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Research Article

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Research of the Potential Radioprotective Activity of Trimethyl Glycine and N-Acetyl-L-Cysteine with Quantitative Analysis Using Real-Time Polymerase Chain Reaction (QRT-PCR)

Racheva G[1]

[1]*Research Laboratory of Radiobiology and Radiation Protection, Military Medical Academy–Sofia, Sofia, Bulgaria.*

***Corresponding Author**
Galina Racheva, PhD, Research Laboratory of Radiobiology and Radiation Protection, Military Medical Academy–Sofia, Sofia, Bulgaria.

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Abstract

The irradiation exposure could affect the cellular genome and specifically target genes, such as TP53 gene. The TP53 gene is known as a "guardian of the genome" and is a tumor suppressor gene. Its role is to keep the cell of malignant transformation. After ionizing radiation exposure, the TP53 could mutate and the cell could continue to active proliferation (tumor cellular transformation). It has been noticed that different type of substances could have radioprotective effect and to protect the cell of malignant transformation, apoptosis, genome injuries or necrosis. Some of the natural metabolites that are proven antioxidants, show significant ability of radiation protection of the cells.

The aim of that study is to analyze the possible radioprotection ability of two origin metabolites (amino acids)–trimethyl glycine (betaine) and N-acetyl-L-cysteine, applied together and in combination, first before irradiation (preventive treatment) and then 2 hours after irradiation (as a therapeutic agents). The research work is done as invitro analysis to peripheral blood cell cultures.

Conclusion: *The conclusion of the study is that both amino acids showed good radiation protection activity after in-vitro performed analysis. The most significant results showed the combination of both natural metabolites.*

Keywords: TP53, QRT-PCR, cDNA, Trimethyl Glycine, Betaine, N-Acetyl-L-Cysteine, Radioprotectors, Antioxidants

Introduction

The function of the gene TP53 (p53) is to suppress the transformation of a normal cell into an active devided cell (tumor cellular transformation). TP53 functions as a tumor suppressor gene. It acts as a regulator of gene expression by binding to the target gene or specifically interacts with the transcription apparatuses and prevents of its transcription. The p53 gene has been defined as the "guardian of the genome", meaning that it ensures genomic stability [1-6].

The TP53 gene could mutate in a lot of different tumor types. Hereditary predisposition to the development of oncological disease is often associated with the presence of a mutant TP53 allele [7-9]. It is suggested that the mutant TP53 allele probably blocks the action of normal, wild-type TP53 and directs the cell towards tumorigenesis [10-12] One of the cell's responses to ionizing radiation is the induction of cell cycle arrest in G1 and G2 phases. Expression of the TP53 gene increases after exposure of cells to DNA-damaging agents such as ionizing radiation. Events at the G1 phase checkpoint can be regulated by p53-dependent induction of the expression of cell cycle inhibitors. The regulation of G2-phase checkpoint events in mammalian cells has been suggested to be p53-dependent, but the mechanism of is not clarify [13,14].

Also, depending on the damage, cells can be directed to apoptosis after exposure to ionizing radiationCould be observed decrease in gene expression of the native wild-type TP53 gene, as a result of gene mutation. Increase in gene expression immediately after exposure to ionizing radiation could be as a result of p53-dependent induction of cell cycle arrest in phases G1 and possibly G2, in order to proceed with processes of repair of the resulting damages [15].

Aim

The specific study aimed to determine whether, upon application of a potential radioprotector (preventive and therapeutic), irradiation with ionizing radiation and cultivation for a period longer than one cell cycle, a change in the expression of the native gene TP 53 (p53) could be observed. The decrease in the expression of the

studied gene directly correlates with the occurrence of mutations in the gene, as a result of the action of ionizing radiation and a potential possibility for the cell to move to tumorigenesis.

Materials and Methods

Quantitative real-time polymerase chain reaction (QRT-PCR) analysis was performed to study gene expression. To carry out QRT-PCR, it was necessary to isolate RNA in advance, which would be used for the synthesis of the cDNA template required for amplification. For RNA isolation, peripheral blood cell cultures of 14 healthy donors with preventive and therapeutic treatment with radioprotector and irradiation with 3 Gy absorbed dose were used. The cultivation period was 28 hours. Cell cultures were grouped into five experimental groups for the study of preventive and therapeutic treatment. When the starting material is RNA, as was the case, one more step is needed, namely reverse transcription to convert the starting RNA into so-called cDNA. The process takes place in the presence of starting RNA, the enzyme reverse transcriptase (RNA-dependent-DNA-polymerase) and a reaction mixture containing the necessary primers and deoxyribonucleotdtriphosphates (dNTP) [16-18]. This step was necessary to synthesize the necessary cDNA from the isolated RNA, which was used as a template to carry out the polymerase chain reaction. The second step was the QRT-PCR amplification process. QRT-PCR is a development of PCR-technology that allows precise identification and quantitative measurement of the products synthesized during each of the cycles of the PCR-process. This analysis is based on natural biochemical processes used by the cell itself [18]. It represents repetitive in vitro replication of a specific region of a cDNA template, in the 5' to 3' direction. The DNA fragment that is multiplied is limited at both ends by two short oligonucleotides-primers complementary to sections of the cDNA matrix. Also present is the so-called a probe that is labeled with a fluorescent substance at one end and a fluorescence "quenching" substance at the other. Deoxyribonucleotide triphosphates were added to a total reaction mixture. The process is catalyzed by a thermostable DNA polymerase isolated from the microorganism Termophylus aquaticus (Taq-polymerase), which allows denaturation of both the DNA template and the newly synthesized fragments. Repeated cycles of heating and cooling ensure that the starting DNA is multiplied exponentially. The reaction proceeds cyclically, each cycle passing through several stages. The data obtained with is measured quantitatively in the exponential phase of the PCR reaction. QRT-PCR amplification was performed using the fluorescent dye SYBR Green, which binds to the small loop of double-stranded DNA and fluoresces 1000 times more strongly when bound than when unbound. As the RT-PCR process generates more double-stranded products, the SYBR Green signal increases significantly. The disadvantage of this method is that it is not highly specific. Primer dimers and non-specific PCR products can give an identical fluorescence signal as the real product. The melting curve may show an incorrect result for the presence of gene expression. To improve the specificity of the method, the choice of highly specific primers that have been selected by PCR optimization and bind highly specifically to the gene for amplification is important [19]. Due to the lower specificity of the method and the possibility of generating non-specific products, it was necessary to carry out a gradient RT-PCR reaction to confirm the specificity of the primers used for the analyzed TP53 gene. Analysis based on the generated melting curve was used.

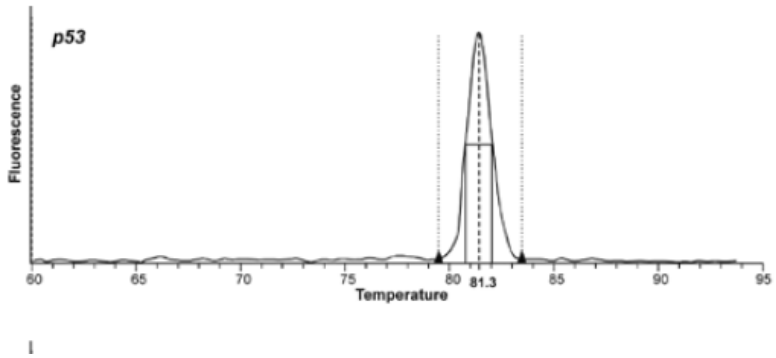

Figure 1: Specific melting curve (melting curve) for gene TP 53 (p53), generated during PCR-amplification - specific melting temperature 81.3ºC.

The result showed a specific melting temperature for the TP53 gene–81.3ºC. Agarose gel electrophoresis confirmed the presence of the two desired products -GAPDH-217 bp and TP53-121 bp. No primer dimers or non-specific products were detected during 45 cycles of PCR amplification. In QRT-PCR amplification, highly specific primers for TP53 were used–primer 1: 5'CCATCTACAAGCAGTCACAGC-3' and primer 2: 5'-GAGTCTTCCAGTGTGGAGATG-3.

In order to compare the results of the study, it was necessary to use a housekeeping gene that has a constant expression in native lymphocytes, regardless of the culture conditions and effects on the cells. GAPDH gene was selected and highly specific primers were used: primer 1: 5'-AAGGTGAAGGTCGGAGTCAA-3' and primer 2: 5'-AATGAAGGGGTCATTGATGG-3'.

A housekeeping gene was used to control for variability in the specificity of reverse-transcription reactions. The expression levels

of the housekeeping gene did not change under the conditions of the previous study.

Results and Discussion

Before conducting the preventive treatment study (two hours before irradiation with 3 Gy absorbed dose) with the amino acids studied for radioprotective properties, validation of the expression of the control (housekeeping) gene was done. This was done by calculating the amount of GAPDH gene mRNA (relative mRNA levels, x104) measured from the melting curve and referred to 1 µg of the total amount of isolated RNA.

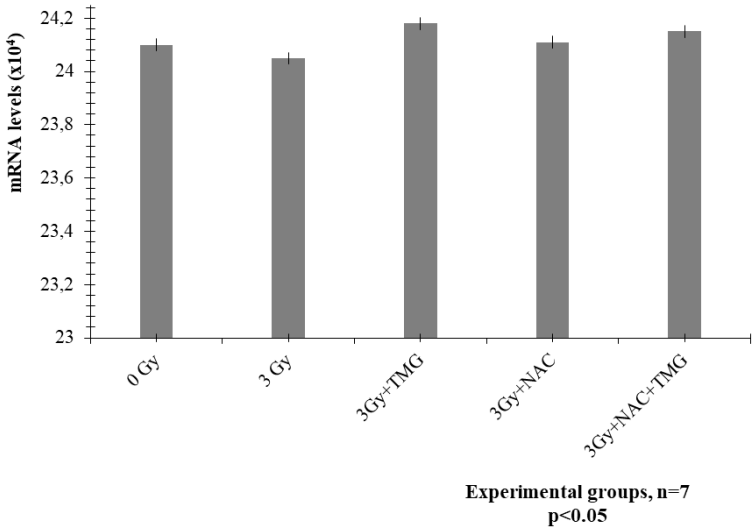

Figure 2: Validation of the control (housekeeping) GAPDH gene for conducting an experiment with preventive treatment of the studied radioprotectors: measurement of relative mRNA levels (x104), n=7, p<0.05;

The housekeeping gene showed almost constant expression for the five experimental groups, conducted in 7 replicates. As a consequence of the shown constant expression regardless of the different conditions of treatment with a potential radioprotector, GADPH gene was chosen as a suitable control gene for the study. Gene fragments were amplified by QRT-PCR using the described primers. QRT-PCR of the target gene was performed for each experimental sample, from each experimental group. The expression levels of the investigated experimental groups from each plate were compared with the expression of the control gene GAPDH, to establish the correct conduct of the experiment.

The result of QRT-PCR amplification of TP53 for all experimental groups, in preventive treatment (two hours before irradiation) are shown in Figure 3.

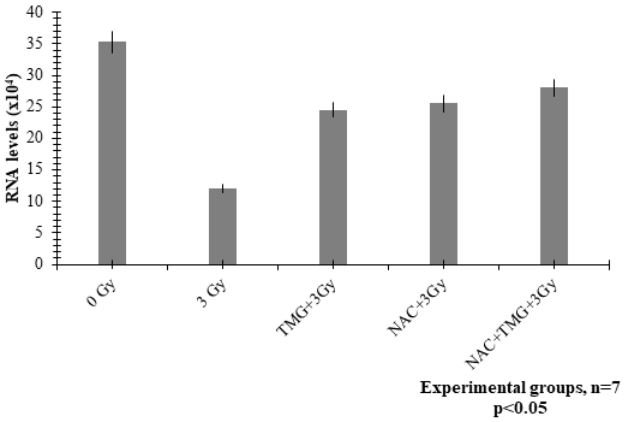

Figure 3: Result of QRT-PCR amplification of TP53 in preventive treatment with radioprotector, n=7, p< 0.05.

No primer dimers or non-specific products were detected during 45 cycles of PCR amplification. CFX Manager Software was used for computer visualization of the results. Data were automatically generated after completion of PCR amplification using the Gene Expression and Melting Curve module. Graphs and tables were exported to a personal computer using the "Export to Excel" function. observed after accounting for the standard error (S.E.M.) that it was within statistically significant limits of p<0.05.

As seen in Figure 3, the highest expression of the native, non-

mutant (wild) type TP 53 gene was reported in the control, non-irradiated and non-radioprotector treated group, while the lowest expression was reported in the non-treated and 3 Gy irradiated group ingested dose experimental group. These results correlate with the expected because they correspond to the assumption of the harmful effects of ionizing radiation on DNA and the occurrence of mutations in the TP53 gene when exposed to a relatively high dose of radiation (3 Gy absorbed dose). As a result of irradiation with a 3 Gy absorbed dose of ionizing radiation, the TP53 gene is affected and damage occurs, which is expressed by a decrease in the expression of the normal, functionally active TP53 gene. As a result of this severe DNA damage located in the TP53 gene region, native gene expression was reduced by half, compared to gene expression in the control, non-irradiated experimental group.

When analyzing the results for the experimental groups treated preventively with the tested substances separately or in combination and irradiated with a 3 Gy absorbed dose, a significant increase in the expression of the target TP53 gene was found. The obtained results correlate with the presence of a potential radioprotective ability of the tested amino acids, which is expressed in the protection of DNA from damage as a result of the action of ionizing radiation. From Fig.3 it is concluded that the best radioprotective ability is shown by the experimental group including preventive treatment with both substances in combination.

Before conducting the study for therapeutic treatment (two hours after irradiation with 3 Gy absorbed dose) with the amino acids studied for radioprotective properties, validation of the expression of the control (housekeeping) gene was done. It was carried out on the same principle as the previous experiment, by calculating the amount of GAPDH gene mRNA (relative mRNA levels, x104), measured from the melting curve and referred to 1 µg of the total amount of isolated RNA. The housekeeping gene showed almost constant expression for the five experimental groups conducted in 7 replicates. As a consequence of the shown constant expression regardless of the different conditions of treatment with a potential radioprotector, GADPH gene was chosen as a suitable control gene for the study. Gene fragments were amplified by QRT-PCR using the described primers. QRT-PCR of the target gene was performed for each experimental sample, from each experimental group. The expression levels of the investigated experimental groups from each plate were compared with the expression of the control gene GAPDH, to establish the correct conduct of the experiment.

The result of QRT-PCR amplification of TP53 for all experimental groups, under therapeutic treatment (two hours after irradiation) are shown in Figure 5.

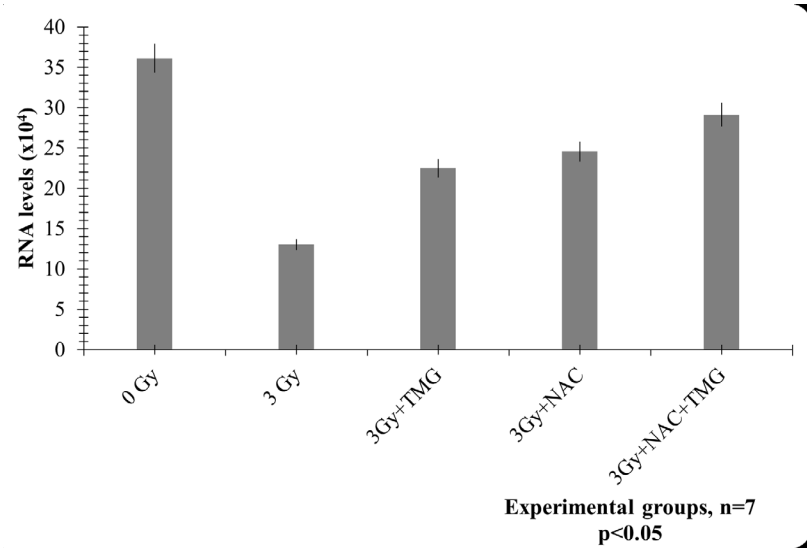

Figure 4: Result of QRT-PCR amplification of TP53 in therapeutic treatment with radioprotector, n=7, p< 0.05.

No primer dimers or non-specific products were detected during 45 cycles of PCR amplification. CFX Manager Software was used for computer visualization of the results. Data were automatically generated after completion of PCR amplification using the Gene Expression and Melting Curve module. Graphs and tables were exported to a personal computer using the "Export to Excel" function. The results were accepted as significantly different after the standard error (S.E.M.) was reported to be within statistically significant limits of p<0.05.

As seen in Figure 4, the highest expression of the native, non-mutant (wild) type TP 53 gene was reported in the control, non-irradiated and non-radioprotectant treated group, while the lowest expression was reported in the non-treated and irradiated with 3 Gy ingested dose experimental group. These results again correlate with what was expected, because they correspond to the assumption of the harmful effects of ionizing radiation on DNA and the occurrence of mutations in the TP53 gene when exposed to a relatively high dose of radiation (3 Gy absorbed dose). As a result of irradiation with a 3 Gy absorbed dose of ionizing radiation, the TP53 gene

is affected and damage occurs, which is expressed by a decrease in the expression of the normal, functionally active TP53 gene. As a result of this severe DNA damage located in the TP53 gene region, native gene expression was reduced by half, compared to gene expression in the control, non-irradiated experimental group. When analyzing the results for the experimental groups treated therapeutically with the tested substances separately or in combination and irradiated with a 3 Gy absorbed dose, a significant increase in the expression of the target TP53 gene was found. The obtained results correlate with the presence of a potential radioprotective ability of the tested amino acids, which is expressed in the protection of DNA from damage as a result of the action of ionizing radiation. When comparing the two experimental groups with therapeutic tertation separately, minimal differences in target gene expression were observed, with slightly higher expression in the experimental group treated with N-acetyl-L-cysteine and irradiated with a 3 Gy absorbed dose. The differences are minimal and cannot be used to establish better protection by one or the other investigated potential radioprotector. Analyzing Figure 4 could make a conclusion that the best radioprotective ability is shown by the experimental group including therapeutic treatment with both substances in combination.

Conclusion

The conclusion of the study is that both amino acids showed good radiation protection activity after in-vitro performed analysis. The most significant results showed the combination of both natural metabolites.

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