

Biosynthesis of silver nanoparticles using *Pterorhachis zenkeri*: Characterization and evaluation of antioxidant, anti-apoptotic, and androgenic properties in TM3 Leydig cells exposed to cyclophosphamide

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Research Article

Keywords: Cyclophosphamide, silver nanoparticles, oxidative stress, testosterone, StAR, TM3 cells.

Posted Date: April 23rd, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4299408/v1>

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Additional Declarations: The authors declare no competing interests.

Abstract

We fabricated silver nanoparticles (AgNPs) using *Pterorhachis zenkeri*, characterized by Atomic Force Microscopy (AFM) and determined their antioxidant potentials *in vitro*. Results confirmed the fabrication of AgNPs by using *P. zenkeri* as a bioreducing agent for the first time. AgNPs possessed potent antioxidant activity *in vitro*. Furthermore, the TM3 cells were treated for 24 h with AgNPs, vitamin E and cyclophosphamide (CP) at different concentrations (25, 50, 100, 250 and 500 µg/ml). The cells morphology, apoptosis, mitochondrial membrane depolarisation (MMD), Reactive Oxygen Species (ROS), caspase 3/9, oxidative stress-related enzymes, testosterone, and the mRNA expression of steroidogenic acute regulatory protein (StAR) were measured. Results revealed that AgNPs, vitamin E and CP decreased the cell viability in a dose-dependent manner, but did not affect the TM3 cells morphology after treatment. The cytotoxicity of CP in TM3 cells was alleviated after AgNPs application. For instance, AgNPs significantly ($p < 0.001-0.05$) reduced the MMD, ROS production, and caspase 3/9 activities, but increased the activities of superoxide dismutase, catalase and glutathione peroxidase in the TM3 cells. Moreover, AgNPs improved testosterone production by activating StAR machineries. These results indicate that AgNPs/*P. zenkeri* could be a potential alternative drug in the management of oxidative stress and androgen deficit associated with CP chemotherapy.

Introduction

Cyclophosphamide (CP) is an anticancer drug widely used in the treatment of various cancers including breast cancer (Kimura et al. 2021), prostate cancer (Li et al. 2022) and ovarian cancer (Handolias et al. 2016), but its chronic use can cause severe damages to normal living cells (El-Emam 2021, Barberino et al. 2023). After administration, CP is transformed into its active metabolites phosphoramidate mustard (responsible for its antineoplastic potential) and acrolein (toxic metabolite of CP) with the action of cytochrome P450 mixed functional oxidase system (Zhang et al. 2005). Acrolein promotes the generation of Reactive Oxygen Species (ROS) and oxidative stress (Jiang et al. 2018, Yasuo et al. 2019). The cytotoxic effects of CP particularly target rapidly proliferating cells like reproductive cells (Elangovan et al. 2006).

Considering the detrimental effects of CP on the reproductive cells, the use of an adjuvant drug that might prevent or alleviate the gonadal damages caused by CP is attractive. For instance, Niakani et al. (2013) demonstrated that Decapeptyl ameliorates CP-induced reproductive toxicity in mice by protecting morphologic and morphometric alterations of the testes tissue. Frankincense extract used to boost fertility potential has the ability to protect the testis against CP cytotoxicity by modulating the apoptotic genes expression (Alharbi et al. 2022). Additionally, some exogenous antioxidant agents such as melatonin and vitamin E are considered useful to decrease oxidative stress caused by CP therapy (Iqtedar et al. 2019, Salim et al. 2019).

The therapeutic potentials of nanoparticles in various aspects of medicine have been reported (Alkilany et al. 2019, Eilers et al. 2020, Wong et al. 2021, Jordi 2023). Among metallic nanoparticles, Silver

Nanoparticles (AgNPs) have received considerable attention due to their pharmacological properties, their effectiveness in the diagnosis and treatment of various diseases, and their unique morphologies and stability (Yin et al. 2020). The use of plants in the bio-fabrication of AgNPs is attractive as it is a safe and ecological method which can be implemented with limited cost. Many studies reported the therapeutic effects of green AgNPs. For instance, the antibacterial properties of AgNPs synthesized using *Mimusops elengi* (Prakash et al. 2013), *Phyllanthus amarus* (Subbaiya et al. 2014), and *Phanerochaete chrysosporium* (Saravanan et al. 2018) have been reported. Studies also reported the *in vitro* antioxidant potential of AgNPs from *Fucus vesiculosus* (Rajeshkumar et al. 2021) and *Azadirachta indica* (Chinnasamy et al. 2021) extracts.

We have previously shown that CP induces testicular toxicity (Watcho et al. 2019) and ovarian toxicity (Watcho et al. 2022) in rats, but its cytotoxicity in TM3 cells has not been studied. Moreover, no data in the literature reported the role of AgNPs on CP-induced cytotoxicity in TM3 cells. In this study, we fabricated AgNPs utilizing the metal reducing potential of *Pterorhachis zenkeri* extract and investigated their antioxidant activities *in vitro* using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals scavenging method. With the hypothesis that the antioxidant properties of AgNPs may protect the Leydig cells against the cytotoxicity of CP, we further investigated the effects of AgNPs on cell viability, cell morphology, apoptosis level, ROS production, mitochondrial membrane potential, caspase 3/9 activities, lipid peroxidation, and antioxidant enzymes (SOD, CAT and GSH-Px), as well as testosterone level, and mRNA level of stAR in TM3 cells.

Materials and methods

Plant material and preparation of aqueous extract of *P. zenkeri*

P. zenkeri roots were harvested in Makenene (Centre Region of Cameroon) and identified at the Cameroon National Herbarium (CNH) in comparison with the specimen deposited under the Voucher number 21553/SRF/Cam. The roots were further cut into small pieces, shade-dried for 7 days, later transformed into powder and used for preparation of aqueous extract. 150 g of *P. zenkeri* powder were macerated in distilled water (1000 ml) for 72 hours and filtered using Whatman no. 1 filter paper and dried at 45°C. 24.96 g of aqueous extract was obtained, giving an extraction yield of 16.64% (Bonsou-Fozin et al. 2020).

Synthesis of AgNPs using *P. zenkeri* and characterization by AFM

The synthesis procedure was done as described previously (Chung et al. 2016). Briefly, in a 100 mL round-bottom flask, 1g of *P. zenkeri* extract was dissolved in deionized water (49 mL) and 85 mg of silver nitrate (0.5 mM) was added. The mixture was stirred for 2 hours at 85–90°C using a magnetic stirrer. Formation of AgNPs was characterised by the instantaneous transformation of the mixture colour from light yellow to dark brown. No colour change was noticed up to the end of the reaction (Chung et al. 2016). The mixture was allowed to cool down and centrifuged for 30 min at 9000 rpm. After centrifugation, the black pellets (AgNPs) obtained was washed 3 times with deionized water and dried for

12 hours at 80°C in an oven, and used for further studies. All stages of the test were implemented in triplicate.

The structural characterization of AgNPs was carried out by using AFM (NTEGRA PRIMA, Ireland) (Kaliaraj et al. 2018).

Evaluation of in vitro antioxidant potential of AgNPs

The antioxidant activities of AgNPs and ascorbic acid (positive control) were determined via their scavenging activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical as described by Koleva et al. (2002) with minor modifications. Briefly, 10 µL of each concentration (25, 50, 100, 250 and 500 µg/ml) of AgNPs and ascorbic acid was added to DPPH (190 µL; 150 µM) in ethanol solution. The mixture was incubated for 30 min in the dark at room temperature and the absorbance was recorded at 517 nm. The percentage of inhibition was calculated (Karanam et al. 2020). The data were analyzed in triplicate.

Culturing of TM3 cells

Mouse Leydig TM3 cells were procured from the National Centre for Cell Science, NCCS, Pune, India. They were cultured in Dulbecco's modified Eagle's medium (DMEM) / F-12 medium supplemented with 15 mM HEPES and L-glutamine, enriched with 2.5% foetal bovine serum, 5% horse serum and 1% penicillin-streptomycin. The cultures were maintained at 37°C under a humidified atmosphere (95% air and 5% CO₂). The cell culture was performed in an aseptic condition to avoid contamination and the passage process was done twice a week at around 80% confluence. The culture media was changed after every 2 days.

MTT assay and treatments

The effects of AgNPs, vitamin E and CP on the viability of TM3 cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This colorimetric test estimates the reduction of soluble yellow tetrazolium salt to water-insoluble blue formazan crystals by mitochondrial succinate dehydrogenase activity in living cells. TM3 cells were seeded in 96-well plates (1.5 x 10⁴ cells/well). After the cells reached good growth, they were incubated with various concentrations (25, 50, 100, 250 and 500 µg/ml) of AgNPs, vitamin E and CP. At the end of each period (1 h, 5 h, 10 h and 24 h), the cell viability was determined as described previously (Deeh et al. 2018, Çınar et al. 2022). All experiments were carried out in triplicates.

Further, the cells were divided into six groups (density of 1x10⁶ cells/flask) and treated as follows: (1) control group: the cells were kept in a flask without any treatment; (2): vitamin E: cells were incubated with vitamin E at 25 µg/ml; (3): AgNPs: cells were treated with AgNPs at dose 100 µg/ml; (4): CP: cells were treated with CP at 250 µg/ml; (5): CP + vitamin E: cells were co-treated with CP (250 µg/ml) and vitamin E (25 µg/ml), and (6): CP + AgNPs: cells were co-treated with CP (250 µg/ml) and AgNPs (100 µg/ml).

The dose of vitamin E (Ghanem et al. 2017, Wang et al. 2022) and CP (Yadav et al. 2020) were chosen from previous studies. At the end of the incubation period (24 hours), cells morphology, apoptosis level, JC-1 level, ROS production, caspase 3/9 activities, oxidative stress related enzymes (MDA, SOD, CAT and GSH-Px), testosterone concentration, and the mRNA expression of stAR in TM3 cells were measured.

TM3 cells morphology

The TM3 cells were exposed to AgNPs, vitamin E in the presence or absence of CP. After 24 hours of treatment, cells were washed with PBS, fixed with 3.7% formaldehyde, and stained using Alexa Fluor 488 phalloidin and DAPI (Invitrogen, UK). Cell morphology was observed and recorded under inverted fluorescence microscope (EVOS FL digital inverted fluorescence microscope (AMG)).

Apoptosis essay

After treatment with AgNPs, vitamin E, and CP for 24 hours, the apoptosis study was carried out by using AO/EB staining as described earlier (Leite et al. 1999, Natesh et al. 2018). Briefly, coverslips were taken and kept on glass slides and stained with 100 μ L of EB + AO (1:1 ratio of each dye) and immediately viewed under inverted fluorescence microscope (EVOS FL digital inverted fluorescence microscope (AMG)).

Determination of JC-1 level, ROS production, and caspase-3 and caspase-9 activities

TM3 cells were treated for 24 hours with AgNPs and vitamin E in the presence or absence of CP. At the end of the treatment period, the cells were detached using trypsin-EDTA, collected in centrifuge tubes and suspended in PBS (500 μ L) containing JC-1 (10 μ g/mL) dye. After incubation (15 min at 37°C), mitochondrial membrane potential was measured using flow cytometry.

After treatment with AgNPs, vitamin E or CP, the cells were trypsinized and collected in separate centrifuge tubes, and ROS level was measured using dihydrorhodamine-123 (DRH 123). Briefly, DRH 123 easily penetrates inside the cells and becomes fluorescent after oxidation. The intensity of fluorescence is proportional to ROS production. In all samples, the intensity of fluorescence was recorded at 488 nm (excitation) and 543 nm (emission) using a professional micro plate reader (Infinite pro200) (Öz et al. 2022).

The measurement of caspase-3 and caspase-9 activities was done by fluorescence microscopy as described earlier (Espino et al. 2010, Nazıroğlu et al. 2019). The data were expressed as fold increase over the pretreatment level (experimental/control).

Measurement of lipid peroxidation and the activity of antioxidant enzymes (SOD, CAT and GSH-Px)

MDA level and Glutathione peroxidase (GSH-Px) activity were measured using a detection kit as per the manufacturer's instructions. The activities of SOD and catalase were estimated as described by Shiraishi et al. (2005) and Sinha et al. (1984), respectively.

Testosterone

Testosterone production in all groups was stimulated with human chorionic gonadotropin (hCG). Indeed, the TM3 cells were incubated for 24 hours with AgNPs, vitamin E and CP in the presence of hCG (6 mIU/200 µl medium) (Opuwari et al. 2015). Testosterone level in all groups was estimated using an ELISA kit (Accubind, Monobind Inc. Lake Forest, USA) following the procedure outlined in the manufacturer's instructions.

RNA extraction and quantitative real-time polymerase chain reaction

After treatment with drugs (AgNPs, vitamin E or CP), total RNA from the TM3 cells was extracted using Qiagen RNeasy Mini Kit as defined according to the manufacturer protocol. The experimental conditions were the same as described previously (Olugbodi et al. 2019). Briefly, total RNA was reverse transcribed using the SuperScript1 VILOTM Kit and RT-qPCR was performed with SsoAdvancedTM SYBR1 and a CFX96 TouchTM devices. Gene specific primers StAR Forward 5'-AGTATTGACCTGAAGGGGT-3', StAR Reverse 5'-TTACCAGTCAGTCCTAGTGT-3' were used for target gene amplification. To enumerate gene expression, the Ct of target gene amplification was regularized to the expression level of a housekeeping gene (GADPH forward 5'-AACTCAGGAGAGTGTTCCTCG-3', GADPH reverse 5'-TGATGGGCTTCCCGTTGATG-3'), based on the relation $R = ECt_{Rpl19}/ECt_{target}$, wherever E is the magnification potency for every primer pair (Olugbodi et al. 2019).

Statistical analysis

The data are presented as the mean $\pm$ SEM. Differences between the means were tested by ANOVA one-way followed by the post hoc Tukey HSD test using STATISTICA software, version 8.0 (StatSoft, Inc., Tulsa, USA). Each experiment was repeated three times. Significance level was set at 0.05 or less.

Results

AFM characterization of AgNPs

2D and 3D images of the AFM revealed a regular morphology with high degree of uniformity and smooth topography with uniform grains distribution. The roughness analysis of AgNPs showed that the average roughness (Sa) and root mean square (Sq) values were 2.37 nm and 3.22 nm, respectively (Fig. 1A and B). Grain size distribution of AgNPs shows that the average grain size is $\approx$ 24 nm (Fig. 1C).

DPPH scavenging activity of AgNPs and ascorbic acid

DPPH scavenging activity of AgNPs and ascorbic acid increased in a dose-dependent manner, ascorbic acid being the most effective at all doses. For both compounds, the values were significantly ($p < 0.001$) elevated at doses 250, 500 and 1000 $\mu\text{g/ml}$, compared to the lowest dose (50 $\mu\text{g/ml}$) (Fig. 2).

Effects of different treatments on TM3 cell viability

AgNPs, vitamin E and CP decreased the cell viability in a time and dose-dependent manner (Fig. 3A-C).

The viability of TM3 cells incubated for 1h with vitamin E was statistically unchanged (Fig. 3B). However, a significant ($p < 0.05 - 0.001$) reduction in cell viability was observed in the cells exposed for 1h to AgNPs (50–500 $\mu\text{g/ml}$) and CP (500 $\mu\text{g/ml}$), compared to the control. At 5 hours of incubation, AgNPs (50–500 $\mu\text{g/ml}$), vitamin E (100–500 $\mu\text{g/ml}$) and CP (250–500 $\mu\text{g/ml}$) significantly decreased the cell viability, compared with control. The cell viability was also decreased in a time and dose-dependent manner after 10 and 24 hours of incubation with AgNPs (all doses), vitamin E (10 hours: 250–500 $\mu\text{g/ml}$ and 24 hours: 50–500 $\mu\text{g/ml}$), and CP (all doses) compared to the control group. Overall, AgNPs was more effective in decreasing the cell viability than vitamin E and CP (Fig. 3A-C).

Effects of different treatments on TM3 cell morphology

As shown in Figs. 9 and 10, we noticed that AgNPs, vitamin E and CP did not affect the morphology of TM3 cells after 24 hours of treatment. Indeed, the cells were normally attached with healthy appearances (polygonal shape). However, when we compare the growth rate of the cells in the presence or absence of CP, we observed that CP appears to inhibit the cell growth (Fig. 4).

2.2. Effects of different treatments on apoptosis level in the TM3 cells

In the absence of CP, the apoptotic cells were relatively low after treatment with vitamin E and AgNPs, compared to the control group. However, the percentage of apoptotic nuclei was increase after CP exposure. Of great interest, vitamin E and AgNPs decreased the percentage of apoptotic nuclei after CP application (Fig. 5).

Effects of different treatments on JC-1 level, ROS production, and caspase-3 and caspase-9 activities in the TM3 cells

JC-1 level and ROS production in AgNPs and vitamin E groups were statistically unchanged ($p > 0.05$), compared with control. However, CP application significantly ($p < 0.05$) increased the JC-1 and ROS levels, compared to the control group. Of great interest, we noticed that AgNPs and vitamin E significantly ($p < 0.05$) reduced the JC-1 and ROS levels, compared to the CP group. The lowest value of JC-1 was recorded in AgNPs group, while the maximum reduction in ROS production was observed in vitamin E group (Fig. 6A and B).

Although no significant changes in caspase-3 and caspase-9 activities were observed in the cells incubated with AgNPs or vitamin E, we noticed that AgNPs and vitamin E significantly ($p < 0.05$) reduced caspase 3/9 activities, compared with CP group (Fig. 6C and D).

Effects of different treatments on MDA level and SOD, catalase and glutathione activities in the TM3 cells

As shown in Fig. 7A, CP significantly ($p < 0.001$) increased the MDA level, compared with control. Compared to the CP group, we noticed that vitamin E and AgNPs decreased the level of MDA in the presence of CP, with a remarkable effect observed in the vitamin E group.

In the absence or presence of CP, we observed that vitamin E and AgNPs significantly elevated the activities of SOD, catalase and glutathione in the TM3 cells, compared to the control or CP group, respectively. AgNPs was more effective in improving the activities of antioxidant enzymes than vitamin E (Fig. 7B-C).

Effects of different treatments on testosterone level and mRNA expression of stAR in the TM3 cells

In the absence of CP, testosterone level in the TM3 cells incubated with AgNPs was significantly ($p < 0.05$) elevated, compared to the control group. However, CP significantly ($p < 0.001$) reduced the level of testosterone after application (control vs CP group). Testosterone concentration was significantly ($p < 0.001$) increased in the cells co-treated with CP and AgNPs, compared with CP group (Fig. 8A).

CP significantly ($p < 0.05$) decreased the mRNA levels of StAR in the TM3 cells, compared with control. Vitamin E and AgNPs up regulated ($p < 0.05$) mRNA of stAR compared to CP group. AgNPs was more effective than vitamin E (Fig. 8B).

Discussion

Long-term CP chemotherapy is a cause of cytotoxicity which may affect healthy cells, including male reproductive cells (Leydig and sertoli cells), leading to sexual dysfunctions (Elangovan et al. 2006, Fusco et al. 2021, Park et al. 2021). To prevent the adverse effects of CP and its metabolites, the use of nanoparticles synthesized using plant materials such as AgNPs is attractive, due to their pharmacological properties, their stability, and their effectiveness and small size (Grace et al. 2007, Yin et al. 2020). *P. zenkeri* is a tropical plant widely used in the treatment of sexual disorders, but there is no study on the green synthesis of AgNPs using this plant. In the present work, we fabricated AgNPs using *P. zenkeri* extract, and determined its antioxidant potentials *in vitro* using DPPH radicals scavenging assay. Further, we investigated the effects of AgNPs on cell viability, cell morphology, apoptosis level, oxidative stress related markers as well as testosterone level and mRNA level of stAR in TM3 cells. This study confirms the ability of *P. zenkeri* for the fabrication of AgNPs for the first time. AgNPs exhibit potent antioxidant activity *in vitro*, modulate cell viability, apoptosis and oxidative stress, and improve testosterone production by up regulating stAR genes in TM3 cells.

During the fabrication of AgNPs using *P. zenkeri*, the reduction of Ag⁺ ion was confirmed by the color transformation of the solution from light brown to dark brown. Indeed, silver nanoparticle surface plasmon excitation induces color change, which is the evidence for the formation of AgNPs (Banerjee et al. 2014, Pirtarighat et al. 2019). AFM is a reliable method to characterize nanoparticles as it can be used to image and evaluate their surface morphology and property (Jayaseelan et al. 2012). AgNPs characterization using AFM revealed a regular morphology with high degree of uniformity and smooth topography with uniform grains distribution, which corroborated the work of Iqtedar et al. (2019) and Ullah et al. (2021). The DPPH scavenging activity of AgNPs and ascorbic acid was increased in a dose-dependent manner, ascorbic acid being the most effective at all doses. The *in vitro* antioxidant activity of ascorbic acid is evident as it has been already shown by previous researchers (Rajeshkumar et al. 2021, Kładna et al. 2021). The potent *in vitro* antioxidant potential of AgNPs could be due to its ability to quench free radicals. Previous works also reported the *in vitro* antioxidant potential of AgNPs synthesised using *Fucus vesiculosus* (Kładna et al. 2021) and *Azadirachta indica* (Chinnasamy et al. 2021) extracts. In the current study, this radical scavenging ability of AgNPs could be responsible of its beneficial effects against CP-induced oxidative stress in TM3 cells.

In the current work, AgNPs, decreased the cell viability of TM3 cells in a time and dose-dependent manner, compared with control. This is the first study to report the effect of AgNPs synthesized using *P. zenkeri* extract on the viability of TM3 cells. This result corroborated that of Zhang et al. (2015) who investigated the effects of AgNPs in Leydig (TM3), Sertoli (TM4) and spermatogonial stem cells (SSCs). In parallel, high concentrations of AgNPs (40–100 µg/ml) synthesized using the root extracts of *Beta vulgaris* reduced the viability of human hepatic normal (CHANG) and cancer (HUH-7) cells (Bin-Jumah et al. 2020). The effects of the treatments on cell morphology were investigated and the results showed that AgNPs, vitamin E and CP did not affect the morphology of TM3 cells after 24 hours of treatment. In all groups, the cells were normally attached with healthy appearances, but CP seems to inhibit the cell growth, when compared to the control (cells cultured without CP) (Fig. 4). Studies have shown that the antineoplastic potential of CP is mediated via its inhibitory action on the Notch signalling pathway (Jin et al. 2021) and its capacity to promote oxidative stress in pathogenic cells by triggering the production of excessive amounts of ROS (Yang et al. 2020).

It has been reported that ROS overproduction induces apoptosis by up-regulating caspase genes (Simon et al. 2000), leading to DNA defects and cell death (Görlach et al. 2015). Our results indicated that, CP application induced cytotoxicity in TM3 cells, characterised by the significantly increase in apoptosis level, mitochondrial membrane potential, ROS production, and caspase 3/9 activities, compared with control. Indeed, ROS overproduction induces apoptosis by increasing the mitochondrial membrane potential (Che et al. 2021) and up-regulating caspase genes (Mu et al. 2020), leading to DNA damage (Srinivas et al. 2019). Although no previous work has described the cytotoxic of CP in TM3 cells, CP was found to be cytotoxic for the murine 4T1 mammary carcinoma cell by increasing the ROS production and apoptosis levels via Bax/Bcl-2 pathway (Kurokawa et al. 2021). Moreover, CP induced cytotoxic in HuH-7 cells, characterized by increased intracellular ROS generation and suppression of antioxidant machineries

(Al-Johani et al. 2022). Of great interest, treatment with AgNPs reversed the adverse effects of CP, probably due to their powerful antioxidant potentials. Similarly, low percentages of ROS production and apoptosis level have been reported in CHANG human hepatic normal cells (compared to HUH-7 cancer cells) after treatment with green AgNPs (Bin-Jumah et al. 2020).

Oxidative stress is the key provider causing CP-induced cytotoxicity (Caglayan 2019). We found in this study that CP induced oxidative stress in the TM3 cells by elevating MDA level and decreasing the activities of SOD, catalase and glutathione peroxidase. The cytotoxicity of CP has been reported in various cells like breast cancer cells (Engin et al. 2021), human hepatoma cells (HuH-7) (Al-Johani et al. 2022), and monocyte macrophage cells (Raw 264.7) (Yadav et al. 2020). Interestingly, oxidative stress associated with CP toxicity was attenuated after AgNPs treatment, which may be due to their inhibitory action on free radicals activators such as xanthine oxidase. More mechanistic studies are required to clarify this action. In parallel, AgNPs was reported to prevent oxidative stress in normal cells such as L-929 fibroblast cells, but had an opposite effect in cancer cells like human neuroblastoma cancer cells (SH-SY5Y) (Zhai et al. 2022), human cervical cancer cells (HeLa) (Yuan et al. 2018) and human epidermoid larynx carcinoma cells (HEp-2) (Muthukrishnan et al. 2019). Moreover, Khorrami et al. (2018) reported that AgNPs are nontoxic toward normal cell line, but are cytotoxic to cancer cells. Thus, the properties of AgNPs on testicular cancer cells are highly needed.

Leydig cell are the main source of production of testosterone in male. StAR acts in the steroidogenesis by controlling cholesterol transfer from the external to internal mitochondrial membrane, thus down regulation of StAR decrease testosterone synthesis (Walsh et al. 2000). CP impaired testicular function by decreasing sex hormones, including testosterone production (Jin et al. 2021, Rezaei et al. 2021). We noticed in the present study that CP significantly decreased testosterone and mRNA levels of StAR in the TM3 cells compared to control. Our findings are consistent with those of Fan et al. (2022) who reported that CP down regulated gene expression of genes related to spermatogenesis and lowered testosterone production by altering STAR, CYP11A1, and PRKACB genes. Of great interest, AgNPs elevated testosterone level and up regulated StAR genes after application. The significant increase in the mRNA levels of StAR in the TM3 cells incubated with AgNPs, indicated that their androgenic activities are due to the activation of StAR machineries. However more mechanistic mechanisms are needed to clarify this action. These results indicate that AgNPs synthesized using *P. zenkeri* could be a potential alternative drug in the management of androgen deficit associated to CP chemotherapy.

Conclusion

This study confirms the ability of *P. zenkeri* for the fabrication of AgNPs for the first time. AgNPs exhibit potent antioxidant activity *in vitro*, modulate cell viability, apoptosis and oxidative stress, and improve testosterone production by up regulating stAR genes in TM3 cells. These findings suggested that AgNPs could be an effective strategy for the management of CP- cytotoxicity.

Abbreviations

AFP: Alexa Fluor 488 phalloidin, DAPI: 4',6-diamidino-2-phenylindole; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CP: Cyclophosphamide; AgNPs: Silver nanoparticles; AFM: Atomic force microscope; DPPH: 2,2-diphenyl-1-picrylhydrazyl; ROS: Reactive Oxygen Species; NPs; Nanoparticles; GSH-Px: Glutathione peroxidase; DMEM: Dulbecco's modified Eagle's medium; DRH 123: Dihydrorhodamine-123; SOD, Superoxide dismutase; CAT: Catalase; Bax: Bcl-2 Associated X-protein; Bcl-2: B-Cell Leukemia/Lymphoma 2; HEPES: (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid); hCG: Human chorionic gonadotropin; JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine; stAR: Steroidogenic acute regulatory protein; AO/EB; Acridine orange/ethidium bromide.

Declarations

Acknowledgements

Authors are grateful to Department of Science and Technology (Government of India) and Federation of Indian Chambers of Commerce and Industry (FICCI) for Research Training Fellowship for Developing Country Scientists (RTF-DCS) given to Dr. DEEH DEFO Patrick Brice for this study, infrastructure was provided by Sathyabama Institute of Science and Technology, Chennai, at Centre for Nanoscience and Nanotechnology.

Grant support or other sources of funding:

This work was funded by Department of Science and Technology (Government of India), and Federation of Indian Chambers of Commerce and Industry (FICCI), (DCS/2018/000004).

Declarations of any conflicts of interest:

The authors declare no conflict of interest.

Acknowledgements

Authors are grateful to Department of Science and Technology (Government of India) and Federation of Indian Chambers of Commerce and Industry (FICCI) for Research Training Fellowship for Developing Country Scientists (RTF-DCS) given to Dr. DEEH DEFO Patrick Brice for this study, infrastructure was provided by Sathyabama Institute of Science and Technology, Chennai, at Centre for Nanoscience and Nanotechnology.

Author Contributions

PBDD: conceptualization, data curation, software, formal analysis, original draft preparation; **VV**: project administration, funding acquisition and supervision; **NSN** and **KA**: methodology and software; **MKA**: writing, review and editing; **RD**: methodology and statistical analyses; **TS**: formal analysis and investigation. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Funding

This work was supported by Department of Science and Technology (Government of India), and Federation of Indian Chambers of Commerce and Industry (FICCI), (grant number: DCS/2018/000004).

Data availability statement

All data and materials are available upon request.

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Figures

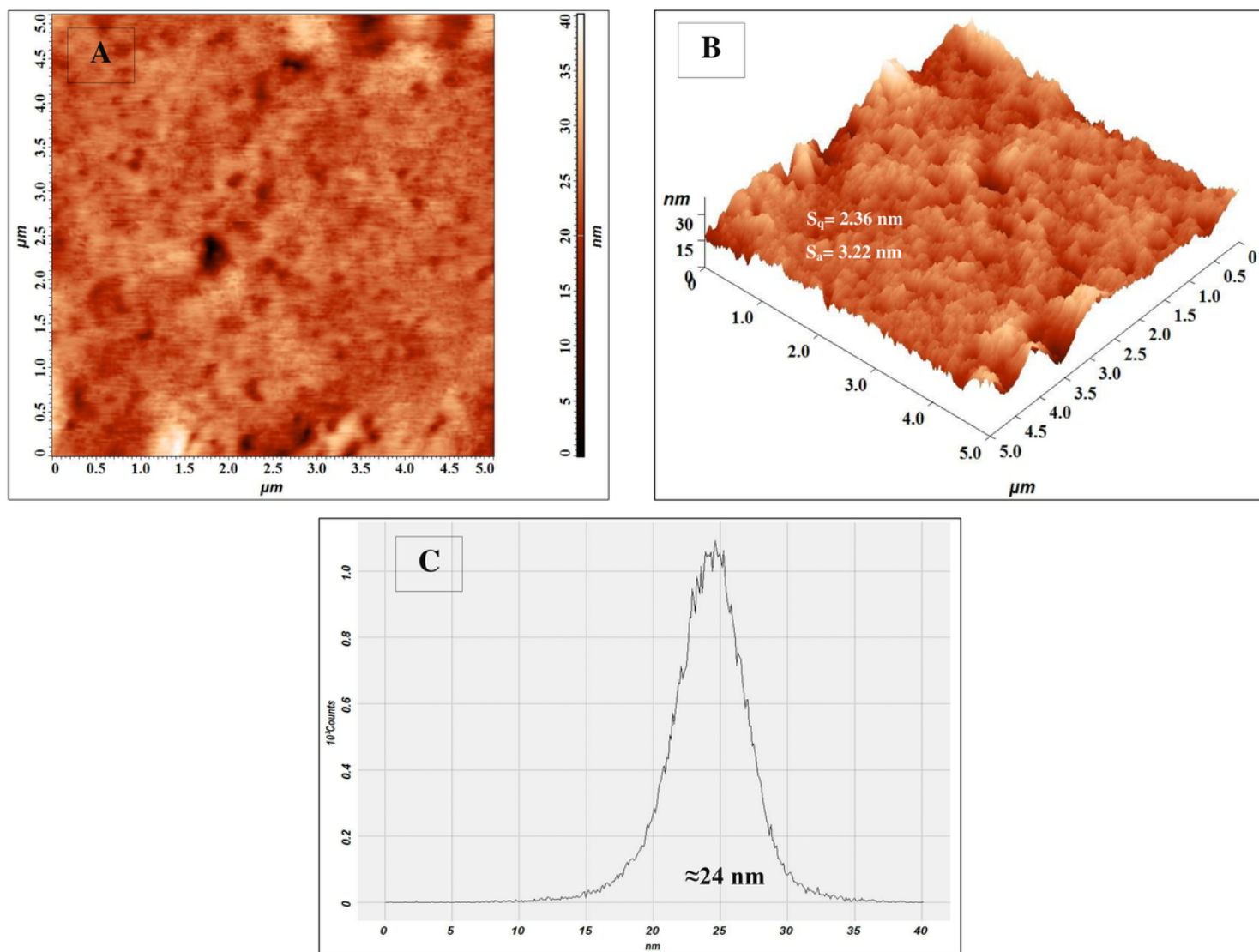


Figure 1

Topographical analysis of AgNPs. (A) and (B): 2D and 3D AFM images, respectively. (C). The grain size distribution of AgNPs. AgNPs: silver nanoparticles; Sa: average roughness; Sq: rootmean square.

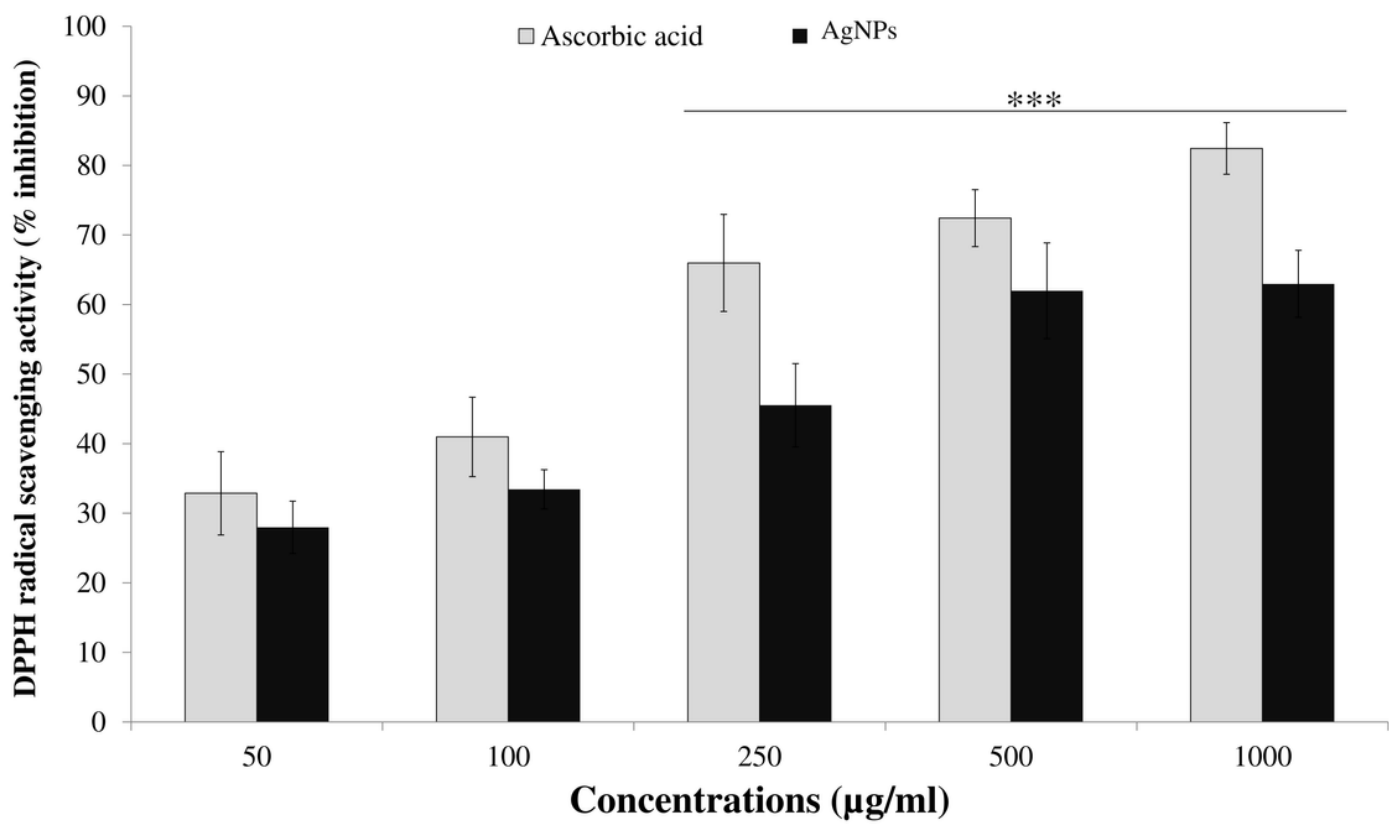


Figure 2

DPPH free radical scavenging activity of AgNPs and ascorbic acid. Number of replicate = 3. ***p<0.001: compared to dose 50 µg/ml. AgNPs: silver nanoparticles.

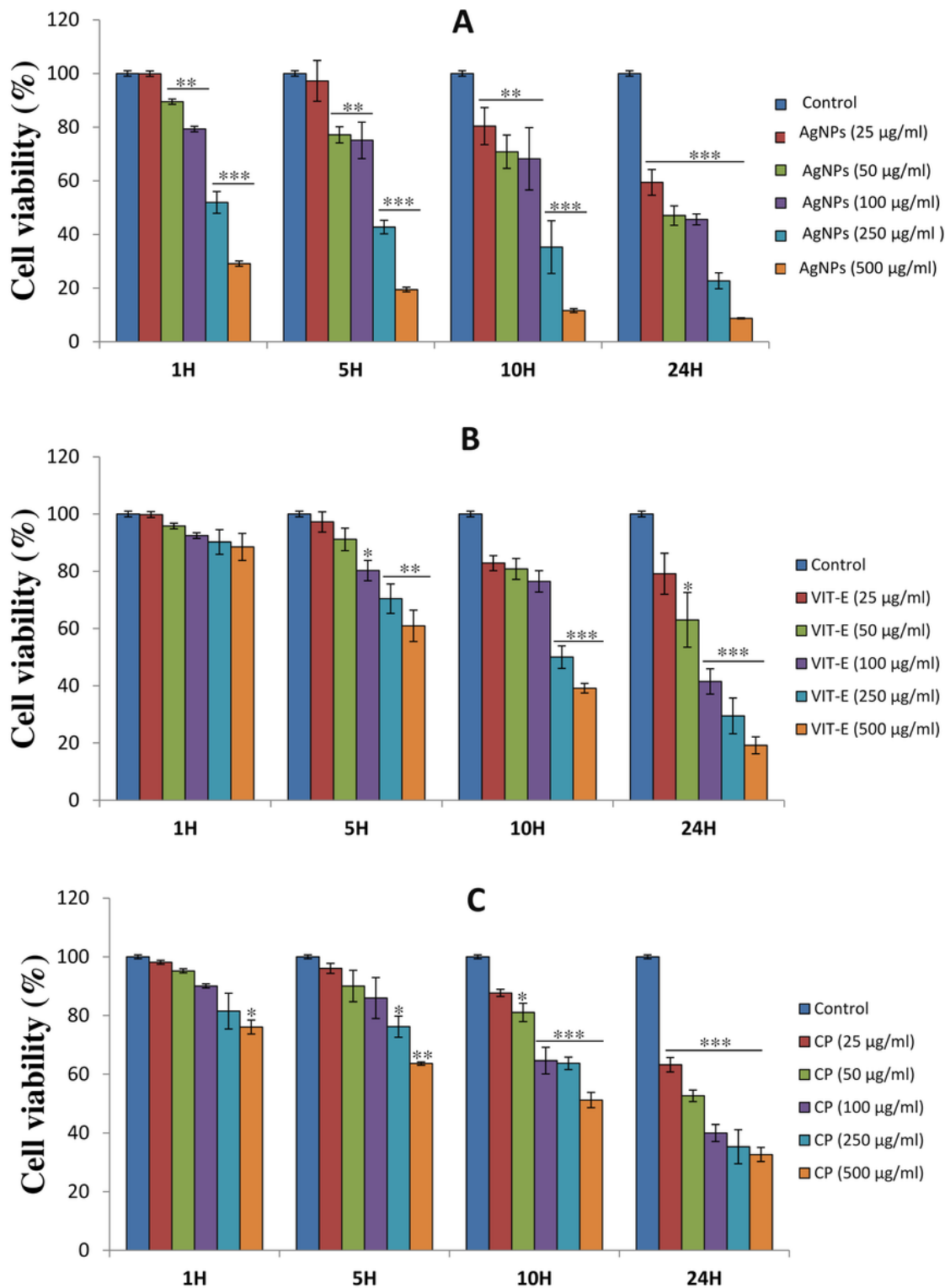


Figure 3

Viability of TM3 cells incubated with AgNPs, vitamin E and CP. The cells were incubated for 24 hours with various concentrations (25, 50, 100, 250 and 500 µg/ml) of AgNPs, vitamin E and CP, and cell viability was estimated as described under “Materials and methods” section. Experiments were performed in triplicate. Values are mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: compared to control. One-way ANOVA+Tukey HSD test. AgNPs: silver nanoparticles; VIT-E: vitamin E; CP: Cyclophosphamide.

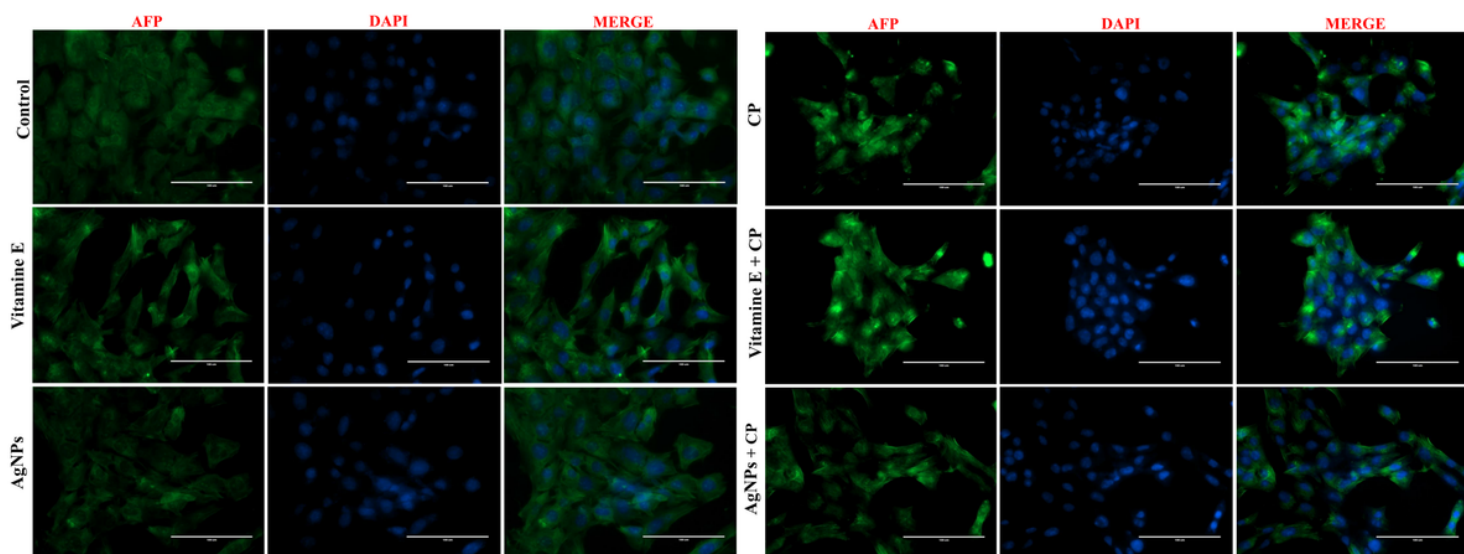


Figure 4

Effects of AgNPs, vitamin E and CP on TM3 cell morphology. Calibration bar: 100 μm ; AgNPs: silver nanoparticles (100 $\mu\text{g}/\text{ml}$); AFP: Alexa Fluor 488 phalloidin; DAPI: 4',6-diamidino-2-phenylindole; CP: Cyclophosphamide (250 $\mu\text{g}/\text{ml}$); Vitamin E (25 $\mu\text{g}/\text{ml}$).

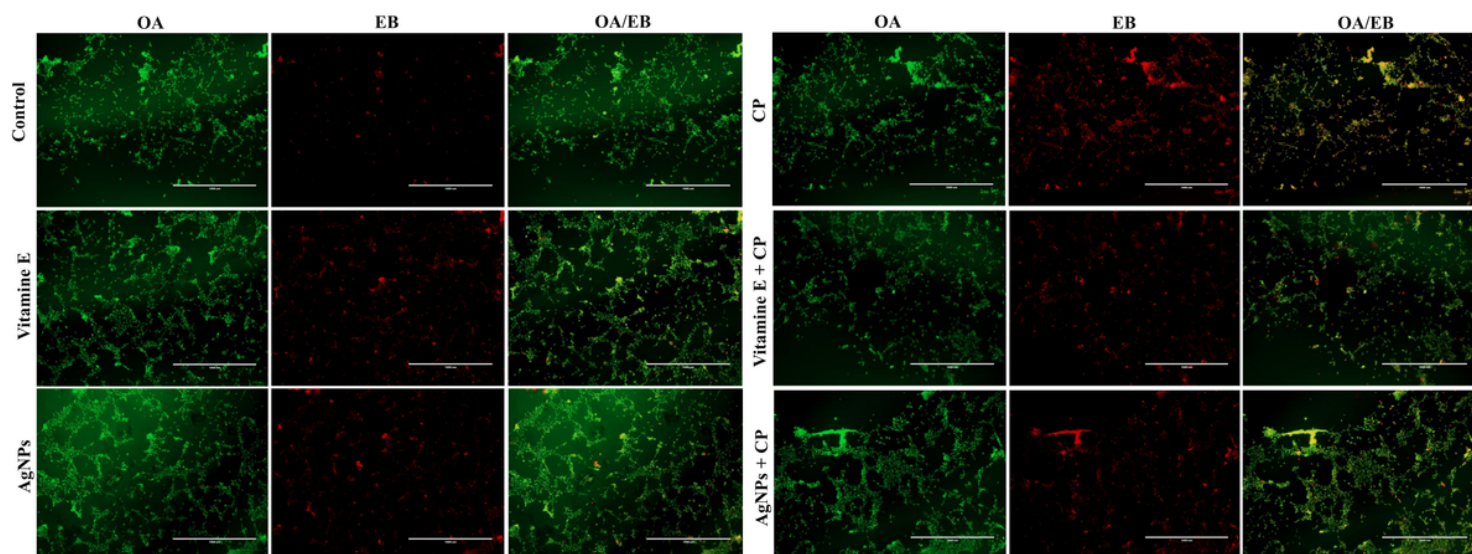


Figure 5

Effects of AgNPs, vitamin E and CP on apoptosis level in the TM3 cells. Calibration bar: 1000 μm ; AgNPs: silver nanoparticles (100 $\mu\text{g}/\text{ml}$); CP: Cyclophosphamide (250 $\mu\text{g}/\text{ml}$); Vitamin E (25 $\mu\text{g}/\text{ml}$); AO/EB; acridine orange/ethidium bromide.

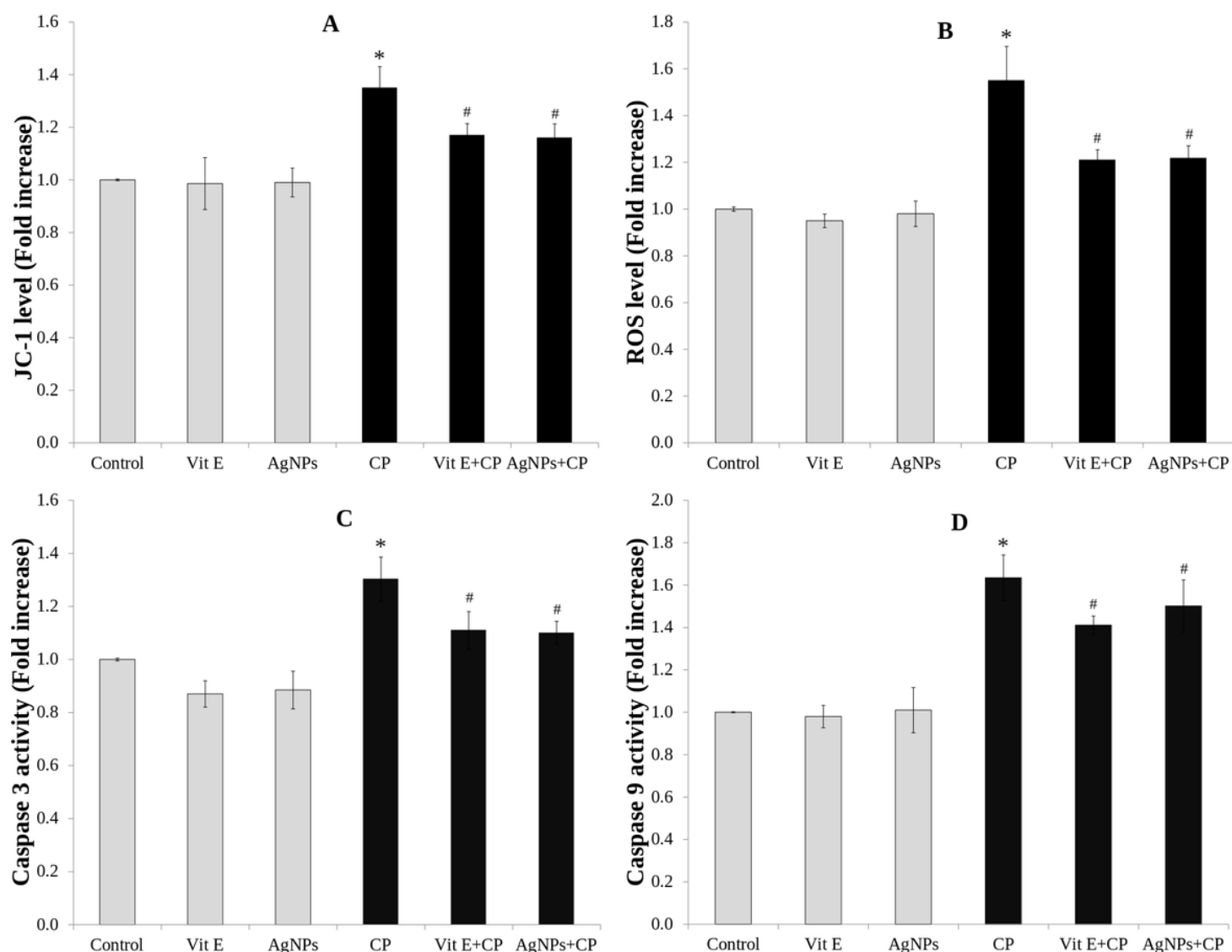


Figure 6

Effects of AgNPs, vitamin E and CP on JC-1 (A), ROS production (B), and caspase 3 (C) and caspase 9 (D) activities in the TM3 cells. Each bar represents the mean $\pm$ SEM. JC-1 and ROS levels were determined as described under "Materials and methods" section. * $p < 0.05$: compared to control. # $p < 0.05$: compared to CP. One-way ANOVA+Tukey HSD test. AgNPs: silver nanoparticles (100 $\mu\text{g/ml}$); CP: Cyclophosphamide (250 $\mu\text{g/ml}$); Vitamin E (25 $\mu\text{g/ml}$).

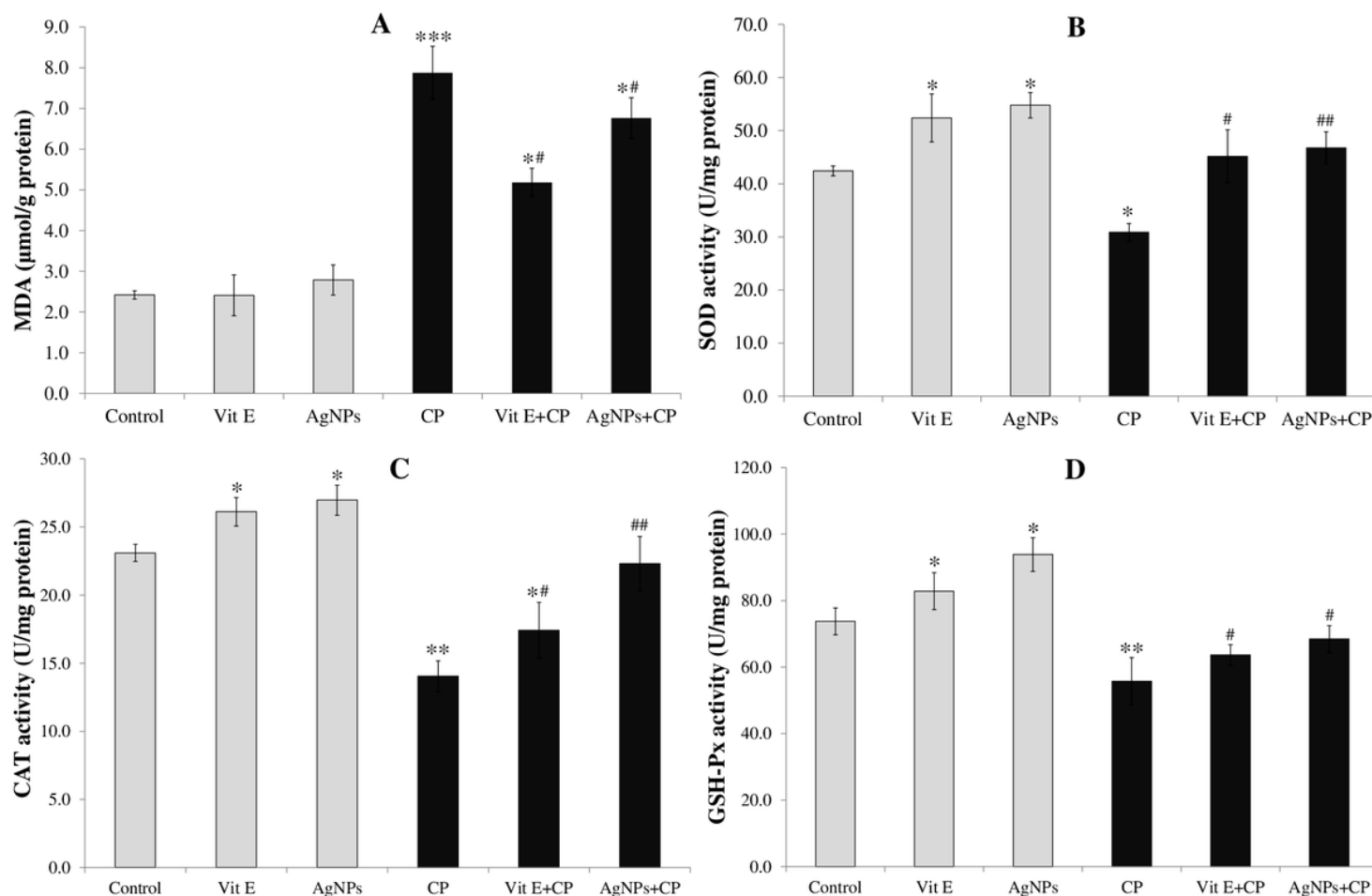


Figure 7

Effects of AgNPs, vitamin E and CP on MDA level (A), SOD (B), catalase (C), and glutathione peroxidase (D) activities in the TM3 cells. Each bar represents the mean $\pm$ SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: compared to control. # $p < 0.05$; ## $p < 0.01$: compared to CP. One-way ANOVA+Tukey HSD test. MDA: Malondialdehyde; SOD: Superoxide dismutase; CAT: Catalase; GSH-Px: Glutathione peroxidase. AgNPs: silver nanoparticles (100 $\mu\text{g/ml}$); CP: Cyclophosphamide (250 $\mu\text{g/ml}$); Vitamin E (25 $\mu\text{g/ml}$).

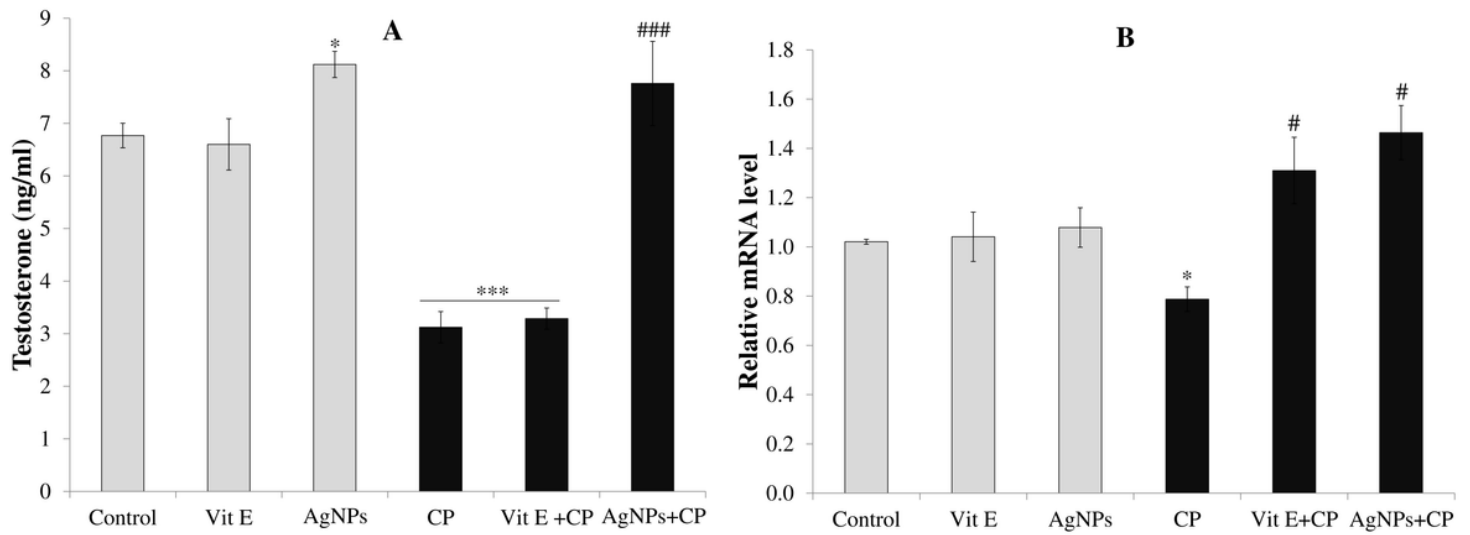


Figure 8

Effects of AgNPs, vitamin E and CP on testosterone level (A) and mRNA expression of stAR (B) in TM3 cells. Gene expression levels represent the mRAN expression levels relative to control levels. Each bar represents the mean $\pm$ SEM. * $p < 0.05$; *** $p < 0.001$: compared to control. # $p < 0.05$: compared to CP. One-way ANOVA+Tukey HSD test. AgNPs: silver nanoparticles (100 $\mu\text{g/ml}$); CP: Cyclophosphamide (250 $\mu\text{g/ml}$); Vitamin E (25 $\mu\text{g/ml}$).