AGE-RELATED CHANGES IN GSH STATUS ON MICRONUCLEUS FREQUENCY OF ALVEOLAR MACROPHAGES, AND BIOPHYSICAL PROPERTIES OF LUNG SURFACTANT IN FISCHER F344 RATS

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The increase in the antioxidant defense capacity of the lung and surfactant system appears to be one of the necessary *in vivo* maturational changes that help to assure successful adaptation of the newborn to its new environment at birth for proper respiratory function [19,20,34,39]. It has been established that 50% of deaths due to exposure of rats to pure oxygen for 60-72 hours is associated with pulmonary edema [13,17]. Both *in vitro* [3,4,5,37,38], as well as *in vivo* [9,10,13,15] studies indicate inactivation of lung surfactant by oxidative damage of phospholipids or thiol groups of surfactant-related proteins [10,33] with adverse effects on surface-tension lowering capacity of surfactant. Surfactant function is suggested to be impaired by inflammatory processes in the lung triggered by ROS, which is produced by various oxidizing agents, such as excessive oxygen, O_3, NO_2 [9,10,12,15,17,40,47].

The lung epithelial lining fluid (ELF) of which surfactant is the main constituent, is the major site of impact of active oxygen species. Accumulated literature supports the concept that the level of oxidative stress increases during aging process [24,25,26] as a result of depletion of levels of endogenous antioxidants [43,46,48,49]. The prooxidants (i.e. H_2O_2, Fe^{2+}), decrease the ratio of GSH/GSSG, and the proportion of oxidatively damaged products are increased, e.g. lipid peroxidation, which itself adversely affects the status of DNA [1,2,18]. It is also estimated that a very high level of oxidative damage to DNA occurs during normal metabolism, thus in each rat cell the steady-state level of this damage is 10^6 oxidative lesions, among which about 10^5 DNA adducts are formed daily [1,2,18]. According to estimates made by Saul and Ames about 20,000 - 40,000 DNA breaks are produced per cell per day, due to distruction of deoxyribose moieties or direct hydrolysis of phosphodiester bonds without depurination process. As a result of permanent exposure of cells to low levels of endogenous, or exogenous oxidants or environmental genotoxic agents, large number of DNA lesions are induced in living cells that lead to cell or tissue

disfunction, and consequently the underlying cause of age-related reduction of homeostasis [1,2,24,25] with increased incidence of cancer and diseases of the elderly [11,29,31,35,43,46,54].

It is interesting that the type II cells, with relatively high antioxidant activity and resistant to hyperoxia, are the major targets for paraquat toxicity [44], and when incubated with paraquat are inhibited in their ability to release superoxide ($0^{-}\cdot 2$), similar with substances that provoke the respiratory burst [17]. Due to selective poisoning effect of paraquat on alveoli and surfactant [36,44], the oxyradical production of paraquat and its secondary effects on chromatin material of macrophages were tested in different age groups of rats.

Based on facts that GSH is essential to life itself in neutralizing free radicals, removing dangerous waste products and toxins from our body, and the results of our *in vitro* studies [3,37,38]; that GSH and ascorbic acid (AA) protect surfactant from oxidative insults of hydrogen peroxide, hypochlorous acid, and $\cdot OH$ produced by Fenton-reaction [3], we tested the hypothesis that depletion of GSH during aging in rats could have inhibitory effects on surfactant function, as well as increase the frequency of micronuclus induction in alveolar macrophages [21,34,45] due to disorientation of chromatin material in cells by ROS.

Material and Methods

Natural lung surfactant was obtained from lung lavage of rats as described previously [5] and in methods. Chemicals, including paraquat dichloride (methyl viologen) were bought from Sigma (St. Louis, MO). An Oscillating Bubble Surfactometer equipped with the hypophase exchanger system (PBS, Electronetics Corporation, Amherst, N.Y.) was used for surface tension measurements.

Experiments were carried out on four age groups of Fischer F344 rats: Group A (4-6 months), group B (8-10 months), group C (18-20 months), and group D (24-40 months old). Each group consisted of at least 7 rats. Animals were placed on sterile wood shavings in an air-conditioned room, 20-24°C, with a relative humidity of 50%, and maintained in an alternating 12*h* light/dark cycle (lights on at 06.30*h*/ lights off at 18.30*h*). The room and surrounding were kept as sterile as possible. Animals were fed on TD80012 diet obtained from Teklad Test Diet (Madison, WI).

The trachea was exposed and an 18-gauge blunt needle inserted horizontally just below the larynx. Below the point of insertion the trachea and the needle were tied with surgical thread. One end of the stopcock was fitted to the needle, through the other end sterile normal saline was infused into the lungs with a syringe. The lavage fluid was withdrown with another sterile syringe fitted to the 3rd end of the stopcock. A total volume of BALF equal to 0.25 x body wt. was used. The process of infusion and withdrawal of the lavage fluid was repeated 3-times, and an average of 8-20 *ml* fluid collected, corresponding to low and high body weights. The collected fluids were immediately centri-

fuged at 1000 *rpm*/5 *min*, and the pellet containing pulmonary alveolar macrophages (PMS) were mixed with few drops of 0.56% KCl for preparing cell smears on slides. The smears were fixed in absolute methanol for 10 min and air dried. After 24*h* the slides were stained in 1/10 dilution of Giemsa buffered at pH 6.8 for 15 *min* [45] or with Cresyl violet [6], rinsed in deionized water, and air-dried, after which permanent preparations were made, following Xylene treatment and mounted in DPX. Slides were examined at 1000 x magnification, number of micronuclei scored were not less than 500 binucleate cells recorded.

The supernatant was centrifuged at 1000 *rpm*/10 *min*, at 4°C, and the pellet containing the surfactant material was devided into two groups from the supernatant to be evaluated for both the phosphorus assay and biophysical properties, which were kept in microfuge tubes at -80 °C.

Urea nitrogen Determination [42]: Sigma diagnostic reagents (Catalog # 640-A) were used for determination of urea nitrogen in serum, and lung lavage fluids from various age groups of rats for calculation of epithelial lung fluid (ELF) volumes [42].

Thus: $$\frac{\text{Total urea in lavage fluid (mg)}}{\text{Concentration of urea in serum}} = \text{Volume of ELF } (mls)$$

From measured volume of ELF, the GSH content of BALF recovered from each rat was calculated using HPLC analysis.

GSH determination : Since GSH and thiols react with monobromobimane (mBBr) to form highly fluorescent compound, the glutathione bimane conjugate was quantitated after HPLC: 100 μl sample + 10 μl DTT (30 *min*) + 100 μl mBBr (100 *mM* mBBr in acetonitrile) + 10 μl TCA was analysed with Std 100 μmol. HPLC apparatus consisted of Waters Associates HPLC with 721 system controller, with 710B WISP autosampler, high pressure pump, a gradient maker, fluorometer, C18 column (10-μm packing materials), µBondpack column from Waters.

Results and Discussion

Demonstration of GSH protective role on rat surfactant function 37,38: To further confim that GSH has a protective effect on surfactant function of rats, in situ $^{\cdot}OH$ radical production by Fenton reaction, on isolated rat surfactants [3] were utilized. Results indicated a significant protection of surfactants from oxidative damage at 50-100 *mM* GSH, when the minimal surface tensions were kept at 2 *mN/m*> in contrast to surfactants containing 10 *mM* GSH or no GSH, when the MST were > 10 *mN/m* (FIG.1).

The effect of aging on surfactant function: The surfactant function, as determined by measurements of surface tension characteristics [3,4,5] of surfactants, were significantly higher in higher age groups (C,D); the MST and EST were significantly higher ($p < 0.05$), about 10 and 42-45 *mN/m* respectively, in higher age groups compared to A group, with 24 and 2m *N/m* respectively (FIG.2).

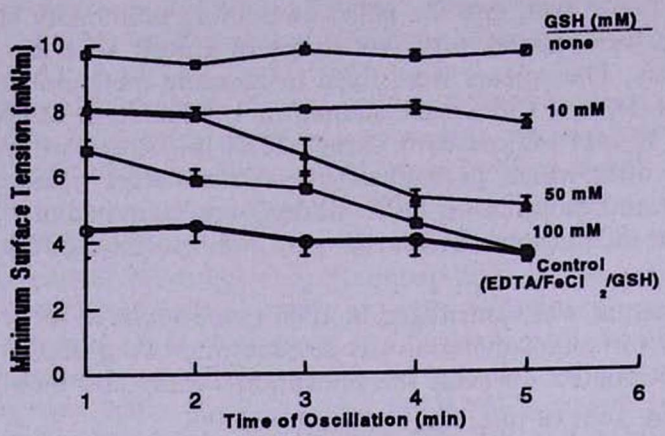

FIG.1. GSH protection of surface characteristics of lung surfactants from Fenton-reaction : Demonstration of effects of *in vitro* exposure of surfactant suspensions (containing 1.25 mg P*l/ml*) in normal saline to Fenton chemistry in the absence (control), and in the presence (test) of serial dilutions of GSH. The quenching effects of GSH on $^{\bullet}OH$ radicals produced by Fenton-reaction is apparent with the increase in the concentration of GSH. The protective effects of GSH against reactive oxygen species were significant at 50-100 *mM* levels ($p < 0.001$)

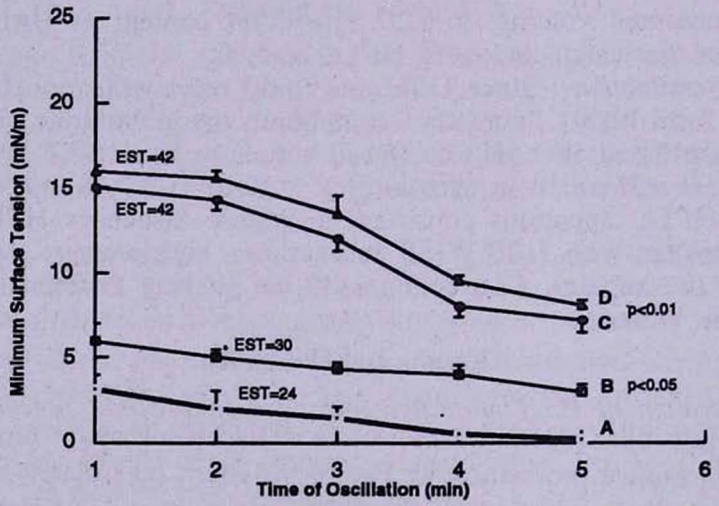

FIG.2. Age-related effects on biophysical properties of rat surfactant: Surface characteristics of surfactants recovered from different age-groups of rats: A=3-6 months, B=8-10 months, C=18-20 months, D=24-30 months old were determined by surfactometer . The surface adsorption of surfactants at equilibrium surface tension (EST) in *mN/m* are shown in parenthesis. After recording the EST at 15 *sec*, the minimum surface tensions (MST, in *mN/m*) were measured at the end of 5 *min* pulsation of the bubble. The EST as well as MST of A-group rats were at normal levels, i.e., below 2 *mN/m* and 24 *mN/m* respectively. The age-groups >10 months have significantly higher surface tensions from the younger rats ($p < 0.05$-0.01).

These effects were more pronounced ($p < 0.01$) amongst the paraquat-treated rats as demonstrated in (FIG.3).

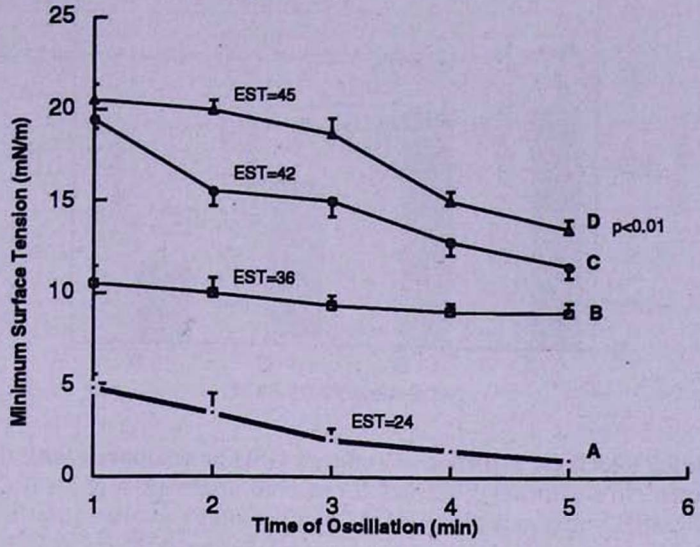

FIG.3. Accentuation of ge-related effects on surfactant function by Paraquat :
Surface characteristics of surfactants recovered from different age-groups of rats: A=4–6 months, B=8–10 months, C=18–20 months, D=24–30 months old. The surface adsorption of surfactants at equilibrium surface tension (EST) in mN/m are shown in parentheses. Both the EST, and minimum surface tensions (MST) of A-group rats are at normal levels, i.e., below 24 *mN/m* and less than 2 *mN/m* respectively, compared to C and D groups (aged and paraquat-treated) with MST >10 and EST >36 *mN/m*, respectively ($p<0.01$).

GSH and total glutathione (GSH+GSSG) values: The mean concentration of reduced glutathione (GSH) in BALF in group A and B rats were almost the same (about 130 μM), when compared to 45 μmol and 90 μM in groups C and D rats ($p < 0.05$) respectively. The results of reduced and total glutathione in different age groups is presented in Fig.4.

Micronucleus frequency in different age groups of rats [21,45]: Pulmonary alveolar macrophages recovered from lung lavage were identified as largest cell types with characteristic prominent nuclei. those macrophages containing one or two micronuclei were identified in the cytoplasm close to nucleus [45]. The counts were expressed as mean and SE of $n > 3$ experiments in each case by using ANOVA for statistical analysis, $p < 0.05$ was considered as significant. The Mann-Whitney U -test was used to compare the mean of micronucleus (MN) scored among different groups for MN frequency in each group. The table presents significant increases in micronucleus frequency in higher age groups ($p < 0.05$) in contrast to the same age groups of paraquat treated and control rats, where highly significant effects ($p < 0.01$) were apparent.

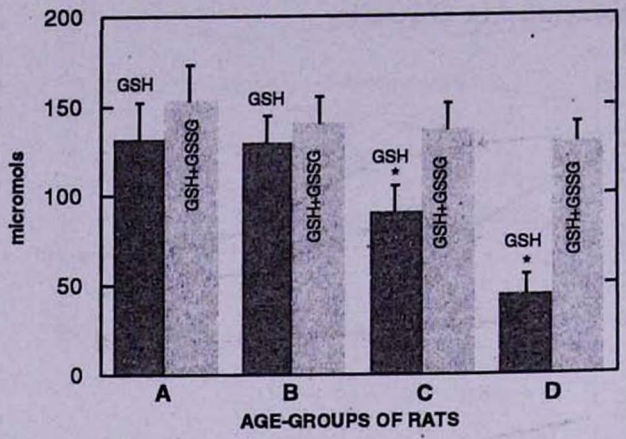

FIG.4. Age-related effects on depletion of reduced GSH as compared with the total glutathione in surfactants: No significant difference is observed in groups A & B (10-20 months old) with almost 130 μM GSH, whereas groups C & D (20-30 months and over) show about 90 and 45 μM GSH respectively, with SEs of 15 and 10 respectively ($p<0.05$)*. It is possible that due to unavoidable errors during assays, or big differences in age of rats within groups the high SEs were registered.

Table

Micronucleus frequencies in pams, isolated from half of three different age groups of rats

Code for Rat groups	Mean of MN cells/1000 cells/Rat of 3 Rats in each sub-groups 1-4				
(months old)	1	2	3	4	Mean (SEM)
A (10)	3.22	2.82	2.80	2.12	2.74 (0.22)
B (20)	3.45	4.52	3.60	2.92	*3.62 (0.33)
C (30)	6.95	5.59	4.20	6.49	*5.80 (0.60)
D (B group) + Paraquat 6 mg/KgWt	7.99	8.20	7.55	8.50	*8.06 (0.19)
E (C group) + Paraquat 6 mg/KgWt	8.89	9.95	9.50	9.35	#9.42(0.21)

* **ANOVA:** Significant at $p < 0.05$ level, from A and intergroups;

\# $p < 0.01$ level from **A or B**

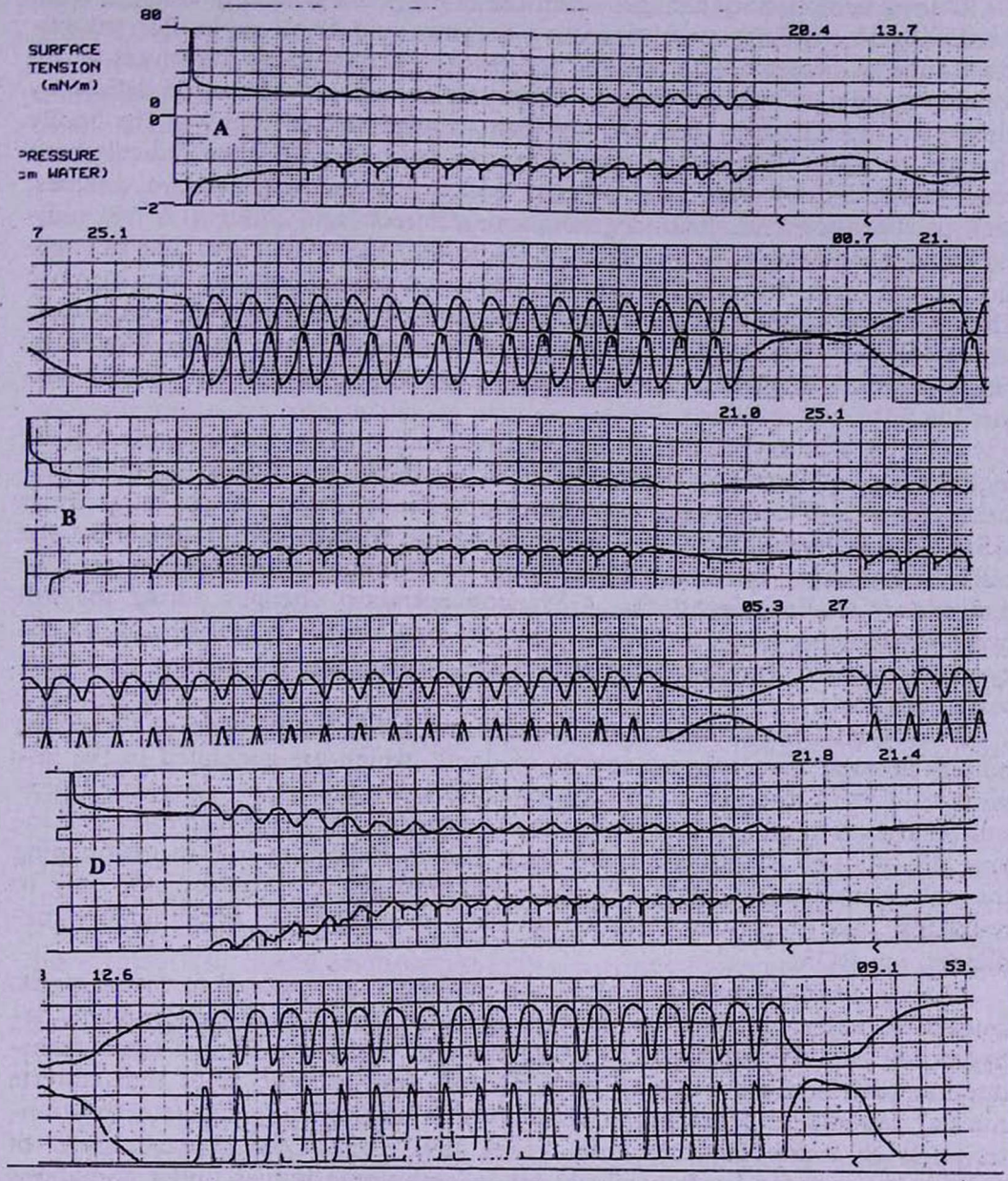

FIG.5. The surfactometer pulsation charts showing age-related surface tension characteristics of surfactants from groups A (4-6), B (8-10) & D (20-30 months old) rats respectively. For each surfactant the first and fifth minutes pulsation is presented. The adsorption at equilibrium surface tension (EST) from zero to 15th second before pulsation is characteristic of each group, which are significantly different from each other. The minimum surface tension (MST) in group A is near zero, whereas in higher age groups (C and D) it is >10 *mN/m*, ($p<0.05$)..

During evolutionary changes when the atmosphere gradually changed from a reducing to oxidizing one, protective enzymes and small molecular antioxidants such as ascorbic acid became the favorite evolutionary life savers [7,18] which are now recognized beyond their role in the prevention of deficiency disease [7,16,18,22,23]. Harman, was first to propose that many of the bodily changes in aging process was due to oxygen radicals [25]. Free radicals have been implicated in most inflammatory conditions and degenerative diseases, such as atherosclerosis, neurodegeneration, cataracts and cancers. A free radical being a molecule with an unpaired electron is highly reactive, and can take an electron from other molecules that in turn become unstable and reactive. Thus a chain reaction insues resulting production of a series of compounds; some with harmful effects to the cell membrane, nucleus and its metabolism by reactions, such as glucose crosslinking to proteins, hormones and growth factors [46,53].

GSH (g-glutamyl-cysteinyl-glycine), is a major redox buffer in the cell for maintenance of its redox potential, which is widely distributed in animal tissues, plants, and microorganisms. Although little is known of the intracellular GSH in mM levels during cell proliferation, or changes that might affect the cell's susceptibility to toxic metals which act through redox mechanisms, the preliminary studies suggest that GSH concentration changes during the cell cycle. GSH being a cystein-based tripeptide participates in several cellular enzymatic reactions; transport of amino acids, neutralizes free radicals and some toxic chemicals.

In vitro studies on the role of catalase and GSH-redox cycle in protecting human alveolar macrophages against oxidants, which are generated in the respiratory birst in response to stimulation by microorganisms or formylated peptide to H_20_2 is implicated as potent reactive metabolite responsible for cell injury [17,39]. The glutathione redox cycle has an important role in maintaining the cell membrane integrity. Alveolar macrophages are exposed not only to pollutants and exogenous oxidants but also to endogenous inflammatory mediators, i.e. ROS.

During perinatal period, fetuses and newborns are exposed to severe stress; caused by uterine contraction, sudden exposure to air, cold environment, dehydration, and starvation [20,50]. A cascade of retinoid-mediated signal transduction pathways are triggered by t-RA (all-*trans*-retinoic acid) that activate nuclear retinoic acid receptors (RAR) which subsequently regulate the transcription of many developmental genes [29,32,35]. Low concentrations of GSH and increased levels of aldehydes in embryonic tissues under conditions of oxidative stress make embryos vulnerable to inhibition of t-RA synthesis during lipid peroxidation. Fetal liver becomes susceptible to oxidative stress, because of lower levels of antioxidants and related enzymes (i.e. α-tocopherol, GSH, SOD, catalase, and GSH-peroxidase), compared to adult liver [50, 52,53]. Thus, a marked change in GSH status around liver cells play a critical role in triggering apoptosis of hemopoietic cells, thereby enhancing the regression of hemopoiesis. Glutamate-induced GSH depletion, and eventual apop-

tosis through endogenously produced ROS, which is accompanied by 1-2 *Mbp* giant DNA fragmentation prior to the intranucleosomal DNA fragmentation has been documented [29]. Preferentially, the specific domains of chromatin in chromosomes become sites, or hot spots sensitive to ROS attacks, thus the possible reason for micronucleus production, which ought to be investigated further.

The present study is a preliminary design of experiments which ought to be executed with a larger number of animals and the distances between age groups reduced further to be able to define the exact line of demarcation between age-groups with small standard deviations. The possibilities explored are by no means exhaustive, but are directly related to the central 'dogma' of cytosolic pool of reduced or oxidized forms of glutathione, that has important bearing on the status of major antioxidants (vitamins E and C). Both vitamin C and E concentrations decrease in tissues of male Fischer rats with age [43,51]. Vitamin C deficiency results in decreased plasma GSH [28], and vitamin E supplementation increases plasma GSH [14], suggesting that GSH may decline with age [16]. We have shown some age-related phenomena with GSH depletion in rats, by inhibition of surfactant function, oxidative damage to DNA of pulmonary alveolar macrophages associated with increases in micronucleus frequency, and accentuation of these phenomena with paraquat – a substance that seems to increase ROS content in alveoli and is responsible for chromatid breakage that lack centromere, and consequently appear as micronuclei in the cytoplasm.

Furthermore, Shay and Wright were the first who manipulated the length of telomeres (the DNA at the ends of chromosomes), and reported that by lengthening the telomere it was possible to extend the life of cell hybrids [27,54]. The telomeres are specialized structures which are important in maintaining chromosomal stability. Each time a cell divides, its telomeres shorten, and eventually, the shortening of telomeres of normal cells affect cell division, while expression of the enzyme telomerase maintains stable telomere length. Since there is a causal relationship between telomere length and proliferative capacity of cells [27,30,54], it would be interesting to examine the length of telomeres of rats at different ages under the above mentioned experimental conditions.

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ՎԵՐԱԿԱՆԳՆՎԱԾ ԳԼՈՒՏԱՏԻՈՆԻ ՎԻՃԱԿԻ ՏԱՐԻՔԱՅԻՆ ՓՈՓՈԽՈՒԹՅՈՒՆՆԵՐԸ ԵՎ ՆՐԱՆՑ ԱԶԴԵՑՈՒԹՅՈՒՆԸ F344 ՍԱՌԵՏՆԵՐԻ ԹՈՔԵՐԻ ԱԼՎԵՈԼՅԱՐ ՄԱԿՐՈՖԱԳԵՐԻ ՄԱՆՐԱԿՈՐԻԶՆԵՐԻ ՀԱՃԱԽԱԿԱՆՈՒԹՅԱՆ ԵՎ ՍՈՒՐՖԱԿՏԱՆՏԻ ԿԵՆՍԱՖԻԶԻԿԱԿԱՆ ՀԱՏԿՈՒԹՅՈՒՆՆԵՐԻ ՎՐԱ

Ջ.Դ. Ամիրխանյան

Ցույց է տրված, որ օքսիդատիվ ստրեսը բերում է օքսիդացված գլուտատիոնի հարաբերության զգալի բարձրացման վերականգնվածի նկատմամբ (1:6) F344 երիտասարդ (6-20 ամս.) առնետների բրոնխո-ալվեոլյար հեղուկում և նրա ավելի նվազ բարձրացման (1:2) ծեր առնետների (25-30 ամս.) մոտ, վերականգնված գլուտատիոնի վիճակի հավաստի տարբերությունների պայմաններում:

Պարակվատի ճմգ/կգ քանակի ներորովայնային ճանապարհով ներմուծման ժամանակ տարբեր տարիքային խմբերի առնետների թոքերի մակրոֆագերում նկատվել է մանրակորիզային ինդուկցման հաճախականության զգալի բարձրացում ծեր առնետների մոտ, երիտասարդ և ստուգիչ (առանց պարակվատի) խմբերի համեմատությամբ:

Վերականգնված գլուտատիոնի քանակը, որ անհրաժեշտ է թոքերում սուրֆակտանտի ֆունկցիայի պահպանման համար, կարևոր ցուցանիշ է ծերացման պրոցեսի մեխանիզմների ուսումնասիրման ժամանակ:

ВОЗРАСТНЫЕ ИЗМЕНЕНИЯ В СОСТОЯНИИ ВОССТАНОВЛЕННОГО ГЛУТАТИОНА, В ЧАСТОТЕ МИКРОЯДЕР АЛЬВЕОЛЯРНЫХ МАКРОФАГОВ И БИОФИЗИЧЕСКИХ СВОЙСТВАХ СУРФАКТАНТА ЛЕГКИХ У КРЫС F344

Дж.Д.Амирханян

Показано, что окислительный стресс приводит к значительному увеличению соотношения окисленного глутатиона, к восстановленному (1:6) в бронхоальвеолярной жидкости у молодых (6—20 мес.) крыс F344 и менее заметному увеличению (1:2) у пожилых (25—30 мес.) при достоверных различиях в состоянии восстановленного глутатиона.

При внутрибрюшинном введении параквата крысам различных возрастных групп в дозе 6 *мг/кг* было отмечено заметное увеличение частоты микроядерной индукции в альвеолярных макрофагах легких у пожилых крыс по сравнению с молодыми и контрольными (без параквата).

Количество восстановленного глютатиона, необходимого для сохранения функции сурфактанта в легких, является важным показателем при изучении механизмов процесса старения.

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