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Original article

Aromatase inhibitors decrease radiation-induced lung fibrosis: Results of an experimental study

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ABSTRACT

Purpose: In experimental and clinical trials, tamoxifen (TAM) has been shown to increase radiation-induced lung fibrosis (RILF). Furthermore, aromatase inhibitors (AI) have been shown to be superior to TAM in the adjuvant setting and preclinical data suggest that letrozole (LET) sensitizes breast cancer cells to ionizing radiation in other studies. In this experimental study, we evaluated whether AI have any impact on the development of RILF in rats.

Materials and methods: 60 female wistar- albino rats were divided into 6 groups: Control (group A), RT alone (group B), RT + TAM (group C), RT + anastrozole (ANA group D), RT + LET (group E), and RT + exemestane (EXE, group F). RT consisted of 30 Gy in 10 fractions to both lungs with an anterior field at 2 cm depth. Equivalent doses for 60 kg adult dose per day of TAM, ANA, LET, and EXE were calculated according to the mean weight of rats and orally administrated with a feeding tube. Percentage of lung with fibrosis was quantified with image analysis of histological sections of the lung. The mean score values were calculated for each group. the significance of the differences among groups were calculated using one way ANOVA test and Tukey HSD post-hoc test.

Results: Mean values of fibrosis were 1.7, 5.9, 6.7, 2.5, 2 and 2.2 for groups A, B, C, D, E, and F, respectively ($p = 0.000$). TAM increased RT-induced lung fibrosis but without statistical significance. Groups treated with RT + AI showed significantly less lung fibrosis than groups treated with RT alone or RT + TAM ($p = 0.000$). RT + AI groups showed nearly similar RT-induced lung fibrosis than control group.

Conclusions: In this study, we found that AI decreased RT-induced lung fibrosis to the control group level suggesting protective effect.

Introduction

In large randomized studies, third generation Aromatase Inhibitors (AI) have been shown to be superior to Tamoxifen (TAM) in the adjuvant systemic therapy of postmenopausal women with endocrine responsive early breast cancer [1,2] and upfront AI have been recommended as a part of standard treatment in this patient population. Postoperative whole-breast irradiation is an essential component of breast conserving surgery and post mastectomy radiation therapy has been demonstrated to drastically reduce

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locoregional recurrences and improve overall survival in high-risk patients [3–5].

The integration of these two common treatment modalities and their use in clinical practice concurrently or sequentially is not well established or known in detail. Insufficient data is available regarding the toxicity of concurrent use of AI and radiation therapy (RT). However in a preclinical study it has been shown that an AI, Letrozole (LET) may have a sensitizing effect on breast cancer cells to ionizing radiation which may lead to increased toxicity when used concurrently with RT in clinical setting [6]. Therefore, in this experimental study, we aimed to study the late effects of concurrent use of AI with irradiation and we evaluated weather AI have any impact on the development of radiation-induced lung fibrosis (RILF) in rats. Lung is chosen as an end organ as it is one of the most radiosensitive tissues to evaluate for late effects of RT [7]. We tested different molecules of AI both steroidal (Exemestane; EXE) and non-steroidal inhibitors (Anastrozole; (ANA) and LET) which interact with the aromatase enzyme differently. In addition we also retested the impact of nonsteroidal antiestrogen TAM with concurrent irradiation which has been shown to increase RILF [8].

Materials and methods

Sixty female Wistar albino rats, weighting approximately 200 g each were used in this study. Animals were bred, raised and housed in the Experimental Animal Breeding and Research Laboratory in X Medical School. Ten animals were housed per cage and maintained under identical conditions with food and water provided ad libitum. All experiments were carried out in compliance with the regulations of our institution and the 3R (reduction, replacement, refinement) ethical guidelines and ethical approval was obtained from the local Experimental Animal Research Ethical Committee. Wistar albino rats were randomized into 6 experimental groups and number of rats per group was 10. The first group of rats were the control group that was kept without receiving any treatment. (Group A). The second group had irradiation to whole thoracic region (Group B). The third group received TAM (Group C), the fourth group had ANA (Group D), the fifth group had LET (Group E) and the sixth group received EXE (Group F) in addition to thoracic irradiation (Table 1).

All five groups, excluding group A were irradiated to the whole thoracic region with Cobalt 60 unit at the Radiation Oncology Department of X Medical School. Whole lungs of the rats were simulated and marked prior to irradiation (Fig. 1). Animals were anesthetized with an intramuscular (IM) injection of Ketamine-HCL at a dose of 50 mg/kg, prior to simulation and irradiation. Animals were held securely on a foam holder in a supine position and plastic bandages were used to immobilize the thoracic region during irradiation. Irradiation was fractionated to analyze the effect of hormonal treatment with concomitant administration. A total dose of 30 Gy in 10 fractions which has been shown to cause RILF in rats was administrated [8] in 5 fractions per week to a 4 × 4 cm anterior single field at 2 cm depth.

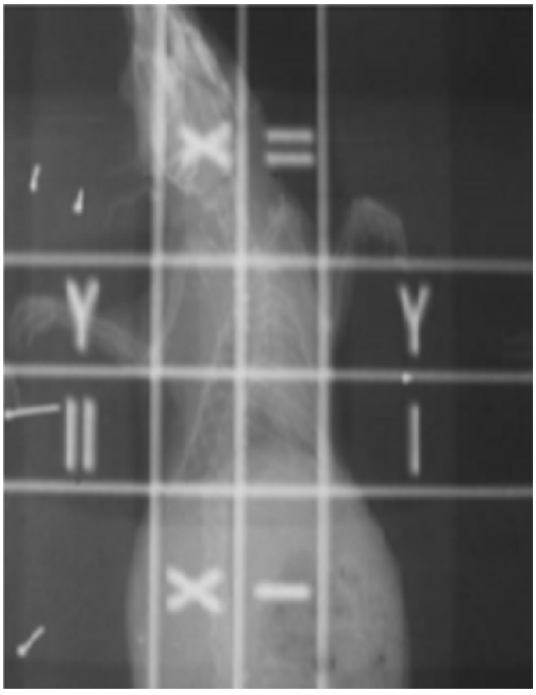

Fig. 1. Simulation of irradiated zone.

Standard dosage of hormonotherapy for adults was correlated to rats on weight basis. Average adult was presumed to be 60 kg and the average weight for subject rats was 200 g. The results of calculations is summarized in Table 2. Equivalent doses for 60 kg adult dose per day of TAM (clinical Nolvadex 10 mg tablet; gift of AstraZeneca pharmaceutical company), ANA (clinical Arimidex 1 mg gift of Astra-Zeneca pharmaceutical company), LET (clinical Femara 2.5 mg gift of Novartis pharmaceutical company) and EXE (clinical Aromasin 25 mg gift of Pfizer pharmaceutical company) were calculated according to the mean weight of rats which was 200 gr and orally administrated with a feeding tube. Administration was started at the first day of RT and continued with a daily single dose, including the week-ends, until the animals were sacrificed. Animals were anesthetized and sacrificed with cervical dislocation 16 weeks after RT which was shown to be a sufficient period for the development of RILF in rats [9]. Both lungs were removed and fixed by tracheal instillation of 10% neutral-buffered formalin and then embedded in paraffin. Four micron thickness of tissue sections were obtained and stained with Masson's Trichrome to observe lung fibrosis which is a late effect of RT. Fibrosis was defined as the thickened alveolar walls with superimposed collagen. As quantitative end point, the area of fibrosis in the alveolar walls was scored by a pathologist blinded to experimental groups, using an image analyzer (an IBM-Pentium II computer and Samba-400 IPS program (Software)) attached to a stereomicroscope on a scale of 0 (normal lung or minimal fibrous thickening) (Fig. 2) to 4 (total fibrous obliteration of the field) (Fig. 3) as described in Table 3.

Table 1
The distribution of animals according to the study groups are shown.

Groups	Description
Group A (n = 10)	Control
Group B (n = 10)	Irradiated group- (10 × 300 cGy)
Group C (n = 10)	Irradiated + Tamoxifen
Group D (n = 10)	Irradiated + Anastrozole
Group E (n = 10)	Irradiated + Letrozole
Group F (n = 10)	Irradiated + Exemestane

Table 2
Drug dosage.

Drug	Adult human dosage (60 kg)	Rat dosage (200 g)
Tamoxifen	20 mg	0.067 mg
Anastrozole	1 mg	0.003 mg
Letrozole	2.5 mg	0.008 mg
Exemestane	25 mg	0.083 mg

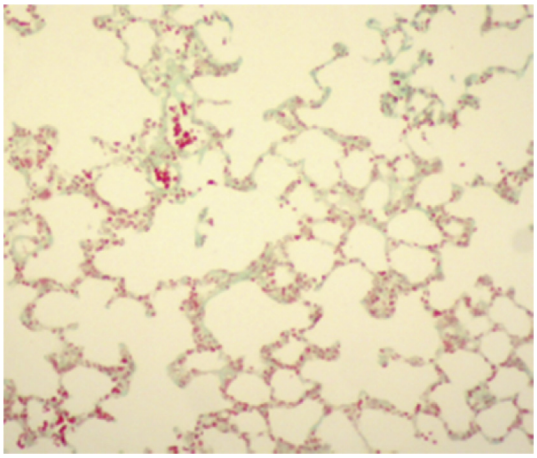

Fig. 2. Normal lung (Grade 0).

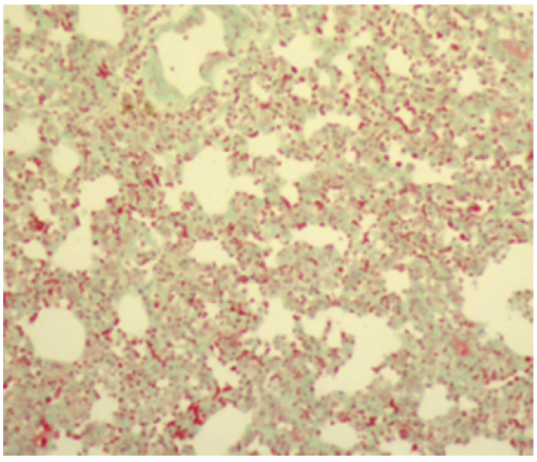

Fig. 3. Total fibrous lung (Grade-4).

For each rat, the total amount of the alveolar tissue was determined by using a threshold pixel value for tissue. The fibrotic scar stained with Masson's Trichrome was counted for each tissue section in 20 different areas with a morph metric method. The fibrotic score for each rat was the area of fibrosis in alveolar tissue and a mean value for each rat was obtained after the calculation in 4 histological tissue sections. After the mean fibrosis score was obtained for each rat, the mean fibrosis score values were calculated for each group. The distribution of the fibrotic score in each group and linearity were tested and when the groups were found as homogenous, one-way ANOVA test and Tukey HSD post-hoc test were used to calculate the significance of the differences among groups [10].

Table 3
Criteria for grading lung fibrosis.

Grade of fibrosis	Histological features
0	Normal lung or minimal fibrous thickening of alveolar or bronchial walls
1	Moderate thickening of the wall without obvious damage to lung architecture
2	Increased fibrosis with definitive damage to lung structure and formation of fibrous bands or small fibrosis masses
3	Severe distortion of the structure and large fibrous areas; "honeycomb lung" is placed in this category
4	Total fibrous obliteration of the field

Table 4
The distribution of animals according to the study groups and mean values of fibrosis scores for each group is shown.

Groups	Fibrosis score mean value
Group A (n = 10) Control	1.70 ± 0.60
Group B (n = 10) Irradiated group	5.88 ± 1.66
Group C (n = 9) Irradiated + Tamoxifen	6.72 ± 2.30
Group D (n = 7) Irradiated + Anastrozole	2.49 ± 0.80
Group E (n = 10) Irradiated + Letrozole	1.99 ± 1.06
Group F (n = 9) Irradiated + Exemestane	2.18 ± 0.90

One-way ANOVA P = 0.000.

Results

By the end of the study all the rats from groups A, B and E were alive. Nine rats survived the study from each of Groups C and F. Group D had seven survivors. The mean scores for each group using the number of surviving rats and histopathological analysis was calculated and given in Table 4. The highest average score was observed in the group of animals which received thoracic irradiation with concurrent TAM (mean value: 6.72 ± 2.30). There was a high statistical difference among groups for fibrosis scores with ANOVA test (p = 0.000). When groups were compared with each other, groups treated with RT + AI (Group D, E and F) showed significantly less lung fibrosis than groups treated with RT alone (Group B) or RT + TAM (Group C). (p = 0.000). The mean fibrosis score values of groups that were treated with concurrent ANA, LET or EXE (Group D, E and F) were not significantly different than each other and were very similar with the control group (Group A).

On the other hand, TAM increased RT-induced lung fibrosis. However, this increase was not found to be of statistical significance (p > 0.05) in this experimental study.

Discussion

Pulmonary fibrosis is a well-known consequence of radiation following the post-operative radiotherapy of breast cancers. Studies so far recorded that irradiated pulmonary volume, radiation dose, fraction size, RT technique and additional treatment applications have implications for the lung injury due to RT [11,12]. Over the years, there have been a number of studies investigating the issue of cellular mechanisms of the injury but conclusive results could not be achieved. Currently, a few mechanisms that explain lung injury due to RT are defined. These are, alveolar-capillary basal membrane injury, re-epithelialization and re-endothelialization for the repair of injured basal membrane resulting in fibrosis and the over-expression of TGF-B, which is a versatile cytokine. TGF-B facilitates the immigration of lymphocytes and fibroblasts to the injury site, which in turn, begin the fibroblast proliferation and this results in the production of collagen and fibronectin. TGF-B also enhances the extracellular matrix which fills in the space left behind by the diminishing normal tissue and results in lung fibrosis, as a late side effect of RT, which is characterized by the loss of normal tissue and increasing fibrous tissue [7,13].

The concurrent use of endocrine treatment and RT is a matter of concern for breast adjuvant treatment particularly due to resultant pulmonary toxicity and subcutaneous tissue toxicity which may lead to adverse effect in terms of cosmetics. The effects of Tamoxifen (TAM) on the lung fibrosis have been investigated in a considerable number of studies while it was the only known agent for hormonotherapy over a long period of time [8,14]. It is a well-established fact that, alongside its anti-estrogenic effects, TAM has non-hormonal activities such as the stimulation of TGF-B production which in turn triggers tissue fibrosis [15,16]. Furthermore,

some clinical studies have shown that TAM increases pulmonary fibrosis when combined with RT [17]. An experimental study evaluated the concurrent vs sequential use of TAM with pulmonary irradiation in wistar-albino rats. The highest pulmonary fibrosis scores were obtained in the concurrent group [8]. Results from the current study suggest that concurrent use of TAM and RT increases the pulmonary fibrosis score compared to RT alone (6.72–5.88 respectively). However, statistical analysis proved the difference to be insignificant. It is presumed that current number of subjects (10 rats per group) is insufficient to derive a statistically significant result.

Number of studies concerning the use of AI alongside of RT is quite limited compared to those that deal with TAM + RT. One such rare studies is by Varga et al. in which the long term effects of systemic therapy on lung fibrosis was investigated in 328 patients [18]. Development of symptomatic and asymptomatic pulmonary fibrosis in breast cancer patients receiving concurrent hormonotherapy (TAM and AI) and sequential taxane-based chemotherapy was evaluated using tomographical methods. The conclusion was that taxane-based chemotherapy and use of AI had no affect pulmonary fibrosis development but use of TAM increased the risk of development. The current study, as previously mentioned, confirms, though not definitively, the TAM conclusion. As for the effect of concurrent AI, the findings of the current study suggest that rather than having no effect, it reduces this undesirable side effect.

Another study concerning AI and RT has been published in 2005, by Azria et al., discussing the radiosensitizing effect of LET on cell culture [5]. The results showed that radiation induced cell deaths was achieved at lower radiation dosages in cultures treated with LET compared to those that were not. Following this breakthrough, a Phase II randomized study was planned by the same team to investigate the acute and late side effects of such usage [19]. In 2010, after a 26-month follow-up, results showing the effects of concurrent and sequential LET treatment on early stage breast cancer patients subsequent to breast conserving surgery [20]. In both groups, 75 patients each, late skin toxicity over grade 2 was not observed except for 2 subjects. A mechanism explaining the surprising lack of side effects from an essentially radiosensitizing molecule was missing. In a third paper, published in 2016, the follow-up period was extended to 76 months and observations on toxicity in pulmonary and cardiac tissues were added on top of late skin toxicity along with measurements of induced lymphocyte apoptosis. Skin toxicity remained the same (did not further develop) in both groups. No pulmonary and cardiac toxicity over grade 2 was observed. In the current study, late pulmonary fibrosis did not develop in the subject rats that received LET and other AI concurrently with RT. On the contrary, introduction of AI developed a protective mechanism that reduced pulmonary fibrosis. Further investigation is required to determine if this effect of AI is due to either one or a combination of known radioprotection mechanisms (rapid removal of free radicals, reduction of intracellular oxygen pressure, phase blocking within the cellular mitotic cycle or causing microcapillary damage) [21] or due to a completely new mechanism, currently unknown.

Conflict of interest statement

None declared.

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