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**Analysis of Predictive Parameters for the Development of
Radiation-Induced Pneumonitis**

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Abstract

Introduction: Prevention and effective treatment of radiation-induced pneumonitis (RP) could facilitate greater use of radiation therapy (RT) for lung cancer. The purpose of this study was to determine clinical parameters useful for early prediction of RP.

Methods: Blood sampling, pulmonary function testing, chest computed tomography (CT) and bronchoalveolar lavage (BAL) were performed in patients with pathologically confirmed lung cancer who had completed ≥ 60 Gy of RT, at baseline, shortly after RT and at one month post treatment.

Results: By three months post-RT, 11 patients developed RP (RP group) and the remaining 11 patients did not (NRP group). RT significantly increased total cell counts and alveolar macrophages in BAL of the NRP group, whereas lymphocyte count was increased in both groups. Matrix metalloproteinase-9 (MMP-9) increased and vascular endothelial growth factor (VEGF) decreased significantly in the BAL fluid of the RP group following RT. Serum surfactant protein D (SP-D) increased significantly in the NRP group. SP-D in BAL fluid from the RP group increased significantly with a subsequent increase in serum SP-D. Pulmonary dilution decreased similarly in both groups of patients.

Conclusions: Increases in MMP-9 and SP-D as well as decreases in VEGF in BAL fluid could be useful biomarkers in predicting RP.

Key words

bronchoalveolar lavage, matrix metalloproteinase-9, radiation-induced pneumonitis, surfactant protein D, vascular endothelial growth factor

Running title.

Parameters for Radiation-Induced Pneumonitis

Abbreviations

A: adenocarcinoma, alveolar macrophages :AMs, bronchoalveolar lavage :BAL, bronchofiberscopy :BF, carbon monoxide :DLCO, computed tomography :CT, hypoxia inducible factors :HIFs, idiopathic pulmonary fibrosis :IPF, interleukin-1 :IL-1, L: large cell carcinoma, lactate dehydrogenase :LDH, matrix metalloproteinase-9 :MMP-9, platelet-derived growth factor :PDGF, radiation therapy :RT, radiation-induced pneumonitis :RP, regulatory T cells :Treg, S: small cell carcinoma, sialylated carbohydrate antigen-6 :KL-6, Sq: squamous cell carcinoma, standard deviation :SD, surfactant protein D :SP-D, transforming growth factor- β :TGF- β , tumor necrosis factor- α :TNF- α , U: unclassified carcinoma, vascular endothelial growth factor :VEGF

Introduction

The incidence of chest radiation therapy (RT) is increasing in association with the increased incidence of lung cancer worldwide. Although RT would ideally be performed without damaging normal tissue, a variety of normal lung tissue damage may occur since lung tissue is highly sensitive to radiation. Lung damage following RT is classified as radiation-induced pneumonitis (RP) when it develops one to three months following RT, and as radiation fibrosis when it develops three to six months following RT. Both types may overlap and may be hard to distinguish. The incidence of RP is increased following ≥ 40 Gy of radiation exposure. With >60 Gy radiation exposure, severe disease may occur. Generally 10% to 20% of radiated patients develop moderate to severe RP and mortality is estimated to be around 50% in severe RP [1-6]. Prevention and effective treatment of radiation-induced pneumonitis (RP) could facilitate greater use of radiation therapy (RT) for lung cancer. Clinical markers for risk of development of RP could be helpful.

Recently, a number of cell types and mediators have been proposed to be involved in the pathogenesis of pulmonary fibrosis, including fibrosis following RT. An animal model demonstrated that radiation directly induces toxic oxidant production from lung tissue, which causes pulmonary cell death. Pulmonary cells highly sensitive to radiation include type II alveolar epithelial cells, vascular endothelial cells and alveolar macrophages (AMs). Following pulmonary damage caused by radiation, inflammatory cells infiltrate lung tissue

and interact with pulmonary constitutive cells, leading to development of interstitial pneumonitis. Fibroblasts increase in the interstitium, as part of the repair process for damaged tissue, and produce extracellular matrix and collagen, which induce fibrosis. These fibrotic cycles are regulated by cytokines and growth factors produced by inflammatory and constitutive cells [7-9]. Among cytokines, tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), platelet-derived growth factor (PDGF) and transforming growth factor-T- β (TGF- β) increase in a murine model and in human peripheral blood in response to radiation [10, 11]. Matrix metalloproteinase (MMP) and vascular endothelial growth factor (VEGF) have also been suggested to be involved in pulmonary fibrosis [12-18]. Recently serum sialylated carbohydrate antigen-6 (KL-6) and surfactant protein D (SP-D) have been considered as useful biomarkers to determine the activity of pulmonary fibrosis at clinic level. Serum KL-6 and SP-D levels are associated with greater sensitivity in the diagnosis of RP than is serum lactate dehydrogenase (LDH) [19, 20]. Additionally, serum KL-6 level significantly correlates with severity and responses to therapy in pulmonary fibrosis [21]. Nonetheless, the role of these parameters in the prediction of RP remains unclear. This study was designed to determine the clinical parameters that are useful for the early prediction of RP.

Material and Methods

Subjects

This study received ethical approval from the special committee of Toho University Ohashi Medical Center (project registration number 23-11) to proceed between 2011 and 2013 and each patient provided written informed consent to participate. Eligible subjects were adults with pathologically confirmed lung cancer stage IIIb or IV who had completed ≥ 60 Gy of RT. Subjects with pulmonary fibrosis before radiotherapy, with systemic severe comorbidities including cardiac and collagen disease, who were current smokers, who had obvious infection, or with medications that frequently cause drug-induced pulmonary diseases, were excluded.

Study design

Blood sampling, pulmonary function testing, chest computed tomography (CT) and bronchofiberscopy (BF) were performed for all patients at baseline, shortly after RT and at one month after RT. To determine RP, additional chest CT was performed when the development of RP was clinically suspected throughout the study period and up to three months posttreatment. Two experienced respiratory physicians and two radiologists evaluated cases for diagnosis of RP. RP was defined as the presence of abnormal shadows in radiated lung fields in the absence of other causes based on clinical examinations. BF (BF-1T30, Olympus Co. Ltd.; Tokyo, Japan) was performed under standard premedication and local anesthesia. Bronchoalveolar lavage (BAL) was performed at a selected site in the tumor-free

area of the radiation fields to minimize the effect of cancer cells. The same segment was lavaged for subsequent BAL. Total cell counts, cellular components and concentrations of TNF- α , IL-1 β , MMP-9, VEGF, KL-6, SP-D in BAL fluid were determined. Simultaneously, serum KL-6 and SP-D were determined and pulmonary function testing (FUDAC-77[®], Fukuda Denshi CO. Ltd.; Tokyo, Japan) was performed. Total cell count was counted using a Bürker chamber and the differential cell count was evaluated in light microscopy on May Grunwald–Giemsa-stained slides. TNF- α were measured by CLEIA chemiluminescence enzyme immunoassay (CLEIA) (R&D Systems; Minneapolis, MN, USA), IL-1 β was measured by enzyme immunoassay (EIA) (R&D Systems). MMP-9 was measured by EIA (Daiichi fine chemical Co. Ltd.; Toyama, Japan). VEGF was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). SP-D and KL-6 were measured by EIA (Eidea Co. Ltd.; Tokyo, Japan). Detection limits were 0.55 pg/mL, 0.125 pg/mL, 3.13 ng/mL, 15.6 pg/mL, 17.3 pg/mL, 0 ng/ml, respectively. These results were compared between subjects who did not develop radiation-induced pneumonitis (NRP) and RP.

Analysis

Results are expressed as means \pm standard deviation (SD). Differences between groups were examined for statistical significance using Wilcoxon signed-rank test and those within groups were examined using Wilcoxon rank sum test by SPSS Statistics (Japan IBM;

Tokyo, Japan). Sex ratio, pathology of lung cancer and concomitant chemotherapy ratio between groups were examined by Fisher exact probability test. *P* values of <0.05 were considered significant.

Results

Patient characteristics

Thirty-four subjects were entered into the study. Five subjects experienced exacerbation of lung cancer during the study period, two subjects had RT interrupted due to pneumonia, and five subjects rejected repeat bronchoscopy and were excluded from the study. Twenty-two subjects completed the study protocol and were analyzed. Of these 22, 11 developed RP (RP group) and the remaining 11 did not (NRP group) by three months after RT. In the RP group, RP developed 59.0 ± 19.9 days after the end of RT. Patient characteristics are shown in Table 1. There were no significant differences between RP and NRP groups in terms of mean age, sex, Brinkman index, pathology of lung cancer and concomitant chemotherapy ratio.

Cellular profiles from BAL

Results of cellular profiles from BAL are shown in Figure 1. In the NRP group, total cellular counts and fractions of lymphocytes in BAL were significantly increased at one

month post-RT compared with pre-RT. Significant differences did not occur in total cellular counts or fractions of AMs in BAL, while lymphocyte fractions increased significantly at one month post-RT in the RP group. AM fraction at one month post-RT was significantly higher in the NRP group compared with the RP group. Lymphocyte fractions from BAL were comparable between the two groups.

Inflammatory markers in BAL fluid

Figure 2 shows the results of testing for inflammatory markers in BAL fluid. Inflammatory markers in the NRP group did not change significantly following RT. In the RP group, MMP-9 in BAL fluid increased significantly at one month after RT compared to pretreatment. By contrast, in these patients, VEGF in BAL fluid decreased significantly at one month after RT compared to pretreatment. SP-D in BAL fluid of patients in the RP group increased significantly shortly after RT compared with pretreatment. At one month after RT, concentrations of MMP-9 and VEGF in BAL fluid of patients in the RP group were significantly higher and lower compared to those in the NRP group, respectively. There were no significant differences in concentrations of IL-1 β and TNF- α in BAL fluid within and between groups (data not shown).

Inflammatory markers in serum

Figure 3 shows the results of testing for serum inflammatory markers. By comparison with pretreatment levels, serum SP-D increased significantly both shortly after RT and one month after RT in the NRP group. At one month after RT, serum SP-D levels in the RP group increased significantly compared with those at pretreatment and shortly after RT.

Results of pulmonary dilution

Figure 4 shows the results of testing for diffusing capacity of the lungs for carbon monoxide (DL_{CO}). Pulmonary dilution of both groups decreased similarly and significantly at one month after RT compared with pretreatment. With the exception of DL_{CO}, other pulmonary functions did not show significant differences within and between groups (data not shown).

Discussion

The major findings of the present study are as follows: i) RT significantly increased total cell and AM counts in the airways of NRP patients and significantly increased lymphocytes in both groups, ii) MMP-9 increased significantly and VEGF decreased significantly in the airways of patients with RP, iii) SP-D, increased significantly in the serum, but not BAL fluid, of patients without RP, whereas SP-D in BAL fluid increased significantly

with a subsequent significant increase in serum SP-D in patients with RP, iv) pulmonary dilution decreased similarly in both groups of patients.

A previous report showed RT increases BAL lymphocytes equally between patients who develop RP and those who do not following RT for breast cancer^[25]. Lymphocyte fractions in BAL fluid following RT were comparable between the two groups in our study. In contrast to effector T cells, which promote immune activation and resultant tissue damage, regulatory T cells (Treg), counterbalance effector T cells and prevent tissue damage, a phenomenon that has attracted attention^[26]. Treg seem to be involved in the pathogenesis of several pulmonary diseases^[27-29]. The depression of Treg significantly correlates with the severity of idiopathic pulmonary fibrosis (IPF)^[30]. It may be possible that Treg dominantly increased as part of the increase in total lymphocytes and prevented the development of pulmonary damage in the NRP group in the present study.

In accordance with previous reports indicating that MMP-9 increases in the BAL fluid of IPF patients^[14, 22], our study also found that MMP-9 increased significantly in the airways of patients with RP. MMP-9 does not constitutively express in normal lung tissue, while its expression is induced in inflamed tissue. MMP-9 activates TGF- β and thus promotes fibrosis^[23]. Although an exact role of MMP-9 in the development of pulmonary fibrosis is not fully established, recent studies have indicated that MMP-9 could be a potential biomarker of fibrotic pulmonary diseases^[24]. Our study also indicates that MMP-9 might be

a representative biomarker of RP.

VEGF is a representative cytokine involved in the pathogenesis of pulmonary fibrosis^[31]. VEGF as well as AMs significantly decreased in the BAL fluid of patients in the RP group compared with those in the NRP group. VEGF also induces angiogenesis, a process in which tissue responses to oxygen play a critical role^[32]. Tissue hypoxia increases the expression of VEGF via stabilization of hypoxia inducible factors (HIFs) and thus causes angiogenesis. Hypoxic AMs express and produce VEGF via HIFs^[33]. VEGF knockout mice develop severe pulmonary fibrosis due to the activation of pulmonary fibroblasts, suggesting the inhibitory role of VEGF produced from hypoxic AMs in regulating pulmonary fibroblasts^[34]. Decreased AMs and VEGF in the airway of patients with RP might have failed to inhibit pulmonary fibroblasts in the present study.

SP-D is a potential biomarker of RP, since it increases in both serum and BAL fluid^[35, 36]. In our study, serum SP-D, but not that in BAL fluid, increased significantly shortly after and at one month after RT in the NRP group. In the RP group, SP-D in BAL fluid increased significantly shortly after RT and serum SP-D increased significantly at one month after RT. Since SP-D is so hydrophilic, it easily moves from tissue to blood leading to correlation between serum and BAL fluid levels^[37]. Nonetheless, concentrations of SP-D in BALF and serum did not simultaneously increase in the present study. SP-D plays a critical role in control of infection and immune regulation in lung tissue. It may contribute to tissue

homeostasis, preventing fibrosis by regulating MMP-9 activity in lung tissue ^[38]. In our study, MMP-9 increased significantly in the airway of RP patients. SP-D might persist in the airway to prevent MMP-9 activity and subsequently diffuse into the blood. It may be more appropriate to determine SP-D in BAL fluid than in serum to predict the development of RP.

A few studies had evaluated pulmonary dilution in RP. Severity of RP significantly correlates with pulmonary dilution ^[39]. Pulmonary dilution testing has been shown to be useful for early detection of the development of RP ^[40]. It has also been reported that radiological abnormalities precede the decrease in pulmonary dilution ^[41]. By comparison with baseline, RT similarly and significantly decreased pulmonary dilution in both groups in the present study, indicating that the utility of pulmonary dilution in the early detection of RP may be low.

Conclusion

In conclusion, our study indicated that increased MMP-9 and SP-D as well as decreased VEGF in BAL fluid could be useful biomarkers in predicting RP. Further analyses utilizing less invasive methods, such as induced sputum, and parameters such as Treg numbers in BAL fluid are required to better establish predictors for RP.

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Nil.

Conflicts of interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure legends

Figure 1. Cellular profiles of BAL fluid. Bars represent mean \pm SD of total cellular counts (upper), fraction of alveolar macrophages (middle) and that of lymphocytes (lower) at before, shortly after and one month after RT (n=11 for NRP and n=11 for RP). Open bar: pretreatment; Hatched bar: shortly after RT; Closed bar: one month after RT. * P <0.05 and ** P <0.01. NRP: subjects who did not develop radiation-induced pneumonitis, RP: subjects who developed radiation-induced pneumonitis, RT: radiation therapy.

Figure 2. Concentrations of inflammatory markers in BAL fluid. Bars represent mean \pm SD of MMP-9 (upper), VEGF (middle) and SP-D (lower) at pretreatment, shortly after RT and one month after RT (n=11 for NRP and n=11 for RP). Open bar: pretreatment; Hatched bar: shortly after RT; Closed bar: one month after RT. * P <0.05 and ** P <0.01. MMP-9: matrix metalloproteinase-9; VEGF: vascular endothelial growth factor; SP-D: surfactant protein-D.

Figure 3. Concentrations of inflammatory markers in serum. Bars represent mean \pm SD of KL-6 (upper) and SP-D (lower) at before, just after, and one month after RT (n=11 for NRP and n=11 for RP). Open bar: pretreatment; Hatched bar: shortly after RT; Closed bar: one month after RT. * P <0.05. KL-6: sialylated carbohydrate antigen-6.

Figure 4. Pulmonary dilution. Bars represent mean \pm SD of DL_{CO} (%) at pretreatment, shortly after, and one month after RT (n=11 for NRP and n=11 for RP). Open bar: pretreatment; Hatched bar: shortly after RT; Closed bar: one month after RT. * P <0.01. DL_{CO}: diffusing capacity of the lungs for carbon monoxide.

Table 1. Patient characteristics*

	NRP	RP
Patients (n)	11	11
Sex male/female	9/2	7/4
Age (yrs)	73.2±8.9	72.0±8.6
Brinkman index	926.4±650.9	673.3±428.6
Pathology (A/Sq/S/L/U)	5/4/1/0/1	3/4/3/0/1
Chemotherapy (n)	6	5

Data represented as mean±SD. * NRP: subjects who did not develop radiation-induced pneumonitis, RP: subjects who developed radiation-induced pneumonitis; A: adenocarcinoma; Sq: squamous cell carcinoma; S: small cell carcinoma; L: large cell carcinoma; U: unclassified carcinoma.

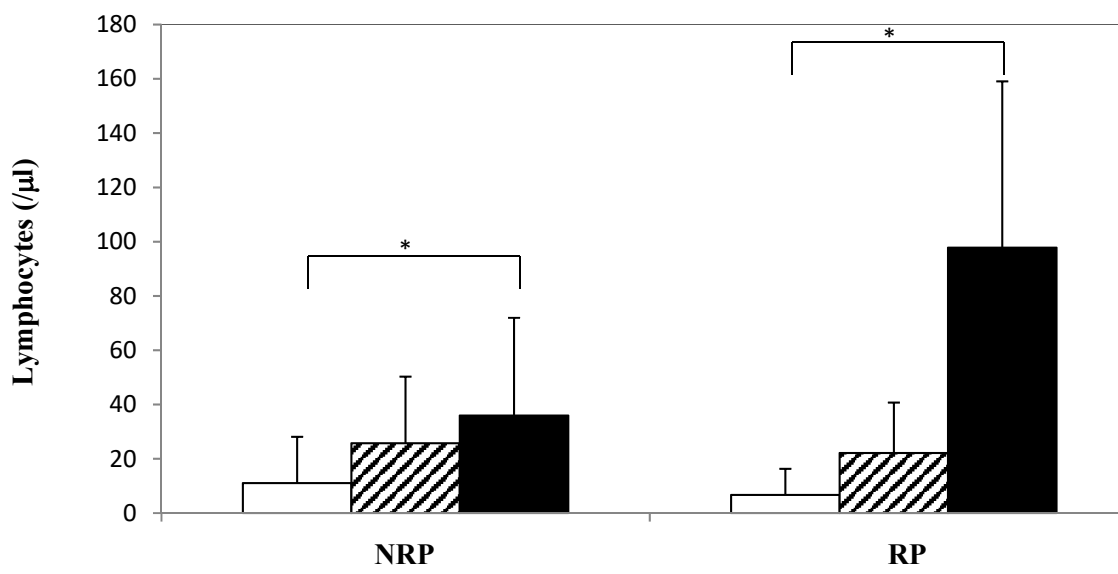
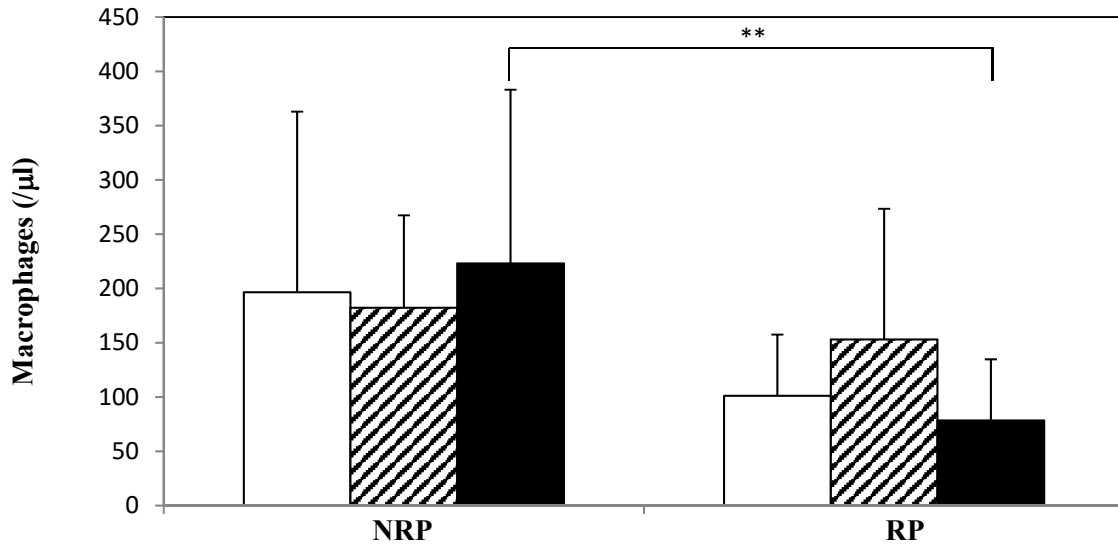
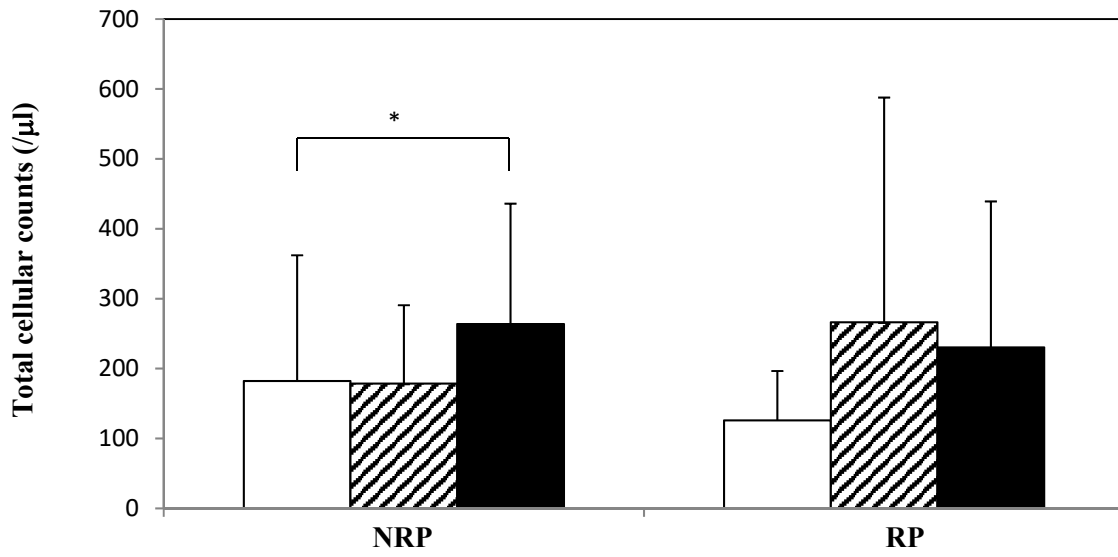


Figure 1

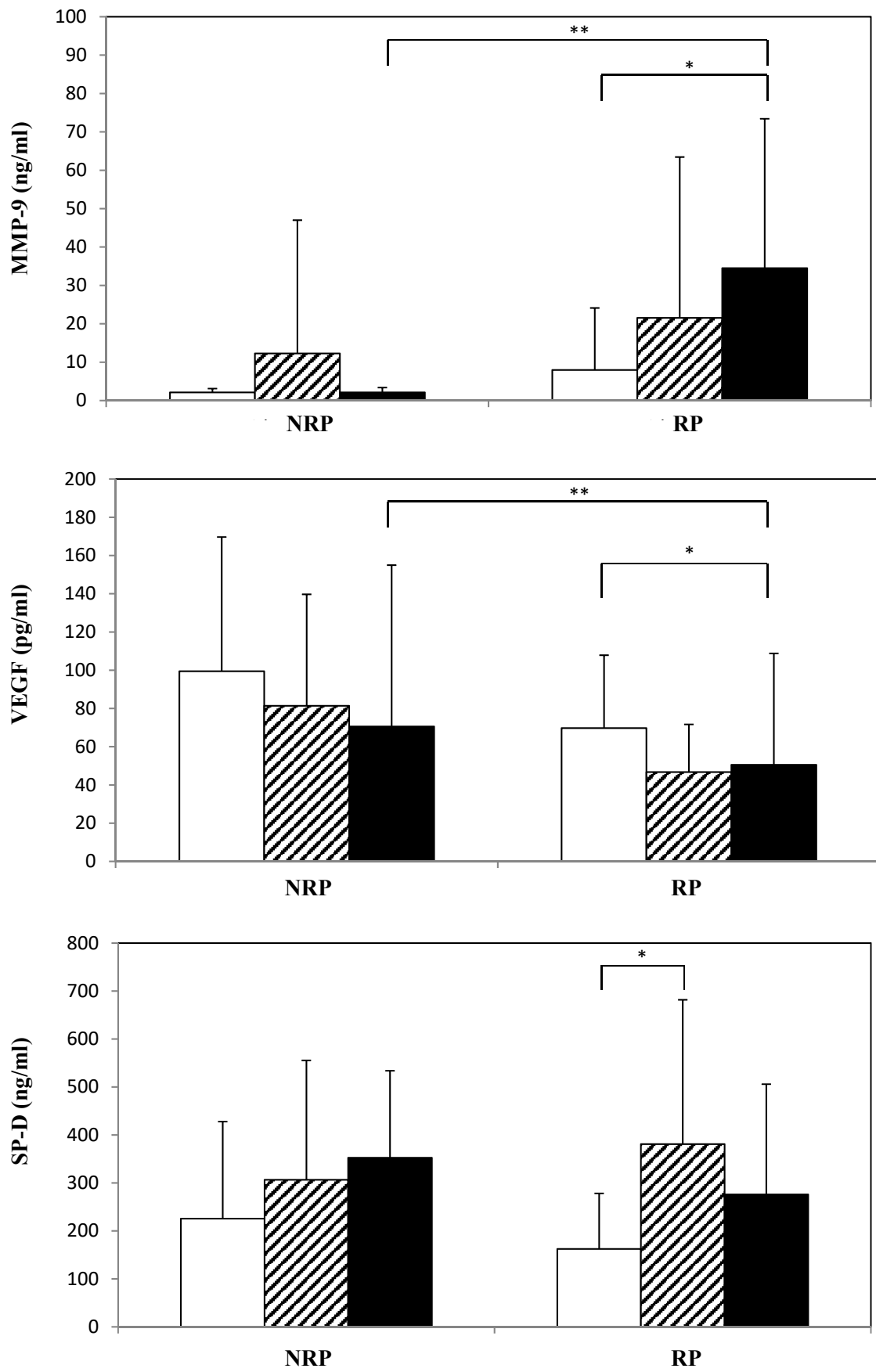


Figure 2

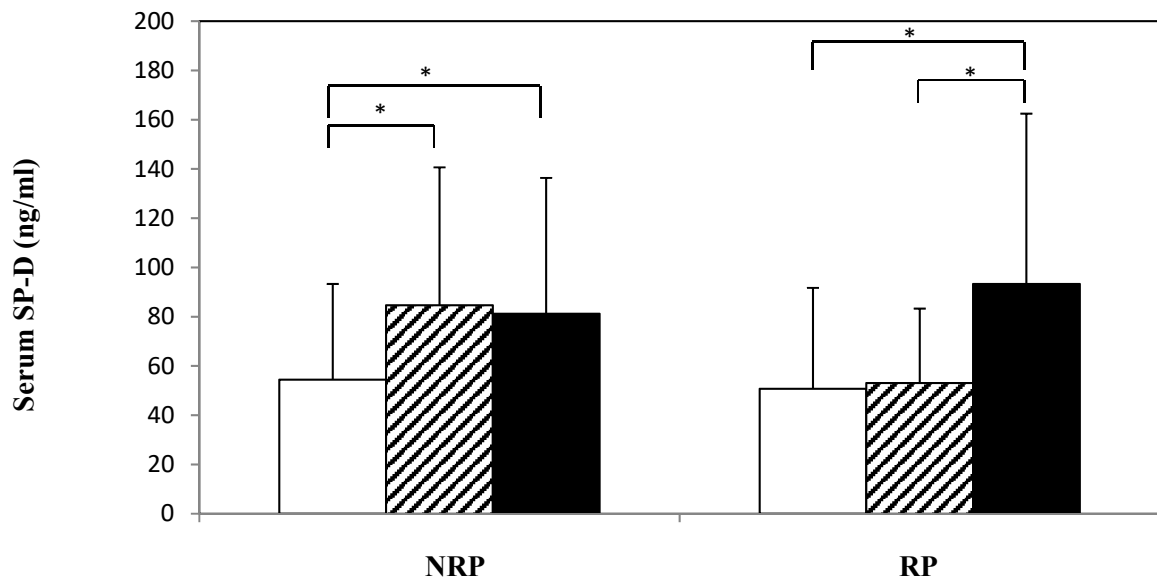
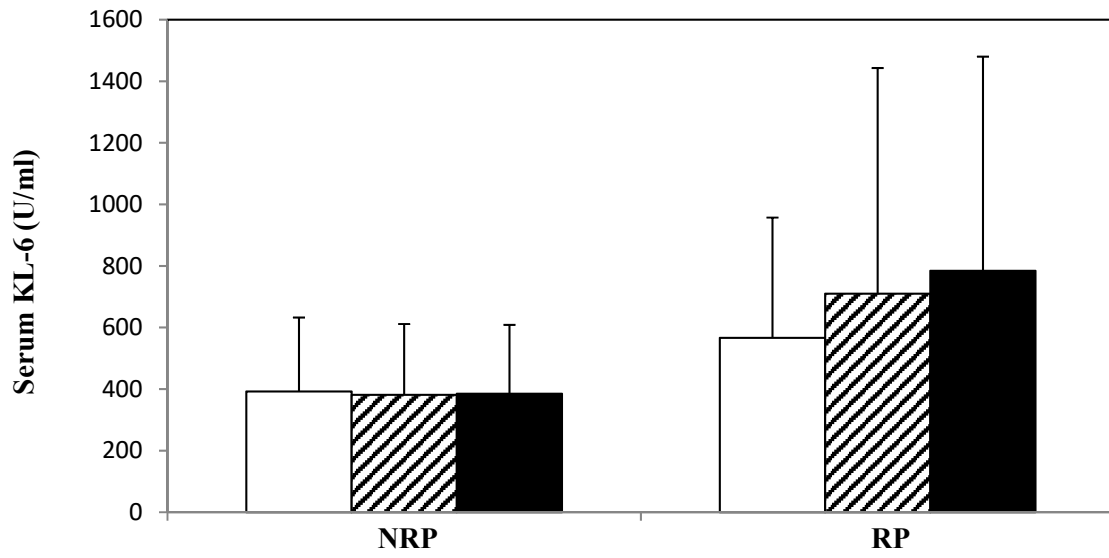


Figure 3

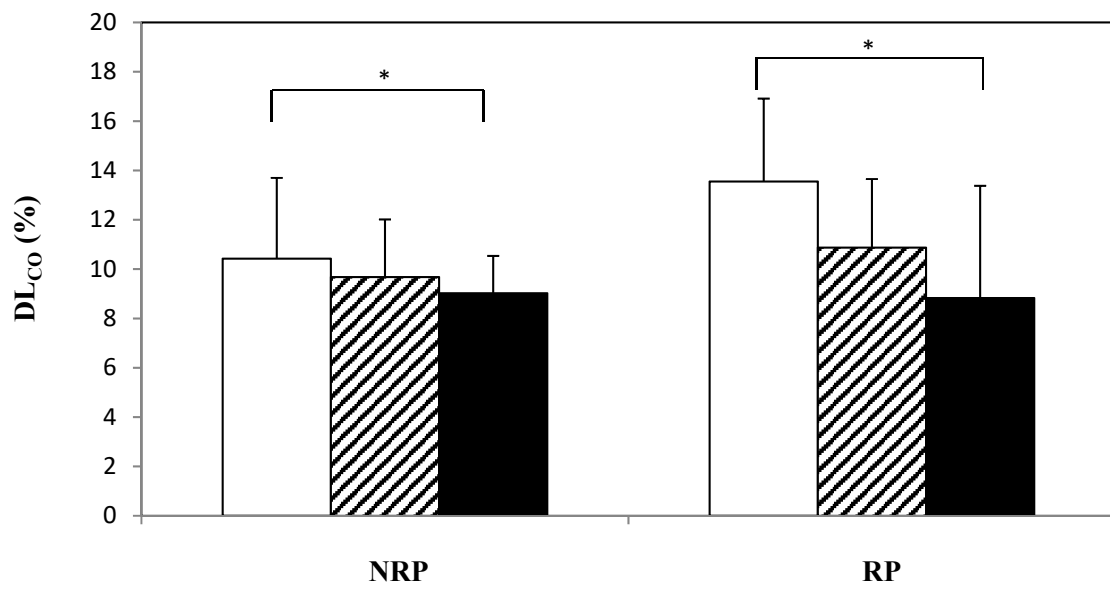


Figure 4