Cellular apoptosis of HFF cells by inorganic nanoparticles not susceptible to modulation by *Toxoplasma gondii* infection *in vitro*

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ABSTRACT

The interaction of nanoparticles with living cells is becoming one of the urgent areas of collaborative research in materials science and biology. Previously, we showed that nanoparticles have promising anti-*Toxoplasma gondii* properties. Meanwhile, *Toxoplasma gondii* has been shown to avert apoptosis in host cells whereas nanoparticles have been implicated for apoptotic tendency. Therefore, in the present study, we assessed the *in vitro* apoptotic properties of inorganic nanoparticles in the absence or presence of *Toxoplasma* infection and/or small molecules used as metabolic modulators. Results showed that inorganic nanoparticles dose-dependently caused cellular apoptosis. However, in the presence of infection by *Toxoplasma gondii*, nanoparticles-induced cellular apoptosis was not mitigated. Likewise, use of several small molecules (anti-metabolites) as metabolic modulators either mildly or nearly failed to abate cellular apoptosis by nanoparticles. Taken together, our findings do not only confirm the apoptotic potential of inorganic nanoparticles but show evidence that cellular apoptosis by inorganic nanoparticles of gold and silver might not be susceptible to modulation by *Toxoplasma gondii* infection. The findings are new and contribute to deepen our understanding of the cellular interaction of nanoparticles.

1. Introduction

One of the relevant and recent advances in material science is the increasing application of nanoparticles (NPs) to transform diverse fields of biomedical science (Adeyemi et al., 2017a). For example, over the past two decades, NPs have been gaining popularity for their usage in drug delivery, therapeutics, diagnostics among others (Adeyemi and Sulaiman, 2015). Among the inorganic NPs with biological significance are silver nanoparticles (AgNPs), gold nanoparticles (AuNPs) and platinum nanoparticles (PtNPs). AgNPs have been implicated for cytotoxic efficacy applicable in cancer therapy (Azizi et al., 2017). AuNPs are inert and biocompatible and these have been applied as theranostics as well as for phototherapy (Moses et al., 2016; Rieznichenko et al., 2012) while PtNPs are noted for antioxidant properties that mimic catalase and superoxide dismutase (Sulaiman et al., 2015; Adeyemi et al., 2018a). As a further testament of the increasing popularity of NPs for biomedical applications, the potential of NPs against *Toxoplasma gondii* infection was recently reported (Adeyemi et al., 2017a; Adeyemi et al., 2017b). In these studies, AgNPs, AuNPs, and PtNPs, exhibited antiparasitic potential which in part was mediated through modulation of

host cellular processes such as the downregulation of hypoxia inducing factor 1 protein (HIF-1α) and activation of kynurenine pathway. Interestingly, NPs and *T. gondii* affect similar cellular targets such as hypoxia inducing factor 1 (HIF-1α) and tryptophan pathway, particularly indoleamine 2,3-dioxygenase (IDO; rate limiting enzyme in the degradation of tryptophan to form kynurenine) (Blader and Saeji, 2009; Adeyemi et al., 2017b; Wiley et al., 2010; Spear et al., 2006; Zinkernagel et al., 2007).

Perhaps, there is a growing need to further our understanding of cellular interactions by NPs. These investigations are warranted if we consider some emerging facts; for example, the anti-*T. gondii* potential of inorganic NPs of silver, gold and platinum has been linked capacity to modulate cellular processes such as the metabolism of tryptophan as well as hypoxia in human foreskin fibroblast (HFF) cells (Adeyemi et al., 2017a, 2017b). Additionally, investigations have shown that NPs including silver and gold nanoparticles are capable of causing apoptosis in a variety of cell types (Selim and Hendi, 2012; Satapathy et al., 2013). On the other hand, *T. gondii* infection has capacity to modulate several host cellular processes in order to aid unhindered growth and replication (Blader and Saeji, 2009; Zinkernagel et al., 2007). In

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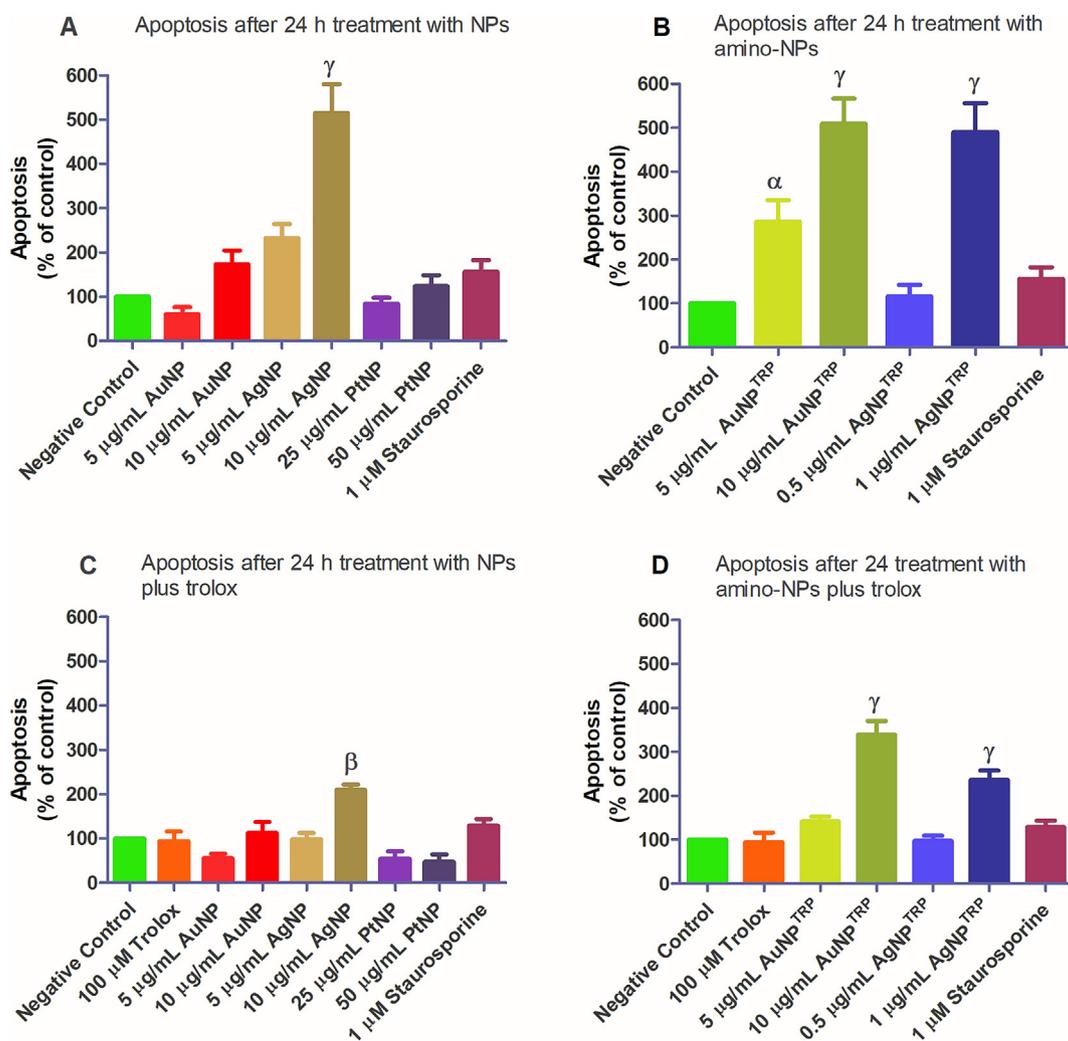
Fig. 1. Cellular apoptosis after 24 h treatment; [A] Nanoparticles (NPs) [B] Amino-Nanoparticles (amino-NPs) [C] Nanoparticles (NPs) plus Trolox [D] Amino-Nanoparticles (amino-NPs) plus Trolox. Data are presented as mean of triplicates ± standard error of mean (SEM). The experiment was performed in triplicate and repeated three times independently. α is significant at $p < .05$, β at $p < .001$ and γ at $p < 0.0001$ versus negative control.
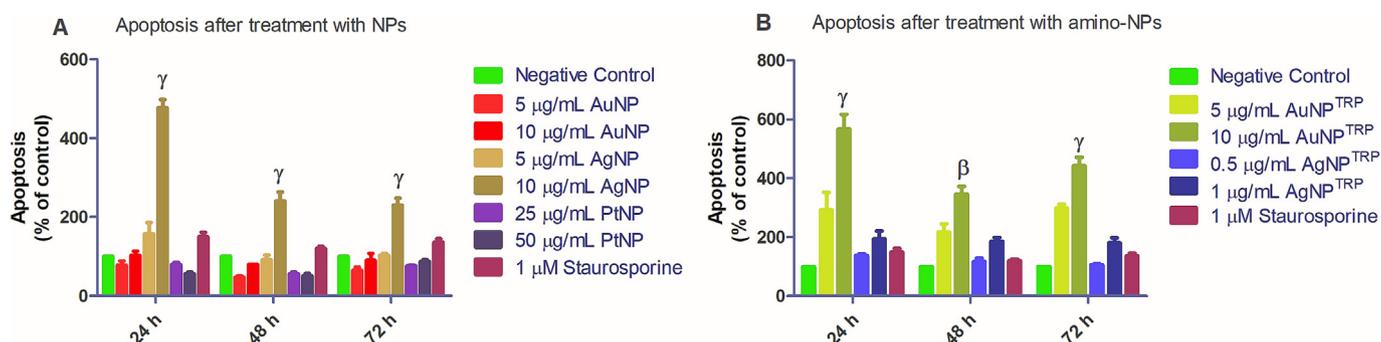
Fig. 2. Cellular apoptosis with time kinetics; [A] Treatment with nanoparticles (NPs) [B] Treatment with amino-nanoparticles (amino-NPs). Data are presented as mean of triplicates ± standard error of mean (SEM). The experiment was performed in triplicate and repeated three times independently. β is significant at $p < .001$ and γ at $p < 0.0001$ versus negative control.

particular, *T. gondii* infection can protect different cell types from apoptosis induced by a variety of pro-apoptotic treatments (Lüder and Gross, 2005; Goebel et al., 2001). Therefore, in the present study we sought to determine how the apoptotic property of NPs are affected in the presence of *T. gondii* infection and other metabolic modulators such as L-tryptophan, cobalt (II) chloride (CoCl$_2$), 1-Methyl-D-tryptophan (DMT), 3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and (±)

3,4-dihydro-3-hydroxy-2,2-dimethyl-4-[(phenylmethyl)amino]-2H-naphtho[2,3-*b*]pyran-5,10-dione (a naphthoquinone derivative – NPQ). All of these metabolic modulators have been demonstrated to either enhance or restrict the growth of *T. gondii* elsewhere (Adeyemi et al., 2017b).
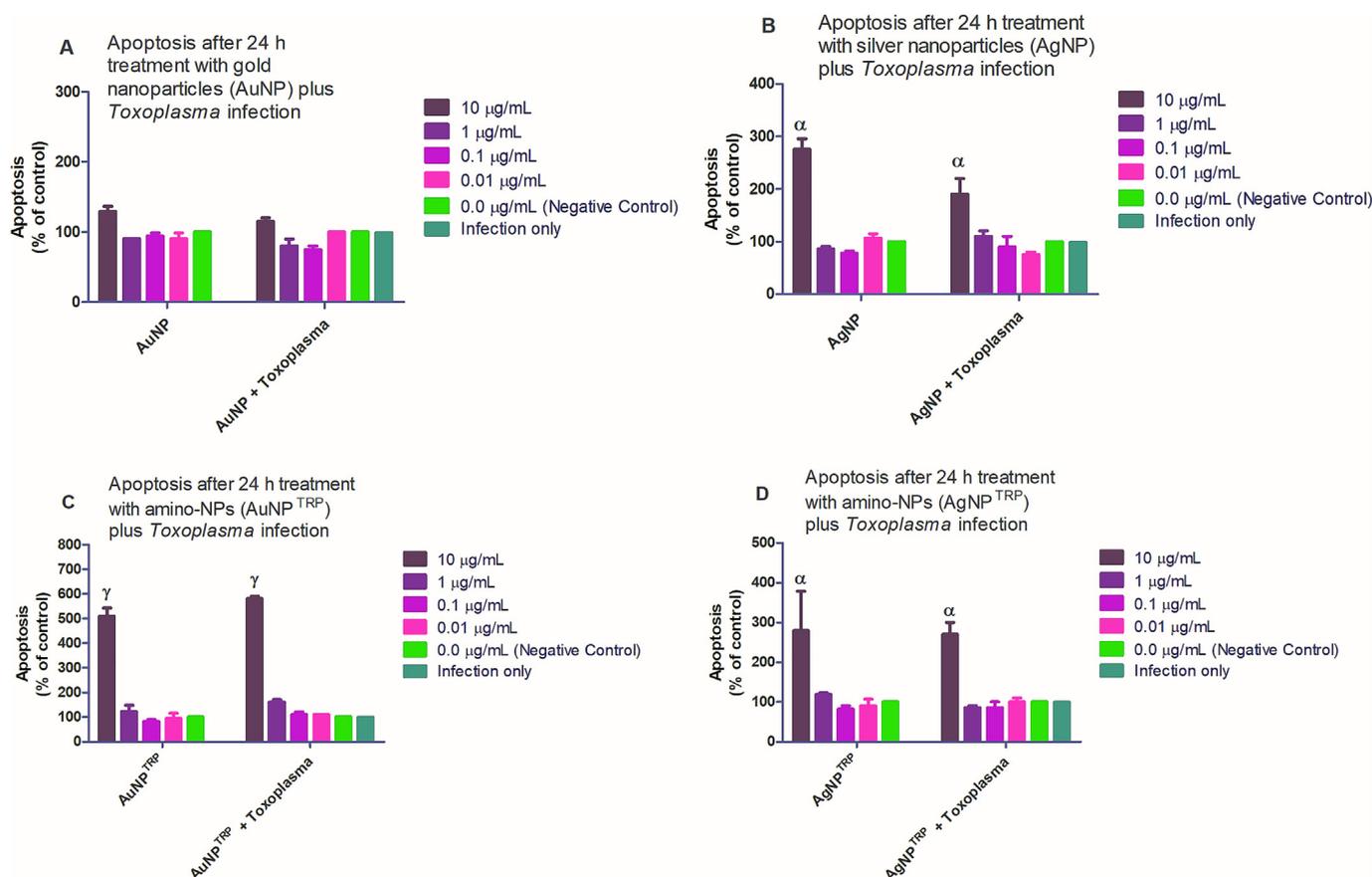
Fig. 3. Cellular apoptosis after 24 h treatment with nanoparticles (NPs) or amino-nanoparticles (amino-NPs); [A] Without *Toxoplasma gondii* infection [B] With *Toxoplasma gondii* infection. Data are presented as mean of triplicates ± standard error of mean (SEM). The experiment was performed in triplicate and repeated three times independently. α is significant at $p < .05$ and γ at $p < 0.0001$ versus negative control and/or infection only.

2. Materials and methods

2.1. Materials and chemical reagents

Nanoparticles (NPs) with diameter size range of 5–40 nm, including silver (AgNP), gold (AuNP), platinum (PtNP) and amino-NPs (silver-tryptophan - AgNP^TRP and gold-tryptophan - AuNP^TRP) were gifted by Global Infection Unit, NRCPD, Obihiro University of Agriculture & Veterinary Medicine, Obihiro, Japan. Their synthesis and characterization have been reported elsewhere (Adeyemi et al., 2018b). The NPs were reconstituted in fresh culture medium prior to each use. L-tryptophan, L-kynurenine, cobalt (II) chloride ($CoCl_2$), 4-(dimethylamino) benzaldehyde, 1-Methyl-D-tryptophan (DMT), and 3-(5'-Hydroxymethyl-2'-furyl)-1-benzyl indazole (YC-1) were obtained from Sigma-Aldrich. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was a product of Wako Pure Chemicals (Osaka, Japan); (±)3,4-dihydro-3-hydroxy-2,2-dimethyl-4-[(phenylmethyl)amino]-2H-naphtho[2,3-*b*]pyran-5,10-dione (a naphthoquinone derivative – NPQ) was obtained from Cayman Chemicals (Ann Arbor, MI, USA). All other reagents were of analytical grade and used as supplied except stated otherwise.

2.2. Anti-metabolites

L-tryptophan is an amino acid required for *T. gondii* growth. $CoCl_2$ is a chemical inducer of hypoxia while YC-1 is a potent inhibitor of hypoxia inducing factor-1 alpha (HIF-1α). Trolox is antioxidant while (±)3,4-dihydro-3-hydroxy-2,2-dimethyl-4-[(phenylmethyl)amino]-2H-naphtho[2,3-*b*]pyran-5,10-dione and 1-Methyl-D-tryptophan are respectively competitive and non-competitive inhibitors of indoleamine 2,3-dioxygenase (IDO) activity.

2.3. Host cells and parasite strain

Human fibroblast foreskin (HFF; ATCC®, Manassas, VA, USA) cells were maintained at 37 °C and 5% CO_2 atmosphere in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Nissui, Tokyo, Japan), GlutaMAX™-I (Gibco, Invitrogen, UK), 10% (*v/v*) fetal calf serum (FCS; Gibco, Invitrogen, UK), penicillin and streptomycin (100 U/mL; Biowhittaker, UK) were maintained. At confluence, cells were harvested as per sub-culture protocols and resuspended to desired cell density.

The luciferase-reporting *T. gondii* RH strain 2F (ATCC® 50,839), expressing β-galactosidase, was used for this study. The parasite was maintained by repeated passages in human foreskin fibroblast (HFF; ATCC® SCRC-1041) cells cultured in DMEM supplemented with GlutaMAX™-I, 10% (*v/v*) FCS; and penicillin and streptomycin. In order to obtain parasite suspension, host cells infected with *T. gondii* tachyzoites were passed through a 27-guage needle to lyse them and the cell lysates were then filtered through a 5-μm filter to obtain a tachyzoite suspension free of host cell debris. The suspension was washed with fresh culture medium. Parasite density was determined with a hemocytometer and adjusted for *in vitro* experimental infection analysis at a multiplicity of infection (MOI) of 0.5.

2.4. Apoptosis assay

Cellular apoptosis was detected and measured with Apoptosis Assay Cell-APOPercentage™ reagent (Biocolor Life Science Assays, Antrim, UK) by following the manufacturer's protocol. Briefly, growing HFF
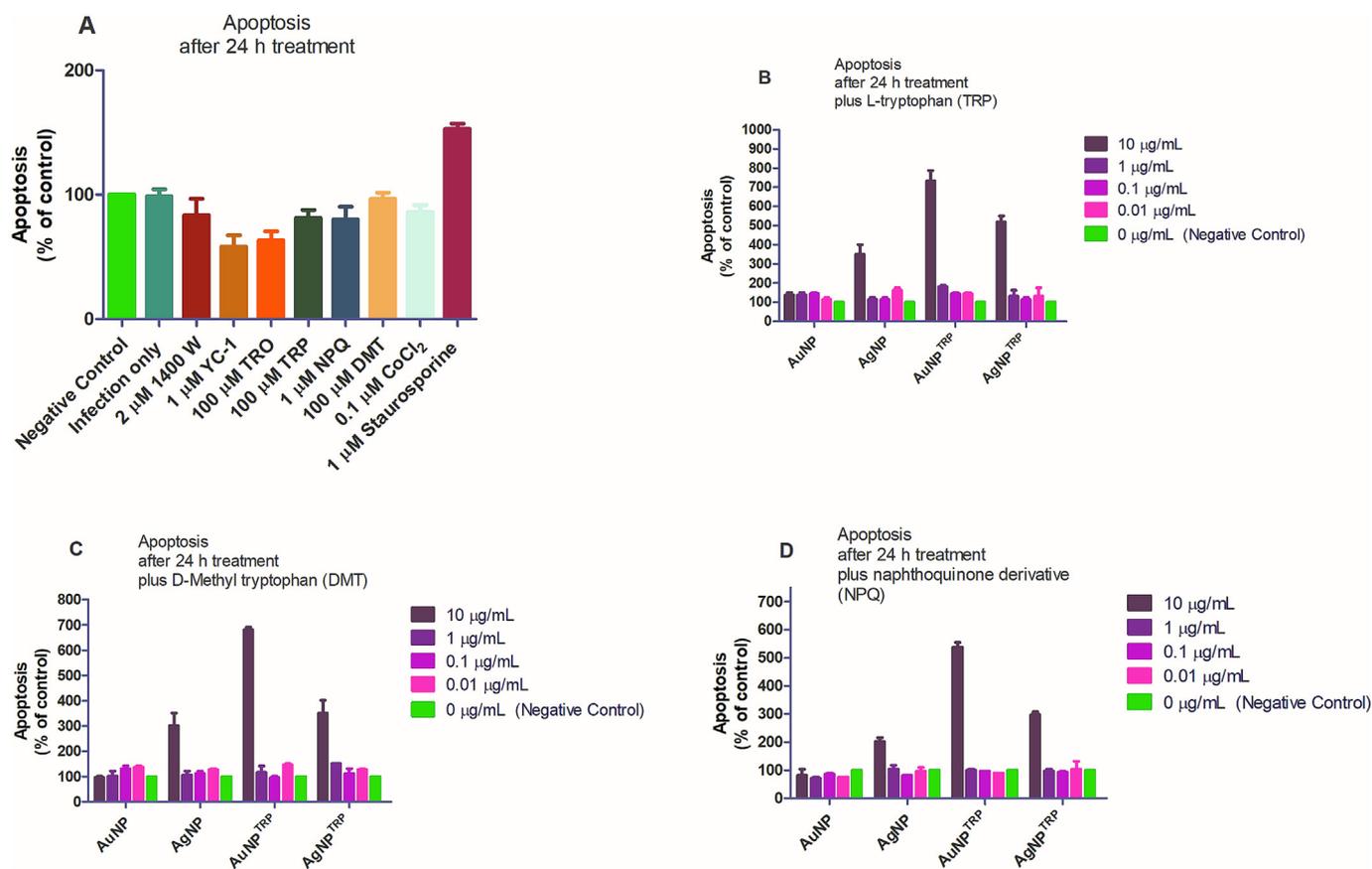
Fig. 4. Cellular apoptosis after 24 treatment; [A] Metabolic modulators only [B] Nanoparticles (NPs) or amino-nanoparticles (amino-NPs) plus L-tryptophan [C] Nanoparticles (NPs) or amino-nanoparticles (amino-NPs) plus 1-Methyl-D-tryptophan (DMT) [D] Nanoparticles (NPs) or amino-nanoparticles (amino-NPs) plus naphthoquinone derivative (NPQ). Data are presented as mean of triplicates ± standard error of mean (SEM). The experiment was performed in triplicate and repeated three times independently.

cells in 96-well plates with or without *T. gondii* infection were treated with the various nanoparticles (AgNP, AuNP, PtNP, AgNP^{TRP} and AuNP^{TRP}) alone or in combination with small molecules as metabolic modulators (anti-metabolites). The cells were then incubated for different time points at 37 °C and 5% CO_2. Thereafter, cellular apoptosis was monitored by colorimetric measurement recorded at 550 nm on a microplate reader (MTP 500; Corona Electric, Hitachinaka Japan). The biological assay was repeated three times, in triplicate. Selection of doses for the nanoparticles as well as for small molecules was premised on our earlier reports (Adeyemi et al., 2017a, 2017b, 2018b) and on preliminary experiments. In all the cases, doses were less than one third of the IC$_{50}$ value.

2.5. Statistical analysis

Data were analyzed by using a one-way ANOVA (GraphPad Software Inc., San Diego, CA) and are presented as the mean ± standard error of the mean (SEM). Comparisons among groups were determined by using Dunnett's post-hoc test. *P*-values < .05 were considered to be statistically significant.

3. Results

AuNP at 5 µg/mL caused cellular apoptosis while AgNP led to a dose dependent apoptosis (Fig. 1a). Likewise, AuNP^{TRP} caused a dose-dependent apoptosis but AgNP^{TRP} only caused cellular apoptosis at the highest dose (Fig. 1b). Meanwhile, addition of trolox appears to mildly mitigate cellular apoptosis by the NPs and amino-NPs (Fig. 1c-d). The cellular apoptosis caused by NPs and amino-NPs do not appear to follow

a definite pattern with time kinetics (Fig. 2a-f). Cellular apoptosis by NPs and amino-NPs peaked within 24 h and further extension of incubation time to 48 and 72 h showed no appreciable but inconsistent increase in level of apoptosis.

In order to determine whether, cellular apoptosis by NPs and amino-NPs was susceptible to modulation by *T. gondii* infection, we treated infected cells with various concentrations of NPs and amino-NPs and cellular apoptosis was determined after 24 h. *T. gondii* infection failed to avert cellular apoptosis by NPs and amino-NPs (Fig. 3a-d). Meanwhile, *T. gondii* infection alone did not cause cellular apoptosis after 24 h.

Furthermore, *T. gondii* and NPs have been shown to affect similar cellular targets (Adeyemi et al., 2017a, 2017b), therefore, we used small molecules as metabolic modulators (anti-metabolites) of these cellular targets to evaluate how cellular apoptosis by NPs and amino-NPs would be impacted. The various anti-metabolites used in the present study did not cause cellular apoptosis at doses investigated (Fig. 4a). However, in order to validate our apoptosis detection assay, we included staurosporine as a positive drug control. As expected staurosporine caused cellular apoptosis thus validating our detection assay. Of the metabolic modulators investigated in this study, none modulated cellular apoptosis by amino-NPs (Fig. 4b-d and Fig. 5a). But trolox (antioxidant), $CoCl_2$ (chemical inducer of hypoxia) and YC-1 (inhibitor of HIF-1α) mildly modulated cellular apoptosis by NPs (Fig. 5b-d). That these metabolic modulators modulate cellular apoptosis by NPs may indicate the relevance of these metabolic processes to the apoptotic action of NPs.
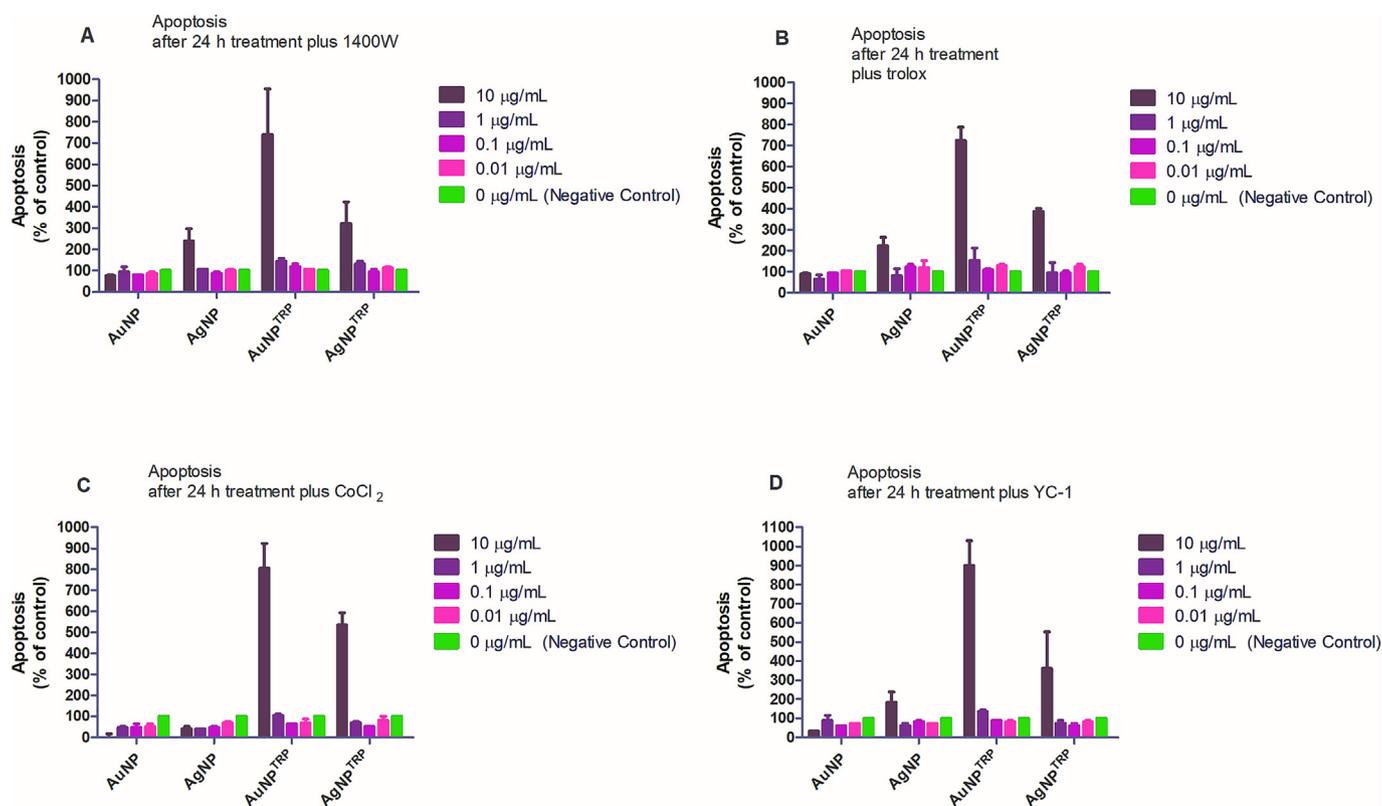
Fig. 5. Cellular apoptosis after 24 treatment with nanoparticles (NPs) or amino-nanoparticles (amino-NPs) plus; [A] 1400 W (inhibitor of nitric oxide synthase) [B] Trolox [C] CoCl$_2$ (chemical inducer of hypoxia) [D] YC-1 (inhibitor of HIF-1α protein). Data are presented as mean of triplicates ± standard error of mean (SEM). The experiment was performed in triplicate and repeated three times independently.

4. Discussion

Our current understanding of cellular interactions by NPs is limited. This is because nanomedicine is still an emerging field. Therefore, in this study, we sought to determine the effect of *T. gondii* infection and/ or various anti-metabolites on cellular apoptosis by NPs. The NPs (AgNP) and amino-NPs (AuNP^{TRP}) caused cellular apoptosis which appeared to be dose-dependent but do not follow time kinetics. Conversely, cellular apoptosis by AuNP and AgNP^{TRP} was not dose-dependent. Taken together, these findings are consistent with existing findings that have demonstrated the apoptotic action of NPs (Azizi et al., 2017; Moses et al., 2016). Interestingly, the NPs and amino-NPs at the sub-cytotoxicity doses used in this study facilitated cellular apoptosis. This finding confirms earlier report which showed that NPs can increase cellular susceptibility to cell death without necessarily causing cytotoxicity (Leite et al., 2015). The PtNPs exhibited poor cellular apoptosis possibly due to the fact that PtNPs have anti-oxidative stress properties similar to those of cellular antioxidant enzymes (Adeyemi et al., 2018a). Nevertheless, studies have demonstrated *in vitro* apoptotic tendency of PtNPs in cancer cells, human fibroblast as well as in bacteria cells (Bendale et al., 2017; Kutwin et al., 2017; bacterial cells and HFF cells (Nejdl et al., 2017).

Meanwhile, in the presence of *T. gondii* infection, cellular apoptosis by NPs and amino-NPs was not mitigated. This finding may not only indicate that cellular apoptosis by NPs and amino-NPs was not susceptible to modulation by *T. gondii* infection but that NPs and amino-NPs have antagonistic impact against anti-apoptotic tendency of *T. gondii*. The *T. gondii* infection has been reported to inhibit pro-apoptotic cellular process, in order to ensure their replication and survival (Hippe et al., 2009). *T. gondii* does this by affecting the apoptotic cascade at multiple levels; the inhibition of pro-apoptotic effector proteins like Bax and Bak, the avoidance of caspase 9 and Apaf-1 binding thus preventing caspase 3/7 cascade, upsetting pro- and anti-apoptotic balance of BCL-2

proteins, impeding the function of poly(ADP-ribose) polymerase expression, and upregulating NF-κB pathway by GRA15, which depresses the host immune system through interleukin 12, thereby preventing apoptosis (Hippe et al., 2009; Besteiro, 2015; Kim et al., 2006; Begum-Haque et al., 2009; Goebel et al., 2001). Thus, it is plausible that NPs and amino-NPs are antagonist in one or more of these same apoptosis effector sites that are targets of *T. gondii*. This finding supports further the prospects of NPs as alternative anti-parasite therapy.

Small molecules used in this study showed marginal apoptotic features when compared with control as the majority of these anti-metabolites failed to interfere with apoptotic potential of NPs and amino-NPs. For example, trolox mildly modulated the apoptotic potential of NPs. Trolox basically functions by mopping up ROS in the cell, thus our finding confirmed previous studies that showed oxidative stress as one of the cytotoxic mechanisms of NPs (Bagherzadeh Homaee and Ehsanpour, 2016; Mateo et al., 2013). This further suggests the culpability of oxidative stress in the apoptotic action of NPs. That trolox modulated cellular apoptosis by NPs may also indicate that cellular reactivity of these NPs differ from that of amino-NPs. In addition, chemical induction of hypoxia by use of CoCl$_2$ also modulated cellular apoptosis by NPs. It's probable that hypoxia might be interfering with the apoptotic action of NPs. This is further supported by mild mitigation of YC-1 on cellular apoptosis by NPs. More so, NPs have capacity to reduce HIF-1α activity (Adeyemi et al., 2017b; Yang et al., 2016) and thus avert hypoxia. Therefore, it is plausible that capacity of NPs to downregulate HIF-1α protein level might be connected to their apoptotic tendency. Taken together, the findings do not only indicate that these metabolic processes (oxidative stress and hypoxia) are relevant to the apoptotic action of NPs but underscore variance in the cellular interactions leading to apoptosis by both NPs and amino-NPs. Data herein support that cellular apoptosis by NPs do not preclude oxidative stress and hypoxia. This is not same for the amino-NPs (AgNP^{TRP} and AuNP^{TRP}). The reason for this variance behavior in cellular apoptosis

induction by NPs and amino-NPs is not known yet but may be attributed to change in surface chemistry of amino-NPs occasioned by conjugation of l-tryptophan. This line of thought is consistent with our earlier findings (Adeyemi et al., 2018b) in which NPs-tryptophan conjugates and NPs lacking this amino acid exhibited dissimilar cellular interactions. Furthermore, NPs and amino-NPs have demonstrated capacity to activate kynurenine pathway *in vitro* (Adeyemi et al., 2017b) by yet unknown mechanism. But in the present study, addition of l-tryptophan to the culture medium failed to ameliorate cellular apoptosis by NPs and amino-NPs. This may indicate that apoptosis by NPs and amino-NPs precludes activation of kynurenine pathway. Moreover, competitive and non-competitive inhibitors of IDO-1 (a rate limiting enzyme in the oxidative degradation of tryptophan to form kynurenine) failed to assuage cellular apoptosis by NPs and amino-NPs.

In conclusion, the present study demonstrates the apoptotic potential of inorganic NPs and amino-NPs of Ag and Au. Further, our findings are evidence supporting that apoptosis by NPs and amino-NPs was not susceptible to modulation by *T. gondii* infection, thus supporting further the prospects of NPs as alternative anti-parasitic agents. Additionally, oxidative stress and chemical hypoxia are partly among the cellular interactions leading to apoptosis caused by NPs. Considered together, the data do not only glean new perspective to deepen our understanding of cellular interactions by NPs and amino-NPs but warrant further investigations in order to fully comprehend mechanistic behavior of NPs.

Acknowledgements

Conflict of interest

Authors declare that there is no conflict of interests.

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