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Determinants Of Oxidant Load In Broncho Alveolar Milieu

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***Abstract*— The lung is exposed to inhaled xenobiotics detemining direct damage , macrophage activation and neutrophils recruitment with an overwhelming oxidant load in broncho-alveolar milieu and alveolar macrophages leading to pulmonary damage .**

Our aim is to assess the biologic activity of oxygen radicals (ROS) and biologic antioxidant potential (BAP) in broncho-alveolar fluids and blood in healthy subjects and in patients affected by COPD under GOLD stage II and III and IPF and to assess the relationship between ROS and macrophage function.In thirteen healthy subjects , twenty patients affected by COPD and twenty affected by IPF we assessed pulmonary function , blood gases , ROS and BAP in blood samples , broncho-alveolar lavage(BAL), expired breath condensate and sovranatant of alveolar macrophages culture.

In patients ROS were increased both in blood (474±177, 345±123 vs 192±46 U Car in COPD , IPF and healthy people respectively) , and in broncho-alveolar lavage (99±24 , 94±33 , vs77±14)and in macrophage culture media(99 ± 37, 106±45 vs 80 ± 15). BAP were mildly decreased in blood (2162±668, 2031±562vs2553±679 mM/lt) , but they were normal in BAL(1064±432, 910±222 vs 882±241). ROS/BAP ratio was significantly increased in blood (0.21±03 vs0.08±.01 U Carr/mM/l) with overlap in BAL (0.09±02 vs0.08 ±.2) . ROS in BAL were significantly related to acid phosphatases and ROS produced by Mf in media culture . ROS load in blood is highly significant under COPD and IPF . ROS and BAP were several folds more concentrated in BAL than in blood by active secretion . Macrophages are the most important source of ROS in BAL .

Index Terms—. *ROS, BAL, EBC , IPF , COPD*

I. Introduction

The respiratory system is exposed to several harmful environmental components such as smoke , pollutants, and micro-particulate (PM10) ; able to induce the generation of oxidants [1 ,2] . The interaction of inhaled xenobiotics with epithelial and broncho-alveolar cells determine the production of oxidants according to different pathways such as the enzymatic activities (NADPH), the oxidative chain, the peroxidases (myeloperoxidase), and the oxidases (xantine oxidase).

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The effects of reactive oxidants species (ROS) upon the pulmonary structures are both direct and indirect (through the production of inflammatory peroxidation derivatives). The ROS determine: a) lipid peroxidation in membranes, sources of isoprostane, 4idrossi 2 noneale, and malondialdeide; b) DNA damage; c) inactivation of enzymatic protein ; e) interference with growth factors receptors (mainly vascular endothelial growth factor VEGF) ; f) inflammation through chinases (MAP JNK and PI-3K), transcription factors (NFKb, AP1) and inflammatory cytokines; g) oxidation of histone deacetylation enzymes; and h) apoptosis (through oxidation of redoxines) [2 - 10].

We may note that the biologic antioxidant potential (BAP) is based upon enzymatic and non-enzymatic substances. Non enzymatic substances for instance are vitamins (C, E), uric acid , glutathione and low molecular weight proteins such as albumin, as well as transferrin and ceruloplasmin; normally present in broncho-alveolar lavage (BAL). Within enzymatic activities, the superoxide dismutase, the catalase, and the glutathione reductase play a major operational role.

In chronic obstructive pulmonary disease (COPD) under stable phase [2-9] an overwhelming oxidant load takes place determined by the imbalance between ROS and BAP , leading to emphysema, airway obstruction , inflammation , increased permeability of bronchi, altered cross –talking between epithelial and mesenchymal or endothelial cells, along with the structural and immunogenic modification of proteins, potentially determinant of autoimmunity. Theerefore, development of emphysema can take place according to three pathways such as through direct damage, the oxidation of anti-elastase proteins (a1AT , SLPI), and apoptosis (by interference with VEGF receptor and oxidation of tioredoxines). Thus, obstruction of airways can be induced through the secretion of mucus, due to the activation of the MUC5AC gene, and the increase of 4idrossi2noneale. Specific literary reports clearly illustrate an imbalance of the redox status in the blood, the expired breath condensate (EBC) in COPD, and within Idiopathic Pulmonary Fibrosis (IPF) [11-17]. Such reporting has asserted that a causal relationship between the ROS load and the pulmonary function exists and can be found. Nonetheless although the suggestions are fully agreed upon and unanimous, an intrinsic weakness is evident in such studies since: a) a single substance is assumed as monitor of the whole redox status; b) the adopted methods are unable to distinguish the contribution of upper airways from that of broncho alveolar structures [18,19,20]; c) no relationship is available between the redox imbalances in the blood and in the broncho-alveolar milieu, and finally, d) the role of broncho-alveolar macrophages(Mf) in the generation of ROS is not clearly stated.

Our aim is to investigate COPD and IPF under a stable phase of the redox status in blood and in the broncho alveolar milieu studied by broncho-alveolar lavage (BAL), and to assess the role of Mf in the generation of ROS .

II. METHODS

Design and selection of patients

Twenty former-smoker patients affected by COPD in II and III stage , according to the GOLD definition, who have expressed the desire for advice in the outpatient ward have been selected to participate. Patients under stage I were excluded due to the initial formation of the disease, and under stage IV because of advanced damage factors. A group of thirteen not smoking healthy people were subsequently used as control . The patients and the control group were observed over an appropriate time frame and all applicable measures were held during a period of clinical stability of symptoms and signs, totalling one month in length.

Physiologic measures

Vital capacity (VC), forced expired volume in one second (FEV1), Total lung capacity (TLC), Diffusion Capacity, by single apnoea method (TlCO), have all been assessed by whole body plethismograph (Autobox 6200, Sensormedics, USA). Blood gas analysis was performed by arterial puncture and an automatic analyzer (Rapidpoint,Bayer Health Care,FRG), with an accurate determination of arterial oxygen tension (PaO2) and arterial carbon dioxide tension (PaCO2).

Broncho alveolar lavage

BAL was obtained according to the ERS suggestions [21] and by instillation of four 50cc aliquots of saline into a segmental wedged bronchus during fiber-optic bronchoscopy (Olympus, Japan), followed by gentle aspiration and collection of fluids in refrigerated bottles. BAL was centrifuged at 1500rpm for 20'; subsequently supernatant was then separated from cells. The overall count was performed by automatic analyzer (Beckman, USA). A differential cell count was made by vital dyes (Simplitest, Behringer,FRG) and cross-checked after fixation and coloration with both ematoxyilin and eosin. Viability was assessed by Trypan Blue vital coloration . Overall protein content, albumin, a1antitripsin, and cerulopasmin were measured by by laser nephelometry (Beckman,USA) and by enzymatic activities (Lactic dehydrogenase (LDH). Further, Acid Posphatase and beta glicuronidase were assayed by standard methods (Roche autoanalyzer, Switzerland). In case of high levels of LDH and potassium, the sample was discharged, to avoid artefacts due to cell death [22].

Expired Breath Condensate (EBC)

Exhaled breath condensate was collected by a condenser (Ecoscreen, Jaeger, Hoechberg, Germany), while patients engaged in breathing displays for a period of ten minutes, using a nose clip at a normal breathing rate and tidal volume through a two way non rebreathing valve. A saliva trap was put in the expiration line and the dosage of amylase allowed the exclusion of samples with mouth secretion contamination. The volume of the condensate (average 2cc) was collected in a plastic tube and kept at a -70°C in Eppendorf tubes. ROS and ASA were determined according to fresh EBC, while the other dosages were examined within three months from collection. The dosages were repeated twice and the measured coefficient of variation was 5%.

Cell cultures

Alveolar macrophages were separated by glass adherence. They were cultured in saline and for one hour at 37'C under micro aerophilic environment (adding CO2 5%) in Tc Hank solution added with fetal calf serum; after centrifugation supernatant was used for assays .

The results from BAL samples were corrected subtracting the activities measured over culture media held in the same environment .

REDOX measures

ROS were measured by the spectrophotometric (505nM) determination of the oxidation of Fe++ in acidic solution of N paraphenilendiamine, and by relevant proteins (d-ROM test, Diacron, Grosseto, Italy). In BAL and EBC the same method was applied in a dynamic mode, allowing the measures over highly diluted media, by means of a prolonged incubation time (30' at 37C; dROMs Exhalation test by Diacron, Grosseto,Italy). Results are expressed as U Carr (1Carr=0.08mg/dl H2O2) with interassay variability of 2% and interassay change of 3%.

BAP was measured by the inhibition operated by the sample upon the reaction of oxidation of ferric ions to ferrous in a solution containing chromogenic substrate(BAP test, Diacron, Grosseto, Italy) . The results are presented as Biologic Antioxidant Potential (BAP) and expressed in mM/lt ; the reaction is linear in the range 150-3000 with a 3% variability.

S*tatistical Analysis*

Comparison between different sets was obtained by T Student's analysis. Their relationship was assessed by linear fitting according to the least square methods. Results are expressed as significant for a p level less than .05 and highly significant for those levels less than .001.

Ethical advice

Informed written consent was obtained from each patient. The ethical committee of the institution approved the protocol.

III. RESULTS

Demographic and clinical data:

The patients affected by COPD exhibited mild characteristics of being overweight, discrete airway obstruction, mild reduction of diffusion and hypoxemia, lung hyper inflation, as well as marked hypercapnia (Tab.I). Twenty patients affected by interstitial pulmonary fibrosis (IPF) were observed under a stable phase. All issued complaints due to dyspnoea under effort and dry cough. The patients all showed normal biometrics, bibasilar rales, interstitial reticular pattern at the base with initial honeycomb lung, mild restrictive impairment, and mild hypoxemia .

BAL features:

The BAL in the patients displayed increased cellularity with a mild increase of neutrophils, mild increase of total protein content, and enzymatic activities (Tab II). The Acid Phosphatase was fairly increased in COPD and it was significantly related to ROS (ROS mf= 15+ 40 AcP ; ± 15 ; $p<0.05, r^2=.45$).

Redox measures:

ROS (Tab III) were markedly and significantly increased in patients as compared to healthy people in arterial, venous, broncho-alveolar, expired breath condensate, and macrophage culture samples. In arterial and venous samples ROS were higher in COPD as compared to IPF. There was no difference between arterial and venous

levels in COPD and healthy people, although in 30% of patients there was a significant transpulmonary gradient with arterial values higher than peripheral venous ones (ROSa = 480±70 vs ROSvp=378±50U) . ROS in sovranatant of macrophage culture were slightly increased in patients and significantly related to ROS in BAL (ROS bal = 30+ .78ROS Mf , p<.000005 ± 5, r2=.45). ROS in EBC were significantly related to the values measured in Mf culture (ROS ebc =30 + .95 ROS Mf, p<.001 ±4 , r2=.47).ROSbal was significantly related to age (ROS bal= 35-9+.21 age , p<.0025 ±7,r^2=.27).

The absolute levels of ROS are halfold in BAL and EBC, than in blood. Taking into account that the recovered BAL is composed of the saline instilled for the lavage and a small and variable amount of broncho alveolar secretions, it is necessary to correct the different dilution dividing the absolute values by the albumine, used as passive test. Relative values indicate that ROS are several folds more concentrated in BAL than in blood (ratio ROS bal/Albumine bal / ROSserum/Albumine serum =56 in HP vs 32 in COPD and 60 in IPF). BAP were mildly decreased in arterial and venous samples in patients, mainly in IPF. Arterial levels were slightly lesser than venous ones. BAPbal were moderately elevated in the patients. BAP were significantly related to ROS (BAP =3501-39ROS ;p>.001 ;±422). ROS/BAP ratio ranged from 0.075 to 0.09 and was relatively stable over venous, arterial, and BAL samples in HP(0.09±02 vs0.08 ±.2), but it increases significantly in patients (0.21±03 vs0.08±.01 U Carr/mM/l) in venous and arterial samples due to the marked increase of ROS and the mild decrease of BAP.

DISCUSSION

These preliminary reports for the first occasion assess: a) an unexpected high amount of ROS and BAP in the BAL ; b) an imbalance of redox status in the blood of patients, and c) the prominent role of Mf secretion in BAL and EBC . Despite the absolute values of ROS and BAP are lower in BAL than in serum, the values corrected by the factor due to the dilution of BAL identify that ROS and BAP are several folds much more concentrated in broncho-alveolar milieu than in blood. In fact, the aspirated BAL is composed mostly by the saline solution used for the lavage and only by a small amount of recovered broncho-alveolar secretions, therefore it is necessary to correct BAL measurements by the values of albumine or potassium, despite these reference substances not always being "passive", but can be modified by inflammation or cellular death.

In COPD and IPF under stable phase, according to experimental data, ROS are increased in BAL as compared to healthy people and are related to age, thus increasing in older people. These observations raise the questions about the origin of such load and the pathways used to fight the potential burden. The origin of the oxidative load is disclosed by the assessment of the relationship between ROS in BAL and ROS over Mf culture media. It suggests that Mf determine most of the oxidant load in BAL. This is confirmed by the relationship between ROS and Ac.P, whose levels are mainly determined by Mf [20,22]. In COPD Mf are increased [24], and show some heightened functions (such as activation, adhesion and secretion of interleukins, I NOS, and ROS) and others depressed (such as as impaired response to stimuli, reduced phagocytosis). Here we may make note of the complex interplay between Mf and ROS, since the functionality of Mf depends upon the availability and the levels of the anti-oxidant glutathione. The Mf produce the ROS, while at the same time, sensing the ROS through a scavenger [24-29].

The protection against such oxidative burden is very effective since the BAP is higher in BAL than in blood, likely due to the presence of an active concentration. The most striking difference between healthy people and COPD is the two fold increase of ROS in blood samples with mild decrease of BAP, leading to a near threefold increase of ROS/BAP ratio. These data are in full agreement with literature reports of increases of oxidants in the blood [4,12,34] , in induced sputum [10] and in EBC [4,36,37]. Several reasons can explain the increase of ROS: a) blood loading of ROS across the deranged alveolar / endothelial layers according to a positive gradient from BAL to blood; b) increased generation of ROS from peripheral muscles; c) decreased endothelial clearance of ROS; and d) decreased availability of anti-oxidants. The fist pathway looks possible, considering the high exchange surface of alveolar/blood vessel surface along with the high BAL to blood gradient. ROS can determine lung damage, interact with endothelial cells, and cross the barrier, loading the blood stream and determining a measurable gradient within circulatory vessels. The transpulmonary gradient across the pulmonary circulation was measured in one third of the patients. Regarding the second pathway, peripheral muscles under COPD show often wasting, different muscle fibre phenotype and increased fatigue with enhanced ROS generation and inflammation [27-32]. The endothelial cells can regulate oxidants levels either clearing or loading ROS. Superoxide anion can be increted by means of NADPH oxidase. NADPH can be over expressed by several stimuli (such as shunts, hypoxia, angiotensin II, endothelin I and TGF1beta), and determine activation of HIF 1 and MAP, as well as secretion of mediators such as serotonin [39-44]. The relationship between ROS and the lung circulatory vessels outlines a feed back loop since ROS can determine wasting of circulatory mesh through an inverse relationship between ROS and VEGF [10]. In turn the reduced pulmonary vascular surface is less able to clear the ROS load. In COPD the decrease of VEGF [10], induced by ROS, leads to the development of emphysema and the oxidant load is significantly related to airways obstruction [38], Decreased availability of anti oxidants is likely in aged people with long standing disease and alimentary or digestive problems.

The limited number of observations indicates that the study must be considered as a preliminary report. The adopted method is limited by the interference of ceruloplasmin [24], that was monitored and did not change significantly. It allowed for only a gross insight in redox balance. The results cannot be extended to the acute phases of COPD, since under inflammation high molecular weight proteins (such as alpha 2 macroglobulin) contribute significantly to the ASA in BAL, while the raised active phase reactant proteins can increase the protection in blood. The ROS also increase in BAL due to the influx of neutrophils and eosinophils [37]. The comparison between COPD and IPF is behind the scope of the paper.

In conclusion the current evidence and the literature reports identify the need of a therapeutic intervention to restore the lower ROS /BAP in the blood of patients affected by COPD. Several drugs such as statines, SOD mimetics, anti-inflammatory, glutathione, n-acetyl cysteine, thioredoxine, and heme-oxygenase [45-48] are used; although the dosage, availability and outcomes are still currently under tria In conclusion the current evidence and the literature reports identify the need of a therapeutic intervention to restore the lower ROS /BAP in the blood of patients affected by COPD. Several drugs such as statines, SOD mimetics, anti-inflammatory, glutathione, n-acetyl cysteine, thioredoxine, and heme-oxygenase [45-48] are used; although the dosage, availability and outcomes are still currently under trial. Furthermore the results identify a delicate homeostasis in broncho alveolar milieu with an high burden of ROS from Mf and an active protection by BAP, indicating the chance to modulate Mf accrual and secretion or rather to improve the BAP shield; which will be the target of future studies.

TAB I DEMOGRAPHICS AND CLINICAL DATA

	Healthy people	COPD	IPF	units
age	60±5	61 ±13	64±13	Years
height	170±4	163 ± 10	163±18	cm
BMI	24±3	26 ± 7	23±5	Kg/cm2
VC	95±5	75 ± 6	65±16	%ERS
FEV1	97±4	42 ± 16	65±17	% ERS
TLC	98±3	120±7	75±7	% ERS
TlCO	95±5	78 ± 15	66±20	%ERS
PaO2	80±4	74 ± 17	65±15	mmHg
PaCO2	40±2	47 ±6	37±13	mmHg

%ERS =values expressed as percentage of ERS normal standards.;BMI=body mass index ;VC=Vital capacity;FEV!=forced expiratory volume in one second; TLC =toltal lung capacity; TlCO=diffusion transfer for carbon monoxide

TAB II BRONCHO-ALVEOLAR LAVAGE FEATURES

		HP	COPD	IPF	Sign. of diff.
CELL	c x10 4	1±.3	3.2±.8	2.2±1	COPD=IPF>H
Mf	%	89±5	80±6	80±8	n.s.
PMN	%	3±2	7±2	10±3	COPD=IPF>H
Ly	%	7±3	10±3	7±3	n.s.
Eo	%	1±	3±2	3±2	n.s.
T pc	mcg/ml	58±9	85±15	75±9	COPD=IPF>H
alb	mcg/ml	25±7	35±10	32±9	n.s.
a1AT	mcg/ml	2±1	2±1	2±1	n.s.
C bal	mcg/ml	1±1	1±1	1±1	n.s.
C ser	mg/dl	30±9	35±20	35±9	n.s.
AcP	U	5 ±.2	2.2 ±. 8	2.0±.9	COPD=IPF>H
Bgli	U/1000	4 ±2	20±5	20 ±5	COPD=IPF>H
	n	13	20	20	

H=healthy people; n.s.=not significant ; bal=broncho.alveolar lavage ; ser=serum levels CELL= Overall cellularity Mf)=macrophages ;PMN=neutrophils;Ly=Lymphocytes, Eo=eosinophils; Tpc=total protein content ; alb=albumin;C bal=ceruloplasmin in broncho alveolar lavage; Cser =ceruloplasmin i serum; Acp=Acid Phosphatase; b Gli =beta glicuronidase

Tab III REDOX STATUS

	HP	COPD	IPF	Sign.of diff.
ROS vp	192±46	474±177	345±123	COPD>IPF >H
ROS a	194±38	435±100	389±117	COPD>IPF >H
ROSbal	77±14	99± 24	94±33	COPD=IPF >H
ROSebc	60±30	72±44	70±34	COPD=IPF >H
ROS mf	80±15	99±37	106±45	COPD=IPF >H
BAP vp	2553±679	2162±668	2031±562	H=COPD> IPF
BAP a	2325±652	2056±637	2016±534	H>COPD= IPF
BAPbal	882±241	1064±432	910±222	n.s.
BAPebc	650±214	822 ±274	785 ±250	COPD=IPF >H

ROS = oxidant radicals ; BAP = anti-oxidant screen ; vp=peripheral venous ; art= arterial ; bal=broncho-alveolar ; mf=levels in macrophages culture sovranatant ;ebc=expired breath condensate ; difference between healthy people and COPD ; H=healthy people ; n.s.=not significant difference ROSS expressed as Carr units/cc ; BAP expressed as mM/lt

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