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Determination of reactive oxygen and nitrogen species in rat aorta using the dichlorofluorescein assay

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Abstract
A method for the determination of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in macroscopic sections of vessels has been developed on the basis of the dichlorofluorescein (DCF) assay. DCF was measured by fluorescence in extracts of vessels. The main artifact of the method is the oxidation of dichlorodihydrofluorescein ($DCFH_2$) which is released from vessels together with DCF during the extraction procedure. This problem was resolved by decreasing pH during the extraction. The optimal conditions and the time for aorta incubation with $DCFH_2$-DA and for the extraction of DCF from aorta have been determined. The ROS/RNS production in different aorta segments and the dependence of ROS/RNS production on rat age have been studied. It was shown that thoracic aorta sections produced the same amounts of ROS/RNS and the intermediate between the thoracic and the abdominal aorta part produced ROS and RNS by 14% more than the thoracic aorta. It was found that ROS/RNS production in aorta increases with rat age: the doubling time of ROS/RNS production rate is 113 days from birth.

Keywords: *Ageing, aorta, dichlorofluorescein, reactive nitrogen species, reactive oxygen species*

Abbreviations: *DCF, 2′,7′-dichlorofluorescein; $DCFH_2$, 2′,7′-dichlorodihydrofluorescein; $DCFH_2$-DA, 2′,7′-dichlorodihydrofluorescein diacetate; RFU, relative fluorescence unit; RNS, reactive nitrogen species; ROS, reactive oxygen species*

Introduction

Oxidative stress is believed to be an important cause of vessel atherosclerosis [1–6]. Numerous methods exist for the determination of individual and total ROS/RNS [7]. These methods are divided into two groups: (1) the methods that determine ROS/RNS outside the vessel (in medium) and (2) those that determine ROS/RNS in vascular cells. The methods of the first group are numerous and can determine individual and total ROS/RNS. The methods of the second group are applied for vessels in the microscopic variant only. To determine the role of oxidative stress in atherosclerosis, it is necessary to measure total ROS/RNS in vessels. The level of ROS outside vessels does not correlate with that in vascular cells because (1) the cell membrane contains NADPH oxidase that produces extracellular ROS and (2) a little portion of intracellularly generated superoxide leaves cells. The dichlorofluorescein assay determines total ROS/RNS [7,8]; however, it is used for vessels in the microscopic variant only. The dichlorofluorescein assay with the use of fluorescence microscopy has limitations, since the indicator undergoes strong photo-oxidation [9–11]. The photo-oxidation of 2′,7′-dichlorodihydrofluorescein ($DCFH_2$) to 2′,7′-dichlorofluorescein (DCF) increases the amount of ROS/RNS being determined; in addition, the photodynamic effect can damage cells. It was shown that the intensity of fluorescence of $DCFH_2$-loaded cells

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increases rapidly (10 s) during fluorescence microscopy, which made it impossible to detect differences in fluorescence between ROS/RNS-generating cells and cells that do not generate ROS/RNS [9]. This artifact also takes place in the determination of ROS/RNS by fluorescent confocal microscopy [11]. Light is kept to a minimum to avoid $DCFH_2$ photooxidation to DCF, which results in the poor quality of image and a low signal-to-noise ratio [12]. Besides, there is another problem associated with ROS/RNS determination in vessels by fluorescent microscopy. This method determines ROS/RNS in the microscopic part of a vessel a few dozen cells in size at a depth of as little as 100 μm from the endothelium surface. Such a small area of the vessel does not give an estimate of the average amount of ROS/RNS in the vessel. Many images (15 or more) should be analysed for overcoming of this defect, and greatly differing images are averaged. It is known that a vessel has heterogeneous cell composition in cross-section and along long axis and it is impossible to choose precisely identical zones of vessels for analysis in control and experimental animals.

The goal of this study was the development of a dichlorofluorescein assay for the determination of ROS and RNS in macroscopic segments of rat aorta for the estimation of oxidative stress in vessels.

Materials and methods

Animals and aorta preparation

A total of 94 male Wistar rats weighing 250–450 g (animal collection of the Institute of Theoretical and Experimental Biophysics, Pushchino, Russia) were used. Rats were held at the animal facilities with free access to water and standard rat chow. The local ethics committee criteria for care and use of laboratory animal were carefully followed. Ether was used for surgical anaesthesia. The thorax was opened and heparin (500 units) was introduced into the heart to prevent blood clotting. A greater part of aorta adherent adventitial fat was cleaned *in situ*. Then the aorta was removed, rinsed with cold (4°C) 10 mm Hanks'-HEPES solution, pH 7.4, and placed in the same solution. Residuary fat was carefully cleaned; care was taken not to damage the endothelium and the adventitial material adjacent to the medial layer.

Determination of ROS/RNS

The aorta from the aorta arc to the point of branching of kidney arteries was cut into seven 5-mm sections. An aortic 5-mm section had a wet weight of ~4–5 mg. Aortic sections were numbered from 1–7, starting with the section near to aorta arc. Sections 1, 3 and 5 were used for ROS/RNS determination without modification and sections 2, 4 and 6 were used for ROS/RNS determination with modification. Section 7 was not incubated with dichlorodihydrofluorescein diacetate ($DCFH_2$-DA). This section was used for the determination of endogenous fluorescent substances leaving the aorta after the treatment with digitonin. The fluorescence of endogenous substances was subtracted from the fluorescence of extracts obtained from sections incubated with $DCFH_2$-DA. Aortic sections were cut lengthwise, turned inside out with the endothelium outside and attached to the tip of the plastic pipette. Then aorta segments were placed in glass flasks in 2.5 ml Hanks'-HEPES solution, pH 7.4, and incubated for 30 min at 37°C with shaking (25 Hz, amplitude 1 mm) for adaptation before the addition of $DCFH_2$-DA. Then 20 μm $DCFH_2$-DA was added, and aorta segments were incubated for 20 min at 37°C with shaking. The $DCFH_2$-containing solutions were removed after the completion of incubation and aorta sections were rinsed twice with cold Hanks'-HEPES solution. Then aorta segments were placed in citrate buffer (2.5 ml), pH 4, containing 0.02% digitonin and incubated for 20 min at 37°C with shaking. Aorta extracts were cooled to room or ice temperature and kept at these temperatures until fluorescence measurement. Fluorescence was measured at 2°C or at room temperature under stirring on an MF44 Perkin Elmer fluorimeter at excitation and emission wavelengths of 475 and 525 nm, respectively. The measurement of DCF fluorescence was made at pH 7, except for the experiments shown in Figures 1–3 . The value of pH during the fluorescence measurement is given in the respective figure legends.

Materials

All chemicals were purchased from Sigma Chemical Co. USA. A 10 mM $DCFH_2$-DA stock solution was prepared in ethanol, stored at −20°C, and diluted in Hanks' solution before use.

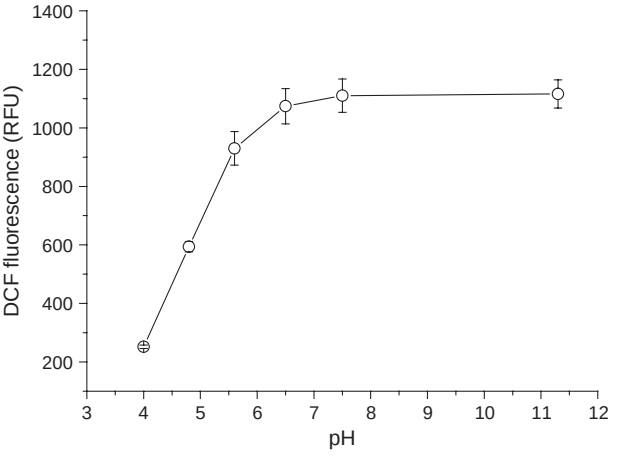

Figure 1. pH dependence of DCF fluorescence ($n = 3$).

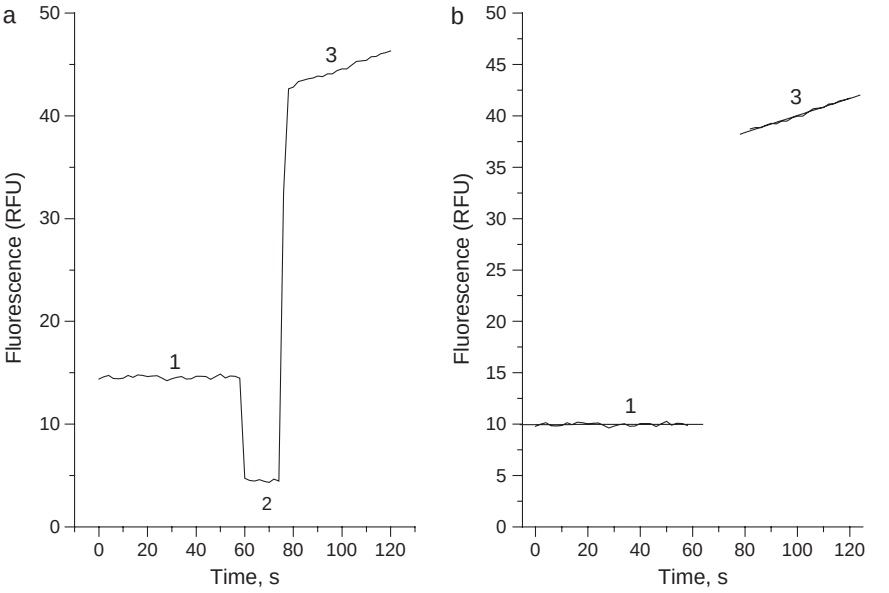

Figure 2. (A) Fluorimeter reading after the addition of aorta extract to a cuvette at room temperature: [1] at pH = 4; (2) with the closed source of light, NaOH was added at this moment; (3) at pH 7. (B) Determination of DCF fluorescence and the rates of DCF oxidation: $A_1 = 8$, $B_1 = 0$, $A_3 = 36.3$, $B_3 = 0.08$.

Statistical analysis

The results are expressed as a mean ± SEM. The numbers of replicates (n) are given in figure legends or in figures. Statistical analysis was carried out using the paired or unpaired Student's t-test. The p-value of less than 0.05 was considered significant.

Results

Dependence of $DCFH_2$ oxidation and DCF fluorescence on pH

$DCFH_2$ is administered for intracellular ROS determination as a diacetate ester ($DCFH_2$-DA), which is rapidly taken up by cells. Once inside the cell, diacetate residues are removed by esterases, liberating $DCFH_2$, which accumulates intracellularly due to low membrane permeability for $DCFH_2$ [13]. $DCFH_2$ reacts predominantly with highly oxidizing ROS/RNS such as hydroxyl radicals ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$) [8,14,15]. The intracellular concentration of $DCFH_2$ is small compared to that of endogenous ROS/RNS scavengers; therefore, a small amount of $DCFH_2$ is oxidized to DCF. However, this amount of DCF is sufficient to reliably determine ROS/RNS in cells if $DCFH_2$-DA is in the concentration range of 20–50 μm. The ROS/RNS determination artifact can arise if DCF is measured in medium after the treatment of cells with digitonin. $DCFH_2$ and DCF go out of cells and $DCFH_2$ can be oxidized quickly with $^{\bullet}OH$ arising in the reaction of the minor iron admixture with H_2O_2 produced by cells in medium where the concentration of other scavengers of ROS/RNS is small. The oxidation of $DCFH_2$ in medium increases the level of DCF and hence of ROS/RNS being determined. The degree of over-estimation of DCF caused by $DCFH_2$ oxidation during extraction depends on the concentration of H_2O_2 in medium that increases in its turn with the amount of cells producing H_2O_2. The amount of cells is much greater in the case of aorta segments than upon determination of ROS/RNS in cultured cells; therefore, ROS/RNS over-estimation caused by $DCFH_2$ oxidation in medium can be considerable, especially in aorta segments. We studied $DCFH_2$ oxidation in medium during extraction to minimize this process.

We have shown earlier [16] that $DCFH_2$ oxidation in medium increases with increasing amount of serum, temperature and pH. We excluded serum from medium in the present work, but the decrease in

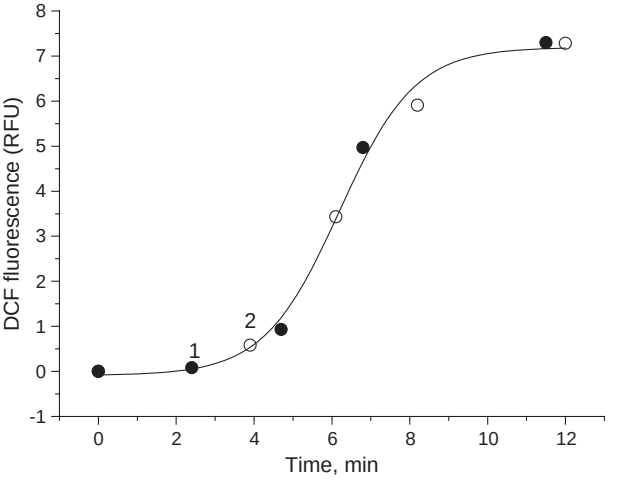

Figure 3. Time dependence of DCF exit from aorta in buffer with (1) 0.02 and (2) 0.04% digitonin (pH 4) at 37°C with stirring in a fluorimeter cuvette. The exit of endogenous substances was subtracted. The fluorescence determination was made at pH 4 ($n = 2$).

temperature was not desirable because a quick extraction of DCF from aorta requires a temperature of 37°C. $DCFH_2$ oxidation ceases at pH 4, but this pH value is not optimal for DCF determination because DCF absorption of excitation light at low pH is small [17]. DCF fluorescence increases with pH ~ 4.4-times as pH changes from 4 to 7 (Figure 1). Figure 2A shows the changes in fluorescence after the addition of an aorta extract to a cuvette at room temperature: *1* is the fluorescence at pH 4; *2* is the fluorescence with the light source closed (NaOH was added at this point); and *3* is the fluorescence at pH 7. It is seen that the fluorescence does not increase at pH 4, indicating that $DCFH_2$ does not oxidize at low pH. The fluorescence increase and DCFH oxidation take place at pH 7. The method of the determination of fluorescence and $DCFH_2$ oxidation rate is depicted in Figure 2B. The value obtained with the closed light source is subtracted from the DCF fluorescence and the fluorescence changes at pH 4 and 7 are approximated by straight lines: $Y = A + BX$, were A is the initial fluorescence value (that is not distorted during determination) and B is the rate of fluorescence change. Subtracting the fluorescence of endogenous substrates from A gives the fluorescence of DCF. The fluorescence of endogenous substrates is ~2–2.5 RFU and does not depend on pH. The B value is 0 at pH 4 and 0.08 RFU/s at pH 7. The fluorescence can increase up to 130 RFU at this rate within 20 min. The fluorescence increase during this time at pH 7.4 and 37°C is much greater (to ~ 490 RFU) (Figure 4). Figure 4 shows the results of experiments that were carried out as follows: all aorta sections were incubated with $DCFH_2$-DA in Hank's solution at pH 7.4, then some sections were extracted at pH 7.4 and the extracts were kept at the same pH value; other sections were extracted at pH 4 and the extracts were kept at pH 4. As is seen in Figure 4, low pH during extraction and storage leads to a sharp decrease in $DCFH_2$ oxidation. The results were the same at pH 4 in citrate buffer and in Hanks' solution (data not shown). However, citrate buffer supports pH more surely in the range of low pH values and it was used for DCF extraction in subsequent experiments. The data (line 5 in Figure 4) show the fluorescence of extracts from aortas that were not incubated with $DCFH_2$-DA. These values were subtracted from the fluorescence of extracts of aortas that were incubated with $DCFH_2$-DA.

Thus, we extracted DCF from aorta with citrate buffer at pH 4 and the extracts were stored at room temperature (for ~ 15 min) until fluorescence determination. Fluorescence was determined at pH 7 and room temperature.

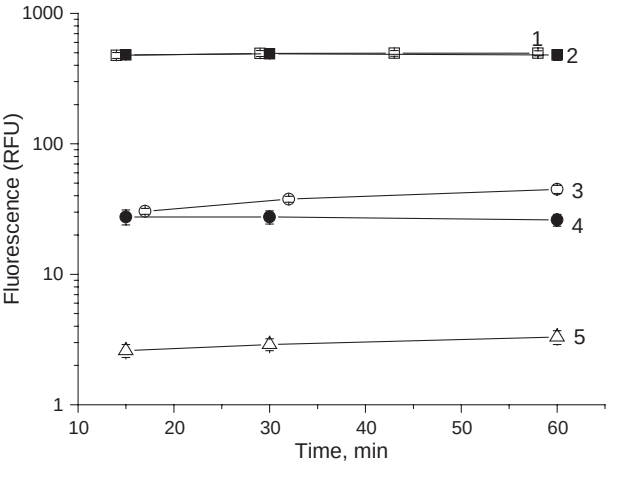

Figure 4. Time dependence of fluorescence changes during the storage of aorta extracts. (1, 2) extraction and storage in Hank's solution, pH 7.4; (1) storage at 2°C; (2) storage at room temperature; (3, 4) extraction and storage in buffer pH 4; (3) storage at 2°C; (4) storage at room temperature; (5) extract of aorta incubated without DCFH-DA ($n = 9$).

Determination of optimal time for the incubation of aorta with $DCFH_2$-DA and for DCF extraction

It is necessary to find the optimal time for the incubation of aorta with $DCFH_2$-DA and for DCF extraction to determine precisely ROS/RNS with the dichlorofluorescein assay. The dependence of DCF increase in aorta and medium during incubation with $DCFH_2$-DA is shown in Figure 5. The DCF content in aorta is maximal at 20 min and then some decrease is seen. The DCF content in medium increases throughout the incubation, with the rate of DCF increase rising 20 min after the onset of incubation. The kinetics of DCF increase in aorta and medium can be explained by a rise in the rate of $DCFH_2$ and DCF exit from aorta after 20 min. The fluorescence values in the aorta and medium can be compared only after taking the volumes of these compartments into account. The volume of an aorta segment is ~ 500-times smaller than the volume of the medium;

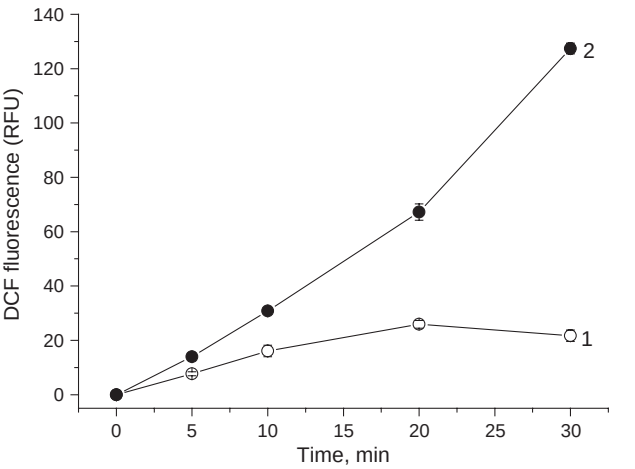

Figure 5. Time dependence of DCF increase in aorta (1) and Hanks' solution (2). Aortas were incubated at 37°C with shaking and 20 μm $DCFH_2$-DA in medium ($n = 3$).

therefore, the DCF concentration in aorta at 20 min (25.9 RFU) is ~200-times higher than in medium (67.2 RFU).

The kinetics of DCF exit from aorta is shown in Figures 3 and 6. The rate of DCF exit at the digitonin concentrations of 0.02 and 0.04% is the same (Figure 3). DCF leaves the aorta more quickly in the cuvette with vigorous stirring (Figure 3) than in flasks with shaking (Figure 6). The DCF exit with shaking is quicker within the first 20 min, after which it is reduced. We restricted the extraction time with shaking to 20 min because more than 70% of DCF was released during this time. The digitonin concentration used for DCF extraction was 0.02%.

Dependence of ROS production on the order number of aorta segment and the age of rat

As was indicated in Materials and methods, aorta sections were numbered from 1–7, starting with the section near to the aorta arc, and sections numbers 1, 3 and 5 were used for ROS/RNS determination in the control. We studied the heterogeneity of ROS/RNS production in different aorta sections. ROS/RNS production of aorta sections 3 and 5 relative to section 1 is shown in Figure 7. ROS/RNS production of aorta section 3 is the same as that of section 1, and section 5 produces ROS/RNS by 14% more than section 1.

The dependence of aortic ROS/RNS production on rat age is shown in Figure 8. ROS/RNS production increases exponentially with age: $Y = Y_0 + A \times e^{x/t}$, where Y is DCF fluorescence (RFU), x is age (days), $Y_0 = 20.6$, $A = 0.79$, $t = 33.7$. With these constants, the doubling of ROS/RNS production takes place 113 days from birth.

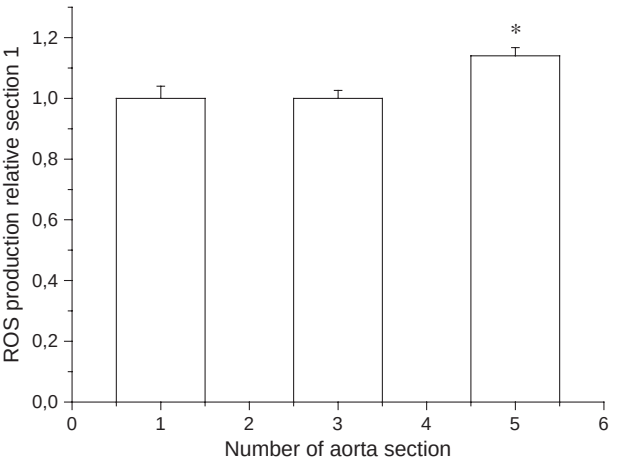

Figure 7. Relative ROS/RNS production by different aorta segments. ROS/RNS production by segment 1 was taken as 1 ($n = 42$). * $p < 0.05$ vs part 1.

Discussion

The main artifact arising in the determination of ROS/RNS by the dichlorofluorescein assay in vessel extracts is $DCFH_2$ oxidation during extraction. We resolved this problem by decreasing pH during extraction. $DCFH_2$ that is released from the aorta is oxidized completely at pH 7.4 within 20 min of incubation at 37°C with shaking. This is evident from the absence of fluorescence increase during a 60-min storage of extracts at pH 7.4 (Figure 4, lines 1 and 2). The fluorescence of extracts at pH 4 is ~ 6% of the fluorescence of extract at pH 7.4. If it is assumed that $DCFH_2$ oxidation is suppressed completely at pH 4, then only 6% of $DCFH_2$ is oxidized in the aorta. The supposition about the complete suppression of $DCFH_2$ oxidation at low pH is supported by the absence of the fluorescence increase during the storage of pH 4 extracts containing a large amount

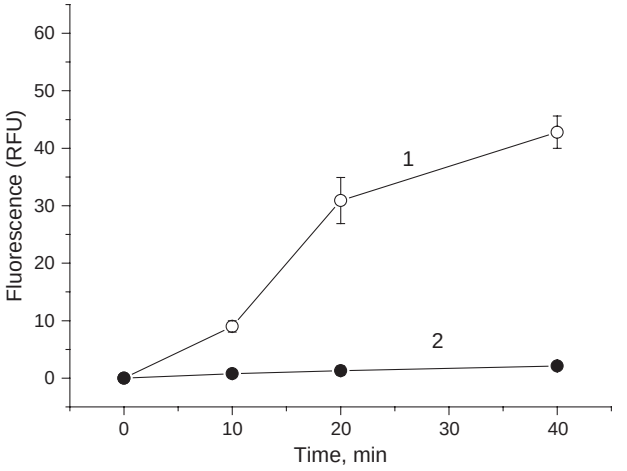

Figure 6. Time dependence of the exit of DCF (1) and endogenous substances (2) after the treatment of aorta with buffer (pH 4) containing digitonin (0.02%) at 37°C with shaking ($n = 6$).

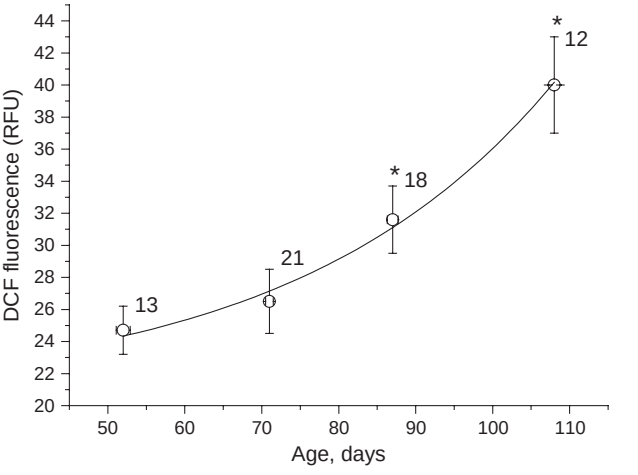

Figure 8. Dependence of aorta ROS/RNS production on rat age. $Y = Y_0 + A \times e^{x/t}$. $Y_0 = 20.6$, $A = 0.79$, $t = 33.7$. The numbers near the mean value indicate the quantity of rats used in averaging. * $p < 0.05$ vs the 52-day-old group.

of $DCFH_2$ at room temperature (Figure 4, line 4). Some increase in the fluorescence of extracts at pH 4 takes place on storage at 2°C (Figure 4, line 3). This effect can be caused by the aggregation of neutral DCF molecules into complexes at low pH and low temperature, which leads to an increase either in light absorption or fluorescence quantum yield. The increase in $DCFH_2$ oxidation with increasing pH is caused by a much more rapid oxidation of phenolates than of phenols [18]. $DCFH_2$ has two pKas (7.9 and 9.2), which is related to the dissociation of phenol substituents [19]. The concentration of phenolates and consequently the $DCFH_2$ oxidation rate should fall ~10 times as pH decreases by one unit in the pH range 7–4 at these pKas values; as a result, DCF increase is not detected in extracts at pH 4 during 30–40 min.

The optimal time for the incubation of aorta with 20 μm $DCFH_2$-DA under shaking at 37°C was determined to be 20 min. The DCF growth stopped in the aorta, but it accelerated in medium after this time. Apparently, the equilibrium of $DCFH_2$-DA concentration between cells and medium was established at 20 min and hence the $DCFH_2$ increase in cells ceased, but the exit of $DCFH_2$ and DCF continued. These processes caused changes in the kinetics of DCF in the aorta and medium.

We have shown that the 5-mm section number 5, which is an intermediate part between the thoracic and abdominal aortas, produces 14% more ROS/RNS than the thoracic aorta. The thoracic aorta also differs from the abdominal aorta by morphological [20] and other features. The rate of Ca^{2+} transport by sarcoplasmic reticulum in the thoracic aorta of rats was greater than in the abdominal aorta [21]. The H_2O_2-induced constriction of the mouse thoracic aorta was less than half of the constriction value for the abdominal aorta [22]. Noradrenaline, endothelin-1 and the thromboxane A_2 mimetic U 46619 increased the formation of inositolphoshate in a concentration-dependent manner and the maximum increase in the rat thoracic aorta was much more pronounced than in the abdominal aorta. Similarly, the maximum contraction evoked by these agents in the thoracic aorta was significantly larger than in the abdominal aorta [23].

The rate of aortic ROS/RNS production, as determined by the dichlorofluorescein assay, is doubled 113 days after birth. Measurements of ROS production, oxidation of macromolecules and the pro-oxidative shift in the cellular redox status showed that ROS production increases with age in different tissues and species [24,25]. It was shown that ROS production by rat vessels also increased with age. For lucigenin chemiluminescence, $O_2^{\cdot -}$ generation in coronary arterioles of 80-week-old rats was significantly greater than of 14-week-old rats [23]. NADH-driven $O_2^{\cdot -}$ generation in vessels of 80-week-old rats was also greater [26]. $O_2^{\cdot -}$ generation in aortas was also significantly higher (2.7-times) in 9–12-month-old compared with 3–4-month-old rats [12]. The oxidative fluorescent indicator 5,6-chloromethyl-2′,7′-dichlorodihydrofluorcein diacetate-acetyl ester was used to study ROS/RNS in vessel endothelium of young (3-month-old) and old (24-month-old) rats by confocal microscopy [27]. An ~3-fold increase in DCF fluorescence in both aged aortas and carotid arteries was shown. Thus, the different methods show the same result: an increase in aorta ROS/RNS production with age.

To summarize, we have developed a method for the registration of total oxidative stress in macroscopic sections of vessels on the basis of the dichlorofluorescein assay. The method was used to study the oxidative stress in different parts of rat aorta and in aortas of rats of different age.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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