Format for ANSWERING REVIEWERS

August 01, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 12213).

Title: The short- and long-term effects of silver nanoparticles on microvascular endothelial cells.

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Name of Journal: *World Journal of Biological Chemistry*

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The manuscript has been improved according to the suggestions of reviewers:

Reviewer 1

(1) *This manuscript lacks clear hypothesis and cohesive experimental design with focusing on characterizing toxicity of AgNP in microvascular endothelial cells per se. As the authors mentioned in the introduction, heterogeneity of vascular endothelial cells is dependent on the origin of cells etc. However, the authors did not state clearly the origin of HMEC in the entire paper. Moreover, some experiments were performed using HUVEC that are not belonged to microvascular EC.*

Our aim was to investigate the response of microvascular endothelial cells to Ag NP. The study of human microvascular endothelial cell behaviour has been limited because of the difficulties to isolate and grow these cells. We have used dermal microvascular endothelial cells and in the revised manuscript we add details about these cells (see methods). All the experiments were performed only on microvascular endothelial cells and this is the first report on these cells. It is true that the cytotoxicity of NP has been described in other cell types, but the implications are rather different, since microvascular endothelial cells are protagonists in angiogenesis and inflammation. Indeed, a study was recently published on the effects of AgNP on HUVEC (ref.5) to demonstrate the induction of endothelial dysfunction with possible implications in atherogenesis.
In addition, we also evaluated the effects of a long-term treatment with NP and the partial reversibility of the effects of sublethal concentrations of NP.

(2) *The toxicity of AgNP has been reported in the peer-reviewed journals. 2. How is AgNP dissolved in the solution is not indicated. It has been clearly demonstrated that solubility of AgNP can affect cytotoxicity. The manuscript indicated that AgNP had been incubated with cells for days. Whether AgNP is truly dissolved in the solution or only suspended for a certain period of time would significantly influence all parameters measured in this study. Likewise, what agents and conditions were used to dissolve AgNP also could produce significant influences.*

Because of their nature, Ag NP can only be in a suspension, they can not be solubilized. As described in the methods, on the basis of results reported in the literature and according to the manufacturer's instructions, AgNP were prepared in culture media and sonicated.

(3) *The rationale for the selection of anti-oxidant agents and their concentrations used in the study is not indicated. The negative results could be caused by which antioxidant agent and what concentration were used.*

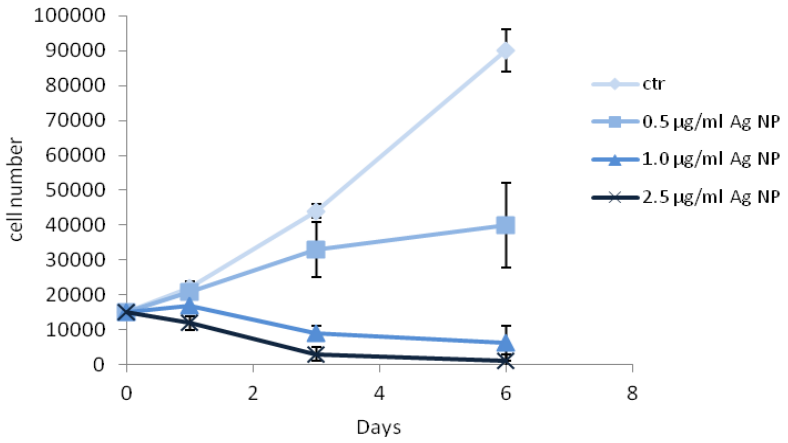
The anti-oxidants used have different chemical structure and different mechanism of action (new figure 2). Trolox is a synthetic cell-permeable analogue of α-tocopherol which scavenges peroxyl and alkoxyl radicals. N-acetyl-L-cysteine (NAC) is a thiol compound that is a precursor of reduced glutathione and increases the activity of superoxide dismutase. Apocynin is an inhibitor of NADPH oxidase. The rational for the selection of the anti-oxidants utilized and the concentration used are reported in the revised manuscript with the new figure showing the results obtained with 0.5, 1 and 2.5 µg/ml AgNP ± anti-oxidants at 24 and 72 h. Briefly, we found that Ag NP induce the formation of free radicals (as detected by DCF), but antioxidants do not prevent Ag NP cyotoxicity (new fig. 2).

Reviewer 2

(1) *Figure 1 Data in A and B are quite different. While in B 1 and 2.5 ug/ml of AgNP appears to completely suppress cell viability, in panel A, cell viability at similar concentrations is 60 and 40% of control. Please, explain these differences and show more uniform data. In B, there is almost no difference in the effect of AgNP at 3 or 6 days. What happens at 24 or 48 hs? Please, show these data as well as cell count numbers. The authors state that at 0.5 ug/ml of AgNP ther is only a slight increase. However after 6 days appears to be a reduction of 50% compared to control samples. Please explain these differences.*

We are aware of the variability that sometimes occurs in our experiments. Apart from times of exposure to Ag NP and concentrations used, we have noticed that also cell density impacts on the response of the cells to Ag NP. It is possible that cell density is slightly different from experiment to experiment, in particular in the case of time course when the cells have to cultured for long times and therefore they are seeded at low density. In the light of the comments of the reviewer, we have repeated the experiments in kinetics adding also an earlier time point (24 h). We now show these results in new fig. 1B.

For reviewer knowledge, we here show the results obtained by counting the cells.



About the results obtained in proliferation assays with 0.5 ug/ml, we corrected the sentence by cancelling the word "slight".

(2) *Figure 2 The values are more like those from Fig. 1 B but quite different from Fig. 1 A so please, uniform data. Please, also include a positive control showing that antioxidants were active.*

The experiment was repeated with 0.5, 1 and 2.5 ug/ml Ag NP at 24 and 72 h. In addition to Trolox and apocynin, we also used NAC. As a positive control, we used 100 uM H_2O_2 for 30 min. While the paper was under review and then after receiving the comments of the reviewers, we performed experiments to determine whether Ag NP induce the formation of free radicals. These results are shown in fig. 2A and discussed in the paper. We conclude that antioxidants can not prevent cytotoxicity, probably because the accumulation of free radicals after hours of exposure to Ag NP is too high to be counterbalanced by antioxidants.

(3*) Figure 3 There is not much information for one figure so Figure 3 can be included in Fig. 1. The effect at 1 and 2.5 ug/ml is quite impressive so it would be important to correlate these data with the MTT values at the same time (16 hs) and to confirm that LDH matches the data from the MTT assay as it is stated in the discussion. What is the total LDH content of the cells?*

We have repeated the experiment at 24 h, so that these results can be compared to the MTT assay at the same time point. In the revised manuscript, these results are shown as fig. 1C. We do not know the total amount of LDH in the cells. We only know that upon treatment with 100 ng/ml of Tumor Necrosis Factor alpha for 24 h, we find much higher amount of LDH in the medium than after exposure to Ag NP (4 times more than with Ag NP).

(4) *Figure 4 A) It is not clear why the authors state that "2.5 µg/ml Ag NP markedly reduced cell number after 4 days treatment". The other two concentrations also induce a marked reduction comparing with non-treated cells. Please explain.*

The sentence has been changed. Our intention was to underscore that 2.5 ug/ml are so cytotoxic that we lose most of the cells at 4 days and, consequently, we can not propagate the culture. We hope the new sentence is clearer.

(5) *It is not evident from the results that removal of AgNP rescues cell growth as it is written in the abstract. More accurate is to state that removal partially rescues cells growth as it is written in the result section.*

The abstract has been corrected as requested.

(6) *Page, 7 line 13: Please, indicate the mean ±SE of the IC50 instead of a range.*

IC50 are now reported as the mean ± standard deviation.

(7) *What is the concentration of the AgNP used in vivo? Please indicate it in the text and compare with that used in this study.*

When injected intraperitoneally in rats (260 g), 1 mg/kg body weight is used (Sarhan OM, Hussein RM, Int J nanomed 2014). When injected intravenously in rats, 5 or 10 mg/kg of AgNPs were used

(Dobrzyńska MM 2t al, Toxicology 2014). It is complex to extrapolate conclusions that could be translated to our results, because it is impossible to know the local concentration of NP in the tissues.

(8) *Minor -Please as they are in vitro studies; use the word "concentration" instead of "dosis". -Abstract line 18 replace to for in angiogenesis. - In Figure 1C and 4 C please indicate the absence of AgNP,*

All these points have been corrected.

Reviewer 3

(1*) Minor concerns: Figures 1C, 4C, 5B: scale bars are missing.*

We appreciate the favorable review of our manuscript. Scale bars have been added.

Sincerely yours,

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