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EFFECT OF ELECTROMAGNETIC FIELD WITH 8 Hz FREQUENCY ON THE CELL INJURY AND PROGRAMMED CELL DEATH CAUSED BY NANOSTRUCTURED SILICON AND HYDROGEN PEROXIDE

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Using the method of double cell staining by fluorescent dyes (Hoechst-33258 and propidium iodide) a content of alive, apoptic and necrotic cells in suspension of isolated rat thymocytes and also their morphological features after three hour incubation with nanostructured silicon, 0.1 mM hydrogen peroxide and exposure to electromagnetic field, considering both individual and combined effects, were studied. The apoptic effect of nanostructured silicon (nc-Si) and hydrogen peroxide as the monoinfluences and in combination with the action of 8 Hz was detected. The dynamics of apoptic and necrotic processes were studied. It was found that the exposure of the thymocyte suspension to electromagnetic fields with frequency of 8 Hz combined with the action of hydrogen peroxide and nanostructured silicon in the presence of light leaded to increased number of apoptic cells mainly due to cells with condensed chromatin.

Ключові слова: apoptosis, extremely low frequenciy electromagnetic field, condensed chromatin, nucleus fragmentation, apoptic bodies, hydrogen peroxide, nanostructured silicon.

INTRODUCTION

The development of nanotechnologies and creation of nanotechnological devices and materials puts the problem of biological activity, reliability and safety of nanotechnological producs. During the last decades more and more frequently nanostructures and porous silicon has been applied for solution of different technical, medical and nanobiotechnological purposes. It has been used to provide active basis for the creation of sensor (for instance, gas sensors) and microreactor configurations. Rapidly responding (≤2s), reversible, sensitive (≤2 ppm), and operating at room temperature, porous silicon gas sensors have been based on a uniquely formed highly efficient electrical contract to the nanopore covered microporous array and have been modified by introducing active nanoparticles for gas selectivity [1]. As the part of the planar micro-pore electroporation technology silicon based micro-pore has been very important for new devices for manipulation with single cells [2]. Ordered mesoporous silica (e.g. SBA-15 [3], TUD-1 [4], HMM-33 [5], and FSM-16 [6]) is applied in catalysis, imaging being filled with a fluorescent dye and is showed potential to boost the dissolution in vitro and in vivo of poorly water soluble drugs [7]. The large surface area of the pores allows to sorb a drugs or a toxins. Some types of cancer cells have taken up more of the particles than healthy cells have, giving researchers to hope that MCM-41 (one of the most common types of mesoporous nanoparticles) would one day be used to treat certain types of cancer [7]. Besides silicon compounds such as silica sand or quartz have been most commonly found in nature as well as in the cell of living organisms. Although silicon has been readily available in the form of silicates, biologists has used it in very limited occasions in the form of silicic acid and soluble silicates. Diatoms, radiolaria and siliceous sponges have used biogenic silica as a structural material to construct skeletons. In higher plants the silica phytolit have been revealed in form of microscopic bodies in the cells [8,9]. Silica manufactured in several forms including fused quartz, crystal, fumed silica (or pyrogenic silica, trademarked Aerosil or Cab-O-Sil), colloidal silica, silica gel, and aerogel. In addition, silica nanosprings produced by the vapor-liquid-solid method at temperatures as low as 350°C [10]. It should be mentioned that inhaling finely divided crystalline silica dust in very small quantities (0.1 mg/m3) over time could lead to silicosis, bronchitis or cancer. This effect could be an occupational hazard for people working with sandblasting equipment, products that contained powdered crystalline silica and so on [11].

The application of nanocrystalline silicon (a powder form in water or other medium) for the effective oxidation of various objects in the presence of dissolved oxygen has been widely known in various fields of science. It should be mentioned that silicon nanocrystals have been efficient sensitizers of spin-dependent excitation process of singlet

oxygen molecules. Under photoexcitation epy silicon nanocrystals could efficiently transfer their energy to the molecules of O_2 adsorbed on the surface of nanocrystals. As a result, the transition of the molecule from the triplet to the singlet state was observed. Certain characteristics of silicon (for example photoconductivity) have been altered under the influence of electromagnetic fields [12].

It was very important to investigate the influence of silicon as an oxidative factor on the cells in different conditions, especially in those with the strengthening impact. That was one of the reasons why the actions of chemical factors (hydrogen peroxide) and low-frequency electromagnetic wave spectrum were chosen in a present studies. Moreover it was shown that hydrogen peroxide and electromagnetic waves of low frequency range can induce cell death, including apoptosis [13]. In order to explore how nonstructural silicon influences cells, we examined content of alive, apoptic and necrotic cells in suspension of isolated rat thymocytes and also their morphological features after three hour of incubation with silicon, 0.1 mM hydrogen peroxide and exposure to electromagnetic fields, considering both individual and combined effects using the method of double cell staining by fluorescent dyes.

As a model for studying the influence of electromagnetic waves of low frequency range (8 Hz) on the processes caused by the action of silicon and hydrogen peroxide (0.1 mM) as an oxidative factor suspension of isolated thymocytes were selected because they were not fully differentiated cells, characterized by greater instability of genome than other cells, and lower activity of DNA reparation systems of single strand breaks, which facilitates the activation of apoptosis while they were exposed to various factors. The use of isolated thymocytes for analysis of apoptosis in vitro allowed us carry out morphological control of thymocytes and to identify cells with fragmented chromatin and apoptotic bodies.

It was significant to examine role of silicon in oxidative cell damage upon extremely low frequency electromagnetic influence as well as at the action of hydrogen peroxide and also possible effects caused by the combined influence of these factors. Thus, we could identify potential approaches to control and regulate the intensity of programmed cell death, and also the location and links involved in the induction, which in turn might be important for preventive, diagnostic and therapeutic developments. So, the purpose of research was to study the viability, early morphological changes of chromatin structure in thymocytes and to assess the level of DNA fragmentation after three-hour exposure to silicon, hydrogen peroxide and extremely low frequency electromagnetic field.

MATERIALS AND METHODS

Thymocytes were obtained from the thymus of Wistar rats with weigh about 120-150 g which were kept in standard vivarium diet. Dedicated thymus was grinded through the filter of synthetic fibers (Ø = 0,1 mm) in buffer solution with following composition (g / l): NaCl - 6,796; KCl - 0,274; CaCl2 - 0,288; NaHCO3 - 2,091; KH2PO4 - 0,299; MgSO4 - 0,144 ; glucose - 1,8; (pH 7.4). The number of cells was counted by light microscope in the chamber of Horyayev using dye (0,4% solution of trypan blue).

Incubation of thymocytes (2-4 × 106 cells / ml) was performed in a water thermostat at 37°C in a stationary medium RPMI-1640 with the addition of 2.05 mM glutamine. Incubation was been carried out for 3 hours with light and without it.

Cells (thymocytes) in the form of suspensions were subjected to effect of the factors thereafter the morphological changes which were identified by double supravital staining using fluorescent dyes were observed. In particular, we used Hoechst-33 258 which easily penetrated the cell membrane and bound to DNA in the nucleus, causing fluorescence in the blue spectral region [14]. We used also propidium iodide that penetrate via membranes only necrotic cells and intercalated in DNA, causing fluorescence in the red spectral region [15].

Samples were subjected to the electromagnetic field treatment which was created by the Helmholtz rings. Impulses were rectangular with different polarity. Frequency of the electromagnetic field was 8 Hz with induction of magnetic component 25 μT. Frequency of the magnetic field was selected due to its ecological and geophysical significance [16]. Magnetic vector was parallel to vector of the geomagnetic field. The samples contained cell suspensions were set down in Helmholtz rings. Control samples were in the conditions of the electromagnetic fields background commonly appropriate to the laboratory (20-65 nT). To assess the reliability of the impact of the electromagnetic fields of low frequencies we used Student's t-test for independent samples linked in pairs.

In this experiment we used the following conditions: control cell suspension; cell suspension that subjected to the effects of nanocrystalline silicon with the size of pores and crystallites of 2-5 nm; the electromagnetic influence with frequency of 8 Hz during incubation with the addition of hydrogen peroxide to a final concentration of 0.1 mM in the incubation medium; a combination of electromagnetic influence with hydrogen peroxide and nanocrystalline silicon.

To evaluate the content of viable, necrotic and apoptotic cells in the suspension we used method of double supravital staining with fluorescent dyes Hoechst-33 258 (the company SIGMA - Germany) and propidium iodide (firm SIGMA - Germany) (final concentration of dyes in suspension was 10 μM). To wash away the incubation medium the buffer was used. Thereafter thymocytes have been stained by fluorescent dyes for 15 min in the dark at room temperature. Stained cells were washed again and have been fixed in the dark with 4% buffed formalin solution (pH 7.4) for 5 min and were

washed away from formalin by same buffer. Aliquots of cell suspension were deposited on the slides; swabs were made and dried in the dark. Morphological assessment of cells was performed using fluorescent microscope Leica DM1000 (eyepiece × 10, object lens × 100). In each sample at least 2000 cells (4 counts of 500 cells) were analyzed. Statistical processing of results was performed by conventional methods of variation statistics using Student's t-test (assessment of reliability differences between statistical sampling).

RESULT AND DISCUSSION

As a result of experimental series the data on the quantitative estimation of living, necrotic and apoptotic cells' content in the suspension of thymocytes in control and after the effect of factors were obtained. Apoptotic cells were divided and classified by morphological characteristics as a result of supravital staining of cells with two fluorescent dyes. This classification was used in the research of apoptosis processes [14, 17,18].

The data presented in Figure 1a indicate that after tree hour exposure of electromagnetic field (EMF) with frequency of 8 Hz, silicon and hydrogen peroxide the reduce of number of viable cells in comparison with control in different ways was observed. The largest decrease in viability (24%) was observed under action of hydrogen peroxide. Under the action of EMF with 8 Hz frequency insignificant decrease of 2.5-3% in viability was observed. The number of viable cells in the presence of nanostructured silicon depended on the presence of light. In the absence of light the quantity of viable cells decreased insignificantly by 2,4%, but when silicon was activated by light it decreased by 5.6% compared to control that might be due to the formation of reactive oxygen species.

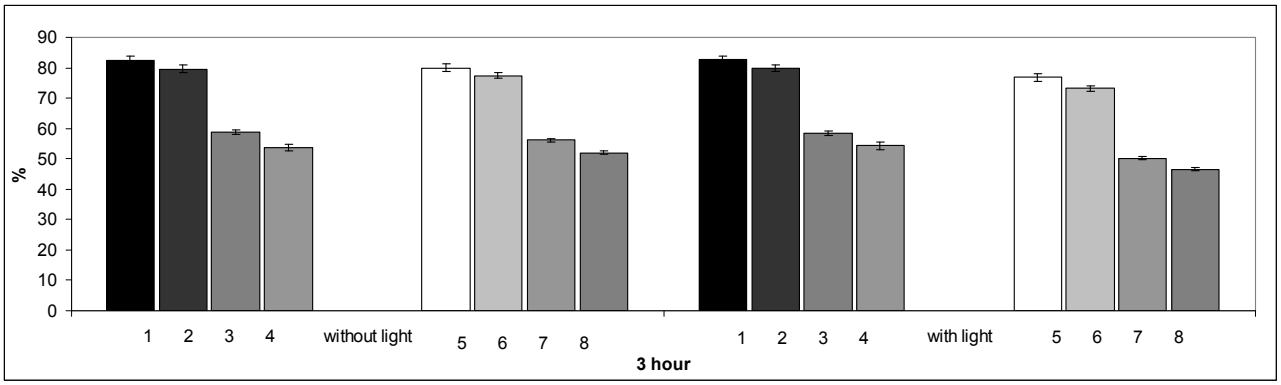
In some cases a significant reduction in the number of viable cells was observed when we combined different factors influence with the action of silicon. Injuries caused by silicon depended on the presence of light. After the combined action of 0.1 mM H_2O_2 and silicon without light the amount of viable cells reduced by 26.2%, and in the presence of light their number reduced by 32,1% compared with control. After the combined action of EMF and silicon the number of viable cells decreased insignificantly compared to the impact of hydrogen peroxide. Namely, in the absence of light the number of viable cells reduced by 5%, while in its availability it decreased by 9,4%. Therefore, we made an assumption that the EMF with frequency of 8 Hz enhanced oxidative cell damage caused by silicon, but only in the presence of light. This fact was also confirmed in the further investigations where we compared the combined effect of H_2O_2 and the EMF and the combined effect of nanostructured silicon with H_2O_2 and the EMF in the presence of light and without it. The combined influence of H_2O_2 and EMF reduced the number of viable cells nearly by 28 % compared with control in the presence of light as well as in its absence. The combined influence of silicon, H_2O_2 and EMF reduced the number of viable cells nearly by 30,4% compared with control in the privation of light, in the presence of light the decline of viable cells was 36%. Thus, the quantity of viable cells was considerably reduced by influence of silicon in the presence of light due to its oxidative properties and the effect was enhanced by the EMF with frequency of 8 Hz.

The number of necrotic cells did not significantly changed relative to the control under both action of nanostructured silicon, EMF, 0.1 mM H_2O_2 and the combined action of aforementioned factors (fig.1b). Only slight tendency of reduction of necrotic cells was marked in the experiment with combined influence of silicon, H_2O_2 and EMF in the presence of the light. After the combined action of 0.1 mM H_2O_2 and silicon in the presence of the light the amount of necrotic cells was 6.5%. When we added to this effect the influence of EMF the number of necrotic cells was not significantly changed (4,3%) comparatively without light.

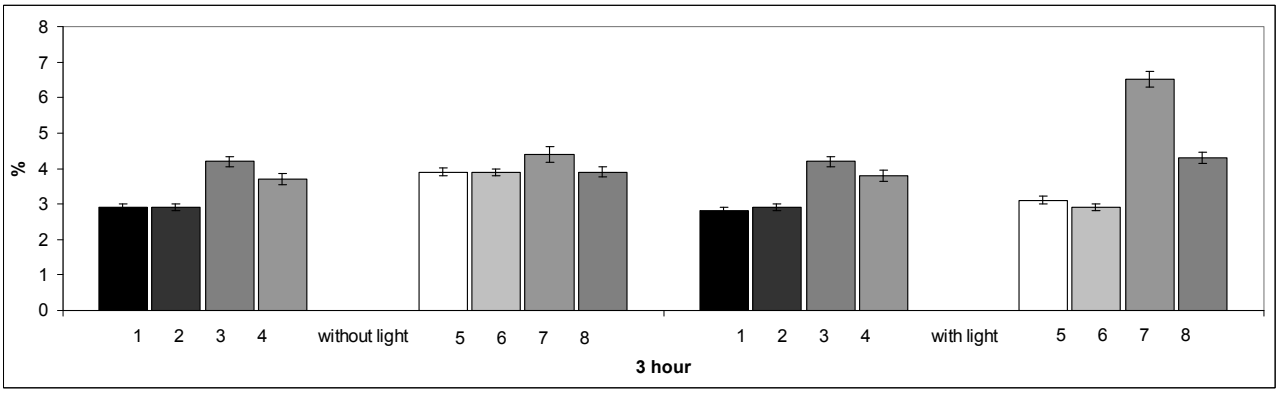
As seen from the data presented in fig.1c the viability of thymocytes was reduced by the apoptotic processes caused by factors of influence. Speaking about influence it should be mention that the largest augmentation of quantity of apoptic cells on 37% was observed under action of hydrogen peroxide both with and without light. Under the action of EMF the amount of apoptic cells was 17,5%. The number of apoptic cells in the presence of nanostructured silicon depended on the availability of light. Thus, in the absence of light the quantity of apoptic cells was 16,1%, when silicon was activated by light it amounted 20,1%, what made 4% difference that might occur due to the formation of reactive oxygen species.

The combined effect of nanostructured silicon and EMF increased the amount of apoptotic cells by 6% and 9.3% respectively in the absence of light and when it was present in comparison with control. It could be assumed that EMF strengthened the influence of nanostructured silicon as an oxidative factor so long as the addition of EMF effect increased the quantity of apoptic cells by 4,6% and 3,8% accordingly in the presence of the light and without it in comparison with the influence of nanostructured silicon only. The combined influence of H_2O_2 and EMF enhanced the number of apoptic cells nearly by 28 % compared with control in the presence of light as well as in its absence. It was interesting to compare the results of the combined action of 0.1 mM H_2O_2 and silicon with the combined action of silicon, H_2O_2 and the EMF in the presence of light and without it. In the absence of light the combined action of 0.1 mM H_2O_2 and silicon augmented the number of apoptic cells by 24,7% compared with control, if the influence of EMF added to the effect of H_2O_2 and silicon the amount of apoptic cells increased by 3,9% in comparison with the combined action of H_2O_2 and silicon. In the presence of light the combined action of H_2O_2 and silicon augmented

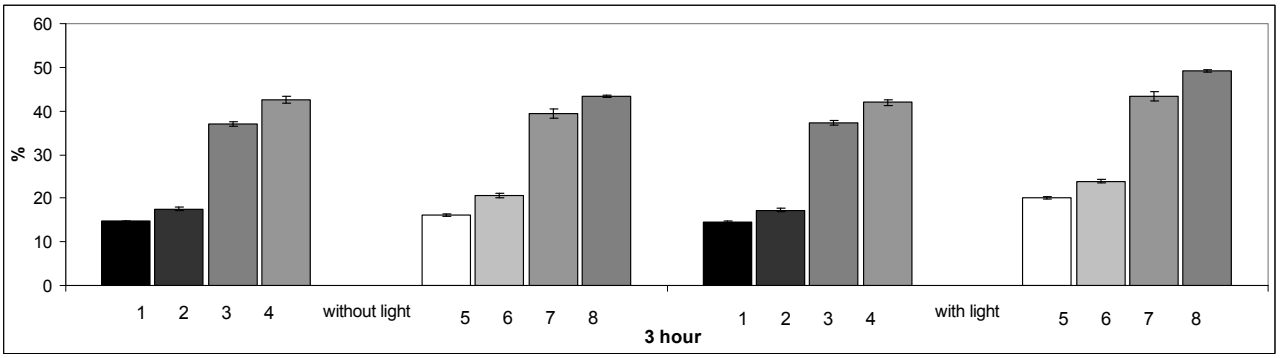
the number of apoptic cells by 28,7% compared with control, if the influence of EMF was added to the effect of H_2O_2 and silicon the amount of apoptic cells increased by 5,8% in comparison with the combined action of H_2O_2 and silicon. These results indicated that cell death occured mainly by apoptosis under the influence of the these factors. The assumption also could be made that under photoexcitation the nanostructured silicon was activated and could effectively transfer their energy to O_2 molecules adsorbed on the surface of nanocrystals, as a result the transition from triplet molecules in the singlet state was possible. This might explain the enhancement of apoptic cells amount under influence of silicon in the presence of light as well as the impact of EMF in combined action, where EMF intensify the oxidative effect.



a) Viable cells

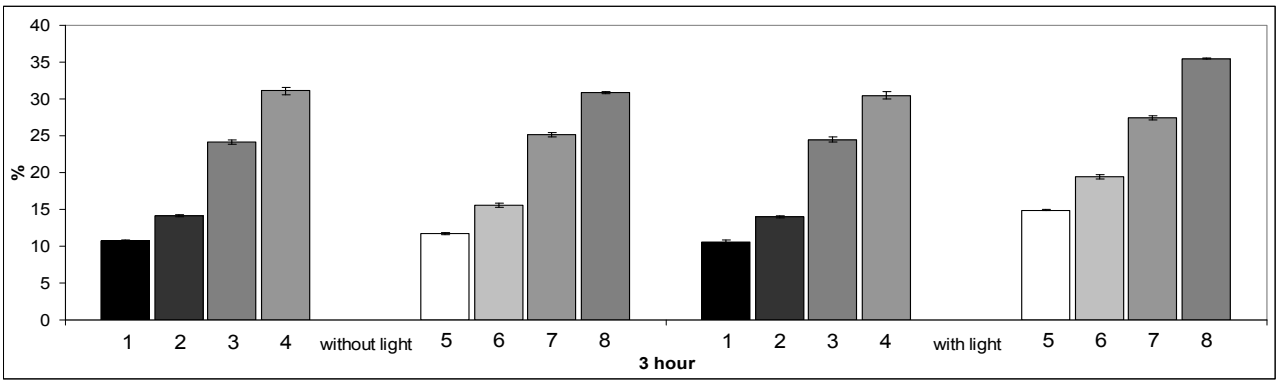


b) Necrotic cells

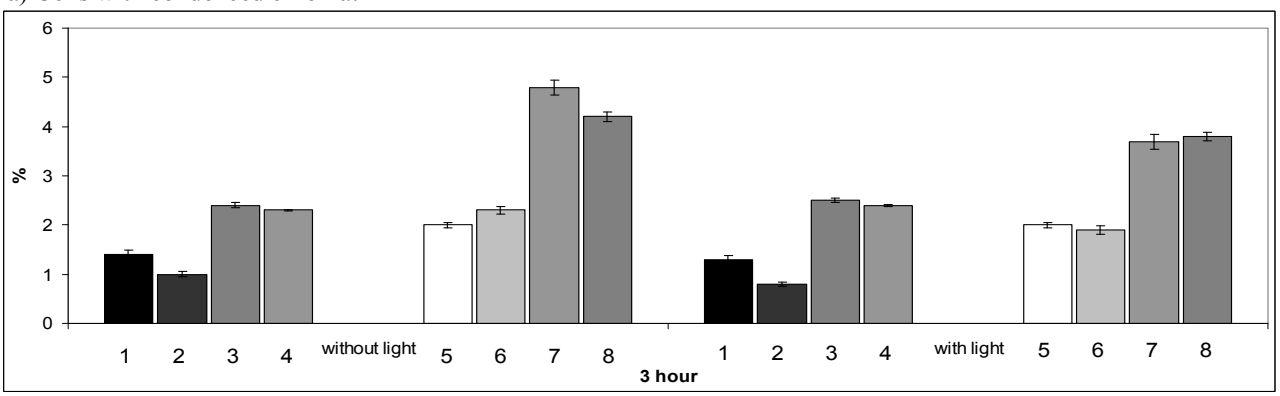


c) Apoptic cells

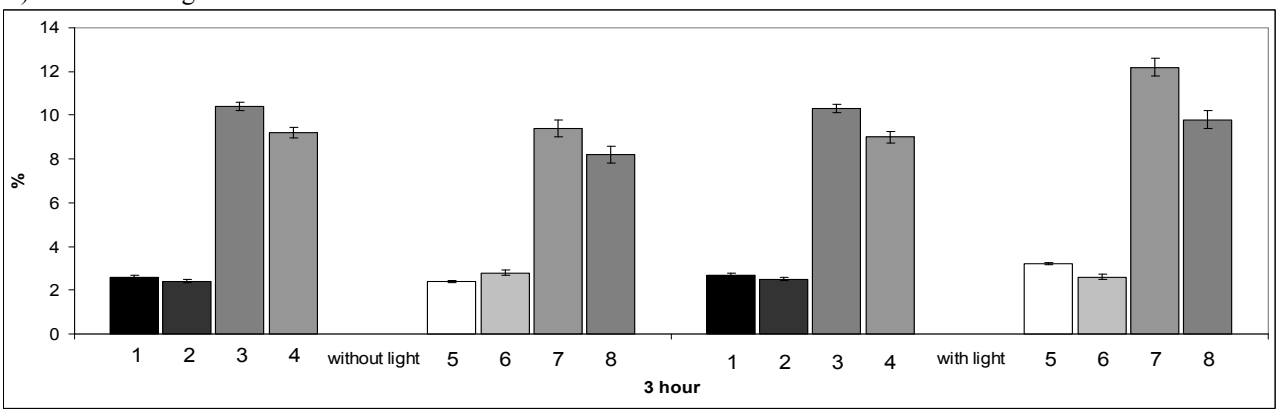
Fig. 1. Cell viability.
1) control; 2) 8 Hz 3) H_2O_2; 4) 8 Hz +H_2O_2; 5) nc-Si; 6) nc-Si +8 Hz; 7) nc-Si + H_2O_2; 8) nc-Si +8 Hz +H_2O_2



a) Cells with condenced chromatin



b) Cells with fragmented nucleus



c) Cells with apoptic bodies

Fig. 2. Apoptic cells.
1) control; 2) 8 Hz 3) H_2O_2; 4) 8 Hz +H_2O_2; 5) nc-Si; 6) nc-Si +8 Hz; 7) nc-Si + H_2O_2; 8) nc-Si +8 Hz +H_2O_2

As known from the literature the main morphological feature of apoptosis in thymocytes caused by the action of H_2O_2 in the relevant concentration is to increase the number of cells with condensed chromatin. And it was common knowledge that chromatin condensation is an intermediate stage of apoptosis that accompanied by its fragmentation and the appearance of apoptotic bodies if there is a significant number of broken DNA [19]. The same trend we observed in our experiment when the number of apoptotic cells increased mainly due to cells with condensed chromatin (fig. 2a). In the absence of light in samples exposed to nanostructured silicon the negligible fluctuations of apoptotic cells amount were observed whereas under influence of EMF and H_2O_2 the number of apoptotic cells with condensed chromatin increased by 3,4% and 13,5% accordingly compared with control. Speaking of the combined effect of the factors it should be said that in the absence of light the addition of nanostructured silicon realised to inconsiderable impact while in the presence of light its contribution was significant. So, in samples exposed to nanostructured silicon the amount of apoptotic cells with condensed chromatin was 4,3% higher than in control, in combination with influence of 8 Hz EMF and H_2O_2 the impact of silicon was considerable: the number of apoptotic cells with condensed chromatin increased by 5% and 2,9% accordingly compared with separated effects of these factors. In the presence of light under combined actions of nanostructured silicon, H_2O_2 and EMF the amount of apoptotic cells with condensed chromatin was 4% higher than under combined action of only H_2O_2 and EMF that proved the importance of nanostructured silicon impact to the combined influence. On the other hand, in the presence of light under

combined actions of nanostructured silicon, H_2O_2 and EMF the amount of apoptotic cells with condensed chromatin was 7,1% higher than under combined actions of only H_2O_2 and nanostructured silicon that proved the importance of EMF impact to the combined influence.

The combined influence of silicon and H_2O_2 in combination of EMF and without it the number of the apoptotic cells increased by 3,4% and 2,8% respectively in the absence of light and by 2,4% and 2,5% respectively in the presence of light (fig. 2b).

There were an increased number of thymocytes with apoptic bodies in the investigated samples exposed to H_2O_2 and combined effects (fig. 2c). Under the influence of H_2O_2 the quantity of thymocytes with apoptic bodies increased by about 8% both in the presence of light and in its absence compared with control samples. The combined effect of nanoporous silicon and H_2O_2 enhanced the number of cells with apoptic bodies by 6,8% and 9,5% respectively in the absence of light and in its presence compared with control samples. The combined effect of H_2O_2 and EMF increased the amount of cells with apoptic bodies by approximately 6.5% both in the presence of light and in its absence and the combined effect of nanoporous silicon with H_2O_2 and EMF augmented the number of thymocytes with apoptic bodies by 5,6% and 6,3% respectively in the absence of light and in its presence compared to control samples.

Obtained results allow as suggest that exposure to EMF with frequency 8 Hz in some way activated several mechanisms of apoptosis via H_2O_2-dependent and H_2O_2-independent ways. Under photoexcitation the silicon nanocrystals could efficiently transfer their energy to the molecules of O_2 adsorbed on the surface of nanocrystals. As a result, the transition of the molecule from the triplet to the singlet state is realised and nanoporous silicon could demonstrate oxidative effect. Thus, the generation of singlet oxygen caused by illumination of ensembles of silicon nanocrystals can be proposed to usisng for the suppression of reproduction of cancer and other cells. Presumably the suppression efficiency would be higher under the combined impact of nanostructured silicon and EMF of 8 Hz frequency. To confirm this assumption a further studies were needed.

Overall it should be noted that the mechanisms of biological effect of extremely low frequency magnetic fields are insufficiently researched but despite this fact the magnetic fields are used quite effectively in modern medical practice. In particular they are used for antiinflammatory therapy, accelerating tissue regeneration and improvement of microcirculation. It was believed that the physical basis of this action was to coordinate the motion of charged particles [20]. The result of this interaction was primarily the change in membrane potential and activity of lipid peroxidation. In addition, the magnetic field influence the physical and chemical properties of water, free-radical chemical reactions, macromolecule of large anisotropic diamagnetic compounds [20].

CONCLUSION

1. The quantity of viable cells was considerably reduced and the amount of apoptic cells was increased by influence of nanostructured silicon in the presence of light due to its oxidative properties.

2. The combined influence of electromagnetic field with frequency 8Hz 25 μT enhanced the effect caused by the action of oxidative factors such as hydrogen peroxide and nanostructured silicon in the presence of light that reduced viability of thymocytes by increasing the number of apoptic cells.

3. The increase of the amount of the apoptic cells exposed to hydrogen peroxide, nanostructured silicon and in combination with electromagnetic field with frequency 8Hz 25 μT was mainly due to cells with condensed chromatin.

4. Slight tendency in reduction of necrotic cells was marked in the experiments with combined influence of silicon, H_2O_2 and electromagnetic field with frequency 8Hz 25 μT in the presence of the light.

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ВПЛИВ ЕЛЕКТРОМАГНІТНОГО ПОЛЯ ЧАСТОТОЮ 8 Гц НА КЛІТИННЕ УШКОДЖЕННЯ ТА ЗАПРОГРАМОВАНУ КЛІТИННУ ЗАГИБЕЛЬ СПРИЧИНЕНУ ДІЄЮ НАНОСТРУКТУРОВАНОГО КРЕМНІЮ ТА ПЕРОКСИДУ ВОДНЮ

Собко В.М. , Мартинюк В.С. Шевченко В.Б., Ратушна О.О.

Методом подвійного прижиттєвого фарбування клітин за допомогою флуоресцентних барвників - Hoechst-33258 та пропідіум йодид, - досліджували вміст живих, некротичних та апоптуючих клітин, а також їх морфологічні особливості у суспензії ізольованих тимоцитів щурів після трьохгодинної інкубації з наноструктурованим кремнієм, 0,1мМ пероксидом водню та при впливі електромагнітного поля, як при окремому, так і комбінованому впливі. Виявлено апоптогенний ефект наноструктурованого кремнію та пероксиду водню, як моно впливу, так і в поєднанні з дією 8 Гц, встановлена динаміка некротичних та апоптичних процесів, зокрема динаміка конденсації хроматину, фрагментації ядра та утворення апоптичних тілець. Встановлено, що вплив електромагнітного поля частотою 8 Гц у комбінації з дією наноструктурованого кремнію та пероксидом водню на суспензію тимоцитів призводить до збільшення загальної кількості апоптуючих клітин, в основному за рахунок клітин з конденсованим хроматином. Зроблено припущення, що вплив 8 Гц в певній мірі активує запуск механізмів апоптозу, але як захисну відповідь на перекисне пошкодження.

Ключові слова: апоптоз, низькочастотне електромагнітне випромінювання, конденсований хроматин, фрагментоване ядро, апоптичні тільця, пероксид водню, наноструктурований кремній.

ВЛИЯНИЕ ЭЛЕКТРОМАГНИТНОГО ПОЛЯ ЧАСТОТОЙ 8 Гц НА КЛЕТОЧНОЕ ПОВРЕЖДЕНИЕ И ЗАПРОГРАМИРОВАННУЮ КЛЕТОЧНУЮ ГИБЕЛЬ ВЫЗВАННУЮ ДЕЙСТВИЕМ НАНОСТРУКТУРИРОВАННОГО КРЕМНИЯ И ПЕРЕКИСИ ВОДОРОДА

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Методом двойного прижизненного окрашивания клеток с помощью флуоресцентных красителей - Hoechst-33258 и пропидиум йодид, - исследовали содержание живых, некротических и апоптических тимоцитов крыс, а также их морфологические особенности в суспензии после трехчасовой инкубации с наноструктурированым кремнием, 0,1 мМ пероксидом водорода и при воздействии электромагнитного поля частотой 8 Гц, как при отдельном, так и комбинированном воздействии. Выявлено апоптогенный эффект наноструктурированного кремния и пероксида водорода, как моно воздействия, так и в сочетании с действием 8 Гц, установлена динамика некротических и апоптических процессов, в частности динамика конденсации хроматина, фрагментации ядра и образования апоптичных телец. Установлено, что в условиях индуцированного наноструктурированым кремнием и пероксидом водорода повреждения клеток воздействие электромагнитного поля частотой 8 Гц на суспензию тимоцитов приводит к увеличению общего количества апоптических клеток, в основном за счет клеток с конденсированным хроматином. Сделано предположение, что влияние 8 Гц в определенной мере активирует запуск механизмов апоптоза, как защитный ответ на перекисное повреждение.

Ключевые слова: апоптоз, низкочастотное электромагнитное излучение, конденсированный хроматин, фрагментированное ядро, апоптические тельца, пероксид водорода, наноструктурированый кремний.