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APPLICATION OF GAMMA RAY TECHNIQUES IN CELL CULTURE MEDIUM STERILIZATION

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Abstract.

Objective of this study was the search for the optimal modes of gamma-ray decontamination of culture media used for virus reproduction. The following culture media were used in the study: Eagle's Minimum Essential Medium (EMEM), medium 199, lactalbuminhydrolyzate medium, Hanks' balanced salt solution (HBSS), plain broth, plain agar, Kitt-Tarozzi medium, thioglycolate medium, Sabouraud agar, bovine serum; as well as transplantable cell culture lines: calf kidney (MDBK), bovine embryo lung (LEK), African green monkey kidney epithelium (Vero). To simulate the artificial cell contamination the asporogenous (*St.aureus*, *E.coli*) and sporogenous (*B.subtilis*) bacteria and infectious bovine rhinotracheitis (IRT) and parainfluenza-3 (PI-3) viruses were used. Simulation of artificial contamination of culture media was performed by adding 0.2 ml of the virus suspension with a titer of virus 6.0 lg of tissue cytopathic dose (TCD 50/ml) and bacterial agents at a dose of 1.5×10^6 CFU/ml to 100 ml culture media. Both native and contaminated culture media were subjected to gamma-irradiation using the device "Issledovatel" IN-1 (Russia) in the dose range of 0.1 to 1.0×10^4 Gy. The results of microbiological studies have shown that the required doses of gamma rays for the decontamination of native dry media ranged 0.5 to 1.0×10^4 Gy, and for liquid media - 1.0 to 2.0×10^4 Gy. A reliable radiosterilization was achieved at artificial contamination at a dose of gamma rays of 3.0×10^4 Gy. The results of karyological and biochemical studies have shown that exposure to culture media at the indicated doses did not result in significant changes in their basic parameters, such as transparency, pH, total protein and its fractional composition, and lipids. The results obtained during the investigation of the influence of irradiated environments on growth performance and reproduction of cell culture lines on the irradiated culture media did not differ from those cultured on the unexposed (control) media.

Keywords: viruses, radiation biotechnology, radiostimulation, genome instability.

Introduction.

The growing scale of production of the diagnostic and therapeutic and preventive drugs requires improvement of the cell cultivation technology, and, above all, the decontamination methods of culture media [1]. It is known that the level of contamination of the bovine blood serum and the cell cultures of animal organs and tissues with mycoplasma, bacteria and viruses accounts for 10 to 82%. Currently, the most common method of decontamination of the growth media and the cell lines is to use microbial antibiotics and surfactants [2,3]. However, the rapid formation of antibiotic-resistant types of microorganisms prevents from efficient use of existing methods of decontamination of cell lines with the use of antibiotics. A number of researchers use resonance-frequency method and radiation sterilization as alternative decontamination methods of these objects, providing for reliable decontamination of culture media by their irradiation with gamma rays at doses of $2.5-3.5 \times 10^4$ Gy [4] and electromagnetic effect at a frequency of 95.56 Hz [5]. The use of low doses of ionizing radiations (0.05-10.0 Gy), as opposed to high doses (sterilizing) ($1.0-3.3 \times 10^4$ Gy) has, on the contrary, growth-stimulating effect and contributes to the increase by 1.5-2 times in the number of the cell population of lymphocytes, fibroblasts and other cells of organs and tissues of animals and humans [6]. A number of investigators have found that the pre-irradiation of lymphocytes at low doses leads to increased resistance of the cells to the subsequent lethal irradiation and improves their proliferative activity [7], which is associated with the induction of radiation-induced genomic instability (RIGI), and may have both negative and positive consequences.

Materials and Methods.

The following culture media were used in the study: 0.5% lactalbuminhydrolyzate (LAH) dissolved in Hanks' solution (RPA "Vector", Russia); Eagle's minimum essential medium (EMEM)(pH 7.5-7.6) with glutamine (RPA "Vector", Russia); synthetic medium 199 (RPA "Vector", Russia); standard Hanks' balanced solution, pH 7.2-7.4; trypsin solution; trypsin and Versene solution; sterile normal bovine serum; bovine fetal serum. Virological studies of the cytopathic effect (CPE) on cell cultures were carried out using a virus of infectious rhinotracheitis (IRT), vaccine strain TK-(ARIEV)-B2 (All-Russian Institute of Experimental Veterinary, Moscow) adapted to growth on MDBK and type I reovirus of Lang reference strain (UK), to which the Vero line is sensitive [8]. The passaged cell cultures were maintained by conventional method of successive passages in accordance with the "Instructions for the preparation of culture media and cell cultures" (1987). For cell dispersion, a mixture of 0.02% Versene solution and

0.25% trypsin solution were applied during passage at a ratio of 9:1 and 3:1 (depending on the type of cultures) at a temperature of $37.5\pm 0.5^{\circ}\text{C}$. The proliferative activity of the cell cultures was determined by microscopic examination, and the proliferation index was calculated by conventional method [8]. Cell cultures grown on the selected nutrient media were exposed to ^{137}Cs gamma-rays at a dose of 0.05; 1; 2; 3; 4; 5; 6; and 7 Gy (depending on the experimental conditions) with the specific absorption rate of $3.43\times 10^{-3}\text{Gy/sec}$. In addition to the analysis of chromosomes in the irradiated cells, the degree of their DNA fragmentation was determined by modified DNA electrophoresis of single cells immobilized in agarose according to the number of double-stranded DNA [7]. The sterility of cells, culture media, sera, solutions and viral materials was determined through inoculation on plain broth, plain agar, Kitt-Tarozzi and Sabouraud media. Both artificially and spontaneously contaminated culture media were irradiated on a gamma-ray device "Issledovatel" IN-1 (Russia) with ^{60}Co radiation source at doses of 1.0×10^3 to $2.5\times 10^4\text{Gy}$. Total protein content in the intact and irradiated blood serum was determined by conventional methods on the biochemical analyzer Stat Fax 3300 (USA). Chromosomal preparations for cytological (karyological) analysis were prepared by standard colchicine method, followed by staining with 2% Giemsa aqueous solution. The number of chromosomes in metaphase plates was counted in 50-100 cells of each line. The virus titer was calculated by the Reed-Muench method and expressed as logarithms $\text{TCD}_{50}/\text{cm}^3$. Immunological competence of viruses was determined by serologic method through creating a hemagglutination inhibition reaction (HGIR) by conventional method. The viral sensitivity was determined by infecting the cell cultures with virus material with subsequent incubation and determination of virus titer in accordance with conventional methods applied in the industrial biotechnology.

Results. The first series included experiments for the determination of the optimum gamma ray doses for the decontamination of both dry and liquid culture media under natural contamination of organisms. The data in Table 1 shows that the reliable decontamination of dry culture media was achieved by irradiation at doses of $0.5-1.0\times 10^4\text{Gy}$. In addition, the reliable decontamination of medium 199 and MEM required irradiation at a dose of $0.5\times 10^4\text{Gy}$, and bovine and LAH sera - $1.0\times 10^4\text{Gy}$.

Table 1. Doses of γ -rays, ensuring decontamination of dry and liquid media.

culture medium	γ -ray dose, $\times 10^4\text{Gy}$					
	0.1	0.2	0.5	1.0	2.0	3.0
dry						
Bovine serum	-	-	-	+	+	+

Medium 199	-	-	+	+	+	+
MEM	-	-	+	+	+	+
LAH	-	-	-	+	+	+
liquid						
Bovine serum	-	-	-	-	+	+
Medium 199	-	-	-	+	+	+
MEM	-	-	-	+	+	+
LAH	-	-	-	+	+	+

MEM – minimum Eagle medium, LAH – lactalbuminhydrolyzate; - the lack of effect, + complete medium decontamination.

Given that the level of medium decontamination depends on the concentration of contaminants, the 2nd series of experiments in the decontamination of culture media at artificial contamination with their asporogenous and sporogenous microorganisms was conducted. The data in Table 2 show that a reliable decontamination of culture media, artificially contaminated with all species of asporogenous and sporogenous microorganisms, was achieved by applying γ -rays at a dose of 3.0×10^4 Gy.

Table 2. Doses of γ -rays, ensuring decontamination of liquid culture media artificially contaminated with test-strains.

culture medium	γ -ray dose, $\times 10^4$ Gy, contaminated					
	1.0		2.0		3.0	
	<i>St.aureus</i>	<i>B.subtilis</i>	<i>St.aureus</i>	<i>B.subtilis</i>	<i>St.aureus</i>	<i>B.subtilis</i>
Bovine serum	-	-	+	-	+	+
Medium 199	+	-	+	+	+	+
MEM	+	-	+	+	+	+
LAH	-	-	+	+	+	+

+ positive, - negative result

Considering the literature data that the irradiation of objects with ionizing radiation induces the formation of toxic products of radiolysis and causes changes in the physico-chemical and biochemical properties of the irradiated objects, the following series of experiments were conducted to study the stability of the basic biological parameters of irradiated media. The indication results of toxic products of radiolysis in the gamma-ray irradiated culture medium (bovine blood serum), depending on the dose of ionizing radiation used in the IHAR test-system showed that an increase in irradiation dose applied to the culture medium causes more intensive formation of toxic products of radiolysis –hemagglutinatingquinoidradiotoxins (HAQRT). The results of parallel biochemical studies have shown

that irradiation of blood serum at a dose of $0.1-3.0 \times 10^4$ Gy did not result in significant changes in the main parameters: neither irradiated nor non-irradiated sera had any significant difference in their transparency, pH, total protein, total lipids, fractional composition of proteins (globulins, albumin). Increase in the dose of radiation up to 6.0×10^4 Gy led to opalescence, increase in the optical density by 1.55 times ($P < 0.05$), acidification of medium (decrease in pH by 1.30 times, $P < 0.05$), decrease in the total protein content by 1.1 times ($P < 0.05$), decrease in globulin - by 1.07 times ($P < 0.05$), total protein - by 1.1 times, albumin - by 1.19 times ($P < 0.05$), and lipids - by 1.16 times ($P < 0.05$). The study of proliferative activity of MDBK culture grown on irradiated with different doses of gamma-rays on culture media showed that their irradiation in a dose range of $0.1-3.0 \times 10^4$ Gy has no significant effect on the growth and reproduction of MDBK cells. Thus, the concentration of growing cells irradiated at a dose of 0.1; 0.5; 1.0; and 3.0×10^4 Gy was slightly behind in that of the control, namely by 1.03; 1.04; 1.06; and 1.08 times ($P > 0.05$). It has been established that the cell proliferation index was 3.58 (on media irradiated with 0.1×10^4 Gy), 3.81 (0.5×10^4 Gy), 3.79 (1.0×10^4 Gy), 3.75 (0.3×10^4 Gy) and 3.11 (6.0×10^4 Gy). Therefore, this index in the cells, grown on $0.1-6.0 \times 10^4$ Gy irradiated culture media, was slightly behind (1.01-1.04 times, $P > 0.05$) of the control, which indicates the absence of toxicity of the media irradiated at the indicated doses.

Discussion. Using different doses of gamma rays for the decontamination of native culture media and artificially contaminated with sporogenous and asporogenous microorganisms showed that the final effect depends on both the physical and chemical state of the objects, and the degree of contamination of the test culture media. For example, irradiation of culture media artificially contaminated with bacteria and viruses at a dose of 1.0×10^4 Gy resulted in a partial decontamination, because only two of four used culture media were sterile in relation to asporogenous microbes (*St.aureus*) and viruses (IRT), namely medium 199 and MEM. Contamination of the tested culture media with spore-forming microorganisms (*B.subtilis*) revealed ineffectiveness of gamma-ray dose of 1.0×10^4 Gy - the growth of sporogenous test microbe was observed in the inoculated samples of the irradiated objects.

The use of radiation dose of 2.0×10^4 Gy resulted in sterilization of all culture media contaminated with bacteria (*St.aureus*) and viruses IRT. However, the bovine blood serum and LAH, artificially contaminated with spore-forming microflora (*B.subtilis*), were sterile - the growth of the test strain was observed in the inoculated samples of the irradiated media. A reliable decontamination of culture media, artificially contaminated with all species of asporogenous and sporogenous microorganisms and viruses IRT, was achieved by using γ -rays at a dose of 3.0×10^4 Gy.

We conducted our experiments on culture media, exposed to a wide range of gamma-ray doses: 0.1; 0.3; 1.0; 3.0; 6.0; 10.0; 20.0; 30.0; 50.0x10⁴ Gy. For this, the bovine blood serum was used as a culture medium, which includes all nutritional qualities and contains all components (proteins, amino acids, polysaccharides, lipopolysaccharides, lipids) providing, on the one hand, the comprehensive growth of microorganisms, cultures of animal cells, and on the other hand, the listed components of serum, by being subjected to radiolysis, contribute significantly to the formation of radiation induced toxic products that have a negative effect on their cultured microorganisms and animal cells [9-11]. Therefore, the first stage of this series of studies involved investigation of the toxicity of bovine blood serum irradiated at different γ -ray doses with the use of the biochemical (MDA) and immunochemical (IHAR) test-systems accepted in radiotoxicology. The indication results of toxic products of radiolysis in the gamma-ray irradiated culture medium (bovine blood serum), depending on the dose of ionizing radiation used in the MDA and IHAR test-systems showed that an increase in irradiation dose applied to the culture medium causes more intensive formation of toxic products of radiolysis - TBA-active compounds (MDA) and hemagglutinatingquinoidradiotoxins (IHAR). Although the content of TBA-active products and quinoidradiotoxins at irradiation doses of 0.1 to 3.0x10⁴Gy was 2.5-3.0 times higher than the same in the unexposed media, these doses of toxic radiolysis products for animal cells, however, are low toxic and do not cause apoptotic cell death. The most dangerous concentrations of toxic radiolysis products in the irradiated serum begin accumulating at irradiation doses of 6-10x10⁴Gy, accounting for 5.1±0.31 log₂ - for quinoidradiotoxin. A further increase in the irradiation dose (20-30x10⁴Gy) to the culture medium induces a significant increase in the synthesis of lipid (MDA) and quinoid (HAQRT) radiotoxins, the content of which is 6.6-6.7 mmol/g (malondialdehyde) and 6.1-7.3 log₂(quinoidradiotoxin). The maximum accumulation of toxic radiolysis products of proteins, lipids and lipopolysaccharides contained in serum is observed at irradiation at a dose of 5x10⁴Gy, when the content of TBA-active products (MDA) was 9.5±0.81 (P<0.001), and the content of haemagglutinatingantigen-quinoidradiotoxin (HAQRT) - 9.9±0.43 log₂ (P<001). The results of parallel biochemical studies have shown that irradiation of blood serum at a dose of 0.1-3.0x10⁴Gy did not result in significant changes in the main parameters: neither irradiated nor non-irradiated sera had any significant difference in their transparency, pH, total protein, total lipids, fractional composition of proteins (globulins, albumin). Increase in the dose of radiation up to 6.0x10⁴Gy led to opalescence, increase in the optical density by 1.55 times (P<0.05), acidification of medium (decrease in pH by 1.30 times, P<0.05), decrease in the total protein content by 1.1 times (P<0.05), decrease in globulin - by 1.07 times (P<0.05), albumin - by 1.19 times (P<0.05), and lipids - by 1.16 times (P<0.03).

Summary: 1. As a result of our studies, we have obtained a new radiostimulated transplantable subline of bovine kidney cells - MDBK-02 grown on gamma-ray decontaminated culture medium.

2. Irradiation of one of the most important components of the culture medium - bovine blood serum with ^{137}Cs γ -rays at a dose of $1 \times 10^4 \text{Gy}$ ensures reliable decontamination from both bacterial and viral microorganisms.

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