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Original Article

Cellular response of MCF-7 and MCF-12A cell line to conventional versus ultra-high dose rate radiotherapy: analysis of proteins involved in the protection of cells against oxidative stress and the regulation of reactive oxygen species (ROS)

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ABSTRACT

In this paper we show our preliminary study results in which it was planned to investigate the response of selected cell lines (MCF-7 and MCF-12A) at the protein level, to doses of ionizing radiation typical for conventional and set towards ultra-high radiotherapy in normoxic and hypoxic conditions. The irradiations were performed with a dedicated linear accelerator for intraoperative radiotherapy (IORT). The cells were irradiated with 0,0833 Gy/s and 10 Gy/s dose rates. Western blot analysis was performed. We also investigated the proteins involved in the protection of cells against oxidative stress and the regulation of reactive oxygen species (ROS) – superoxide dismutase 1 (SOD 1), catalase and thioredoxin (Trx). In general, no significant differences were observed between conventional and ultra-high dose rate types of radiotherapy, but comparing the results of cells cultured in normoxia with those cultured in hypoxia, we noted a difference in the level of thioredoxin expression. We will definitely continue our research with a beam with a dose rate of at least 40 Gy/s considered to be required for FLASH radiotherapy but, by this study, we demonstrate that FLASH-related effects could be observed at a lower dose rate.

KEYWORDS: radiobiology; conventional radiotherapy; ultra-high dose radiotherapy; electron beam; breast cancer; biological response; proteomic research; oxidative stress.

INTRODUCTION

Radiotherapy (RT) with other treatments, such as surgery, chemotherapy and immunotherapy is used to treat over 50% of cancer patients. It remains highly efficient at eliminating cancer cells and regardless of significant technological improvements related to tumor targeting by image-guidance, the dose required for tumor control is still limited by normal tissue toxicity. Furthermore, some tumors remain very

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radioresistant, escaping modern treatment plans to recur and metastasize [1]. Modern radiotherapy technologies, such as volumetric modulated arc therapy, tomotherapy, and proton radiation therapy, can optimize the dose distribution and reduce the toxicity in normal tissues, but despite that the enhancement of anti-tumor effect is limited [2-4]. Ultra-high dose rate radiotherapy (also called FLASH-RT) delivers RT in milliseconds and has been associated in preclinical studies with less side effects on healthy tissues, when compared to conventional (CONV) dose rate RT delivered in minutes, while the effect on tumors appeared similar [5, 6]. Although the specific mechanisms by which FLASH protects the normal tissues are yet to be defined, it has been suggested that FLASH IR may cause rapid consumption of local oxygen, eliciting a transient hypoxia in tissues and producing larger quantities of reactive oxygen species (ROS), which could be eliminated much more efficiently in normal tissues during steady-state metabolism than in tumors [7, 8]. Spitz *et al*. [9] indicated that the FLASH effect is related to the instantaneous generation of free radicals augmenting the influence of differences in redox and free radical chemistry between normal and tumor tissue. The study suggested that the content of unstable iron in normal tissue cells is less than that in tumor tissue cells, and therefore, the production of cell destructive ROS was more easily restricted, reducing cell damage [9, 10]. In this paper we show our preliminary study results in which it was planned to investigate the response of selected cell lines (MCF-7 and MCF-12A) at the protein level, to doses of ionizing radiation typical for conventional and set towards ultra-high radiotherapy in normoxic and hypoxic conditions. We also investigated the proteins involved in the protection of cells against oxidative stress and the regulation of reactive oxygen species (ROS) – superoxide dismutase 1 (SOD 1), catalase and thioredoxin (Trx). We report here the outcome of these experiments.

MATERIALS AND METHODS

Cell culture

The human breast cancer MCF-7 and normal MCF-12A cell lines were donated from The Greater Poland Cancer Centre. MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific) with high glucose (4.5 g/l), sodium pyruvate and glutamine supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific), 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific) and 0.01 mg/ml human insulin (Gensulin R, Bioton S.A.) at 37 °C in a humidified atmosphere of 5 % CO_2. MCF-12A cells were maintained in Dulbecco's Modified Eagle Medium:Nutrient Mixture F-12 (DMEM / F-12) (Gibco, Thermo Fisher Scientific) supplemented with 5% horse serum (Gibco, Thermo Fisher Scientific), 1% penicillin/ streptomycin (Gibco, Thermo Fisher Scientific), 0.01 mg/ml human insulin (Gensulin R, Bioton S.A.), 0.02 µg/ml human epidermal growth factor (Gibco, Thermo Fisher Scientific), 0.5 µg/ml hydrocortisone (Sigma-Aldrich, Merck) and 0.1 µg/ml Cholera toxin (Sigma-Aldrich, Merck) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. 1 x 10^6 cells from each cell lines (MCF-7 and MCF-12A) were seeded in T-25 cell culture flasks (Googlab Scientific) and left in CO_2 incubator (NU-5710E model, NuAire).

Chemical induction of hypoxia – preparation of $CoCl_2$ stock solution and hypoxia treatment

Stock solution (25 mM) of cobalt chloride ($CoCl_2$) (Cobalt(II) chloride hexahydrate, Sigma-Aldrich) was prepared in sterile distilled water and further diluted in medium in order to obtain the final desired concentration. MCF-7 and MCF-12A cell lines were treated with 100 µM concentration of $CoCl_2$ and incubated under CO_2 incubator for 24 hours before irradiation.

Irradiation and dosimetry

The irradiations were performed with a dedicated linear accelerator for intraoperative radiotherapy (IORT), manufactured in an EU project in collaboration with the Greater Poland Cancer Centre and available at the National Centre for Nuclear Research (Otwock, Poland). The device works with two available modes. The conventional intraoperative mode generates a beam with energies from 4 to 12 MeV, with a dose rate of 5 and 10 Gy/min. In the conventional mode, cell culture flasks were irradiated with a 6 MeV

electron beam at a dose rate of 5 Gy/min. Doses in conventional mode were measured using a Markus Advance chamber (type 34045 PTW Freiburg, Germany) and a dose of 2.45 ± 0.02 Gy was obtained. The beam quality parameters obtained for the conventional 6 MeV beam were in accordance with the requirements of the International Electrotechnical Commission (IEC). The accelerator has been adapted to operate in the higher dose rate than in conventional dose rates for two beam energies of 6 and 9 MeV (measured for source to surface distance (SSD) of 60 cm and a circular field diameter of 10 cm). In the higher dose rate mode, measurements were performed using radiochromic film dosimetry - Gafchromic EBT-XD films. Due to the placement of the film under the cell, a correction factor for the attenuation of the beam after passing through the medium and the bottom of the cell was determined and applied. After correction, the bottle dose distributions obtained by the film method were compared using gamma (γ) analysis. Figure 1 shows the gamma index map with a 2 mm/2% criterion.

The results confirm the agreement of the dose distributions between the irradiations with the accuracy required for medical devices, and the γ index for the 2 mm/2% criterion was 98.5%. The dosimetric measurements showed that the cells were irradiated by a 6 MeV beam in a higher dose rate mode with a dose rate of 10 Gy/s and delivered a dose of 2.35 ± 0.11 Gy.

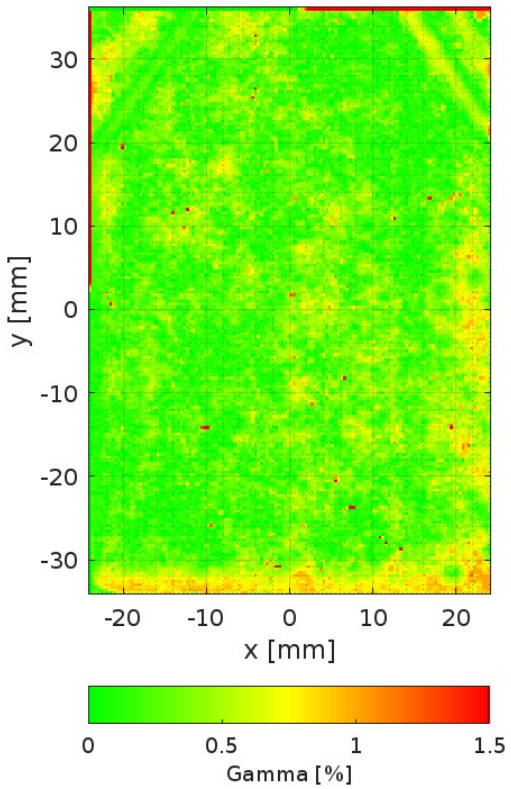


Figure 1. Analysis of the gamma index map with a 2 mm/2% criterion measured by film dosimetry.

Cell lysis and protein extraction for western blotting

At several time points after cell irradiation (15 min., 30 min., 1 h, 3 h, 6 h, 24 h, 48 h, 72 h) cells were washed twice in PBS pH 7.4 and trypsinized with trypsin-EDTA (0.25%) solution (Gibco, Thermo Scientific) and then centrifuged (5 min., 200 x g). After centrifugation the supernatant was discarded and tubes with the pellet fraction were put on ice without pellet resuspension. Then the cells were lysed in RIPA lysis and extraction buffer (Thermo Scientific). To protect proteins from degradation during extraction the Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) was added to lysis buffer. The lysates were kept on ice for 30 minutes with the RIPA buffer, and soluble proteins recovered after centrifugation (20 000 x g for 15 min.) were quantified by Bradford assay and analyzed by Western blot. Additionally, lysates from MCF-7 and MCF-12A cells not subjected to irradiation were prepared in the same way as a negative control.

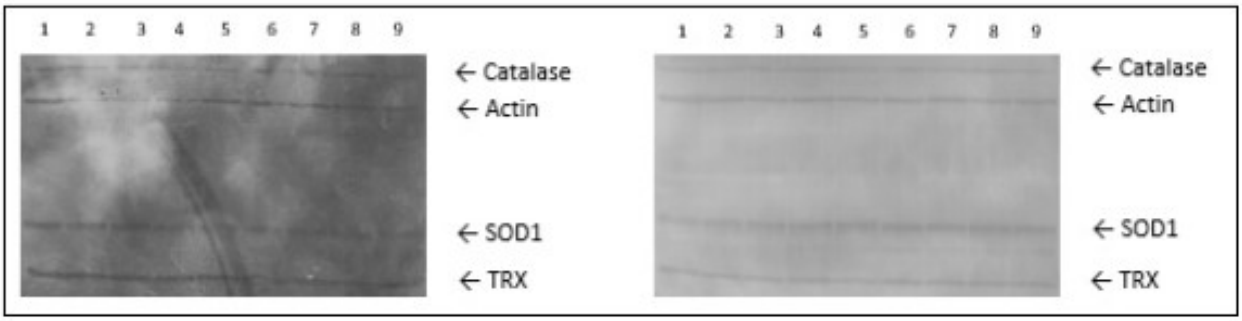
Western blot analysis

Oxidative Stress Defense Western Blot Cocktail (ab179843, Abcam) was used for analysis. This set of antibodies is designed to determine the relative abundance of several important proteins involved in the protection of cells against oxidative stress and the regulation of reactive oxygen species (ROS). Superoxide dismutase 1 (SOD1) scavenges harmful superoxides (O_2-) within cells protecting them from harmful oxidation of lipids, proteins and nucleic acids. Similarly, the hydrogen peroxide (H_2O_2) scavenging enzyme, catalase, also regulates ROS concentrations within cells by reducing H_2O_2 into less reactive O_2

and water. Thioredoxin is a small enzyme (12 kDa) that facilitates the reduction of other enzymes via cysteine thiol-disulfide exchange. Thioredoxin is used by cells to reduce ROS amounts and in redox signaling processes. Finally, alpha smooth muscle actin was included in the cocktail as a loading control. The protein samples were denatured in Laemmli sample buffer, boiled for 5 min. at 100 °C, and analyzed by Western blot. 50 µg of protein was loaded into each well and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to polyvinylidene fluoride (PVDF) (Bio-Rad). Consequently, after the blocking step primary antibody for each protein like anti-smooth muscle β-actin, anti-SOD1, anti-catalase and anti-TRX was added in blocking solution (5% skim milk/PBS+0.05%Tween 20) with dilution (1:250) and incubated for 16-18 h at 4 °C. The membrane was washed five times in TBST and then incubated for 2 h with secondary antibody (Goat anti-rabbit IgG H&L HRP) (ab205718, Abcam) (secondary antibodies have been used with 1:20,000 dilution). Subsequently membrane was washed five times in TBST and exposed to X-ray film (CL-Xposure film, Thermo Scientific). Relative protein expressions were estimated against β-actin. Results reported were derived from at least 2 independent experiments.

RESULTS

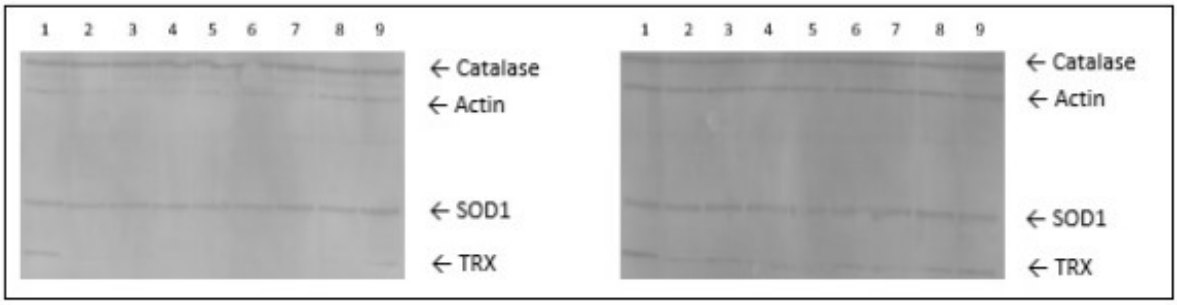
To compare the cellular response to conventional and ultra-high dose rate radiotherapy, we used human breast cancer MCF-7 and normal breast MCF-12A cells. We have also conducted previous research with human breast cancer cells preparing the electron accelerator for radiobiological research [11]. Since it has been show that the FLASH effect of ultra-high dose rate radiotherapy depends on oxygen concentration [12] we divided both cell lines into two groups: half of the cells were cultured under normoxic conditions and the other half under chemically induced hypoxia. In result, we had total four groups of cells to irradiate: 1) MCF-7 and MCF-12A / normoxia / conventional dose rate; 2) MCF-7 and MCF-12A / chemically induced hypoxia / conventional dose rate; 3) MCF-7 and MCF-12A / normoxia / ultra-high dose rate; 4) MCF-7 and MCF-12A / chemically induced hypoxia / ultra-high dose rate. Cells were treated with a single fraction of about 2.5 Gy electron radiation delivered using 2 different modalities: (1) conventional dose rate delivering 5 Gy/min. (0,0833 Gy/s) and (2) ultra-high dose rate delivering 10 Gy/s. Appropriate controls were unirradiated cells (MCF-7 and MCF-12A) cultured under normoxic and hypoxic conditions. Cells were detached and cell lysates were prepared at 15 minutes, 30 minutes, and 1, 3, 6, 12, 24, 48, and 72 hours after irradiation. The expression of proteins involved in the protection of cells against oxidative stress and the regulation of reactive oxygen species (catalase, superoxide dismutase 1 and thioredoxin) was assessed in both normal and cancerous human breast cell lines. The results of Western blot analysis are shown in Figures 2A-D. All proteins included in Oxidative Stress Defense Western Blot Cocktail (ab179843,



(2 A)

Figure 2

Figure 2 continued..



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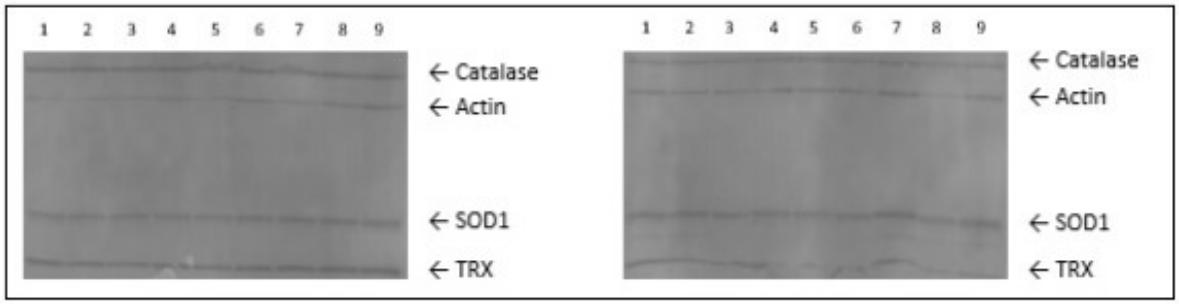

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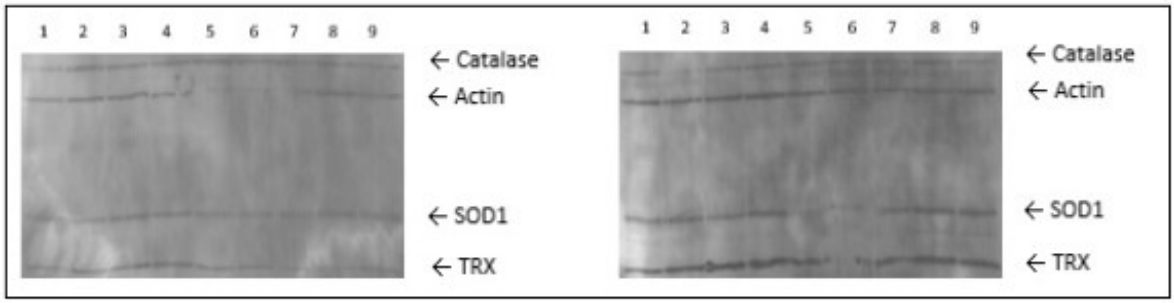

(2 D)

Figure 2. Western blot analysis for the expression of proteins involved in the protection of cells against oxidative stress and the regulation of reactive oxygen species (ROS) (catalase, superoxide dismutase 1, thioredoxin) in treated MCF-7 (left panel) and MCF-12A (right panel) cells: (A) conventional dose rate; hypoxia. (B) conventional dose rate; normoxia. (C) ultra-high dose rate; hypoxia. (D) ultra-high dose rate; normoxia.

Abcam) are visible on the blots (with more or less intense bands). In general, no significant differences were observed between conventional and ultra-high dose rate types of radiotherapy, but comparing the results of cells cultured in normoxia with those cultured in hypoxia, we noted a difference in the level of thioredoxin expression. Under conventional radiotherapy, in cells cultured with the addition of cobalt chloride (chemically induced hypoxia), we have stronger thioredoxin bands compared to cells cultured in normoxia (very weak or no bands) (Figures 2A and B). However, under ultra-high dose rate conditions, there is a difference in thioredoxin

expression between MCF-7 and MCF-12A cells cultured in normoxia (stronger bands in the case of healthy MCF-12A cells and weaker in MCF-7 cells) (Figure 2D).

DISCUSSION

In recent years, ultra-high dose rate radiotherapy (UHDR-RT) has gained attention in preclinical studies as a potential advancement in cancer treatment. The great advantage of UHDR-RT is the ultra-fast, high dose delivery of radiation that has a similar or greater effect on cancer cells than conventional mode of delivery, while sparing normal, healthy tissue surrounding the tumor site. Several hypotheses have been proposed to explain the biological mechanisms contributing to the selective FLASH effect, including differences between healthy and cancer cells in the production of reactive oxygen species and free radicals, limitation of Fenton reaction caused by high $Fe^{2+/3+}$ levels in tumor cells, and impaired DNA damage repair mechanisms occurring in cancer [12]. It was shown that a mechanism, depending on oxygen depletion, causes reduction in the produced amount of ROS and free radicals [13]. Therefore, we wanted to examine the expression level of proteins involved in the protection of cells against oxidative stress and the regulation of reactive oxygen species in healthy and cancer cells exposed to conventional and ultra-high dose rate radiotherapy conditions. Instead of reducing oxygen pressure in cell culture we used chemically induced hypoxia method with a cobalt chloride solution ($CoCl_2$) which is a commonly used hypoxia-mimetic agent, that artificially induces hypoxia and can block the degradation and thus induce the accumulation of HIF-1α protein [14]. In our results there were no significant differences in cellular response between the two types of radiotherapy administered. The responses / differences were so small that they can be explained by the fact that we used too low dose rate (10 Gy/s) to allow significant differences in the levels of the tested proteins to occur. Theoretically the FLASH effect occurs at a dose rate equal to or greater than 40 Gy/s. Nevertheless we assumed that 10 Gy/s may also elicit desired difference in cancer and healthy tissue response, although to a lesser extent. We speculate that a subtle cellular response difference in reactive oxygen species (ROS) elicited by higher dose rate radiation is augmented at the level of several important proteins involved in the protection of cells against oxidative stress and the regulation of ROS. Indeed, there are different responses when comparing thioredoxin expression between MCF-7 and MCF-12A cells cultured in normoxia, irradiated under ultra-high dose rate conditions. At the moment of oxidative stress thioredoxin (Trx), to some extent, reduces intracellular proteins and lowers levels of ROS as part of the antioxidant defense. Trx also translocates to the nucleus in response to oxidative stress where it can exert regulatory functions of transcription factors via redox modifications [15]. *In vitro* human tumor cell lines have been found to produce significantly greater amounts of ROS than nontransformed cell lines, thereby keeping these cells under persistent oxidative stress [16]. These higher levels of ROS in cancers are usually associated with extremely high levels of Trx and other redox control proteins. We will definitely continue our research with a beam with a dose rate of at least 40 Gy/s considered to be required for FLASH radiotherapy but, by this study, we demonstrate that FLASH-related effects could be observed at a lower dose rate. We will also change the research model and instead of 2D cell cultures we will use 3D spheroids. The concept of 3D spheres is based on the creation of spheroid structures in which cells form various layers. This structure mimics the physical and biochemical features of a solid tumour mass [17]. We will also want to replace the chemiluminescence-based western blotting method with a fluorescence-based method to precisely determine the amount of individual proteins. Fluorophore signal more linearly correlates with protein concentration, likely because it is a more direct assessment of protein abundance than enzymatic function in chemiluminescent method. It also provides a greater dynamic range, offering superior sensitivity for both low and high protein amounts [18, 19].

CONCLUSION

We show our preliminary study results in which it was planned to investigate the response of

selected cell lines (MCF-7 and MCF-12A) at the protein level, to doses of ionizing radiation typical for conventional and set towards ultra-high radiotherapy in normoxic and hypoxic conditions. The cells were irradiated with 0,0833 Gy/s and 10 Gy/s dose rates. Western blot analysis was performed. We also investigated the proteins involved in the protection of cells against oxidative stress and the regulation of reactive oxygen species (ROS) – superoxide dismutase 1 (SOD 1), catalase and thioredoxin (Trx). In general, no significant differences were observed between conventional and ultra-high dose rate types of radiotherapy, but comparing the results of cells cultured in normoxia with those cultured in hypoxia, we noted a difference in the level of thioredoxin expression. We will definitely continue our research with a beam with a dose rate of at least 40 Gy/s considered to be required for FLASH radiotherapy but, by this study, we demonstrate that FLASH-related effects could be observed at a lower dose rate.

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AUTHOR CONTRIBUTIONS

Marcin Zieliński: Conceptualization, Methodology, Investigation, Resources, Writing – Original draft preparation, Project administration, Funding acquisition.

Aleksandra Lenartowicz-Gasik: Conceptualization, Methodology, Investigation, Writing – Original draft preparation.

Agnieszka Misiarz: Methodology, Writing – Reviewing and Editing.

Jacek Rzadkiewicz: Validation, Writing – Reviewing and Editing, Supervision.

Janusz Kocik: Conceptualization, Writing – Reviewing and Editing, Supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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