# Administration of a Perfluorochemical Emulsion Plus Carbogen Breathing Does Not Alter Radiation Pneumonitis (43858)

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*Abstract.* **The effects of treatment with a perfluorochemical emulsion plus carbogen on radiation pneumonitis were examined in a rat model system. Rats received thoracic irradiation (15 Gy) and radiation reactions in the lungs were assessed 25 and 35 days later using bronchoalveolar lavage and histologic assessments. The irradiated lungs showed the expected evidence of acute radiation pneumonitis, including protein leaks and also alveolar infiltrates and interstitial infiltrates. Administration of a perfluorochemical emulsion (Fluosol; 15 ml/kg) plus carbogen breathing for 30 min before and during irradiation did not enhance the reactions seen in the irradiated lungs.**
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Preclinical studies (1–13) and clinical trials (14–16) at several institutions have examined the potential value of using perfluorochemical emulsions (PFC-E), combined with oxygen, carbogen, or hyperbaric oxygen breathing, to improve tumor oxygenation during irradiation and thereby increase the efficacy of radiotherapy. For such an approach to improve the therapeutic ratio and produce therapeutic gain, the effects of the adjunctive treatment on the tumor must be greater than its effects on the critical normal tissues within the irradiated field. The experiments reported here were part of a series of studies examining the effects of PFC-E on the radiation responses of normal tissues (4, 7–10) and were performed to assess whether administration of a clinically tested PFC-E (Fluosol) before irradiation, combined with breathing an $O_2$-enriched atmosphere before and during irradiation would alter the nature or severity of the reactions in irradiated lung.

## Materials and Methods

**Animals.** These experiments were performed using male WAG/rij Y rats that were 8 weeks old at the start of the experiments. Rats were bred and maintained as specific pathogen-free animals and were housed in a moderate security barrier throughout the experiments. Rats were treated with the PFC-E and radiation in a small laboratory within this barrier facility. Rats used in these studies were from the same strain and colony as those used in our studies examining the effects of PFC-E and oxygen enriched atmospheres on the oxygenation and radiation response of BA1112 rhabdomyosarcomas (2, 3, 5, 7, 8). All studies were prereviewed and approved by the Yale Animal Care and Use Committee and were performed in full accord with the principles expressed in the USPHS Guide.

**Experimental Design.** To ensure that temporal variations in the rats or the assays used to evaluate lung injury did not compromise the comparisons made

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in these experiments, rats within each experiment were randomized into four treatment groups: controls (receiving no treatment); rats treated with Fluosol, carbogen, anesthesia, and sham irradiation; rats receiving radiation alone; and rats treated with Fluosol plus carbogen plus irradiation. Rats from each of the four treatment groups were handled together. Rats from each group were then randomly allocated for 25 day BAL, 35 day BAL, or histologic analysis. BAL assays on sham-irradiated rats were performed only on Day 25. Data for control rats and for rats receiving radiation alone were compared with data from previous studies (20) in which the time course of radiation pneumonitis in WAG/rij Y rats had been examined to ensure that the experimental system had remained stable; no significant differences were observed.

**Radiation and PFC-E Treatments.** For irradiation, rats were anesthetized with a single ip injection of sodium pentobarbital and positioned on their backs in a Lucite positioning jig. The thorax was irradiated with 15 Gy of 250 kV x-rays, delivered at a dose rate of 1.6 Gy/min using a Siemens Stabilipan. The remainder of the body, including the long bones, was shielded with lead and received a dose less than 5% of that delivered to the lungs. Sham-irradiated animals were treated with Fluosol and carbogen, and were anesthetized and positioned as if for irradiation for a time similar to the duration of the irradiation.

PFC-E-treated rats received 15 ml/kg of Fluosol, by a slow iv injection into the tail veins, exactly as described previously (2, 3). Fluosol was provided without cost for these studies by Alpha Therapeutic Corp. (Los Angeles, CA). The formulation and characteristics of this PFC-E are described elsewhere (6, 17, 18), as are the results of studies examining the effects of Fluosol and carbogen breathing on tumors and on other normal tissues in rodents (1–7, 9–11, 13, 18, 19) and in human patients (14–16). The dose of Fluosol used in these studies is similar to that used in many preclinical studies in rodents and to the largest doses used in human patients (16). Immediately after injection of Fluosol, rats were put into a gassing box flushed continuously with carbogen (95% $O_2$/5% $CO_2$). Rats were anesthetized in this box and continued to breath carbogen until they were positioned in the irradiation jigs. During positioning and irradiation, carbogen was administrated to the rats via nose cones. Rats breathed carbogen for 30 min before irradiation and during the irradiation process.

**Assessment of Radiation Reactions.** Bronchoalveolar lavage (BAL) was performed as described previously (20–22). Rats were given an overdose of sodium pentobarbital (100–150 mg/rat, ip), then a cervical incision was made and blunt dissection was performed, exposing the trachea. A 14-gauge angiocatheter was inserted into the trachea and sutured in place. Seven individual 7-ml aliquots of physiologic saline were instilled and removed. Effluent was recovered by gravity drainage, and all aliquots from a single rat were pooled. The BAL fluid was centrifuged at 400g for 15 min. The cell pellet was suspended in 1 ml of $Ca^{++}$/$Mg^{++}$ free Hanks' balanced salt solution (GIBCO, Grand Island, NY) and cells were counted with a hemocytometer. Differential cell counts were performed by scoring 200 cells on slides prepared by cytocentrifugation (Shandon Southern Instruments, Sewickley, PA) at 600 rpm and stained by the DiffQuick method (Scientific Products, McGaw Park, IL). The total numbers of macrophages, neutrophils, lymphocytes, and eosinophils recovered were then calculated by multiplying the total nucleated cells recovered by the differential percentages of the different cell types (20). Total protein in the BAL supernatant was determined by a colorimetric assay, using Coomassie Blue as the dye and bovine serum albumin as the standard (23). Four rats were examined in each group.

In the same experiments, whole lungs (3–4/treatment group) were removed from nonlavaged rats and were fixed in buffered formalin. The lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin, Masson trichrome, and Leder stains. Slides were graded for alveolar cellular infiltrate and protein exudate, interstitial cellular infiltrate, and fibrosis by an observer blinded to the treatment regimens (20). Fibrosis was graded from the Masson's trichrome stain. Alveolar exudate was graded from 0 to 3 on the basis of the proportion of alveoli filled with eosinophilic material. Cellular infiltrate in alveolar spaces was graded on a scale of 0 to 4, for each of five cell types (pneumocytes, macrophages, lymphocytes, plasma cells, and polymorphonuclear leukocytes) with 1 representing occasional cells and 4 representing a marked alveolar filling. Interstitial cellular infiltrates were graded analogously for four cell types (macrophages, lymphocytes, plasma cells, and polymorphonuclear leukocytes). The vasculature and the bronchi were also examined for any changes from normal appearance.

## Results

The effects of Fluosol injection (15 ml/kg) plus carbogen breathing on the radiation response of the rat lung were examined 25 days (Table I) and 35 days (Table II) after treatment with 15 Gy of x-rays. These times were chosen as a result of our more detailed studies examining radiation pneumonitis in this same experimental system (20), which showed maximal changes in protein and recovered cells in the BAL fluid at these two times after irradiation.

In the two experiments reported here, the changes in the BAL fluid seen with irradiation alone at 25 days

**Table I.** Cells and Protein in Bronchoalveolar Lavage Fluid at 25 Days After Irradiation

| | Total Nucleated Cells ($\times 10^5$) | Alveolar Macrophages ($\times 10^5$) | Lymphocytes ($\times 10^4$) | Neutrophils ($\times 10^5$) | Total Protein |
|---|---|---|---|---|---|
| Control | 2.9 ± 0.5 | 2.9 ± 0.5 | 0.9 ± 1.8 | 0 ± 0 | 26 ± 2 |
| F + C + Sham X | 3.0 ± 1.3 | 2.98 ± 1.3 | 2.73 ± 2.8 | 0 ± 0 | 23 ± 2 |
| X Only | 1.3 ± 0.2[a] | 0.8 ± 0.1[a] | 6.0 ± 4.2[a] | 3.9 ± 0.9[a] | 122 ± 25[a] |
| F + C + X | 1.5 ± 0.8[a] | 1.2 ± 0.6 | 1.5 ± 0.7[b] | 3.1 ± 1.4[a] | 91 ± 29[ab] |
| Control | 2.6 ± 0.3 | 2.5 ± 0.4 | 0.4 ± 0.4 | 0.2 ± 0.2 | 12 ± 2 |
| F + C + Sham X | 2.4 ± 0.3 | 2.4 ± 0.3 | 1.8 ± 0.7 | 0.1 ± 0.1 | 15 ± 0.3 |
| X Only | 1.6 ± 0.3 | 1.1 ± 0.2 | 3.5 ± 1.3 | 5.0 ± 1.4 | 324 ± 216 |
| F + C + X | 3.2 ± 1.4 | 2.3 ± 1.1 | 3.9 ± 2.3 | 8.8 ± 3.6[a] | 73 ± 6 |

*Note.* Regimens for administering irradiation (X), sham irradiation (Sham X), and Fluosol plus carbogen breathing (F + C) are detailed in the text. Values are means ± SEMs of values for four individual rats. Data from two independent experiments are shown. Significances of differences between groups in each experiment were tested by ANOVA and were corrected for multiple comparisons.
[a] $P < 0.05$ relative to control.
[b] $P < 0.05$ relative to X only (this comparison is shown only for F + C + X).

**Table II.** Cells and Protein in Bronchoalveolar Lavage Fluid at 35 Days After Irradiation

| | Total Nucleated Cells ($\times 10^5$) | Alveolar macrophages ($\times 10^5$) | Lymphocytes ($\times 10^4$) | Neutrophils ($\times 10^5$) | Total Protein |
|---|---|---|---|---|---|
| Control | 2.6 ± 0.7 | 2.6 ± 0.7 | 0.7 ± 1.4 | 0.06 ± 0.12 | 19 ± 7 |
| X Only | 5.0 ± 2.4 | 3.9 ± 1.8 | 2.9 ± 2.2 | 10.5 ± 7.7[a] | 282 ± 182[a] |
| F + C + X | 5.6 ± 1.0 | 5.4 ± 1.0 | 0 ± 0[b] | 2.0 ± 1.2[b] | 252 ± 199 |
| Control | 2.8 ± 0.9 | 2.8 ± 0.3 | 0 ± 0 | 0 ± 0 | 11.7 ± 0.6 |
| X Only | 6.4 ± 2.6 | 5.2 ± 2.2 | 19.4 ± 8.1[a] | 9.3 ± 2.9 | 219 ± 75[a] |
| F + C + X | 6.0 ± 2.2 | 4.5 ± 1.9 | 10.0 ± 5.5 | 13.6 ± 6.9 | 179 ± 10[a] |

*Note.* Regimens for administering irradiation (X) and Fluosol plus carbogen breathing (F + C) are detailed in the text. Values are means ± SEMs of values for four individual rats. Data from two independent experiments are shown. Significances of differences between groups in each experiment were tested by ANOVA and were corrected for multiple comparisons.
[a] $P < 0.05$ relative to control.
[b] $P < 0.05$ relative to X only (this comparison is shown only for F + C + X).

(Table I) and 35 days (Table II) after irradiation were similar to those reported previously (20). BAL protein was increased over control levels at both time points. The numbers of lymphocytes and neutrophils recovered in the BAL fluid was also increased at both time points. Administration of 15 ml/kg Fluosol plus carbogen breathing and sham irradiation produced no significant changes in any BAL parameter measured on Day 25; treatment with this PFC-E plus carbogen breathing plus the anesthesia and positioning needed for irradiation therefore produced no discernible direct lung injury in these rats. Fluosol/carbogen-treated, sham-irradiated controls were not included in the matrix examined at 35 days. The BAL samples taken after irradiation were similar in the Fluosol/carbogen-treated animals and in rats receiving radiation alone. In the few cases where the differences between these two irradiated groups were statistically significant, the Fluosol/carbogen-treated rats showed less radiation injury than the rats receiving radiation alone. These BAL data therefore provide no evidence that adjunctive treatment with Fluosol and carbogen injures the lung or enhances radiation pneumonitis.

Histologic assessments of radiation injury support these findings. None of the specimens examined in these experiments showed evidence of fibrosis. The vasculature and the bronchi were likewise similar to controls in rats receiving Fluosol and/or irradiation. Moderate alveolar exudates were noted in the lungs irradiated 25 and 36 days previously; the severity of the exudates was similar in Fluosol/treated and nonpretreated rats (Table III). Moderate alveolar cellular infiltrates were also noted in the irradiated lungs, with excess numbers of macrophages and lymphocytes, and occasional neutrophils noted; the infiltrates were similar at 25 and 36 days. Less marked cellular infiltrates were noted in the interstitia of the irradiated lungs (data not shown), maximum median scores of 1+ were seen for lymphocytes; median scores for other cells types, including macrophages, were 0. There were no significant differences in the scores recorded for cellular infiltrates in rats irradiated with and without Fluosol/carbogen pretreatment. The scores recorded for the lungs of unirradiated animals treated with Fluosol plus carbogen plus sham irradiation were indistinguishable from the scores for untreated control animals. Overall, the histologic changes noted in the irradiated lungs were similar to those described and

**Table III.** Histologic Findings

| | Alveolar Exudate | Alveolar Cells | | | |
|---|---|---|---|---|---|
| | | Macrophages | Lymphocytes | Pneumocytes | Polymorphonuclear Leukocytes |
| Control | 1 (1–2) | 1 (0–1$^+$) | 0 (0–1$^+$) | 0 | 0 (0–1) |
| F + C + Sham X | 1 (1–1$^+$) | 0 (0–1$^+$) | 0 (0–1$^+$) | 0 | 0 |
| X Only | 2$^+$ (1$^+$–3$^+$) | 2 (1$^+$–2$^+$) | 1 (0–2$^+$) | 0 (0–1) | 0 (0–2$^+$) |
| F + C + X | 2 (1$^+$–2$^+$) | 1$^+$ (1–2$^+$) | 1 (0–1$^+$) | 0 | 0 (0–1$^+$) |

*Note.* Alveolar exudate was scored on a scale of 0–3; alveolar cells were scored on a scale of 0 (no cells) to 4 (marked filling). No plasma cells were seen in any specimen examined. Values shown are medians and ranges for animals in each group. Because the scores for 25 and 36 day animals were not different, the two time points have been combined.

illustrated previously in independent studies using the same experimental system (20). Administration of Fluosol plus carbogen did not alter the appearance of the lungs of unirradiated animals and did not alter the nature or magnitude of the histologic changes in irradiated lungs.

## Discussion

Radiation pneumonitis can be a serious, sometimes life-threatening side effect of radiation therapy, not only in the treatment of primary carcinoma of the lung and metastases in the lung, where lung tissue must be irradiated to sterilize embedded tumors, but also in the treatment of other malignancies, such as breast carcinoma, Hodgkin's disease, and certain head and neck cancers, where the tumors lie so near the lung that they cannot be irradiated successfully without including lung tissue in some irradiated fields. Lung injury is also a significant problem after treatment with many widely used antineoplastic drugs, including bleomycin and mitomycin C. Because the tolerance of lung tissue frequently limits the intensity of the therapy which can be administered, it is important to ensure that changes in radiation therapy, such as adjunctive use of perfluorochemical emulsions, do not compromise the tolerance of the lung to irradiation.

The radiation reactions in the lungs of the rats irradiated in these experiments were similar to those we described in detail previously in WAG/rij rats receiving radiation alone (20). These changes include a small initial protein leak at 24 hr, occurring without concomitant cellular changes and resolving by 48 hr. This is followed ~2 weeks later by an inflammatory cell recruitment and a more significant protein leak and by a third phase of increase in inflammatory cells and further increase in protein levels at 35 days. These changes are accompanied by significant cellular infiltrates, which can be seen in histologic sections. These changes can be modulated by administration of γ-interferon (20) and by levels of dietary vitamin A (24). These early processes in irradiated lung are thought to be an important part of the inflammatory reactions which lead to the development of symptomatic radiation pneumonitis and lung fibrosis (25–30). The biologic basis of radiation pneumonitis and the relationships between this damage and injury from other toxic agents (hyperoxia; drugs; asbestos) is an area of active investigation (20–22, 24–31).

Perfluorochemical emulsions are being examined as potential adjuncts to radiotherapy and chemotherapy in laboratory studies and in clinical trials (1–16); some past and planned clinical studies have included tumor sites requiring irradiation of lung tissue. The primary premise underlying the use of PFC-E as adjuncts to radiotherapy is that the combination of the circulating perfluorochemical droplets (which carry very large amounts of $O_2$) plus breathing oxygen at high partial pressure (e.g., carbogen, 100% $O_2$, or hyperbaric oxygen) should alter the transport and delivery of $O_2$ to tumor tissue (1). Unlike healthy normal tissues, which are supported by rich vascular beds which maintain the tissue $pO_2$ in a range of ~20–40 torr, solid malignancies have inadequate, poorly functional vascular beds and include large areas which exist at oxygen levels well below the physiologic range (i.e., at $pO_2$'s of 0–20 torr) (1, 11, 12, 32). Because $O_2$ is potent chemical radiosensitizer, cells in severe hypoxia are approximately three times more resistant to radiation than aerobic cells. Delivering $O_2$ to these radioresistant hypoxic tumor cells increases their sensitivity to radiation and improves the ability of radiation to cure the tumor (1, 32). Because the radiosensitizing effect of $O_2$ saturates and plateaus at $pO_2$'s of ~20 torr, increases in the oxygenation of normal tissues theoretically, should not alter their radiation sensitivities significantly (32). Thus, in principle, PFC-Es could be used to increase the effect of radiation on tumors, without compromising the radiation tolerance of normal tissues, such as lung, and could thereby improve the success of cancer treatment.

This approach has been examined through mathematical modeling (1) and through extensive studies with tumors in mice and rats (2–13). Appropriate treatments with PFC-E plus oxygen breathing have been

shown to increase tumor oxygenation, to increase the killing of naturally hypoxic tumor cells by radiation, and to improve the cure of tumors by radiation (2, 3, 5–8, 10–13). However, to ensure that regimens combining PFC-Es with radiation can be used safely, it is important to confirm the theoretical expectation that these regimens will not increase injury to normal tissue. Preclinical studies with hemaeopoetic tissues (8, 10), intestine (4), and skin (7, 8) suggest that these regimens can be used safely. The experiments reported here extend these studies to lung.

The effect of PFC/E/$O_2$ regimens on lung was of special concern, because dissolving useful amounts of $O_2$ in the circulating PFC droplets as they pass through the lung requires that the patient breath 100% $O_2$ for a few minutes before and during radiotherapy. Although short regimens of $O_2$ breathing are generally nontoxic, long $O_2$ exposures can injure lung tissues (31). $O_2$ breathing therefore could increase lung injury, either by producing damage additional to radiation-induced injury or by oxygenating marginally hypoxic, poorly ventilated areas of lung and thereby increasing their radiosensitivity. In addition, the perfluorocarbon droplets are removed from the circulation by the reticuloendothelial system and therefore are sequestered to a certain extent in lung, as well as to larger extents in liver, spleen, and marrow (17–19). The perfluorocarbons are removed from the body by expiration through the lung (17). One can therefore envision possible pathways by which PFC-Es could compromise the radiation tolerance of the lung (e.g., by directly injuring alveolar cells or altering tissue oxygenation) or could influence the development of radiation pneumonitis (e.g., by activating alveolar macrophages). The studies reported here provided no evidence that treatment with Fluosol in a high dose plus carbogen breathing before and during irradiation injured the lung or increased the severity of the radiation reactions in irradiated lung.

The studies are in agreement with our studies of EMT6 tumors in BALB/C mice (9, 19), which examined the effect of pretreatment with Fluosol plus carbogen on the radiation-enhanced increase in the development of lung tumors from intravenously injected tumor cells. Those studies showed a linear increase in the number of lung tumors forming from intravenously injected tumor cells as the radiation dose to the lungs increased from 0 to 5, 10, and 15 Gy (9). Pretreatment with 15 ml/kg Fluosol plus 30 min of carbogen breathing before and during irradiation did not alter the number of lung colonies developing after these doses of radiation. Data using three different endpoints and two different experimental rodent systems therefore suggest that adjunctive treatment with PFC-E plus an enriched oxygen atmosphere before and during irradiation does not increase significantly the effects of radiation on the lung.

These laboratory data are in agreement with data from Phase I/II clinical trials testing Fluosol as an adjunct to radiotherapy for carcinoma of the head and neck. The apices of the lungs are included in the superclavicular fields used to treat these tumors. Follow-up studies of patients in these trials have not reported excess rates or severities of radiation pneumonitis or lung fibrosis in patients receiving Fluosol plus radiotherapy (14, 15).

Since these studies were performed, second generation PFC-E have been developed (17) which appear from studies in animal tumor systems to be superior to Fluosol as potential adjuncts to radiotherapy (7, 8). As these emulsions move toward clinical trials, it will be necessary to ensure that these newer PFC-E, like the first generation emulsion tested here, do not compromise the radiation tolerance of the lung.

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