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The effect of 3-methylcholanthrene and butylated hydroxytoluene on glycogen levels of liver, muscle, testis, and tumor tissues of rats

Fikriye POLAT[1,*], Egemen DERE[2], Eylem GÜL[3], İzzet YELKUVAN[4], Öztürk ÖZDEMİR[5], Günsel BİNGÖL[6]

[1]Department of Education, Faculty of Education, Kocaeli University, Kocaeli, Turkey
[2]Department of Biology, Faculty of Arts and Sciences, Uludağ University, Bursa, Turkey
[3]Forensic Medicine Institute, Cerrahpaşa Faculty of Medicine, İstanbul University, İstanbul, Turkey
[4]Department of Medical Biology, Faculty of Medicine, Cumhuriyet University, Sivas, Turkey
[5]Department of Medical Genetics, Faculty of Medicine, Çanakkale Onsekiz Mart University, Çanakkale, Turkey
[6]Health Sciences, Dialysis Program, Nişantaşı University, İstanbul, Turkey

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Abstract: This study examined the effects of separate and combined applications of 3-methylcholanthrene, a polycyclic aromatic hydrocarbon and potent carcinogenic agent, and butylated hydroxytoluene, the antioxidant food additive, on the glycogen levels of liver, muscle, testis, and tumor tissues in rats. Adult male Wistar albino rats weighing 100–110 g at 8 weeks of age were used in this study. This study consisted of a control group (n = 9) and 3 different experiment groups in which rats were chronically treated with 3-methylcholanthrene (n = 9) or butylated hydroxytoluene (n = 11) or a combination of these agents (n = 14). Rats were intraperitoneally injected with a 200 mg kg^{-1} dose of butylated hydroxytoluene and a 40 mg kg^{-1} dose of 3-methylcholanthrene. At the end of the 26-week experimental period, tissues of rats killed via cervical dislocation were placed in 10% trichloroacetic acid for glycogen determination. Our results showed that the administration of 3-methylcholanthrene, butylated hydroxytoluene, and 3-methylcholanthrene + butylated hydroxytoluene caused statistically significant changes in the glycogen levels of liver, muscle, and testis tissues, and glycogen was stored in tumor tissue.

Key words: Glycogen, 3-methylcholanthrene, butylated hydroxytoluene, rat

1. Introduction

3-Methylcholanthrene (MCA) is a polycyclic aromatic hydrocarbon (PAH) common in polluted urban air and is a potent carcinogenic agent often used in experimental cancer studies. For many years, butylated hydroxytoluene (BHT) has been used as an antioxidant to preserve and stabilize the freshness, nutritive value, flavor, and color of foods for human consumption and animal feed products. Some studies claim these agents cause cancers in the human liver, lungs, colon, breast, stomach, and urinary tract (1–3).

PAHs, found in cigarette smoke, charbroiled foods, and polluted air, are known potent carcinogens that travel through the placenta to directly stimulate cytochrome P450 enzymes. The most common sources for PAH exposures are found in heating systems and vehicle motors, and are the result of the inadequate combustion of solid and fuel oils. These sources are regarded as the biggest factors responsible for air pollution (1,2,4). It is thought that 10% of cancer phenomena related to the respiratory system are the result of air pollution. Additionally, there is a report of increased leukemia incidence in some populations that are exposed to these kinds of substances (5).

Synthetic phenolic antioxidants are added to foods to retard the auto-oxidation of lipids. BHT, a major antioxidant, is widely used in foods all over the world (6,7). It has played a role as a typical promoting agent for tumorigenesis in biological systems (8). Even though BHT is not genotoxic or carcinogenic, it may regulate tumor formation in mouse lung tumorigenesis in a 2-staged carcinogenesis. Formation of lung tumors can be either prevented or enhanced by BHT in mice, depending on the strains and ages of the test subjects. Following exposure to a single dose of a carcinogen, BHT treatment with a repeated weekly dose is necessary to achieve tumor promotion (9,10).

The increase in blood glucose level also elevates the risk of cancer. Especially in women, (chronic) high blood

* Correspondence: fikriyepolat@gmail.com

glucose level is known to play an important role in the development of certain cancer types. Under the age of 49 bad eating habits increase the risk of pancreas, skin, uterus, and bladder cancers. Glycogen, the storage form of blood glucose, has an important role especially in liver and muscle tissues. In various types of cancers, significant changes in the activity of glycogen synthase have been reported (11).

The purpose of this study is to contribute to cancer research by examining the effects of separate and combined applications of MCA and BHT on the glycogen levels of the liver, testis, muscle, and tumor tissues of rats. This research contributes to the understanding of the effects of BHT on MCA.

2. Materials and methods

2.1. Animals

Fifty nontransgenic adult male rats (Wistar albino strain), weighing 100 to 110 g, were received at 8 weeks of age and allowed 1 week for adjustment to their new environment. The rats were kept in optimal laboratory conditions, fed with standard rat food, and given tap water ad libitum. They were randomized into 1 control and 3 experimental agent administration groups. The rats were first injected with individual agents, and then together in various dose combinations. After 26 weeks of injection, the control and experimental group rats were killed by cervical dislocation. Liver, muscle, and testis tissues were removed from all groups, except soft tissue tumors originating in the muscle at the point of injection in rats receiving MCA. All procedures on animals were carried out in accordance with the guidelines of the Animal Ethics Committee of Cumhuriyet University School of Medicine.

2.2. Experimental design

The rats were exposed to potential carcinogens of MCA (as a cancer initiator) and BHT (as a progressive agent) in tissue tumorigenesis in the rat model. MCA (Aldrich-Chem) and BHT (Sigma–B1378) were dissolved in corn oil. In the chronic MCA and BHT groups, rats were injected every week with either 40 mg/kg of MCA or 200 mg/kg of BHT, and the treatments were repeated for 6 weeks. In the other experiment group (MCA + BHT), following a single dose of MCA (40 mg/kg), rats were treated with 200 mg/kg of BHT injections for 6 consecutive weeks. Rats in the control group (n = 9) only received weekly corn oil injections for 6 weeks. Following their last treatments, 26 weeks later, rats were sacrificed by cervical dislocation and their tissues were surgically removed. In the chronic MCA group, we observed the development of soft tissue tumors in the inguinal regions of 5 rats. The tumors were collected and they were evaluated as a separate group for glycogen level assessment.

The tissues of the liver (500 mg), muscle (1000 mg), and testis were quickly harvested and perfused in 10% trichloroacetic acid. Until the homogenization procedure was performed, the tissues were stored at +4 °C. Tissues were homogenized in trichloroacetic acid (1/3 mass/volume) at 15,000 rpm for 5 min for liver and testis and 15 min for muscle in a tissue homogenizer (Teflon Glass Homogenizer, B. Braun). Thereafter, homogenates were centrifuged at 1400 × *g* for 15 min as described by Roe et al. (12) and designated according to Carroll et al. (13). In this study, Beckman–Model spectrophotometry was used.

Calculation

$$\frac{\text{DU}}{\text{DS}} \times 0.1 \times \frac{\text{volume of extract}}{\text{g of tissue}} \times 100 \times 0.9 =$$

mg of glycogen per 100 g of tissue

DU: optical density of the unknown,

DS: optical density of the standard

0.1 = mg of glucose in 2 mL of standard solution

0.9 = factor for converting glucose value to glycogen value

Analysis of variance was used to evaluate the data and any difference between the average values higher than F value at a 0.05 significance level was deemed significant (14).

3. Results

During the 26-week period, 6 rats in the chronic MCA treated group, 1 rat in the chronic BHT treated group, and 1 rat in the MCA + BHT treated group died. Evaluation of glycogen levels was performed on 9, 11, and 14 rats in the chronic MCA, chronic BHT, and MCA + BHT groups, respectively (Table). Tumoral developments were observed in 5 rats in the chronic MCA injected group. Those subcutaneous malignant mesenchymal tumors were 4 × 4 cm in diameter and localized at the injection sites. Fusiform cell proliferation and multinuclear tumoral giant cells were also detected at the injection site of the chronic MCA treated animals after histopathologic examination (Figure 1). Immunohistochemically, desmin and vimentin were stained positively in those soft, malign tumoral tissue cells (15).

Glycogen values in rats' livers administered MCA were observed to be lower than in control animals, and the difference was statistically significant (Figure 2). The liver tissue glycogen level in BHT treated animals, compared with the control group, was approximately 4 times higher (Figure 3). Although the hepatic glycogen level in the MCA + BHT treated group was higher than in the control group (~1.76 times), this level was lower in the BHT treated group (Figure 3). In the MCA, BHT, and MCA + BHT treated groups, differences in hepatic glycogen levels were found to be significant at the 0.05 probability level (Table).

Table. Effects of MCA, BHT, and MCA + BHT application on the glycogen levels (%) of liver, muscle, testis, and tumor tissues.

	Control (n = 9)	MCA (n = 9)	BHT (n = 11)	MCA + BHT (n = 14)
Liver	256.3 ± 4.1 *	22.26 ± 0.36 †	1020.7 ± 100.56 ‡	452.91 ± 3.2 ©
Muscle	22.62 ± 0.35 *	5.85 ± 0.90 †	131.13 ± 4.32 ‡	11.24 ± 0.22 ©
Testis	2.68 ± 0.22 *	1.51 ± 1.82 †	1.39 ± 0.14 ‡	0.51 ± 0.05 ©
Tumor tissues		5.93 ± 0.07 †		

* Data shown with the same letter in each group in the horizontal axis are not different from each other at a 0.05 probability level (*, †, ‡, ©).

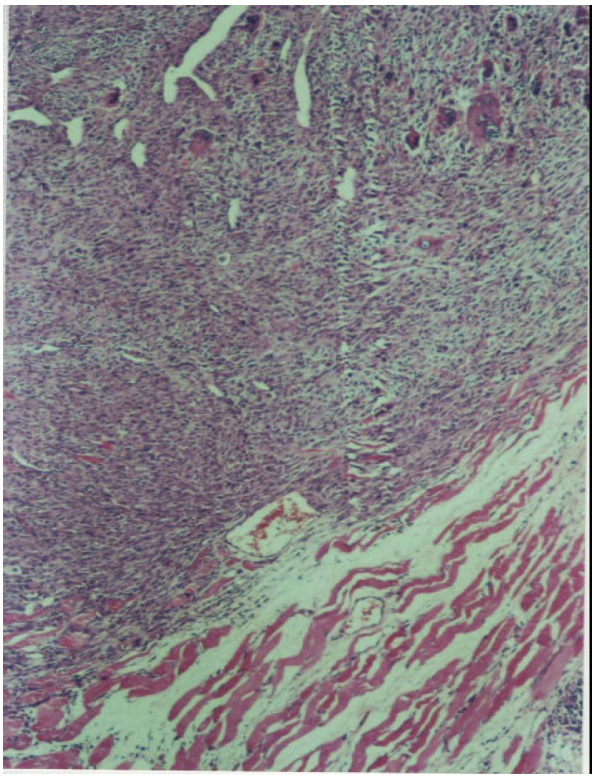

Figure 1. Malign soft tissue tumor with atypical fusiform cells, giant tumoral cells (H & E 25) (Polat et al. (13)).

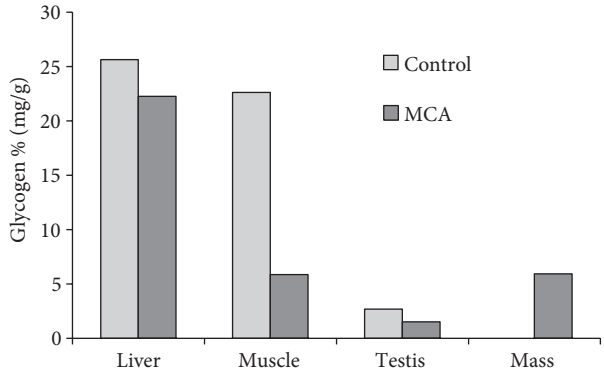

Figure 2. Comparison of glycogen levels (%) in liver, testis, muscle and tumor tissues among MCA applied in rats. (Control liver glycogen level is given as $\times 10^1$).

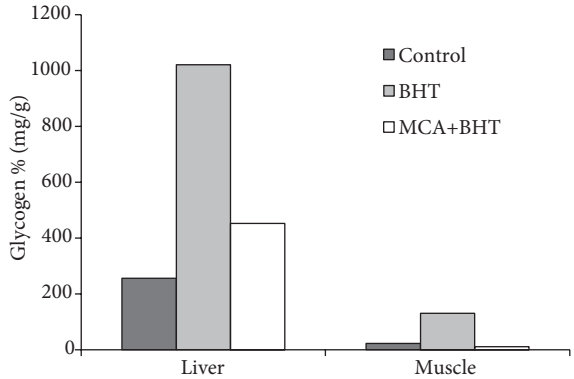

Figure 3. Comparison of glycogen levels (%) in liver and muscle tissues among BHT and MCA + BHT applied in rats.

In the MCA treated group, the apparent decrease in the glycogen level in the muscle tissue was found to be significant at a 0.05 probability level (Figure 2). When the glycogen values obtained after BHT application were compared with both the MCA group and the control group, a considerable increase was observed in the BHT group. This increase was 5.8 times more than the control group, and 22.4 times more than the MCA group (Table). When compared to the control group, this difference in the glycogen level was found to be significant at a 0.05 probability level (Figure 2). Even though there was an increase in the muscle glycogen level of the MCA + BHT treated group, the statistical comparison to the

MCA treated group did not show any significant change. Conversely, the glycogen level of the MCA + BHT treated animals was found to be 91.43% lower than that of the BHT treated group ($P < 0.05$).

Another important result of this study was that the glycogen level induced with BHT alone, compared to the value induced with MCA alone, was significantly higher in both liver and muscle tissues; however, administration of MCA + BHT combination, comparing with BHT alone, decreased the glycogen level in both liver and muscle tissues (Table).

The lowest glycogen level percentage in testis tissue was 0.51 ± 0.05 in the MCA + BHT treated group. Comparing the MCA + BHT treated group with the control, MCA, and BHT treated groups, the glycogen level was significantly reduced at 0.05 probability level. When MCA and BHT were administered separately, they showed reductions in glycogen levels, when compared to the control group. These reductions were found to be significant at a 0.05 probability level (Figures 2, 3; Table).

The glycogen level obtained from the tumor tissues was almost the same as the glycogen level obtained from the muscle tissue in the MCA group. There was no difference in glycogen level between these tissues; however, there was a decrease in the muscle tissue of the control group (Table; Figure 2).

4. Discussion

In this study, MCA was used as a cancer initiator and BHT was used as a tumor-promoting agent. BHT has a significant role as a tumor-promoting agent even though it is commonly known for its use as a food antioxidant. In a previous study, Polat et al. (15) studied carcinogenesis with a similar approach and subsequently in this study we would like to evaluate the changes in the glycogen levels in muscle, liver, testis, and tumor tissues. In most of the carcinogenesis studies in which rats and mice have been used as subjects, initial tumor development was induced with the administration of a single dose of carcinogenic agents. Following tumor induction, the growth of the tumors was promoted by the chronic administration of various agents (16–20). In this study, we intended to generate lung cancer especially in MCA + BHT treated rats. In other experiment groups, we planned to evaluate the individual effects of the chronic administration of the chemical agents. We were unable to generate lung cancer in the MCA + BHT administered rats. However, the chronic administration of the MCA induced soft tissue tumors in the inguinal region of 5 rats. Twenty-six weeks after the completion of the injections, animal deaths in our experiment groups prompted us to discontinue the experiments.

Our literature search for the chemical agents used in this study and the levels of glycogen and various other biochemical parameters revealed no study performed within the last 10 years.

It was observed that the reductions in glycogen levels were remarkable in the liver, muscle, and testis of the MCA treated groups ($P < 0.05$). One reason for these reductions may be the fact that the tumoral cells, which result from applications of MCA, utilize glucose. These tumoral cells using glucose do not break down the molecule to CO_2 or water (hydrolysis), and they produce lactic acid. In other words, they are fermented. Thus, cancer cells need energy. They provide this energy need by using more glucose. The glucose requirements of tumoral cells are 20 times more than the needs of normal cells. Consequently, the glycogen levels in tissues decrease as a result of the increasing glucose need and occurring anorexia. In the meantime, lactic acid levels also increase. Increased amounts of lactic acid cause pain. In a study conducted on Lewis-Wistar rats, Shearer et al. (21) reported an increase in gluconeogenesis rates in rats carrying tumors and found that the increase in the oxidation of CO_2 of pyruvate and the conversion of this molecule into lactic acid and glucose were related to the increased clearance of pyruvate and uptake. The findings from Shearer et al. (21), and our findings of the occurrence of soft-tissue tumors and the marked reductions in glycogen levels in the MCA treated group, all demonstrate that increased glucose needs in tumor tissues and anaerobic respiration are met by producing lactic acid. Doi et al. (22) subcutaneously administered MCA to F344 rats and histochemically examined glycogen levels in the liver. As glycogen stores in the liver cells of rats with tumors were significantly reduced, compared with the control group, the researchers concluded that glucose is expended, cyclically.

Another reason for glycogen-level reductions may be an increase in activities of enzymes taking part in the glycogenolysis metabolism. Kleeberg et al. (23) administered doses of 1, 10, and 100 mg/kg[1] MCA to 7-day-old rats and examined aryl hydrocarbon (benzo[a]pyrene) hyroxylase (AHH), succinate dehydrogenase, and glucose-6-phosphatase enzyme activities in the liver and kidney. They found that AHH activity increased, depending on the dose. However, they reported that they did not find any changes in other biochemical parameters, nor did their investigations using light and electron microscopes show morphological changes in liver cells of rats treated with the highest MCA dose.

Safer and Al-Nughamish (24) fed Sprague-Dawley rats antioxidant BHT, a food additive, for 6-, 12-, 18-, and 24-week periods in different concentrations and then compared the results against the control group. They

reported a significant increase in liver weight. The electron microscopic study showed an increase in lipid drops, as well as abnormalities in hepatocytes, but a reduction in glycogen level. Contrary to the study conducted by Safer and Al-Nughamish (24), our study of Wistar albino rats given 200 mg/kg[1] dose of BHT per week determined significant increases in the glycogen levels in the liver.

In the experimental design described by Siman and Eriksson (25), 1 group of female rats was fed BHT containing vitamin E and 1 group of female rats was given only BHT for 4 weeks. An increase in the liver weights of rats treated with just BHT was observed. It was also determined that BHT metabolized with cytochrome P450 system in the liver and transformed into pro-oxidative compounds.

Wong and Rao (26), in a study on female rats fed a diet of BHT for 5 weeks, showed hepatosplenomegaly, a reduction in body weight, a significant inhibition in glucose-6-phosphotase activity, hepatic microsomal protein and cytochrome P-450 induction, and an increase in cholesterol levels.

Takahashi and Hiraga (27) studied hepatic lipids in male Sprague-Dawley rats given 1.20% BHT for 1 week. They found that BHT increased cholesteryl esters and phospholipids while reducing triglyceride, nonesterified lipid acids, and diglyceride. They also reported that BHT increased the amount of phosphatidylethanolamine, whereas it reduced phosphatidylinositol and lysophosphatidylcholine amounts.

Sondergaard and Olsen (28) found that feeding rats BHT at doses of 500 and 5000 ppm for different lengths of time, up to 90 days resulted in an increase in thyroid weight with both doses, and an increase in liver weight for the 5000 ppm dose. Rhin et al. (29) stated that a single injection of MCA produced genotoxic effects immediately, within a 3-day period. In their study of MCA, they observed increase trends in G:C → T:A and C:G → A:T transversions in the DNA. In addition, following the stimulation by BHT, MCA started mutations in Ki-ras and p53 genes in various tissues.

It is obvious that MCA + BHT application increases glycogen level in the liver but reduces it in the muscles and testis, when compared to the control group (Figures 3, 4). In comparing MCA + BHT groups against the MCA groups, glycogen levels were observed to increase in the liver and muscles, and are at the lowest level in the testis (Figure 4). These results suggest that BHT reduces the effect of MCA in muscles while it does not affect levels in the testis. Even though we did not evaluate hormone levels, we think that MCA may directly, or indirectly, affect different hormones. Likewise, Chance et al. (30) reported increases and reductions in insulin and glucagon levels in F344 rats

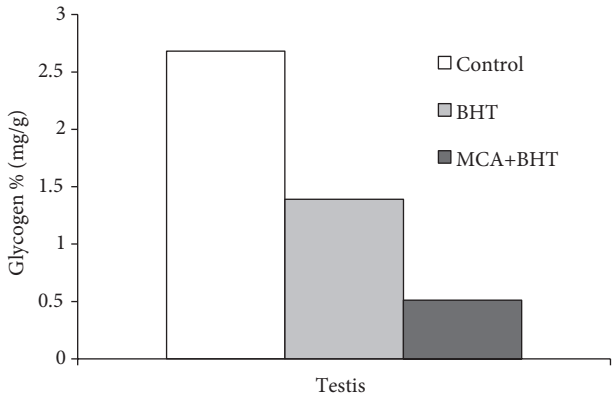

Figure 4. Comparison of glycogen levels (%) in testis tissue among control, BHT, and MCA + BHT groups.

given MCA. It was found that corticosterone and glucagon levels were increased in tumorous rats. While an increase was determined in glycogen levels of brain and skeletal muscles, in spite of hypoglycemia and hypoinsulinemia, a decrease was determined in glycogen level in the liver (31). Thus, affected hormones will, although indirectly, lead to changes in glycogen levels. The sensitivity of tumor tissues to insulin explains why they collect glycogen.

When we examine the Table, we observe that glycogen levels in the MCA + BHT group are lower than those in the BHT group. When we compare MCA with the control group, we see that glycogen levels in the MCA group are lower than those in the other. In this case we mention that glycogen levels in the groups including MCA will be lowered. Thus, in a study by Besedovsky et al. (32), it is observed that insulin levels are lowered in rats given MCA. A decrease in the level of insulin gives rise to an increase in the blood glucose level and, accordingly, to the discharging of glycogen stores. In our research in the groups administered MCA it is observed that there is compliance between reducing glycogen level in tissues and reduction of insulin levels in rats and mice given MCA, as reported by Besedovsky et al. (32). The same researchers declared that increases in corticosterone and prolactin levels were observed after the inoculation of MCA in mice, and a decrease in insulin, thyroxin, and sexual steroid levels.

In conclusion, as cancer cells reproduce very quickly and use high amounts of glycogen, glycogen levels in tumoral tissues decrease from time to time. Future studies should investigate enzyme activities with respect to glycogen metabolism.

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