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Low dose gamma radiation controls let -7a and miR-21 in solid tumor model

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ABSTRACT

Cyclophosphamide and doxorubicin are often used as chemotherapy in Cancer treatment. However, these medications can cause harmful side effects, which may lead to disease progression. To address this issue, this study was conducted to evaluate the effect of combining cyclophosphamide and doxorubicin chemotherapy with low-dose gamma irradiation on the immune response and antitumor efficacy in a tumor mass animal model. Ehrlich ascites carcinoma cells (EAC) were implanted intramuscularly in the right thigh of female albino mice. The mice were then treated with doxorubicin (D) at a dose of 10 mg/kg body weight once a week for four weeks, and low-dose gamma radiation (0.25 Gy) (LDR) in the third and fourth weeks. The current study discovered that radiation might regulate angiogenesis and proliferation in solid tumors more effectively than cyclophosphamide and doxorubicin by themselves. considerable regulation of miR-21 and Let-7a fold change. Additionally, heat shock proteins 70 and 90 were decreased and the apoptosis marker caspase-3 was increased by the chemotherapy and radiation combination. These results suggest that low-dose gamma radiation combined with doxorubicin and/or cyclophosphamide might be a useful therapeutic regimen for the treatment of cancer.

INTRODUCTION

Cancer is a multifaceted disease that has been treated with several standard modalities for decades. Nowadays, many types of cancer are treated with combination therapies that include immune-, chemo-, and radiotherapy. Combination therapy is more effective than monotherapy, as it reduces drug resistance and has some drawbacks [1].

Radiotherapy (RT) is commonly used in the treatment of solid tumors, either pre- or post-surgery. Furthermore, RT damages the DNA of tumor cells and normal cells in the irradiation area in a way that cannot be reversed. However, over the past few decades, RT has seen significant advancements. It has been suggested that low-dose radiation (LDR) (in the range of 0.1–0.5 Gy) may have some physiological benefits [2]. LDR has been shown to boost DNA repair rates, activate the radical detoxification system, and boost immunological competence [3], promoting the growth of a diverse range of cytotoxic cells and reducing the incidence of metastatic cancer [4]. Chemoradiotherapy aims to improve treatment efficacy while minimizing harm by increasing local control, reducing the risk of distant metastases, and prolonging survival [5]. In addition, a greater median survival length is linked to chemotherapy followed by radiation [3]. Cyclophosphamide (C) is a member of the

oxazaphosphorine group of alkylating agents, which has been used in clinical settings for more than forty years. It possesses immune-regulating and immunosuppressive qualities, which make it a useful drug for treating immune-mediated and autoimmune illnesses as well as cancer [6]. Because of its selectivity for T cells, cyclophosphamide is also being widely used in cancer vaccination programs [7]. Doxorubicin (DOX) is the primary component of anti-cancer therapy schedules currently in use. However, the specific mechanisms of DOX activation remain unknown. DOX has a pleiotropic anticancer action, which includes contributing to DNA damage, reactive oxygen species (ROS) generation, induction of apoptosis, senescence, autophagy, ferroptosis, and pyroptosis, as well as an immunomodulatory effect [8]. Ehrlich ascites carcinoma (EAC) is a highly transplantable, 100% malignant tumor that lacks tumor-specific transplantation antigens. It is administered as ascites or in solid form. EAC has been used for chemotherapeutic investigations since its description [9].

This study aims to explore the significance of low-dose radiation (LDR) in boosting the anticancer impact of DOX and C in solid tumor models. Additionally, the study aims to explore the processes underlying the ability of LDR to regulate let-7 and mir-21 in oncogenic pathways associated with cancer.

MATERIALS AND METHODS

Materials: Doxorubicin was utilized as an injectable commercial product (Adriplastina vials), with each vial comprising freeze-dried powder doxorubicin hydrochloride. Each vial's contents were freshly dissolved in a sterile saline solution just before use. It was administered at 10 mg/kg body weight, while Cyclophosphamide was administered at a rate of 100 mg/kg body weight as a single i.p. dose once a week for four weeks. All chemicals were of analytical quality and acquired from Sigma-Aldrich VR (St. Louis, MO).

Animals: Fifty Female Swiss Albino mice (20–25 g) were used in this study; they were acquired from the breeding unit of the Egyptian Holding Company for Biological Products and Vaccines.

The animals were kept in standard cages with free access to water and a standard laboratory diet. They were all kept under standard conditions of temperature and light-dark cycle. This study was conducted following the guidelines set by the Research Ethics Committee (REC) for experimental studies involving human and animal subjects at the NCRRT-Egyptian Atomic Energy Authority in Cairo, Egypt (Ref. No. 212A/21). The study adhered to international guidelines for the proper care and use of laboratory animals and complied with relevant legislation outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, 1996).

Radiation protocols: At the NCRRT in Cairo, Egypt, whole-body gamma irradiation was performed using Canadian gamma cell-40 (¹³⁷ Cesium). According to the Protection and Dosimetry Department's requirements, the unit's ventilation holes align with ventilation portions via the main shield to offer small animals with uniform irradiation at a dosage rate of 0.67 Gy / min.

Animal Category: Female albino mice injected intramuscularly in the right thigh with EAC cells at the start of the experiment. The viability test was performed on Ehrlich ascites carcinoma-bearing cells [10]. In addition EAC was subsequently maintained with repeated intraperitoneal (IP) transplantation of 2.5x 10⁶ EAC/0.2 ml ascitic fluid/mouse [11]. When the tumors developed to a size of around 100 mm³ nine days following the injection,

the mice were randomly categorized into five equal groups ten mice each as follows:

- 1- **Control group:** normal mice just received saline intraperitoneal.
- 2- **E group:** mice injected intramuscularly in the right thigh with 2.5 x10⁵ EAC cells
- 3- **EDC group:** mice were injected I.P. with a drug mixture of cyclophosphamide (100 mg/kg body weight) and Doxorubicin (10 mg/Kg body weight) at a single dose once weekly for 4 weeks.
- 4- **ER group:** mice injected with EAC cells and then exposed to 0.25 Gy gamma-radiation (R) twice at the 3rd and 4th weeks from EAC injection.
- 5- **ERDC group:** mice received both treatments cyclophosphamide + doxorubicin, followed by exposure to gamma-radiation.

Tumor volume monitoring

The change in tumor volume was measured regularly, once a week, using Vernier calipers. It was determined using the formula: Tumor volume (mm³) = 0.52A²B, where A represents the minor axis and B represents the major axis, as described by Papadopoulos et al. [12]

Blood and tissue sampling: Intracardiac blood samples were collected, and serum was centrifugated (3000 rpm) and stored at -80°C until analysis. Tumor tissues were kept for biochemical investigation. Tissue samples were washed with saline, and a known weight was homogenized. After centrifugation, the supernatant was collected for further biochemical and molecular analysis.

Quantitative real-time polymerase chain reaction

The gene expression of different microRNAs (miRNAs) was measured as described by Livak and Schmittgen [13]. The sequences of PCR primer pairs that were applied for each gene of MicroRNAs (miRNAs) are revealed in Table 1. Data were analyzed with the ABI Prism sequence detection system software and quantified through the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). The comparative threshold cycle method elaborated on the relative expression of worked genes. All data were normalized to the endogenous control U6.

Table (1): List of primers used in the qRT-PCR

Gene symbol	The sequence of the primers used for real-time PCR	Accession number
miR- let7a	F: 5'-GCTGAGGTAGTAGGTTGTATAG-3' R: 5'-GAGCAGGGTCCGAGGT-3'	AJ459692.1
miR-21	F:5'-GCCGCTAGCTTATCAGACTGATGT-3' R:5'-GTCGTATCCAGTGCAGGGTCCGAG-3'	MIMAT0000790
U6	F:5'-GCTTCGGCAGCACATATACTAAAAT-3' R:5'-CGCTTACGAATTTGCGTGTTCAT-3'	

Enzyme-linked immunosorbent assay ELISA:

The levels of HSP-70, HSP-90, VEGF, and caspase 3 in tumor tissue were determined. Following the manufacturer's instructions, a rat kit from RayBiotech, Peachtree Corners, GA, US was used to analyze HSP-70 and HSP-90, and a rat ELISA kit from Glory Science Co., Ltd. (USA) was used to detect caspase 3 and VEGF.

Statistical analysis:

The data were analyzed using SPSS (version 20). One-way analysis of variance (ANOVA) was applied and followed by a Tukey post hoc test (LSD alpha) for multiple comparisons. The data were expressed as mean \pm standard error (SE). P values < 0.05 were considered to be statistically significant.

RESULTS

Therapeutic impacts on solid tumor volume

The tumor size was calculated during the experiment, starting with the inoculation of Ehrlich carcinoma cells intramuscularly to the mice's right thighs. In group (E), the tumor size was significantly elevated as compared to the normal control group. Tumor volume was considerably decreased ($p \leq 0.05$) by all treatments. Furthermore, a significant tumor size regression was observed in the group treated with the chemotherapeutic drugs followed by radiation (ERDC) (Figure 1)

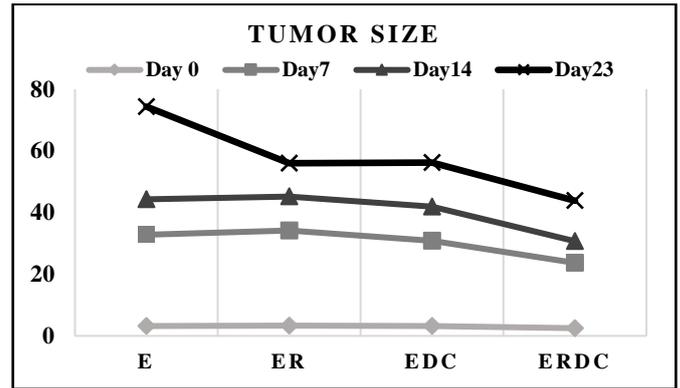


Fig (1): Effect of doxorubicin /cyclophosphamide, and γ -radiation on tumor size in different time intervals. Each value represents the mean \pm SE (n=10). Significance level at $p < 0.05$

Influence of combination cyclophosphamide plus doxorubicin followed by radiation on caspase-3 activity and VEGF protein in mice bearing EAC

Caspase-3 activity was significantly decreased ($P < 0.05$) in the Ehrlich carcinoma (E) group compared to the corresponding normal control group (Fig. 3a). On the other hand, treated with a combination of cyclophosphamide and doxorubicin significantly increased caspase-3 activity ($P < 0.05$) compared to the E group. A potent elevation was caused by the treatment with chemotherapeutic drugs plus R (ERDC). Exposure to low-dose radiation showed a significant influence in the ER group. Moreover, it exhibited a more pronounced effect in the combination group (ERDC) rather than the EDC group (Fig. 2). Conversely, the activity of VEGF showed an inverse profile that increased in the E group.

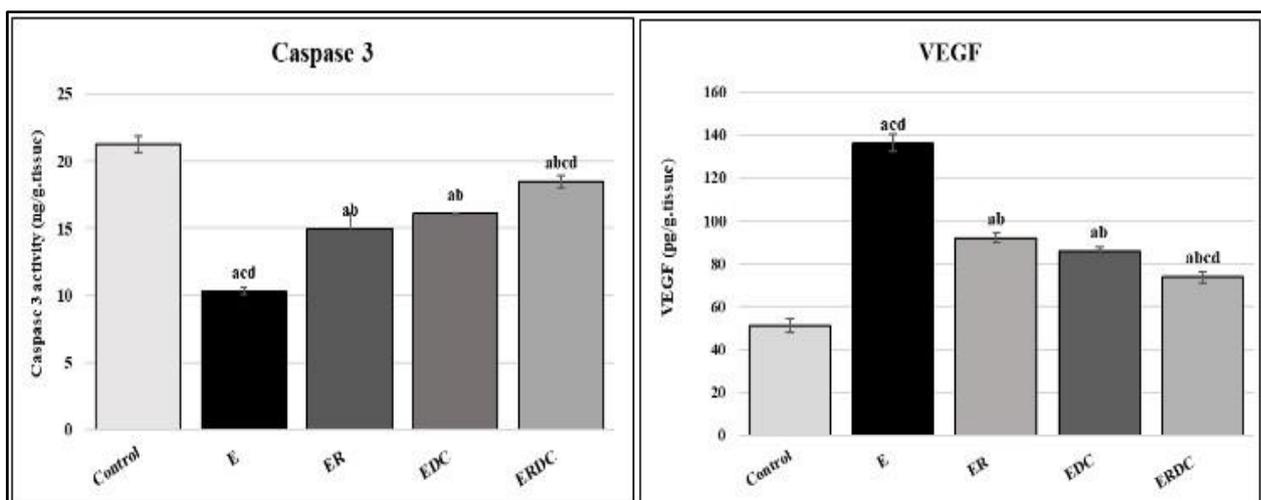


Fig (2): Effect of doxorubicin /cyclophosphamide, and γ -radiation on Caspase 3 and VEGF. Each value indicates the mean \pm SE (n=10). At $p < 0.05$, the mean difference is significant, a) significant from control b) Significant difference from E, c) Significant difference from ER

Impact of combination cyclophosphamide plus doxorubicin followed by radiation on heat shock proteins 70 and 90 in mice bearing EAC

Figure (3 A, B) illustrates the heat shock proteins 70 and 90 levels exhibited a significant rise in mice bearing EAC (E) group. The treatment with doxorubicin/cyclophosphamide showed a substantial decrease in HSP 70 and 90 expressions. Conversely, mice exposed to R revealed a potent decrease in their levels compared to the E and EDC mice group

The effect of cyclophosphamide plus doxorubicin followed by radiation on the expression of miR-Let-7 and miR-21 genes in mice inoculation EAC.

Figure 4 illustrates the gene expression of miR-21 and miR-Let 7a. The expression of miR-21 was significantly higher ($P < 0.05$) in the E group compared to the normal

control N. However, treatment with Dox/C (EDC) resulted in a significant decrease ($P < 0.05$) in its expression compared to the E group. Interestingly, after irradiation, there was a more pronounced decline ($P < 0.05$) in miR-21 expression (ERDC). Additionally, the miR-Let 7a gene expression was significantly lower ($P < 0.05$) in the E group compared to the normal control group. Conversely, mice with EAC receiving chemotherapy (EDC) showed a significant increase in miR-Let7a ($P < 0.05$) compared to the E group. Furthermore, the combination of chemotherapeutic medicines and R resulted in a strong increase in the tumor-suppressor gene miR-Let 7a ($P < 0.05$) compared to the E group. Radiation exposure had a significant impact on the ER group, which was more noticeable in the combination group ERDC (Figure 4B) than in the EDC mice group.

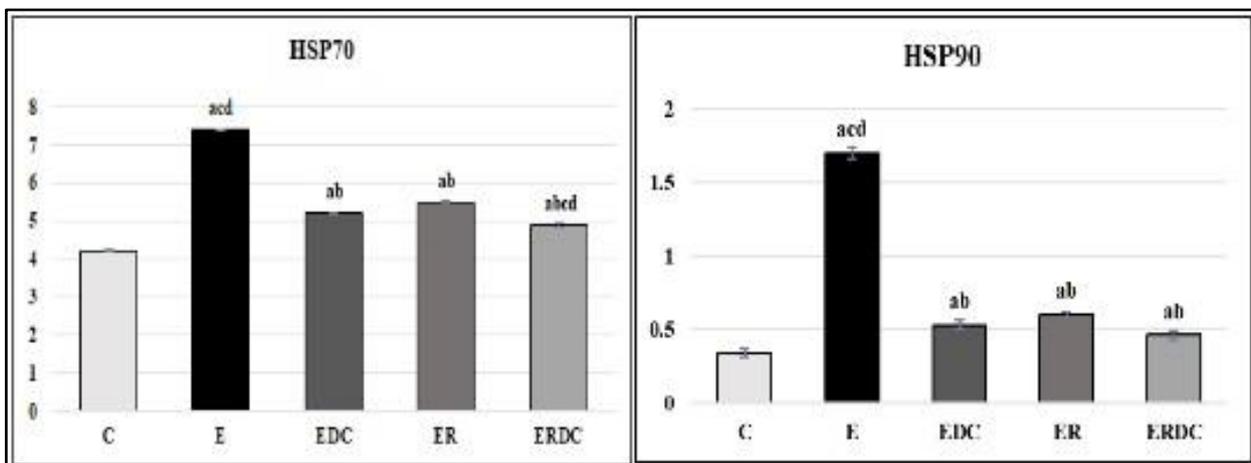


Fig (3 A, B): Effect of doxorubicin /cyclophosphamide, and γ -radiation on HSP levels expression. Each value represents the mean \pm SE (n=10). At $p < 0.05$, the mean difference is significant, a) significant from control b) Significant difference from E, c) Significant difference from ER

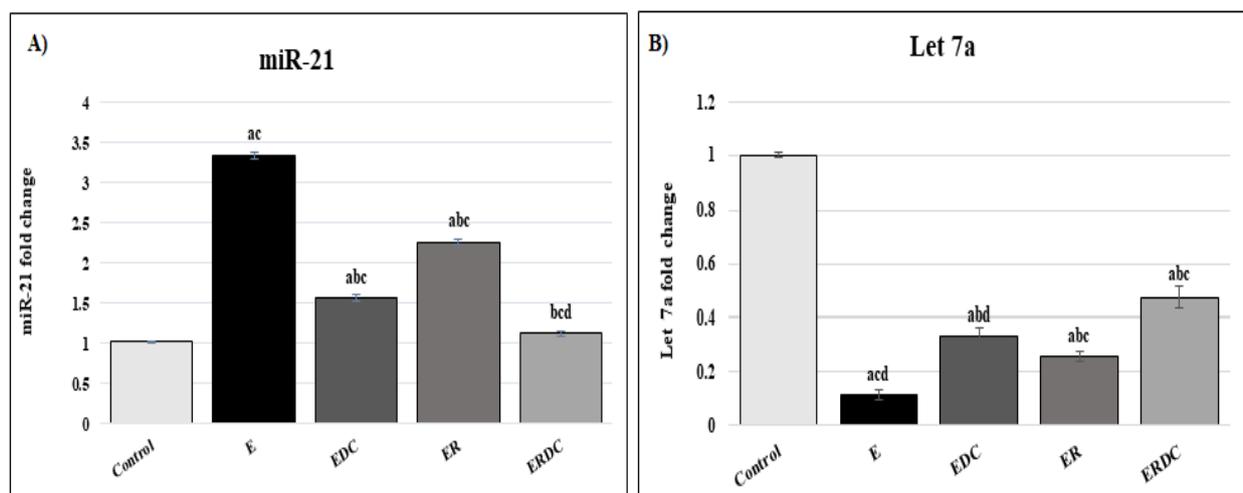


Fig (4 A, B): Effect of doxorubicin /cyclophosphamide, and γ -radiation on miR-21 and miR-Let a gene expression. Each value represents the mean \pm SE (n=10). At $p < 0.05$, the mean difference is significant, a) significant from control b) Significant difference from E, c) Significant difference from ER.

DISCUSSION

Humans and other living organisms are naturally exposed to low doses of radiation from natural sources such as the sun, soil, and water [14]. It's been found that exposure to LDR can benefit unstressed cells that are far away from the source of radiation. This phenomenon is known as radiation hormesis. The management and control of malignancies now include chemotherapy and radiotherapy [1]. Our research found that using a combination of doxorubicin/cyclophosphamide and low-dose gamma-radiation significantly reduced the proliferative capacity, induced apoptosis, inhibited angiogenesis, and regulated gene expression and inflammatory response, leading to the regression of solid tumors. All treatments reduced tumor development, but the combination of therapies (ERDC) was more effective than EDC or ER alone. Even though radiation can cause systemic immunosuppression, it can also enhance anti-cancer efficacy when combined with immunotherapies by lowering tumor-induced local immunosuppression through cancer cell debulking [15]. All the treatments used were able to lower the levels of VEGF protein, the primary marker of cell angiogenesis, but only Dox/C followed by irradiation increased caspase-3 activity, a key regulator of apoptosis [16], which was linked with a reduction in tumor volume, indicating that tumor proliferation is suppressed. Radiotherapy, which reduces cell proliferation and restores apoptosis, can exhibit an antitumor effect because cancer cells rely on high proliferation rates and resistance to apoptosis to survive [17].

Heat Shock Proteins HSP90 and HSP70 are two highly efficient chaperone mechanisms that participate in almost all stages of tumor development. They are highly expressed and contribute to the folding and stabilization of the human proteome [18]. HSP90 and HSP70 homologs are involved in various cellular processes, including apoptosis regulation, lipid metabolism, innate and adaptive immune responses, autophagy, angiogenesis, and metastasis through different signaling pathways [19]. Although the intracellular function of Hsp70 is critical for the proper folding of nascent proteins, its antiapoptotic action can protect cancer cells from environmental stress [20]. Interestingly, in the present study, it was observed that the use of LDR or D/C as immunotherapies resulted in a decrease in Hsp70 and 90, which correlated with tumor reduction. However, a better response was obtained by the combined treatment of D/C+ R. Additionally, **Rothammer et al.** [21] found that exposure to radiation-induced death of tumor cells can lead to the production of

damage-associated molecular patterns (DAMPs), such as released Hsp70, which can activate inflammatory immunological responses.

Several pieces of evidence have demonstrated the critical role miRNAs play as either tumor growth process activators or inhibitors [22]. Their role can be achieved through the regulation of tumor suppressor genes, or oncogenes, which in turn modulate carcinogenesis and many cellular biological processes associated with malignancy [23]. Combining chemo- and radiotherapy significantly reduces miR-21 levels since it prevents apoptosis. MiR-21 is considered an oncogene for breast cancer as it is overexpressed in various human cancer tissues such as multiple myeloma, glioma, ovarian, cervical, prostate, bladder, lung, and breast cancer, making it a proto-oncogene. It plays a crucial role in the differentiation, proliferation, and death of cells, besides being strongly associated with the incidence, growth, invasion, and metastasis of tumors [24]. MiR-21 is a significant regulator of miRNAs in various cellular pathways. This miRNA plays a crucial role in regulating metastasis and can control cell viability. According to **Liang et al.** [25], exposure to LDR can increase miR-21 expression in liver tissue. This particular miRNA is oncogenic and plays a role in the cellular response to ionizing radiation, as demonstrated by **Halimi et al.** [26]. It was reported that the upregulation of miR-21 and subsequent downregulation of PTEN affected MCF-7 breast cancer cells' sensitivity to doxorubicin [27]. Moreover, Zare et al [28] reported that miR-21 declined after exposure to 0.2 Gy radiation in the MCF7 cells. In addition, reports indicate that radiation-induced changes in miRNA expression are transient, dose-dependent, and cell type-specific. miR Let-7 has been shown to play a role in cell proliferation and differentiation in both human and animal cell lines, and it was reported to inhibit tumor pathogenesis [29]. Let-7, interestingly, has been linked to the suppression of cancer cell development [30]. Our findings indicate that doxorubicin/cyclophosphamide + LDR therapy stimulated miR Let-7a gene expression. Moreover, **Thammaiah and Jayaram** [31] documented the link between Let-7 overexpression and the enhancement of apoptosis and that is clear in the elevation of caspase 3 levels after the treatment in the present study. Let-7 targets several signaling pathways, such as the JAK/STAT3 pathway that is activated in many types of tumors [32]. Numerous studies have been reported that let-7a down-regulated Myc mRNA and protein by binding to its 3' UTR [33]. The tumor-suppressive function of let-7 is

achieved by inhibiting several oncogenic pathways [34]. Furthermore, Let-7a was found to downregulate breast cancer cell invasion and migration by regulating RAS and HMGA2 oncogenes [35]. Additionally, reduced levels of let-7a were found to be linked to elevated RAS expression in lung squamous carcinoma [36]. For instance, let-7 family miRNAs exhibit common patterns of radiation-induced deregulation across various cell types [37]. Moreover, the current work suggested that LDR modifies the immune response in a model of EAC-bearing mice, augmenting or prolonging the antitumor efficacy of doxorubicin/cyclophosphamide therapy

Declaration of Conflicting Interests:

The author(s) have stated that they have no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

Ethics Approval:

Ethical approval for this study was obtained from the Research Ethics Committee (REC) for experimental studies (involving human and animal subjects) at the NCRRT-Egyptian Atomic Energy Authority, Cairo, Egypt (Ref. No. 212A/21).

Animal Welfare:

The present study adhered to international, national, and/or institutional guidelines for the humane treatment of animals and complied with relevant legislation.

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Data Availability Statement:

The data is accessible upon request.

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