRI 1.5T scan (RF-64 MHz, Achieva & Intera; Philips Healthcare 2012, Holland), closed-type and using coil spine when scanning at a room temperature of 20 - 22ºC. The scanning was conducted at Alkarkh Hospital in Baghdad.

Draw blood samples

Following the exposure, 2 ml blood samples (whole blood) were drawn by vein puncture from the ear of rabbits for all groups. The drawn blood was immediately put into EDTA-K3 tubes (China) and kept in a small ice portable fridge (box) before being carried back to the laboratory. Samples were kept in a freezer and handled under yellow or dim light to avoid damaging DNA from UV radiation. The samples were processed within 24 hours.

Comet analysis

The period between exposure to MRI scans and examination of the comet ranged from 16 to 18 hours. Blood was placed in a refrigerator at -20°C until processed. The time interval between the end of exposure and blood withdrawal was 45 minutes. An external laboratory, the Rawan laboratory in Karrada, Baghdad, performed comet analysis, and the lymphocytes were isolated from the whole blood [21]. Singh et al. (1988) method was used as an alkaline comet assay with slight modification (pH >13) [22]. The Comet Assay Silver kit (TA4251-050 K.50 test, Trevigen, USA) was used, and the Comet slides were covered with 1% normal agarose. Each sample was analyzed accordingly (50 cells per slide). Adding 2 ml of blood to a standard tube and completing 10 ml of 1X red blood cell (RBC-preparation) lysis, the tube was easily stirred by hand for less than a minute and then centrifuged (Hettich, Germany) for 10 minutes at 10,000 rpm to separate the cellular suspension and to remove the supernatant (repeated three times). Following that, the pellets were washed once with 1X phosphate-buffered saline (PBS) (Trevigen, Inc., USA). Ca^{2+} and Mg^{2+} free, pH 7.4 added 500 µl cool 4° C, a good mix using a vortex mixer device (VM-300 Griffin, Germany) at 10 minutes. Then a 10 µl cell sample was placed in Eppendorf, combined with agarose 250 µl, at 37ºC (mixed). A mixture drop (50 µl/well) was quickly placed onto a comet slide. Then the slide was covered. The slices were then quickly frozen on an ice block. As the agarose solidified, the coverslips were transferred into a small basin containing and submerged in 1X lysis solution 25 ml/ slide buffer 4ºC, pre-chilled lysis buffer remained submerged for 24 hours, put in a cool place and far from light. Afterwards, any surplus lysis solution was rinsed off the slides, and they were immersed in a newly prepared alkaline solution with a pH greater than 13. The slides were left in this solution for 10 minutes at room temperature in a dark room. Then, the slides were washed with distilled water and transferred to alkaline electrophoresis solution pH >13 in a horizontal chamber (Bio-Rad Horizontal, China), power supply set at 300 mA and 25 V/cm for 40 minutes. After, excess alkaline was gently tapped off for 5 mins at 37ºC with an air dryer. The cells were stained with SYBR Green (diluted-deionized) 50 µl and applied to fluorescent DNA imaging on the slide. A fluorescence microscope (Primo Star Zeiss, Germany) with a green maximum excitation filter was used to view the slides. Complex systems used, including microscopes, computer Lenovo (Windows 10), Microsoft-365 packages, and mobile cameras (A52 Galaxy). Numerous Comet parameters were determined using the Tri Tek Comet Score™ (2006 programme, USA).

In this study, the data were quantitatively analyzed, and three parameters were used for Comet analysis: percentage tail DNA, tail length (µm), and tail moment. The damaged cells have comets, while unharmed cells resemble entire nuclei without tails. DNA length migration is an estimate of DNA damage and is assessed by detecting increased DNA migration of exposed rabbit lymphocytes [23].

Statistical analysis

The IBM SPSS version 24 (IBM Corp., Armonk, NY, USA) was utilized for the statistical analysis. The data were expressed as mean ± standard error (SE). To compare the differences in DNA damage among the four treatment groups, One-way analysis of variance (ANOVA) was conducted, followed by the Least Significant Difference (LSD) post-hoc test. A significance level of $p<0.05$ was adopted to determine statistical significance [24].

RESULTS

Characterization of ZnO NPs by FESEM and EDX analysis

The FESEM was used for nano-ZnO 40 nm of 0.1 M solution to examine the morphology of ZnO NPs solutions. Most of the observations were mixed spherical and rod-shaped forms [25] of the magnification 200X at 400 nm, as shown in figure 1. The ZnO NPs sizes ranged from 39 - 63 nm. Similar outcomes were seen in other research. As an illustration, a study by Fuad *et al.* (2017) observed that elevating the precursor's concentration from 30 - 35 mM resulted in an enlargement of the nanoparticles' size from 44.6 to 58.9 nm [26]. Figure 2 presents the energy-dispersive X-ray (EDX) analysis results conducted on the sample from the same area. The mapping analysis confirmed that the ZnO NPs were highly purified, with approximately 78.45% Zn and 21.55% oxygen composition [27]. Thus, Zn and O_2

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were identified as the predominant components of the ZnO sample. The higher the peak in a spectrum, the more concentrated the element is in the specimen.

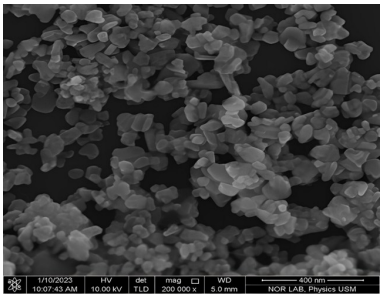


Figure 1. FESEM morphology of zinc oxide nanoparticles 40nm.

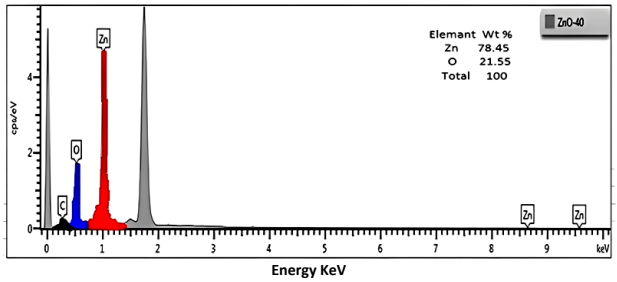


Figure 2. EDX analysis of zinc oxide nanoparticles components.

Effects of contrast medium on DNA single-strand following exposure to 1.5T MRI for 20 minutes

In table 2, the mean DNA single-strand for the control group was 5.58 ± 0.92. The mean % damage of DNA SSB was significantly higher in the Gd group (15.32 ± 0.89; p<0.001) and the alternative ZnO NPs group (10.09 ± 0.18; p=0.006) compared to the control group. However, no significant change in the mean % damage of DNA SSB in the alternative contrast, the $MgCl_2$ group (7.00 ± 1.15; p=0.277). These were also shown in figures 3 and 4.

Table 2. Effects of contrast medium on DNA single-strand following exposure to 1.5T MRI for 20 minutes.

Contrast Medium	SSB (Mean ± SEM)	*p*-value
Control	5.58 ± 0.92	1
Gadolinium	15.32 ± 0.89***	<0.001
$MgCl_2$	7.00 ± 1.15	0.277
ZnO NPs	10.09 ± 0.18**	0.006

Keys: n=3, SSB=single-strand break; SEM=standard error of the mean; MRI=magnetic resonance imaging; RF=radiofrequency; Significant at *=p<0.05, **=p<0.01, ***=p<0.001 compared to control using One-way ANOVA followed by LSD post-hoc tests.

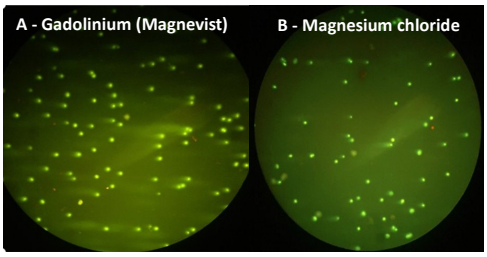


Figure 3. Effect of contrast medium on DNA single-strand on exposure to MRI 1.5T at 20 minutes and viewed under fluorescence microscope power 10X. **(A)** Gd contrast comet tail for DNA damages seen in the picture. **(B)** alternative MgCl2 group.

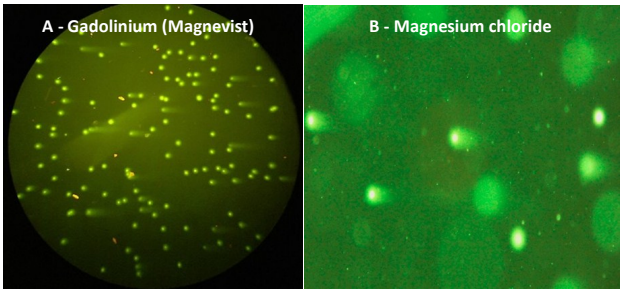


Figure 4. Effect of alternative contrast ZnO NPs on DNA single-strand following exposure to MRI 1.5T at 20 minutes. **(A)** Taken by the mobile camera from the microscope zoom 10X. **(B)** Screenshot from computer to take an image of the slide under the fluorescent microscope zoom 20X.

Effects of contrast medium on DNA single-strand following exposure to MRI 1.5T scan at 20 min using three comet scores

Table 3 shows quantitatively the mean values of three parameters, which tend to be highly significant in the Gd group. DNA tail fraction measurement is probably the most useful parameter in the comet assay regarding the mean % of DNA in the tail [28]. The Gd (p=0.001) and alternative ZnO NPs (p=0.001) groups significantly increased the mean tail length. Conversely, there was no significant change in the mean tail length for the alternative $MgCl_2$ group (p=0.074) compared to the control. Regarding the mean % of DNA in the tail, both the Gd (33.05 ± 0.93; p<0.001) and the alternative ZnO NPs group (26.48 ± 1.25; p=0.048) significantly increased the values than the control group. However, the mean % of DNA in the tail for the $MgCl_2$ group (22.27 ± 1.21; p=0.838) did not significantly change. Furthermore, the mean tail moment in both the Gd (p=0.001) and alternative ZnO NPs (p=0.001) groups was significantly higher than that in the control group. However, there was no significant change in the mean tail moment for the alternative $MgCl_2$ group (p=0.720).

Table 3. Effects of contrast medium on DNA single-strand following exposure to MRI 1.5T scan at 20 min using three comet scores.

	Groups	Mean ± SEM	*p*-value
Tail Length (px)	Control	3.50 ± 0.25	1
	Gd	10.60 ± 0.76***	0.001
	$MgCl_2$	6.07± 1.03	0.074
	ZnO NPs	9.70 ± 1.20***	0.001
%DNA Tail	Control	21.85 ± 1.99	1
	Gd	33.05 ± 0.93***	0.001
	$MgCl_2$	22.27± 1.21	0.838
	ZnO NPs	26.48 ± 1.25*	0.048
Tail Moment	Control	0.99 ± 0.12	1
	Gd	6.58 ± 0.68***	0.001
	$MgCl_2$	1.32 ± 0.48	0.720
	ZnO NPs	9.77 ± 0.89***	0.001

Keys: n=3, Gd=Gadolinium; SSB=single-strand break; SEM=standard error of the mean; MRI=magnetic resonance imaging; RF=radiofrequency; Significant at *=p<0.05, **=p<0.01, ***=p<0.001 compared to control using One-way ANOVA followed by LSD post-hoc tests.

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[DOI: 10.61186/ijrr.22.1.131]

DISCUSSION

The present study demonstrated that the enhanced contrast gadolinium (Gd) and alternative zinc oxide nanoparticles (ZnO NPs) cause significant DNA single-strand breaks following exposure to MRI 1.5T (RF-64 MHz) in rabbit lymphocytes with Gd causing more damage. However, the alternative contrast $MgCl_2$ did not significantly affect the DNA single-strand. Similar to our finding, Yildiz *et al.* (2011) reported the genotoxicity effects of contrast-enhanced Gd 1.5T MRI in human lymphocytes using the alkaline comet assay [29]. Another study by Fiechter *et al.* (2013) found double-strand breaks on DNA in human cells due to contrast-enhanced Gd after a cardiac MRI 1.5T [9]. In line with our findings, Cho *et al.* (2014) demonstrated heightened cytotoxicity and genotoxicity effects of Gd in conjunction with extremely low-frequency electromagnetic fields generated during an MRI scan. This was observed in human peripheral blood cells obtained from healthy donors [8].

In contrast, other studies reported no enhanced contrast Gd was found on the genes during cardiac MRI 1.5T in human lymphocytes [10, 30]. A study has shown that unbound Gd can have cytotoxic effects. To minimize the potential toxicity associated with free Gd^{3+}, Gd is administered in MRI contrast agents in a chelated form [31]. Owing to their restricted stability, Gd-based contrast agents (GBCAs) have the potential to undergo transmetallation when exposed to specific cations, such as zinc, iron, copper, and calcium. Consequently, this process can lead to the release of free Gd^{3+}. Subsequently, the liberated Gd^{3+} ions can bind with endogenous anions such as carbonate, phosphate and hydroxide. Consequently, the Gd^{3+} can accumulate in tissues as insoluble compounds [32, 33], leading to oxidative and immune effects [34]. These mechanisms of Gd retention and their biological consequences have significant safety implications for biological systems [7].

Furthermore, our study revealed that ZnO NPs induced DNA single-strand breaks, making them unsuitable as an alternative contrast medium. According to a prior study, ZnO NPs induce oxidative stress, leading to DNA damage and apoptosis in rat liver [35-37]. Additionally, the progressive accumulation of ZnO NPs in the body could present further concerns [19]. Our study used a dose of 1.1/2 ml and a concentration of 0.1M. Although ZnO NPs are thought to be protective in living organisms. According to previous studies, ZnO NPs are promising biocompatible and biodegradable nanoplatforms, making them a subject of investigation for potential applications in cancer treatment [38, 39]. A recent study reported ZnO NPs exhibit some cytotoxicity in cancer cells, most due to their enhanced intracellular release of dissolved Zn^{2+}, increased generation of reactive oxygen species (ROS), and promotion of cancer cell death through the apoptosis signalling pathway [40].

The current study demonstrated the alternative contrast $MgCl_2$ does not affect DNA single-strand. Previous studies have shown that Mg^{2+} is essential for maintaining DNA stability in the human body and supporting key enzymatic interactions with genetic material [41, 42]. Mg^{2+} also has good electrochemical characteristics, acting as an antioxidant in blood medium [12]. For these reasons, the alternative contrast group $MgCl_2$ did not affect the DNA single strand. A recent study reported that magnesium supplementation significantly reduced the number of cells with severe DNA damage to protect DNA from alkylation and function as a cofactor of nucleic acid metabolism enzymes. It plays a role in DNA replication, DNA repair, and gene expression [1, 43, 44]. Part of its functions, Mg^{2+} protects the DNA, and its essential compound is prospectively applicable in clinical diagnosis. $MgCl_2$ does not affect DNA single-strand despite the exposure group to MRI.

Our findings reveal the potential DNA-damaging effects of Gd contrast medium, which pose a significant risk to overall health. Numerous studies have already reported the various health risks associated with Gd. However, our research highlights a novel aspect by demonstrating that ZnO NPs also impact DNA damage. This discovery expands our understanding of the potential risks of different contrast agents. Interestingly, our study shows that the alternative contrast medium, $MgCl_2$, yielded favorable results without causing any significant effect on DNA single-strand. These promising outcomes suggest that $MgCl_2$ could be a viable contrast medium in the future, replacing Gd in MRI scans.

Limitations of the study include the small sample size of only twelve rabbits, which limits generalizability, and the use of an animal model that may not fully represent human responses. The short duration of exposure of 20 mins and the *in-vitro* study design also present limitations. Additionally, the study did not compare the contrast agents with other commonly used ones and did not assess functional or long-term effects. Patient-specific factors were not considered, and human studies were absent. Nevertheless, the study's notable strength resides in its comparative design, which offers valuable insights into the genotoxic effects of various contrast agents (Gd, ZnO NPs, and $MgCl_2$) and underscores $MgCl_2$ as a safer alternative for MRI scans.

CONCLUSION

The contrast medium gadolinium and alternative zinc oxide nanoparticles were demonstrated to cause significant DNA single-strand breaks, with Gd causing

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[DOI: 10.61186/ijrr.22.1.131]

more damage, thus raising critical concerns regarding their suitability for routine MRI scanning. However, the alternative contrast $MgCl_2$ was found to be safer with no effect on the DNA single-strand, suggesting it to be a more suitable alternative contrast media during the MRI scanning. These findings underscore the importance of evaluating the genotoxicity of contrast agents and provide valuable insights for selecting appropriate contrast media in medical imaging.

ACKNOWLEDGEMENT

We want to thank the academics, authors, and researchers at Universiti Sains Malaysia for their support in making this research work successful.

***Funding*:** None.

Conflicts of interests: The authors declared no conflict of interest.

***Ethical consideration*:** The animal ethical approval was obtained from the Universiti Sains Malaysia, Institutional Animal Care and Use Committee (IACUC), with reference No. IACUC/(128)(1140) dated 24 May 2021. Another approval was obtained from the Central Committee for Bioethics, University of Kufa in Iraq, with reference No. CCB/6667 dated 12/04/2021. All methods in the study were carried out following standard guidelines and that of the IACUC.

***Author contribution*:** RMS and NAN: Conceptualization, Methodology. MMR, NBS and MMD: Data curation, Software. RMS and NAN: Writing the original draft. MMR, NBS and MMD: Reviewing and editing the draft. RMS and NAN: Visualization, Investigation, Validation. NAN, MMR and NBS: Supervision. All the authors revised and approved the final manuscript.

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