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A toxicological evaluation of 8–28 nm gold nanocrystals

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ABSTRACT

Gold nanocrystals (AuNC) are gold nanoparticles (AuNP) relatively homogenous in size at 8–28 nm with clean surfaces and crystalline structures. There are concerns and a lack of consensus in the scientific literature and major regulatory bodies regarding not only the safety of nanoparticles when consumed by humans, but exactly how to determine their safety and whether evidence from a nanoparticle with one set of physiochemical properties extends to one with a different set. Additionally, there are few general long-term toxicity data on AuNP. To our knowledge, the potential toxicity of AuNC specifically, with the above characteristics, or otherwise, has not been investigated in preclinical studies; thus, we conducted a battery of genetic toxicity tests and an oral repeated-dose toxicity test to further explore their safety. AuNC were not mutagenic or clastogenic in bacterial reverse mutation and in vitro mammalian chromosomal aberration tests, respectively, and did not exhibit in vivo genotoxicity in a micronucleus test in mice. In a 60-day, repeated-dose oral toxicity study, rats were administered 0, 2.5, 5, or 10 mg/kg bw/day of AuNC by gavage. No toxicity was identified. Therefore, a no observed adverse effect level was determined as 10 mg/kg body weight/day.

1. Introduction

Nanotechnology holds great promise for biomedical applications (Deng et al., 2017) and is an emerging technology in food manufacturing (CFSAN et al., 2014). However, due to their unique attributes nanoparticle (NP) safety in biological systems is not well understood. The very same qualities that make them unique in industry give rise to uncertainty around their potential for toxicity in a biological context. Their reduced size can lead to changes in behavior, which may result in altered toxicological effects. Other physiochemical characteristics including shape, surface functionalization, charge, and whether they are coated can contribute as well (CFSAN et al., 2014; EFSA, 2018; Fratoddi et al., 2015; Soenen et al., 2011). The variety of characteristics make the direct comparison of results between different nanoparticle substances nearly impossible (Soenen et al., 2011) and research using nanoparticles is conflicting regarding their toxicity (Fratoddi et al., 2015). Consequently, there is a large knowledge gap in the biological mechanisms arising from nanoparticle interactions with human systems.

Possible reasons for NP toxicity are their large surface area to volume ratio which leads to an alteration in biological activity compared to the parent bulk materials (Yildirimer et al., 2011) and their unique surface chemistry (Chen et al., 2009). For instance, gold in its bulk form is considered an inert, non-toxic, biocompatible noble metal For instance, gold in its bulk form is considered an inert, non-toxic, biocompatible noble metal (Fratoddi et al., 2015) and has been used clinically for years (Fratoddi et al., 2015; Yildirimer et al., 2011). Reducing gold's size to the nanoscale (1–100 nm) results in behavior changes (Fratoddi et al., 2015; Yildirimer et al., 2011), particularly when very small (below 4–5 nm), potentially through direct genotoxicity by penetrating the nuclear compartment and binding to DNA (Magdolenova et al., 2014a; Soenen

Abbreviations: ANOVA, analysis of variance; AuNC, gold nanocrystals; AuNP, gold nanoparticles; BRMT, Bacterial Reverse Mutation Test; FOB, functional observation battery; GLP, good laboratory practice; IP, intraperitoneal injection; MPCE, micronucleated polychromatic erythrocyte; OECD, Organisation for Economic Co-operation and Development; T3, triiodothyronine; TSH, thyroid stimulating hormone.

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et al., 2011), crossing the blood brain-barrier (BBB) (Yildirimer et al., 2011) or indirectly by promoting endogenous oxidative stress and inflammation (Magdolenova et al., 2014a).

Gold nanoparticles (AuNP) with diverse physiochemical characteristics have been studied to determine their toxicological profile. The majority, if not all, are chemically synthesized (commonly using the Turkevich method). A 2011 review by Yildirimer et al. discussed multiple studies, using similarly sized AuNP that reported contrasting results regarding whether pro-inflammatory cytokine release enhanced BBB permeability and toxicity in rat brain microvessel endothelial cells. They further summarized that multiple in vitro and in vivo studies suggest smaller-sized AuNP (3 nm) seem to have more access to the brain than larger ones (5 nm) (Yildirimer et al., 2011). In a later article, Magdolenova et al., 2014a, 2014b, reviewed the genotoxicity of nanoparticles in in vitro and in vivo studies. They noted that the majority of in vitro comet studies, using different cell lines, had positive results while negative results were observed an in vivo comet assay and micronucleus test in male Wistar rats, as well as a bacterial reverse mutation test (BRMT) (Magdolenova et al., 2014a). In a 2009 investigation, Chen et al. (2009), performed a four-week toxicity study using naked AuNP with diameters from 3 to 100 nm, given to BALB/C mice by intraperitoneal injection (IP). Severe systemic effects and death were induced by 8–37 nm particles, while particles sized 3, 5, 50, or 100 nm revealed no significant toxicity.

In light of these conflicting results and because, to our knowledge, the potential toxicity of gold nanocrystals (AuNC) specifically, non-chemically synthesized 8–28 nm AuNP with clean highly faceted, non-functionalized crystalline surfaces have not been investigated in pre-clinical studies; thus, we conducted a battery of genetic toxicity assays and an oral repeated-dose toxicity test in order to further explore its safety.

2. Material and methods

2.1. Test article

The AuNC colloidal solution (4Life Research USA, LLC), the test article, is produced from 99.99% gold wire electrically reacted in USP grade deionized water and USP-grade potassium bicarbonate ($KHCO_3$) (0.04 mg/mL, as a processing enhancer). Manufacturing results in highly faceted AuNC with a mixture of crystalline structures that have clean surfaces (i.e., no surface contaminants) that are relatively homogenous in size (hydrodynamic radiuses of 8–28 nm) with a pH of 8.3–8.4, zeta potential −48 to −47 mV, and UV–Vis peak of 521 (see Fig. 1).

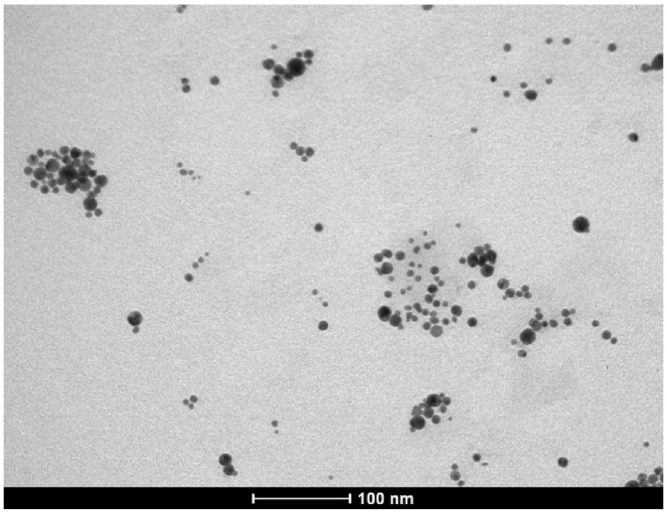

Fig. 1. TEM image of AuNC.

Due to the nature of the test article, the sponsor (4Life Research USA, LLC) provided three concentrated batches for direct use as test solutions and a vehicle control. The provided solutions contained 0, 250, 500, or 1000 ppm (nominal) AuNC suspended in purified water with up to 0.04 mg/mL $KHCO_3$ (batch numbers 60-1-31, 60-1-28, 60-1-29, and 60-1-30, respectively). Each batch containing AuNC was a light to dark red/purple liquid and was provided with a certificate of analysis verifying its concentration and homogeneity.

2.2. Care and use of animals"

The animal studies were conducted under the permission of the Institutional Animal Care and Use Committee of Toxi-Coop Zrt. During the acclimation and treatment periods, ad libitum access was provided to food (ssniff® SM R/M-Z + H, ssniff Spezialdiäten GmbH, Soest, Germany) and potable water. The 60-day study was additionally conducted according to the National Research Council Guide for Care and Use of Laboratory Animals (National Research Council, 2011) and in compliance with the principles of the Hungarian Act 2011 CLVIII (modification of Hungarian Act, 1998 XXVIII) and Government Decree 40/2013 regulating animal protection.

2.3. Bacterial reverse mutation test

BRMT was performed to GLP (good laboratory practice) requirements and was conducted in accordance with OECD test guideline 471 (OECD, 1997), except for the deviation regarding higher treatment volume discussed below. The BRMT was based on methods previously described (Ames et al., 1975; Kier et al., 1986; Maron and Ames, 1983; Venitt and Parry, 1984) according to the standard operating procedures of the laboratory. *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 (MOLTOX, Inc., NC, USA), histidine auxotrophs, and *Escherichia coli* WP2uvrA (MOLTOX, Inc., NC, USA), a tryptophan auxotroph, were used in the BRMT using the three ready-to-use AuNC test formulations, described in section 2.1, as stock solutions. Initial solubility and cytotoxicity tests were performed using the 1000 ppm test article and determined the concentrations of 25, 50, 100, 250, 500, and 1000 μg/plate of AuNC that were used in the main experiments. The stock solutions were used undiluted for the three higher concentrations. For the three lower concentrations, the vehicle control was used to dilute each of the three stock solutions 9:1. It was necessary to deviate from the OECD 471 test guideline (OECD, 1997) proposal for usual treatment volumes from 0.05–0.1 mL–1.0 mL in order to reach the maximum test article concentration of 1000 μg/plate. Therefore, the experiments were designed such that all plates received a 1.0 mL volume of their respective test or control solution. To accommodate this larger volume, the top agar composition was adjusted to avoid significant dilution. The controls, investigated in parallel at a 1.0 mL treatment volume, confirmed the applicability of the higher treatment volume.

The tests were performed with and without S9-mix metabolic activation (+S9 and -S9, respectively). The S9-mix was prepared in the laboratory according to standard procedures using activated rat liver supernatant (S9, Moltox, Inc., Boone, NC), the sensitivity, reliability, and promutagen activation potential, of which, were verified by the supplier and confirmed in the laboratory with 2-aminoanthracene (2-AA). In the -S9 experiments, 4-Nitro-1,2-phenylenediamine (4 μg/plate) was used as a positive control with TA98, sodium azide (2 μg/plate) was used with TA100 and TA1535, 9-aminoacridine (50 μg/plate) was used with TA1537, and methyl methanesulfonate (2 μL/plate) was used with the *E. coli* strain. In + S9 experiments, 2-AA was used as a positive control at 2 μg/plate with all *Salmonella* strains and at 50 μg/plate with the *E. coli* strain. The negative controls used were dimethyl sulfoxide, ultrapure water (ASTM 1) (for above listed positive controls), and the vehicle control for the test articles.

The mutagenicity tests were performed as an initial mutation test

(plate incorporation test) and a confirmatory mutation test (pre-incubation test) done in triplicate. After the incubation period colonies were manually counted and mean values, standard deviations, and mutation rates were determined. A result was considered positive if a dose-related increase in revertant colonies occurred and/or a reproducible biologically relevant positive response for at least one dose group occurred in at least one strain with or without metabolic activation. A result was considered biologically relevant if the number of reversions was twice that of negative controls for strains TA98, TA100, and *E. coli* WP2 uvrA or three times for strains TA1535, TA1537.

2.4. In vitro mammalian chromosomal aberration test

An OECD 473 (OECD, 2016) in vitro mammalian chromosomal aberration test (CAT), based on methods previously described (Brusick, 1989; Preston et al., 1981), was performed to GLP requirements. Cultured male Chinese hamster lung cells (V79, European Collection of Cell Cultures) were exposed in duplicate to 0 (vehicle control), 25, 50, and 100 μg/mL of the test article and positive and negative controls, with and without metabolic activation, in the main CAT. One mL of the undiluted test solutions and vehicle provided by the sponsor was added to the medium at concentrations of 10% (v/v) to obtain the treatment concentrations. Pre-testing of test article cytotoxicity was assessed for the purpose of selecting these concentrations. For the -S9 experiments ethyl methanesulfonate, a known mutagen and clastogen described in the literature (Preston et al., 1981) and verified by the historical database of the laboratory, was used as a positive control, while cyclophosphamide was used for the +S9 activation experiments.

The main test was divided into two independent experiments. In Experiment A, V79 cultures were exposed to the test solutions for a 3-h period with and without metabolic activation. Sampling was subsequently made at 20 h (1.5 cell cycles) after the start of treatment. Experiment B had three experimental arms. The first two were -S9 with incubation/harvest cycles of either 20/20 or 20/28 (2 cell cycles in case a mitotic delay occurred) hours. The third arm was performed + S9 with an incubation/harvest cycle of 3/28 h.

2.5. In vivo mammalian micronucleus test

An OECD 474 (OECD, 2016b) in vivo mammalian micronucleus test (MMT), based on methods previously described (Salamone and Heddle, 1983), was performed to GLP, except where otherwise noted below. Specific pathogen-free (SPF) Win:NMRI mice aged eight weeks and weighing 34.3–38.6 g at the beginning of the study were used in this MMT. A non-GLP preliminary oral toxicity test was conducted in which two male and two female mice were given two doses of the 1000 ppm test solution at 20 mg/kg body weight (bw). The preliminary test determined the oral dosing for the main experiment at 5, 10, and 20 mg/kg bw and that testing could proceed with only male mice as no toxicity differences between the sexes were observed. The pre-formulated test solutions, with nominal concentrations of 250, 500, and 1000 ppm, and vehicle were used undiluted at a volume of 20 mL/kg bw. The highest dose administered to the animals was the highest feasible dose using the most concentrated of the pre-formulated test solutions (1000 ppm; the highest achievable concentration due to the physiochemical properties of the test article) and the maximum dose volume recommended in the OECD guideline. The male mice were randomly divided into five groups of five; a negative and positive control and three test groups. The test solutions and vehicle control were administered twice, 24-h apart. The positive control, 60 mg/kg bw cyclophosphamide (Sigma-Aldrich Chemie GmbH, Germany) dissolved in sterile water (Naturland Kft, Hungary), was given once by IP.

The mice were examined for visible signs of reactions to treatment immediately after dosing and at regular intervals. All animals were sacrificed by cervical dislocation 24 h after their only/last treatment and bone marrow smears were prepared immediately.

2.6. 60-Day repeated-dose oral toxicity study in rats

A GLP, 60-day Repeated-Dose Oral Toxicity Study in Rats was conducted in general accordance with OECD 408. This study was originally intended and designed to be a standard OECD 408 90-day study; however, a deviation from the guideline was made due to a miscalculation in the amount of test material needed. As the mistake was not realized until after the study was underway and because of the long amount of time necessary to produce more test material, the decision was made to terminate the study early. Thus, the male and female animals were treated with the test article for 60- and 61-days, respectively.

The undiluted pre-formulated test solutions and vehicle control were given by gavage once per day. Just prior to administration each day, the dosing formulations were inverted a minimum of five times to mix the solutions. Doses of 0, 2.5, 5, and 10 mg/kg bw/day were administered using a constant dosing volume of 10 mL/kg.

A total of eighty healthy 44–46-day-old animals were randomly divided into four groups of 10 males and 10 females each, with equal weight distributions among the groups. Males weighed 201–217 g and females 126–154 g at the start of the trial.

Body weights were measured twice during the eight-day acclimatization period. During treatment they were measured twice each week for the first four weeks, once per week for the duration of the study, and immediately prior to sacrifice. Food consumption was determined, and food efficiency was calculated by weekly interval. Mortality and morbidity checks were done twice each day. Clinical observations were done twice during the acclimatization period. General clinical observations were made cage-side during and after each treatment, while detailed hands-on clinical observations were recorded on the day prior to the start of treatment and once per week thereafter. On Day 54, during the last week of the study, a functional observation battery (FOB; a modification of the procedure described by Irwin (1968)) was performed. Ophthalmological exams were performed on all animals before the first treatment and on those in the control and high dose groups on Day 54, during the last week of treatment.

After their last treatment, animals were fasted for approximately 16 h and placed under anesthesia using Isofluran CP® (Medicus Partner Kft., Biatorbágy, Hungary) so that blood samples for clinical pathology could be taken from the retro-orbital venous plexus. Sacrifice by exsanguination from the abdominal aorta immediately followed blood sample collection. The blood samples were analyzed for hematologic, blood coagulation and clinical chemistry parameters and thyroid hormone measurements. At necropsy, gross pathological examinations were conducted, organs were removed organ weight measurements were made, and tissues were preserved in buffered 4% formaldehyde solution (the testes and epididymides were first fixed in modified Davidson solution for one day then preserved in 4% formaldehyde solution). The fixed tissues were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on standard microscope slides, stained with hematoxylin and eosin, and examined by light microscopy. Full histological exams were done on the preserved organs of the control and high dose groups. When there were macroscopic findings in low and medium dose groups specific histological evaluations were also performed. Male reproductive organ morphology as well as sperm enumeration, morphology, and motility analyses were made on the control and high dose animals.

2.7. Statistics

Statistical analyses were performed with SPSS PC + software (SPSS, Inc., Chicago, IL, USA), and Microsoft Excel (Microsoft, Hungary) was used to check for linear trends. A P-value of <0.05 was considered statistically significant in all tests.

2.7.1. In vitro mammalian chromosomal aberration test

The Chi^2 test was utilized for statistical analysis and evaluated the

number of metaphase aberrations and cells with aberrations, both with and without gaps. A linear trend in the number of cells with aberrations (without gaps) was tested for using adequate regression analysis.

2.7.2. In vivo mammalian micronucleus test

The statistical frequency of micronucleated polychromatic erythrocytes (MPCEs) observed was analyzed using the Kruskal-Wallis nonparametric analysis of variance (ANOVA) test. A linear trend for the frequency of MPCEs was tested for using adequate regression analysis.

2.7.3. 60-Day repeated-dose oral toxicity study in rats

Statistical analysis was completed separately on male and female animals. Analyses of quantitative data including body weight, food consumption, feed efficiency, clinical pathology parameters, and absolute and relative organ weights and sperm parameters were done with SPSS PC + software. Variance between groups was checked with Bartlett's homogeneity of variance test. When no significant variance was found a one-way ANOVA was done, which when positive was followed up with Duncan's Multiple Range test to assess the inter-group differences for significance. Where Bartlett's found significant variance the normal distribution of data was examined by Kolmogorov-Smirnov test, which was followed up with the non-parametric Kruskal-Wallis ANOVA when a non-normal distribution was found. If the ANOVA was positive, the Mann-Whitney *U* test was performed for inter-group comparisons. Frequency of qualitative data including clinical signs, ophthalmoscopy, pathological and histopathological findings by sex and dose was calculated.

3. Results and discussion

3.1. Bacterial reverse mutation test

None of the AuNC test solutions caused a concentration-dependent increase in revertant colonies following treatment of the auxotrophic tester strain bacteria in the presence or absence of metabolic activation (see supplementary tables). Sporadic increases, when observed, remained within the corresponding historical control data range and below the threshold of a biologically relevant positive response. Positive controls induced the expected biologically relevant increases in revertant colonies.

As discussed in the introduction, it is difficult to practically compare results from studies using AuNP with different characteristics and to our knowledge, these are the first studies using 8–28 nm AuNC made from 99.99% gold wire, USP-grade water, and USP-grade $KHCO_3$ with clean surfaces and crystalline structure. Therefore, this and all subsequent comparisons should be considered with that in mind, even when not stated directly. That said, assays on nanoparticles of similar size with different physiochemical attributes have also been found negative for mutagenic potential using the BRMT. George et al., performed testing without metabolic activation using 14 nm citrate-stabilized AuNP that did not induce gene mutations (George et al., 2017). Interestingly, the authors went on to discuss that because their test article did not enter the bacterial cells, as determined by high-resolution transmission electron microscopy and Cytoviva dark-field image analysis, the BRMT was not a reliable mutagenicity study for nanoparticles. The European Food Safety Authority (EFSA) and others agree in general that the BRMT is not suitable for assessing nanomaterials and/or AuNP specifically as they may not be able to penetrate the cell well and be internalized by the bacteria (EFSA, 2018; George et al., 2017; Hadrup and Lam, 2014; Magdolenova et al., 2014a), implying that negative results are not necessarily due to a lack of mutagenicity. A 2014 review by Magdolenova et al. extends this discussion noting that if a nanoparticle actually does enter a bacterial cell it could interfere with histidine synthesis and induce false negative (down-regulation) or positive (up-regulation) BRMT results (Magdolenova et al., 2014b). Nonetheless, EFSA notes that in specific circumstances the test may still be informative, citing the example of "indirect genotoxic effects due to extracellular induction of reactive oxygen species" (EFSA, 2018).

3.2. In vitro mammalian chromosomal aberration test

Regarding the test article, there was no observed precipitation or relevant changes to pH or osmolality at any concentration. The vehicle and positive control responses were as expected relative to the historical database and concurrent negative control. None of the AuNC concentrations in any experimental group, with or without metabolic activation and up to the maximum concentration of 100 μg/mL, caused a significant increase in the numbers of cells with structural chromosomal aberrations relative to the vehicle control or in comparison with historical controls, nor was a concentration relationship observed (see supplementary tables). No polyploid or endoreduplicated metaphases were observed in any of the experiments.

After confirming that Chinese Hamster Ovary cells internalized their 14 nm citrate-stabilized AuNP, George et al., 2017, exposed them at 6.2, 12.5, 25, and 50 μg/mL for 20 h, resulting in minimal non-statistically significant chromosomal DNA damage (George et al., 2017). Xia et al. (2017) used 5, 20, and 50 nm AuNP in 0.1 mM PBS on Chinese hamster lung fibroblasts and also reported no significant difference in the incidence of chromosomal aberrations compared to the negative control at the maximum concentration of 12.5 μg/mL after 4 h of incubation with or without S9 (Xia et al., 2017).

3.3. In vivo mammalian micronucleus test

No mortality or adverse reactions were observed in the preliminary toxicity test in male or female Win:NMRI mice. No animals died during the main study, and no adverse reactions were observed. No statistically significant increases in MPCEs were observed in any treatment group compared to the negative vehicle control group, and the MPCEs found were compatible with the negative historical control data. The proportion of immature to total erythrocytes was slightly decreased in the 20 mg/kg body weight group compared to the negative vehicle control group. As expected, the positive control showed a large, statistically significant increase in the MPCEs number compared to the negative and historical controls (see supplementary tables).

Similarly, Xia et al. investigated the genotoxicity of commercially available, spherical 5, 20, and 50 nm AuNP each given intravenously (IV) in doses of 0.02, 0.1, and 0.5 mg/kg in male Kunming mice and also reported no increase in the frequency of chromosomal damage (Xia et al., 2017). It is interesting that their results were similar to those of this investigation considering the potential differences in exposure and toxicity of AuNP by oral versus IV administration routes (particularly systemic exposure) and because contrasting results were seen previously. For instance, Zhang et al., (2010), observed higher toxicity via the oral (and IP) route compared to administration by tail vein injection of 13.5 nm citrate coated AuNP (Zhang et al., 2010). Xia et al. also hypothesized that the relatively short exposure time in the standard in vivo micronucleus test might underestimate genotoxicity, so they followed up with an extended test using the 5 nm AuNP alone. They delivered 0.17 and 0.5 mg/kg to mice for 14 consecutive days, presumably by IV, as discussed in the shorter-term study above. From this longer study, they reported a significant increase in micronucleus formation in the high dose group relative to the negative control, suggesting that genotoxic effects of AuNP may depend on the time of exposure.

3.4. 60-Day repeated-dose oral toxicity study in rats

3.4.1. Mortality, clinical observations, and ophthalmology

No unscheduled deaths occurred, and no adverse clinical signs were observed at any dose in male or female animals. With respect to the controls, the FOB was negative for differences in treatment group

reactions to different types of stimuli, grip strength, or motor activity. There were no test article-related alterations in the eyes in any male or female animal.

3.4.2. Body weights and food consumption

No biologically significant differences resulted, relative to the control group, due to ingestion of the test article at any dose in the mean daily food consumption, feed efficiency, mean body weight, or body weight gain in either sex. Higher mean body weight gain was measured in males of the 2.5 mg/kg bw/day group between days 35–42 and lower mean body weight gain was measured in males of the 10 mg/kg bw/day groups between days 10–14. These changes, shown in Table 1, were statistically significant but were not considered toxicologically relevant as they were transient and did not affect the overall body weight development of the male animals, shown in Fig. 2.

3.4.3. Clinical pathology

The hematological and blood coagulation parameters were similar in both males and females of the control and all test groups (see supplementary tables). A significantly lower mean hemoglobin concentration of −3% was detected in both the female 2.5 and 10 mg/kg bw/day groups. These differences were considered minor and indicative of biological variation rather than toxicologically relevant as they lacked dose dependency and were well within the historical control range.

Clinical chemistry parameters were also unaffected by the test article at any dose in both sexes (see supplementary tables). Female animals in the 2.5 and 10 mg/kg bw/day groups exhibited slightly higher mean glucose concentrations that were statistically significant at +17 and + 18%, respectively, but minor (remaining well within the historical control range) and due to the lack of a dose relationship were judged to be of no biological significance.

Regarding thyroid chemistries, slight, but statistically significant, increases of +24 and + 33% were observed compared to control in mean free T3 levels of the low- and mid-dose group males, respectively. On the other hand, free T3 levels were comparable between the control and high-dose group males, showing only a non-statistically significant 4% change. Additionally, there were no correlating statistically significant changes in free T4 or TSH levels. In fact, while non-statistically significant, TSH levels were increased +2 and + 16% compared to the control in the low- and mid-dose group males, respectively. In contrast, when pathological increases in free T3 occur, the TSH levels decrease, suggesting that the slight, but statistically significant, changes in free T3 levels observed in the low- and mid-dose males were indicative of normal biological variation. No statistically significant differences in free T3, free T4, and TSH compared to control were observed in the treated group females. Additionally, there were no histopathological findings in the thyroids of high-dose males, and while thyroid histopathology is not the most sensitive marker of hormone changes it does, at least in the rodent, reflect that a significant and potentially adverse functional change has been initiated in the thyroid.

3.4.4. Necropsy and histopathology

Gross necropsy revealed macroscopic findings in the control and in all the test article treated groups. The findings observed are common to the species and/or occurred with similar frequency in control animals and accordingly were considered background findings not related to the test article (see supplementary tables). Regarding organ weights, males had a slightly higher mean pituitary weight (absolute and relative to body and brain weights) in the 2.5 mg/kg bw/day group, a higher mean pituitary weight (relative to body weight) in the 10 mg/kg bw/day group, and a lowered mean thyroid weight relative to the brain weight in the 5 mg/kg bw/day group when compared to the control. In females, the absolute mean heart weight slightly exceeded the control value (see supplementary tables). As these differences in organ weights were slight, not dose-related, and not accompanied by histological changes or relevant alterations in clinical pathology parameters they were not considered toxicologically relevant.

The sperm morphology (separated head and tail), motility (motile and immotile sperms) and sperm count were considered similar in animals in the control and high dose test groups (see supplementary tables) There was a significantly higher mean sperm count and higher mean percentage of motile sperm with a corresponding lower mean percentage of immotile sperm that were considered indicative of biological variations as the high dose group means remained within the ranges of control group values, the magnitudes of variation were minor (within 4% of control values in all cases), and the differences in means were in

Table 1
Summary of Bdy weight gain between days, 60-day study.

Group		Body weight gain (g) between days													
mg/kg bw/day		0–3	3–7	7–10	10–14	14–17	17–21	21–24	24–28	28–35	35–42	42–49	49–56	56–59	0–59
Male															
0 (Control)	Mean	18.8	25.9	15.8	19.9	13.7	13.6	13.6	11.2	20.6	17.6	15.9	11.3	9.3	207.2
	SD	3.9	3.9	2.7	3.9	2.5	3.3	2.6	3.7	5.2	5.0	3.2	3.7	3.2	25.5
2.5 (n = 10)	Mean	20.8	27.4	15.2	20.0	13.7	14.8	13.2	11.0	23.3	22.3	13.8	12.8	7.9	216.2
	SD	3.8	4.4	3.8	2.7	3.2	2.5	3.0	1.8	3.5	4.7	2.8	4.1	1.3	17.6
	SS										*				
5 (n = 10)	Mean	19.3	25.1	15.4	18.4	12.0	12.4	13.1	10.5	21.6	16.2	14.9	12.7	7.6	199.2
	SD	2.3	3.3	1.9	3.3	2.7	3.8	3.2	2.6	4.7	3.9	5.7	4.3	2.6	26.3
10 (n = 10)	Mean	19.3	22.6	15.5	16.2	13.7	13.0	12.0	11.1	20.2	17.7	13.9	11.7	7.2	194.1
	SD	4.7	2.8	2.7	3.5	2.6	3.6	2.9	3.4	3.8	3.4	3.5	3.4	4.0	20.1
	SS				*										
Test for Significance		NS	NS	NS	DN	NS	NS	NS	NS	NS	DN	NS	NS	NS	NS
Female															
0 (Control)	Mean	9.0	11.1	8.8	9.2	5.6	5.9	5.8	3.7	9.3	9.4	4.6	4.2	0.8	87.4
	SD	2.7	2.7	2.5	2.3	4.8	3.0	5.7	2.0	3.5	4.4	4.8	4.4	5.7	9.1
2.5 (n = 10)	Mean	9.2	12.3	6.3	9.9	7.7	8.2	5.2	4.8	9.2	8.3	6.9	5.9	2.1	96.0
	SD	2.3	3.3	3.1	2.7	4.3	2.1	4.1	2.4	2.6	3.6	2.9	3.8	2.3	15.4
5 (n = 10)	Mean	9.9	13.8	9.9	9.8	6.4	6.8	6.0	4.0	10.8	8.2	2.4	5.9	2.6	96.5
	SD	4.2	2.5	3.8	1.7	3.8	1.9	4.4	1.8	4.1	3.6	4.2	4.4	3.4	9.8
10 (n = 10)	Mean	11.1	12.5	6.5	7.8	5.3	5.9	7.0	3.5	9.5	5.4	4.8	5.7	3.1	88.1
	SD	3.7	3.9	3.4	3.4	4.7	2.7	3.8	3.0	4.3	3.6	3.8	4.3	2.7	10.0
Test for Significance		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Abbreviations: DN, Duncan's multiple range test; NS, Not Significant.
* = $p < 0.05$.
** = $p < 0.01$.

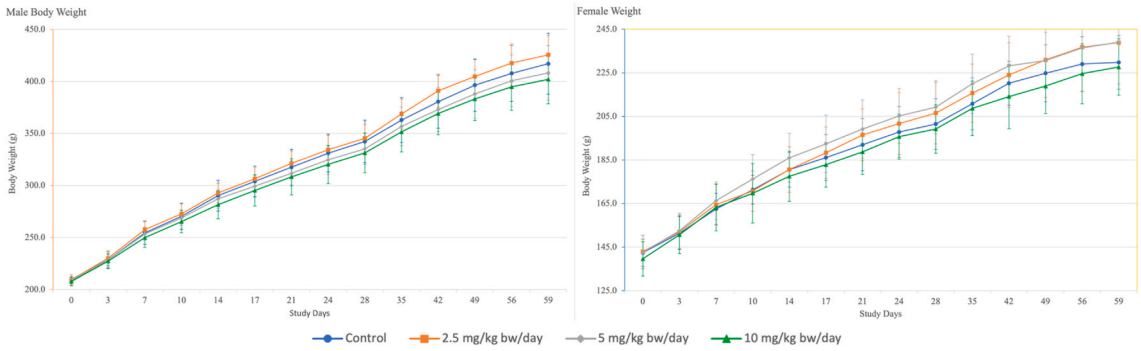

Fig. 2. Male and female body weight development.

the direction opposite of an adverse effect on fertility.

Full histopathological examinations were performed on animals of the control and high dose groups. There were no lesions found in the organs of animals in the high dose group that were not also found in the controls and no evidence of degeneration, inflammation, or necrosis was observed. Additional evaluation was performed on the kidney of one male from the 2.5 mg/kg group and on the diaphragm with liver from one male animal in the 5 mg/kg bw/day on the basis of observations during necropsy (one-sided dilation of the renal pelvis and diaphragmatic hernia, respectively). Microscopic findings were consistent with the macroscopic observations, which are common findings in laboratory rats.

To our knowledge, this is the only sub-chronic repeated-dose oral toxicity study in rats using highly-pure, clean-surfaced AuNC produced without synthetic chemistry at 8–28 nm in size. Chen et al. (2009), performed a subacute toxicity study using 8 mg/kg/week of chemically synthesized purified, naked AuNP with diameters of 3, 5, 8, 17, 12, 37, 50, and 100 nm, given to BALB/C mice by IP for four weeks. Similar to this study, toxic effects were not induced by the 3, 5, 50, or 100 nm particles. Conversely, perhaps due to physiochemical differences in the test articles other than diameter, the 8–37 nm particles induced fatigue, loss of appetite, weight loss, and dramatic fur color changes with underlying skin rashes, bruising, and hemorrhaging. Starting from day 14, mice in this group exhibited a camel-like back and crooked spine. The majority of mice in these groups died within 21 days. Histological assessment of the in vivo toxicity of AuNP indicated Kupffer cell activation in the liver, splenic white pulp diffusion, and emphysema-like deformities in lungs, consistent with gold deposition at these sites. In the end, they noted that cellular uptake of AuNP was a function of time, particle size, and concentration (Chen et al., 2009; Yildirimer et al., 2011). Sun et al., performed a 90-day study in mice using 53 nm citrate-coated spherical AuNP. The animals were given the AuNP at 0.2, 2, and 20 mg/kg orally daily. Compared to this work, their results were similarly negative without significant toxicity, although the authors noted changes in platelet parameters in females that they attributed to sex without adequate explanation. As this was not seen in the current work, it could represent an effect specific to their test item or normal biological variation as indicated by the authors.

4. Conclusion

AuNC did not induce frameshift or base substitution mutations in the BRMT, cause chromosomal damage in the CAT, or induce the formation of micronuclei in Win:NMRI mice during the MMT. Therefore, they were not considered to have genotoxic potential under the applied experimental conditions. In a repeated-dose oral toxicity investigation, gavage administered 8–28 nm AuNC given in doses of 2.5, 5, or 10 mg/kg bw/day did not cause adverse effects, and no target organs were identified, in male or female Han:WIST rats after 60/61 consecutive days as determined by clinical observations, functional observations, body weight development, food consumption and feed efficiency, and clinical and organ pathology. Based on the observations made in this toxicity study the no observed adverse effect level was determined as 10 mg/kg bw/day, the highest dose tested, in both male and female Han:WIST rats. While these are favorable results leaning toward the safety of 8–28 nm AuNC, due to the novelty of nanomaterial, questions about the utility of the BRMT for the evaluation of mutagenicity of nanomaterials and duration of exposure required to produce chromosomal damage, and anomalous data regarding route toxicity of gold nanomaterial a need for a more developed framework related to the toxicological evaluation of nanomaterials in general especially with regards to genetic toxicity and toxicokinetics is indicated. Additionally, due to the inadvertently shortened timeframe of the repeated-dose toxicity study in the current work further repeated-dose studies with the same or similar AuNC could be considered.

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Data availability

The data sets generated during these studies (Tables S1–S20) are available in the supplementary data files.

CRediT authorship contribution statement

Vickie Modica: Writing – original draft, Visualization. **Róbert Glávits:** Formal analysis, Data curation, Writing – review & editing. **Timothy S. Murbach:** Writing – review & editing. **John R. Endres:** Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Gábor Hirka:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. **Adél Vértesi:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization, Supervision, Project administration. **Erzsébet Béres:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization, Supervision, Project administration. **Ilona Pasics Szakonyiné:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization, Supervision, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

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