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Oxidation of Guanine in Liver and Lung DNA of Prematurely Aging OXYS Rats

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Abstract—Immunofluorescence assay was applied for determination of 8-oxoguanine (8-oxoG) in DNA. The 8-oxoG content in liver and lung DNA of 2- and 18-month-old Wistar rats was compared with that of prematurely aging OXYS rats. It was shown that for rats of both strains, 8-oxoG content in lung DNA compared with liver DNA was 1.7-2.0-fold and 1.3-1.7-fold higher for 2- and 18-month-old rats, respectively. However, the degree of oxidative damage in liver DNA of OXYS rats was 2.4- ($p < 0.01$) and 1.5-fold ($p < 0.05$) higher for 2- and 18-month-old animals, respectively, than that in liver DNA of Wistar rats. Oxidation of guanine in lung DNA of OXYS rats was 2- ($p < 0.01$) and 1.7-fold ($p < 0.05$) higher for 2- and 18-month-old animals, respectively, than that in lung DNA of Wistar rats. The data indicate that elevated DNA oxidative damage in various organs of OXYS rats may be an important factor of accelerated aging and progression of age-related diseases—cataract, macular dystrophy, hypertension, osteoporosis, cognitive and behavioral dysfunctions, and also lung and liver pathologies.

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Key words: oxidative stress, 8-oxoguanine, premature aging, OXYS rats

According to current concepts, the main origin of aging is the interaction of various endogenous and exogenous damaging agents with cell genetic material and progressive accumulation of mutations in the genome of somatic cells [1, 2]. Reactive oxygen species (ROS) including superoxide and hydroxyl radicals, hydrogen peroxide, and single oxygen are such agents [3, 4]. Oxidizing various cell components including nucleic acids, ROS cause DNA mutations in various organs and tissues; accumulation of these mutations with aging is assumed to be the main origin of development of age-related pathologies. The 8-oxoguanine (8-oxoG) formed by the action of endogenous and exogenous ROS on nucleic acids is an important biomarker of DNA oxidative damage [5-7]. The 8-oxoG is a mutagenic DNA modification; appearance of this modification results in replacement of the G–C by the T–A pair in DNA [8, 9].

Such important biological processes as mutagenesis, carcinogenesis, aging, and development of certain age-related pathologies are closely correlated with formation of 8-oxoG in cell DNA [10, 11]. ROS genotoxic action stimulated development of methods allowing detection of cell oxidative damage [12]; of these, immunological methods are important [13, 14]. Since immunological assay is highly specific and universal, anti-8-oxoG antibodies are ideal reagents for detection of DNA oxidative damages in cells [13-15].

Oxidative stress—violation of balance in the systems of DNA generation and ROS detoxication—is known to be an origin as well as a consequence of aging and related diseases (see above) [16-18]. Using OXYS rats, a biological model developed in the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy

Abbreviations: Ab) antibodies; FITC) fluorescein isothiocyanate; IFA) immunofluorescence assay; 8-oxoG) 8-oxoguanine; ROS) reactive oxygen species.

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of Sciences, gives unique possibilities for study of the role of oxidative stress in these processes. These animals were obtained by selection and inbreeding of Wistar rats sensitive to cataractogenic action of galactose [19] and are registered in the International Database with key characteristics "innate hyperproduction of free radicals" [20]. The maximal lifetime of OXYS rats is 28% less than that of Wistar rats [21]. OXYS rats can be characterized by early cataract accompanied by dystrophy of the *macula lutea*, early involution of the thymus, increased arterial pressure, decreased reactivity of the cellular immune system, osteoporosis, and cognitive changes typical of aging people and animals [22-26]. The well-known ability of antioxidants for lifetime increase and for age-related disease prevention is considered as good evidence for ROS participation in aging pathogenesis. As we showed earlier, inclusion of antioxidants (vitamin E, bilberry extract, etc.) into the rations of OXYS rats decreased the level of oxidative modifications of tissue proteins and lipids and prevents manifestation of features of premature aging [24, 27, 28]. The data indicate that oxidative stress is included in accelerated aging pathogenesis of OXYS rats. We compared the relative activities of repair enzymes in nuclei, cytosol, and mitochondria of OXYS and Wistar rats [29]. As found, in liver cell nuclei and mitochondria of OXYS and Wistar rats, activity of the repair enzymes removing modified nucleotides (deoxyuridine, hypoxanthine, 8-oxoG) from DNA increases with aging. Activity of the repair enzymes in liver cells of OXYS rats is higher than that of Wistar rats. Increased activity of antioxidant enzymes, superoxide dismutase and catalase, is also detected in cytosol and mitochondria of liver cells of 6- and 12-month-old OXYS rats as compared to that of Wistar rats [28]. However, increased activity of all these enzymes does not prevent the progress of oxidative stress—the content of oxidized proteins in liver and especially in brain of OXYS rats increases more rapidly with aging than that of Wistar rats. Also, for OXYS rats, most manifestations of premature aging described above have progressed before the increased content of oxidized proteins in animal tissues is recorded. As noted above, aging and pathogenesis of age-related diseases are closely correlated with accumulation of oxidative DNA damages and enhanced somatic mutagenesis; however, for OXYS rats these indicators were not studied earlier.

Age-related changes in 8-oxoG content in the genomic DNA may be an objective criterion of accelerated aging of OXYS rats related with somatic mutagenesis. To evaluate these changes, we developed a method of immunofluorescent determination of the relative content of 8-oxoG in DNA. In animals the liver is most subject to oxidative stress [30]. Using monoclonal antibodies against 8-oxoG, we evaluated the relative content and dynamics of 8-oxoG accumulation in liver and lung of OXYS rats with aging as compared with that of control Wistar rats.

MATERIALS AND METHODS

Isolation of genomic DNA from rat liver. In this study, we used two groups (2- and 8-month-old) of male OXYS rats and control Wistar rats, respectively, 7-8 animals in each group. All rats lived under the standard conditions with free access to food and water.

During 2 min after decapitation, samples of rat liver and lung were placed in liquid nitrogen and stored at −70°C (the organs were not perfused). To obtain liver DNA preparations, we took a lateral segment (~500 mg) of the upper right sector of the large hepatic lobe; to obtain lung DNA preparation, we took a segment of right lung top (~300 mg). We did not reveal any noticeable morphological distinctions between the samples of appropriate tissues taken from the various rats.

DNA was isolated from liver and lung tissues according to a protocol described in the QIAGEN Genomic DNA Handbook (QIAGEN Inc., USA). After precipitation, DNA was resuspended in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and 1 mM dithiothreitol (DTT) was added to this solution. DNA concentration was determined spectrophotometrically via the optical density at 260 nm.

Preparation of control DNA. DNA preparations with various amounts of modified 8-oxoG were obtained by treatment of the DNA with methylene blue under illumination according to [31]. To DNA solution (100 μg/ml in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5), 10 μl of 0.1% methylene blue was added, and the mixture was placed in an ice bath and irradiated with visible light (tungsten lamp, 400 W/m^2, irradiation time from 0 to 30 min). Under these conditions, the amount of 8-oxoG accumulated in DNA is proportional to irradiation time [31]. Then DNA was precipitated with ethanol, resuspended in TE buffer, pH 8.0, and DTT was added to 1 mM concentration.

To prove formation of 8-oxoG in DNA, irradiated and non-irradiated DNA preparations were treated with *E. coli* 8-oxoG-DNA glycosylase (Fpg protein) according to [32]. The products of Fpg-dependent DNA hydrolysis were analyzed by electrophoresis in 0.7% agarose gel in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA).

Determination of 8-oxoguanine in the genomic DNA of OXYS and Wistar rats. In this study, we used monoclonal antibodies (Ab) against 8-oxoG (mAb483.15) obtained as described earlier [15]. For experiments, we used culture fluid of cell hybrid clones expressing mAb483.15 in dilutions given below.

Genomic DNA of 2- and 18-month-old Wistar and OXYS rats isolated as described above was denatured in a boiling water bath for 5 min and placed in an ice bath for 4 min. Aliquots (50 μl) of denatured DNA at concentration 1.5 μg/ml were placed in wells of plates for enzyme immunoassay with increased sorption capacity of well

surface from Vektor (Russia) and immobilized for 12 h at room temperature. After DNA immobilization, wells were washed with TBS (0.15 M NaCl, 0.02 M Tris-HCl, pH 7.5) containing 0.05% Triton X-100 (1 time) and with TBS without Triton X-100 (2 times). To decrease nonspecific sorption, well surface was blocked by ovalbumin (0.2% solution in TBS). The assembled strips were washed free from excess protein using bidistilled water (2 times with 200 μl) and TBS (1 time with 200 μl). Hybridoma extracellular fluid containing antibodies against 8-oxoG, 1 : 500 dilution in TBS containing 0.05% Triton X-100 and 0.2% ovalbumin, was placed into wells (100 μl aliquots) and incubated for 2 h at 37°C. Excess Ab was washed out with bidistilled water (10 times with 200 μl) and TBS. The strips surface was blocked by addition of 0.2% ovalbumin solution in TBS (100 μl), and the plate was incubated for 1 h at 37°C. The strips were washed with water (2 times with 200 μl) and TBS and then blocked for the second time. Then 100 μl of rabbit Ab to mouse immunoglobulins conjugated with fluorescein isothiocyanate (FITC) (1 mg/ml) from Sigma (USA) in 1 : 1000 dilution in TBS containing Triton X-100 and 0.2% ovalbumin was placed in strips and incubated for 12 h at room temperature. After incubation, solution was placed in a quartz cuvette and its relative fluorescence was recorded using a Hitachi MPF-4 spectrofluorimeter (Japan). Fluorescence of Ab-bound FITC was excited at the maximal excitation wavelength (490 nm) and recorded at the maximal fluorescence wavelength (525 nm). In control samples treated exactly as experimental ones, Ab against 8-oxoG were replaced by Ab without affinity to this base. DNA treated with Fpg protein was sorbed in strips of samples used as the zero standard; DNA concentration was exactly the same as that for untreated DNA in experimental samples.

We performed preliminary experiments on choosing conditions for quantitative immobilization of high-polymeric rat DNA (DNA content was varied from 0.5 to 5 μg), optimal dilutions of hybridoma culture fluid (from 1 : 100 to 1 : 5000), and FITC-labeled rabbit Ab against mouse immunoglobulins (from 1 : 100 to 1 : 2000), and blocking protein, including its amount and incubation time (from 0.5 to 2 h). All the above-mentioned parameters were optimized in order to provide linear dependence of relative change in fluorescence of samples (ΔF) (due to binding of FITC-labeled rabbit Ab with monoclonal Ab against 8-oxoG specifically sorbed on 8-oxoG-containing DNA in plate strips) on amount of immobilized DNA. Optimal saturation amounts were determined for monoclonal antibodies against 8-oxoG and FITC-labeled polyclonal rabbit Ab.

Data were statistically processed using the Statistica 5.0 program and Student's *t*-criterion. Distinctions were considered reliable at $p \leq 0.05$.

Chemicals. In this study we used Tris, EDTA, NaCl, and Triton X-100 from ICN Biomedicals (USA); dithiothreitol from Serva (Germany); agarose from Amresco (USA); ovalbumin, methylene blue, boric acid, and hydrochloric acid of extra pure grade from Reakhim (Russia). Isolation and characterization of Fpg protein was described earlier [33].

RESULTS

Development of assay for detection of 8-oxoguanine in DNA. Relative amount of 8-oxoG in genomic DNA of animal liver and lung was determined by immunoadsorption conjugated with detection of specifically adsorbed Ab by fluorescent assay. To optimize conditions for assay, we obtained control DNA preparations with varied amount of 8-oxoG. Formation of 8-oxoG was induced by irradiation of Wistar rat DNA solutions by visible light in the presence of methylene blue, varying irradiation time as in [31].

To prove formation of 8-oxoG in DNA under the above-described conditions, DNA preparations were treated with 8-oxoG-DNA glycosylase (Fpg protein), which specifically removes 8-oxoG base from DNA and then cleaves DNA at the 5′- and 3′-ends of the formed carbohydrate base-depleted residue. The products of specific DNA hydrolysis were analyzed by electrophoresis in agarose gel. It was shown that the relative quantity of Fpg-dependent DNA breaks increases with increasing irradiation time. This indicates that under the DNA irradiation conditions used by us, 8-oxoG is formed efficiently.

We optimized conditions of immunofluorescence assay. As shown, among the blocking proteins most often used for minimization of nonspecific adsorption (bovine serum and human serum albumins, ovalbumin, casein), ovalbumin is the optimal blocking agent when 8-oxoG is determined by immunofluorescence assay (IFA). After addition of monoclonal mouse Ab against 8-oxoG into strips, the well surface was twice blocked with 0.2% ovalbumin. After single blocking, and the subsequent addition of anti-mouse rabbit Ab conjugated with FITC, negligible nonspecific sorption of FITC-Ab was observed.

For quantitative evaluation of relative ΔF values, it was necessary to choose conditions when ΔF linearly increased on increase in 8-oxoG-containing DNA concentration. For this, we analyzed the dependence of ΔF on the concentration of 8-oxoG-containing mouse DNA and showed that when 50 μl of DNA at concentration 5 μg/ml was placed in strips, linear dependence of ΔF on its concentration was not observed (Fig. 1). This can be related with the fact that at large amounts of high-polymeric DNA, the latter is sorbed on well surface not as a monolayer but as several layers. Use of monoclonal mouse Ab against 8-oxoG and rabbit FITC-labeled Ab in excess to the modified DNA units in dilution 1 : 500 and 1 : 1000, respectively, appeared to be optimal. At relative-

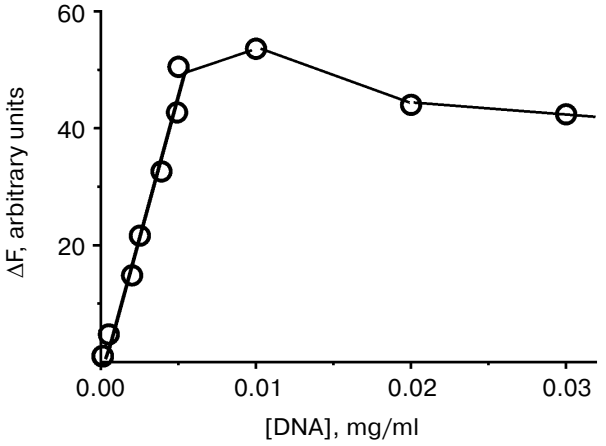

Fig. 1. Relative fluorescence (ΔF) of rat DNA solution versus concentration of DNA placed in strips of immunological plate (determination of 8-oxoG content by Ab against this base). On immobilization, 50 μl of DNA solution was placed in plate wells.

ly large (dilution less than 1 : 150) or small amounts of these Ab (dilution more than 1 : 10^4), ΔF values were underestimated. When primary and secondary Ab were used at concentrations corresponding with the ranges given above, ΔF linearly increased on increase in DNA concentration.

As a result of the IFA, the relative content of Ab bound to 8-oxoG in DNA was determined via the difference in fluorescence (ΔF) of solutions corresponding to preparations of anti-mouse Ab conjugated with FITC added in wells containing sorbed DNA after their treatment with Ab against 8-oxoG and in wells untreated with these antibodies. The relative amount of 8-oxoG (as ΔF values) contained in rat DNA versus time of irradiation by visible light is presented in Fig. 2. As shown, binding of fluorescent Ab with adsorbed complex DNA–Ab against 8-oxoG increases with time of DNA irradiation; this agrees with increase in the relative amount of oxidized bases in DNA composition, according to the data on its cleavage by Fgp protein. It is known that DNA of any living organism is subject to oxidative stress. That is why DNA of Wistar rats also contained a certain amount of 8-oxoG residues (see below) and could not be used as a control. To exclude nonspecific sorption of monoclonal mouse Ab and then also of FITC-Ab, we used Wistar rats DNA treated with Fpg protein. It was shown that ΔF value characterizing nonspecific sorption of FITC-Ab does not exceed 10% ΔF observed for rat tissue DNA not treated with Fpg protein. The data indicate that mouse monoclonal Ab against 8-oxoG specifically react with 8-oxoG in DNA composition and thus are suitable for evaluation of the relative amount of modified bases in its composition.

Determination of 8-oxoG content in rat DNA. According to the previously published data, obvious external changes of OXYS rats as compared with Wistar rats are related with cognitive and visual functions as well as disturbance of memory [22-26]. It is also known that DNA of liver cells of various animals is most often subject to enhanced oxidative stress [30]. Oxidative disruptions of lung and liver of OXYS rats are so far not well studied. Nonetheless, according to our preliminary data, there are significant morphological distinctions between liver and lung of OXYS rats and the analogous organs of Wistar rats less subject to oxidative stress. Earlier we also obtained data that the relative activities of repair enzymes in liver of these rats drastically differ [29]. Accounting for this, we used the method developed by us for determination of 8-oxoG content in DNA isolated from liver and lung cells of 2- and 18-month-old OXYS and Wistar rats (7-8 animals in each group). Values of ΔF characterizing the relative 8-oxoG content in DNA were determined, taking into account ΔF characterizing nonspecific Ab sorption on DNA (see "Materials and Methods").

The data on 8-oxoG content in rat liver DNA are presented in Fig. 3a. The relative 8-oxoG content in liver DNA of OXYS rats was 19.9 ± 3.9 and 26.5 ± 4.2 arbitrary units for 2- and 18-month-old animals, respectively. For 2-month-old Wistar rats, the relative 8-oxoG content in liver DNA was 2.4 times lower (8.4 ± 4.9 arbitrary units) and for 18-month-old Wistar rats it was 1.5 times lower (17.9 ± 3.2 arbitrary units) than that of OXYS rats of the same age.

The data on the relative 8-oxoG content in rat lung DNA are presented in Fig. 3b. For OXYS rats, the relative 8-oxoG content negligibly changed with age: 40.7 ± 5.2 and 44.8 ± 1.5 arbitrary units for 2- and 18-month-old animals, respectively. For 2-month-old Wistar rats, the relative 8-oxoG content in lung DNA was 2.9 times lower

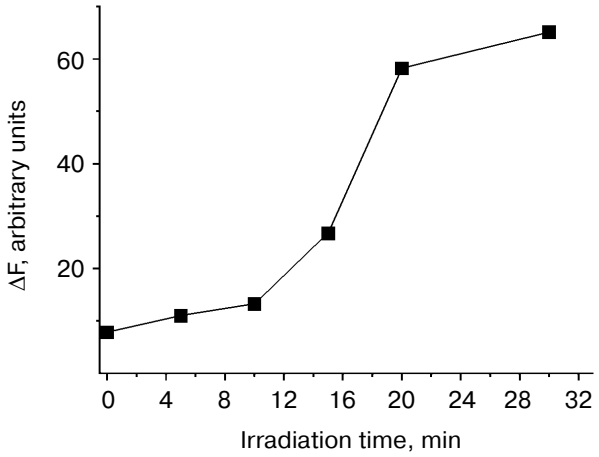

Fig. 2. Relative amount of 8-oxoG in DNA (in ΔF values, average of three measurements) isolated from liver of 2-month-old Wistar rats versus irradiation time by visible light in the presence of 0.1% methylene blue. ΔF is calculated according to the data for control DNA not containing 8-oxoG bases.

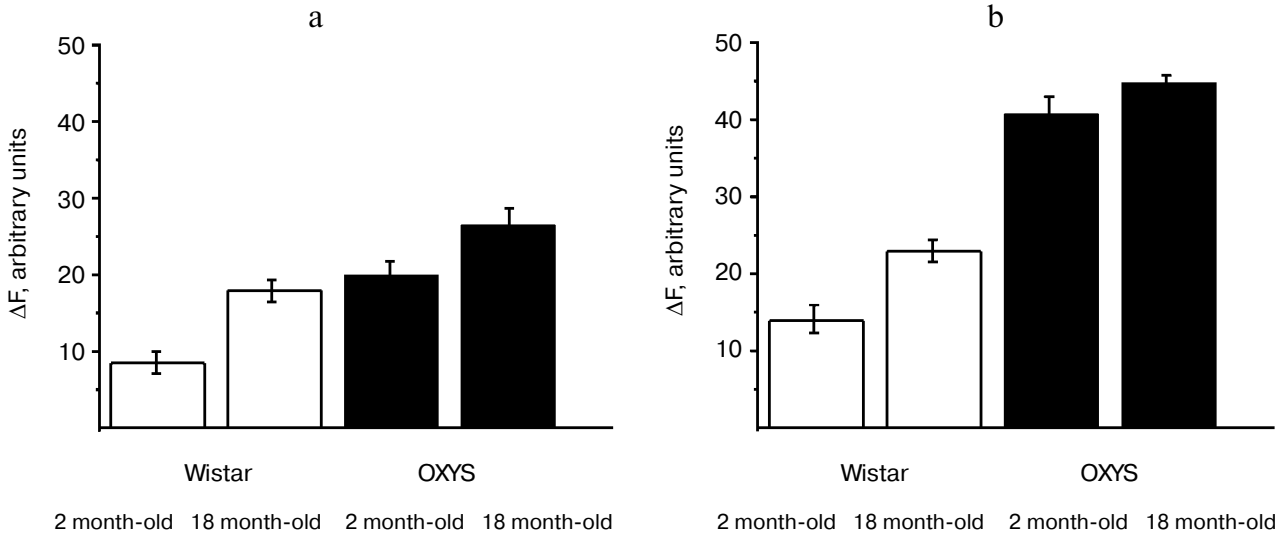

Fig. 3. Relative 8-oxoG content in liver (a) and lung DNA (b) of OXYS and Wistar (control) rats. Values of ΔF average for 7-8 animals are given with standard deviations. ΔF corresponds to relative fluorescence of FITC-labeled rabbit Ab adsorbed by DNA complex with monoclonal Ab against 8-oxoG.

(13.9 ± 4.3 arbitrary units) and for 18-month-old Wistar rats it was 2 times lower (22.9 ± 3.3 arbitrary units) than that of OXYS rats of the same age.

It is interesting that lungs of both OXYS and Wistar rats are subject to the oxidative stress to a greater extent than liver. In lung DNA of 2-month-old OXYS rats, the relative 8-oxoG content was 2 times higher than in liver. The degree of guanine oxidation in lung DNA of 18-month-old OXYS rat negligibly increased; however, it was 1.7 times higher than in liver DNA. An analogous ratio was also observed for 2-month-old Wistar rats: the relative content of oxidized guanine in lung DNA was 1.7 times higher than that in liver DNA, but for 18-month-old Wistar rats, this ratio decreased to 1.3 times. All the estimations of intra- and interlinear distinctions were statistically reliable.

DISCUSSION

The free-radical theory of aging allows explanation of not only aging mechanisms but also of pathogenesis of related diseases: cardiovascular, oncological, neurodegenerative, and so on. The data prove that OXYS rats are unique model objects for studying the role of oxidative stress in aging processes. It is important that clinical picture of progress of "aging diseases" of OXYS rats does not differ from that typical to people with the same pathologies; this allows use of this animals for studying mechanisms of the participation of oxidative stress in the progress of aging and related diseases [16-24].

In this work, we developed a method allowing evaluation of the relative 8-oxoG content in DNA. Conditions for immunofluorescence assay using monoclonal Ab against 8-oxoG possessing high specificity to the oxidized base were optimized. We showed that these Ab are suitable for detection of 8-oxoG in rat genomic DNA using rat DNA containing various amounts of the modified bases and also 8-oxoG-depleted DNA as controls.

This method allows comparison of the relative content of DNA oxidative damages in liver and lung of Wistar and OXYS rats of various ages. The data indicate that oxidative stress first of all attacks lungs of rats of both lines. The amount of mutagenic 8-oxoG in DNA of OXYS and Wistar rats increases with aging. However, the relative 8-oxoG content in lung DNA of 2-month-old OXYS rats was 2.9 times higher than in lung DNA of Wistar rats of the same age; the analogous ratio for 18-month-old animals was 2.0. Distinctions in oxidative damage of liver DNA of rats of both lines are somewhat lower. The relative 8-oxoG content in liver DNA of 2-month-old OXYS rats was 2.4 times higher than in liver DNA of Wistar rats of the same age; for 18-month-old animals, the analogous ratio is 1.5 times.

It should be noted that 8-oxoG is an oxidative modification of DNA which results in appearance of mutations. Thus, enhanced degree of DNA oxidative damages in liver and lung cells of OXYS rats accounts for the relative amount of pre-mutational damages which then are "fixed" in constant gene mutations. Since such fixation of "pre-mutations" constantly occurs, they can accumulate with aging. That is why enhanced accumulation of DNA oxidative damages of OXYS rats may be an important factor of accelerated genetic aging of somatic cells of OXYS rats. It is possible that accumulation of such mutations in OXYS rats manifests itself in early progress of age-related

diseases [21-26]. These data and possible prevention of progress of these diseases by antioxidants [24, 27-29] indicate that there is a direct relation between accelerated aging of OXYS rats and oxidative stress.

As shown above, the maximal distinction in oxidation of guanine residues is observed for lung DNA of OXYS and Wistar rats. According to Esposito et al., DNA of animal liver cell is most often subject to enhanced oxidative stress [30]. However, this statement may be due to the fact that in most cases DNA of lung cells were not analyzed. It is noteworthy that accumulation of DNA oxidative damages in lung DNA of prematurely aging SAMP mice is also higher than in other organs [31-36]. According to our preliminary data, lung and liver of OXYS rats are significantly different in morphological sense from lungs of Wistar rats less subject to oxidative stress.

It should be specially noted that repair enzymes and enzymes of antioxidant protection play an important role in ROS inactivation and correction of damages caused by ROS. As mentioned above, the activity of these enzymes in cells of OXYS rats drastically increases with aging. However, even enhanced activity of these enzymes cannot provide efficient protection of these rats from genetically determined enhanced ROS formation and/or increased sensibility to oxidative stress. This results in accelerated accumulation of oxidative damages to proteins, lipids, and DNA and as a consequence, to accelerated aging.

It should be noted that by now there is only one commonly accepted model of premature aging, SAM mice (senescence accelerated mouse), which is represented by 14 sublines [37]. These sublines differ from each other by character of emotional dysfunction, circadian rhythms, degree and nature of cognitive dysfunctions, and also other aging features. As shown by us, OXYS rats are valuable model objects for study of fundamental mechanisms of premature aging and development of the efficient methods for its maintenance. OXYS rats differ from SAM mice in a complex of manifestations of premature aging, which begins to develop already in the first months of life. So, these rats can be one more unique model for study of molecular mechanisms of animal and human aging.

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