Peer Review File

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Review Comments-Reviewer A

The paper titled "Temporal relationship of the orphan receptor TR3 translocation and expression with zinc-induced apoptosis in prostate cancer cells" is interesting. Zinc-induced apoptosis could be regulated through TR3 protein translocation from the nucleus to the mitochondria in prostate cancer cells without notable changes in TR3 mRNA levels. The direct effect of zinc on the mitochondria was associated with the release of cytochrome c into the cytosol through the mitochondrial targeting of TR3 protein. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) What are the roles of TR3 in androgen receptor expression and signaling in prostate cancer cells? It is suggested to add relevant contents.
   Reply: we have modified our text as advised
   Changes in text: Page 11, line 362.
2) There have been many studies on prostate cancer. What is the difference between this study and previous studies? What is the innovation? These need to be described in the introduction.
   Reply: we have modified our text as advised
   Changes in text: Page 3, line 91.
3) The A and B marks are not shown in Figures 1, 2 and 5. Please check carefully and improve them.
   Reply: we have added A and B markers in Figures 1, 2 and 5.
4) Can zinc be used as a potential adjuvant in the treatment of prostate cancer with chemotherapy drugs? It is suggested to add relevant contents.
   Reply: zinc has been proved to be as an pro-apoptotic agent in prostate cancer, however zinc could be used as an chemotherapy drugs or not and the range of safe dose still need a lot of clinical drug research. We have added relevant contents in this article.
   Changes in text: Page 12, line 396.
5) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "Effect of taurine on the proliferation, apoptosis and MST1/Hippo signaling in prostate cancer cells, PMID: 35836537". It is recommended to quote the articles.
   Reply: we have cited the aboved paper in the introduction part.
   Changes in text: Page 3, line 86.
6) It is suggested to increase the research progress of zinc signal transduction in prostate

cancer.

Reply: In this study, we found zinc could induce apoptosis of prostate cancers through TR3 protein translocated from the nucleus to the mitochondria and was accompanied by cytochrome c release into the cytosol from the mitochondria. This study indicated zinc could be used as a potential target for prostate cancer treatment, it is a good recommendation that increases the research progress of zinc signal transduction in prostate cancer, more in-depth research will be proceeded.

Review Comments-Reviewer B

The paper proposes zinc as an anticancer agent against metastatic stages of prostate cancer, placing the orphan receptor TR3 as the primary inducer of the apoptotic pathway.
The data shown do not bring any further advance of knowledge in compliance with the state of the art. Specifically, there are already a vast number of articles in the literature investigating zinc-induced apoptosis, exploring it in a more accurate and in-depth manner. Furthermore, no valid experimental results are shown to support the involvement of TR3 in the apoptosis induction. In fact, the proposed experiments are uniquely related to the localization of TR3 and its expression, leaving several gaps regarding the proposed interaction with cytochrome-c.

I have the following comments:

1. The data shown in Figure 3 conflict with that of Figure 2. Specifically, the apoptosis rates resulting from the observation of nuclei fragmentation are higher than the amount of dead cells shown in Figure 3.

Reply：In Figure 3, prostate cells were double-stained with PI and Annexin-FITC to determine the level of apoptosis induced by zinc, the results showed that the lower right quadrant representing apoptotic cells with positive Annexin-FITC staining; the upper right quadrant indicated necrotic prostate cancer cells with positive PI staining. In thus, in 8h treatment with zinc, the apoptotic level of PC-3 cells began to increase, significant accumulation of apoptotic cells observed in 24h, meanwhile some necrotic PC-3 cells were also observed, the results also showed that the amounts of apoptotic PC-3 cells were higher than necrotic PC-3 cells, the similar results was showed in LNCaP cells. Furthermore, in Figure 2, the apoptotic level of PC-3 and LNCaP cells was observed with Hoechst staining, the results showed with a time-dependent manner, the apoptotic cells of PC-3 and LNCaP cells increased, significantly higher in 24h.

2. The fields shown in the immunofluorescences of figures 2A, 5A, 5B and 6 should be selected with greater accuracy as they are unclear. I would also suggest paying more

attention to the size indicated by the scalebars, which does not appear to be correct in many images.

Reply: The clearer figures have been uploaded separately.

Review Comments-Reviewer C

The paper presents results of the pro-apoptosis roles of zinc in prostate cancers cells, through treating two prostate cancer cell lines with exogenous zinc for different times between 0 and 24 hours, then performed various assays. This study showed that apoptosis, necrosis, and nuclear fragmentation rates increased with extended zinc exposure. At the molecular level, TR3 protein translocated from the nucleus to the mitochondria, while cytochrome c was released into the cytosol from the mitochondria. It is a topic of interest to the researchers in the related areas. My comments are as follows:

1. Both PC-3 and LNCaP prostate cancer cells are commonly used in prostate cancers experiment. But PC-3 and LNCaP represent different characteristics, PC-3 cells represent hormone refractory prostate cancer and LNCaP represents hormone sensitive prostate cancer. In the method section, the different characteristics of PC-3 and LNCaP should be described.

Reply: The description of different characteristics of PC-3 and LNCaP were added at page 4, line 113 and 114.

2. The conclusion should be concise and only summarize the most important contribution of this paper.

Reply: the part of conclusion was revised at page13, line 414.

3. It is noted that the manuscript needs careful editing by someone with expertise in technical English editing paying particular attention to English grammar, spelling and sentence structure so that the study is clear to the reader.

Reply: The article has been checked by a professional English editing company, and the wrong words and grammar have been corrected.

Review Comments-Reviewer D

Prostate cancer development is associated with a marked decrease in tissue zinc levels. However, the mechanisms of zinc involvement in prostate tumorigenesis remain unclear. This study showed the impact of zinc treatment on apoptosis signaling pathways in two prostate

cancer cell lines. The results indicated that intracellular zinc ion concentrations increase following ZnCl2 exposure, as do nuclear fragmentation, apoptosis, and necrosis rates. TR3 protein translocation rate from the nucleus to the mitochondria increased with zinc exposure, as did cytochrome c release rate. In contrast, the mitochondrial membrane potential decreased after zinc exposure. TR3 mRNA levels were unaffected by ZnCl2

My comments are as follows:

1. Zinc can induce PC-3 and LNCaP prostate cancer cells through regulate the mitochondrial membrane potential in a time-dependent way. Indeed, the mechanism is involved in mitochondrial apoptosis. This statement should describe directly in the part of conclusion.

Reply: the part of conclusion was revised at page13, line 414.

2. The results showed that zinc induce apoptosis in a time-dependent manner, whether there was other study focus on a dose-dependent manner of zinc. If yes, please add related references and describe in discussion section.

Reply: In our study, the results showed that zinc induced apoptosis of PC-3 and LNCaP cells in a time-dependent manner. Through consulting literatures, as far as I know, no literature reported the dose-dependent manner of apoptosis induced by zinc.

Changes in the text: none.

Review Comments-Reviewer E

1. Highlight box

Please provide what is known and what is new in two parts.

What is known and what is new?

Intracellular zinc ion concentrations increasingly rose following $ZnCl_2$ exposure. Notably, nuclear fragmentation increased in a time-dependent manner over the 24-h exposure time, as did apoptosis at 4–8 h in both cell lines, and necrosis ultimately occurred at 24 h.

Reply:

- Zinc has been shown to be present at higher concentrations in the peripheral zone of healthy prostate tissue. the zinc levels are strikingly decreased and inversely correlated with disease progression of prostate cancer. The mechanism of zinc-mediated mitochondrial apoptosis in prostate cancer has not been exhaustively investigated.

- Zinc-induced apoptosis could be regulated through TR3 protein translocation from the nucleus to the mitochondria in prostate cancer cells without notable changes in TR3 mRNA levels, TR3 protein targeted the release of cytochrome c into the cytosol and lead to the apoptosis of prostate cancer cells. zinc can play an inhibitory role in prostate cancer by inducing apoptosis, and TR3 is expected to be a new target for the development of novel treatment methods for this disease.

Changes in the text: Page 15, line 481-491

2. Figure 1

a) Please provide the observational method of Figure 1A in the legend.

Reply: The observational method of Figure 1A has been provided in legend.

Changes in the text: Page 17, line 514

b) Please send us the latest version of figure 1A with scale bar.

Reply: The latest version of figure 1A with scale bar has been sended.

3. Figure 2

a) Please add “h” in after 24.

Reply: The figure 2A has been revised and renamed in “Figure 2A-revised ”.

Changes in the text: Page 18, line 524

b) Please provide the staining method of Figure 2A in the legend.

Reply. The staining method of Figure 2A has been provided in legend.

Changes in the text: Page 19, line 532

c) Please send us the latest version of figure 2A with scale bar.

Reply. The latest version of figure 2A with scale bar has been sent.

4. Figure 5

Please provide the scale bar in the figure or magnification in the legend.

Reply: The scale bar has been provided in the figure and sent to the editorial email.

5. Figure 6

Please send us the latest version of figure 6 with scale bar.

Reply: The latest version of figure 6 with scale bar has been sent.