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PROTECTIVE EFFECT OF FULLERENOL NANO PARTICLES ON COLON CANCER DEVELOPMENT IN DIMETHYLHYDRAZINE RAT MODEL

MARTINA PERŠE*, RADE INJAC[a], ALEKSANDAR DJORDJEVIC[b], BORUT ŠTRUKELJ[a], ANTON CERAR

Institute of Pathology, Medical Experimental Centre, Medical Faculty, University of Ljubljana, Korytkova 2, 1000 Ljubljana Slovenia

[a]Faculty of Pharmacy, Institute of Pharmaceutical Biology, University of Ljubljana, Askerceva 7, 1000 Ljubljana, Slovenia

[b]Faculty of Sciences, Department of Chemistry, University of Novi Sad, Trg Dositeja Obradovica 3, 21000 Novi Sad, Serbia

Colorectal cancer (CRC) is one of the most common cancers world-wide, with highest incidence rates in western countries. In recent years, much effort has been dedicated in search for natural or pharmacological preventive agents, which would block or attenuate CRC process. In search for new pharmacological agent, the effects of fullerenol $C_{60}(OH)_{24}$ nano particles (FNP) on liver oxidative status and promotion or progression phase of colorectal carcinogenesis in dimethylhydrazine-induced rat model of CRC were investigated. Our results demonstrate that FNP effectively inhibited formation of dysplastic aberrant crypt foci, which are regarded as early histopathological lesions in the pathogenesis of CRC. FNP treatment also improved activity of antioxidant enzymes in the liver. Since this was the first study investigating FNP effects on colon carcinogenesis further studies are needed to evaluate its protective effect also in other phases of carcinogenesis and to investigate whether its inhibitory activity was due to modulation of carcinogen-induced oxidative stress or another yet unknown anticarcinogenic activity.

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1. Introduction

Colorectal cancer (CRC) is one of the most common cancers world-wide, with highest incidence rates in western countries [1]. In recent years, growing attention has been paid to environmental and food components, with the hope of identifying its preventive or carcinogenic effects [2,3]. Much effort has been dedicated in search for natural or pharmacological preventive agents, which would block or attenuate CRC process [4,5].

Colon carcinogenesis is a multistage process, involving multiple genetic and epigenetic changes that provide tumour cells with a selective advantage to expand their clones [6]. Recent evidence has indicated that the generation of reactive oxygen species (ROS) may play important role in the initiation, promotion and progression stages of carcinogenesis [7]. It has been already shown that vitamin E has antiproliferative property in cancer cell lines, while different natural antioxidants such as gallic acid [8], polyphenols [9], vitamin D [10], vitamin A [11] as well as pharmacological compounds like bis-1,7-(2-hydroxyphenyl)-hepta-1,6-diene-3,5-dione (BDMCA) [12] have potential to inhibit CRC development in dimethylhydrazine (DMH) model. DMH rat model is well establish CRC animal model and possess many characteristics found in human sporadic CRC, which makes it an important tool for studying different aspects of step-wise development of CRC under defined experimental conditions [13]. DMH administration has been

* Corresponding author: martina.perse@mf.uni-lj.si

shown to affect oxidative status in the circulation [12] and different tissues of rats, such as heart [14], liver [15] as well as small and large bowel [16], which enable this model to be used for an evaluation of antioxidant activities of natural or pharmacological compounds and their involvement in colon carcinogenesis [8,17,18].

Fullerenes are a relatively new group of compounds and represent a class of sphere-shaped molecules made exclusively of carbon atoms. Since their discovery in 1985, many aspects of both fullerene and its analogues have been intensively studied to reveal their physical and chemical reactivity, as well as potential use in biological systems. Both *in vitro* and *in vivo* studies have shown that polyhydroxylated fullerene derivatives, fullerenols ($C_{60}(OH)_n$, n=2-44), can be potential antioxidative agents in biological systems [15,19-26].

Previous studies demonstrated toxicity of pure C_{60} against dermal fibroblasts and liver carcinoma HepG2 cells, in comparison to fullerenol nano particles (FNP) and other water-soluble fullerenes. It was also shown that the cytotoxic activity of C_{60} colloid was caused by ROS-mediated cell membrane lipid peroxidation [27]. In addition, Isakovic et al., confirmed that pure C_{60} and fullerenols $C_{60}(OH)_x$ apparently employ different cytotoxic mechanisms resulting in preferential induction of necrosis and apoptosis, respectively [28]. In addition, C_{60} induces production of oxygen radicals, which are involved in lipid peroxidation, and accordingly stimulates necrotic cell death. In contrast, $C_{60}(OH)_x$-triggered apoptosis seems to be ROS independent [28]. Pro-oxidant activity of pure C_{60} is related to its chemical structure. It was found that derivatization of C_{60} decreases ROS generation and cytotoxicity [27]. The powerful ability of fullerenes to induce production of ROS makes them promising candidates for the photodynamic killing of cancer cells. There have been several studies confirming the efficient photodynamic action of various water-soluble C_{60} derivatives against different types of cultured cancer cell lines (cervical, larynx, lung) and malignant tumors *in vivo* [29].

Therefore, the aim of the present study was to evaluate potential inhibitory effect of FNP on colon carcinogenesis in DMH rat model.

2. Materials and methods

2.1 Animals and experimental design

In experiment 28 male Wistar (HsdRccHan^{TM}:WIST) rats (Medical Experimental Centre, Ljubljana, Slovenia) were used. They were quarantined and housed 5 per cage (1825 cm^2 floor space) on Lignocel ¾ bedding material (Germany) at a 22-23°C room temperature, 55 ± 10% humidity and a 12 h light/dark cycle. Animals had free acces to diet (Altromin, Germany) and tap water. The experiment was approved by the National Animal Ethical Committee of the Republic of Slovenia and was conducted in accordance with the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123).

At 7 week of age rats were randomly divided into three groups and started to receive i.p. applications of DMH (three DMH treated groups; n=10/group) or saline (instead of DMH, i.e. one control group; n=8) once a week for 15 weeks. Two weeks after the last i.p. DMH/saline injection rats were treated with three i.p. applications of FNP (100 mg/kg) or saline (DMH and saline control groups), which were administered one week apart. The dose for FNP was chosen according to preliminary studies of antioxidant activity of FNP on healthy adult Wistar rats [30] and furter examinations on cancer rat models [15,23-26]. Seven days after the last application of FNP (27^{th} week of age) the rats were euthanized by CO_2.

We began with FNP administration two weeks after the last DMH application, i.e. during the promotion or progression phase, because this was the first time that FNP has been tested for its effect in DMH-induced colon carcinogenesis and thus we wanted to exclude any potential modification of DMH metabolism by FNP [13,31].

2.2 Carcinogen and Fullerenol $C_{60}(OH)_{24}$ nano particles

Colon tumours were induced by DMH (Fluka Chemie; Switzerland) prepared according to the standard method as previously reported [14]. DMH was dissolved in 0.001 M EDTA and pH value adjusted to 6.8 using 0.1 M NaOH solution and administered subcutaneously in the medial thigh area of rats at a dose of 20 mg/kg body weight once a week for 15 weeks. Fresh solutions were prepared prior to application.

FNP (Novi Sad, Serbia) [32] was dissolved in a sterilized and apyrogenic NaCl (0.9%): DMSO (80:20; w/w) solution (10 mg/mL) inside a laminar flow cabin immediately before use.

2.3 Morphological methods

At 21th week after the first DMH or saline treatment rats were sacrificed by CO_2. At autopsy all internal organs were removed, weighted and macroscopically examined. The intestine was opened longitudinally, flushed with tap water, pinned on cardboard, and examined macroscopically for the presence of tumours. The location, number and size of the tumours were recorded and the intestine, liver and all detected lesions were fixed in 4% buffered formalin. Large intestine was cut longitudinally and sent to histological examination in total length along with all the macroscopically visible lesions. All tissue samples of large intestine were embedded in paraffin, serially sectioned at 4-5 μm and stained with Kreyberg-Jareg method. Aberrant crypt foci (ACF) with dysplastic epithelium, adenomas and carcinomas were assessed by histological criteria described elsewhere [13].

After fixation liver were embedded in paraffin, serially sectioned at 4 μm and stained with Haematoxylin-Eosin (H&E). Liver sections were assessed according to intensity and diffusion of degeneration and pleomorphism in hepatocytes, proliferation in bile duct, parenhymal necrosis, congestion and thrombosis in central vein, and inflammation in portal space. Scoring was done as follows: (-): showing no changes, (+): mild focal changes, (++): moderate changes, (+++): severe widespread changes. The histological evaluation of tissue samples was carried out blindly without knowledge of the treatment status of each animal.

2.4 Blood cell count and serum enzyme determination

At the end of the experiment animals were sacrificed 7 days after FNP application. The blood for the analysis was taken by a heart puncture after opening thoracic region. One portion of venous blood was put into eppendorfs with K-EDTA (15 %) carefully mixed 20 min and used for blood cell counting. The second portion of blood (8 mL per animal) was kept at room temperature for approximately 2 h and allowed to clot. Serum was prepared by centrifugation and stored at -80ºC before use. The assay for alanine transferase (ALT), aspartate transaminase (AST) was caried out according to the methods described in the commercial kits (Chema Diagnostica, Jesi, Italy), using Tecan Saffire Microplate Reader (Tecan UK, Milton Keynes, UK) as described previously [24]. The results for all enzymes were expressed as U/L.

2.5 Liver MDA and antioxidant defense system determination

Immediately after sacrifice liver were quickly removed from the sacrificed rat, placed in ice-cold solution and trimmed of adipose tissues. Each organ was then homogenized in Tris-buffer solution (pH 7.4; organ: buffer 1:10; w/w) and used for malondialdehyde (MDA) determination. MDA levels were measured with a Chromsystems Diagnostic commercial kit (Munchen, Germany) using HPLC Agilent HP 1100-model system equipped with an autosampler and a fluorescence detector (Waldbronn, Germany) [14,24].

To determine antioxidant status of the liver the second portion of homogenized organ was centrifuged at 13,000 x g for 20 min at 4°C (Beckman refrigerated, Ultracentrifuge). The supernatant was used for the assays of total protein (TP; Sentinel Diagnostics, Milan, Italy) and glutathion concentration, GSH (Chromsystems Diagnostic, Munchen, Germany), glutathione peroxidase (GPx; Ransel, Crumlin, UK), glutathione reductase (GR; Crumlin, UK), catalase

(CAT), superoxide dismutase (SOD; Ransod, Crumlin, UK) and lactate dehydrogenase (LDH; Chema Diagnostica, Jesi, Italy) activity as described previously [14,33]. CAT activity in the tissue homogenate was assayed by monitoring decomposition of hydrogen peroxide according to the method by Aebi [34]. The enzyme activity was expressed as the rate constant of hydrogen peroxide decomposition.

2.6 Relative weight of liver

The relative weight of liver was calculated as the ratio of tissues (wet weight, mg) to body weight (g).

2.7 Statistical analysis

Body weights, relative weight of organs, biochemical parameters and multiplicity of tumors and ACF between groups were compared by ANOVA and a significant difference among treatment groups was evaluated by Multiple range test according to Duncun method (DMRT). All statistical analyses were made using Statgraphics® Centurion XV computer program. Differences were considered significant at $P < 0.05$. All results were expressed as the mean ± SEM.

3. Results

All the animals were in good health during the entire experiment. Their body weights were increasing with advancing age. Administration of DMH resulted in a decrease in body weights and in an increase in relative liver weights as compared to body weight and relative liver weight of untreated control rats. FNP treatment increased relative liver weight and serum ALT when compared to both control and DMH group and the number of white cells in the blood when compared to control rats. Tendency toward increased albeit insignificant circulating lymphocyte concentration after FNP treatment was also seen (Table 1).

Table 1. Hematological findings, body weights and relative liver weights of rats.

Parameter	Control	DMH	DMH+FNP
WBC ($x10^9$/L)	5.87 ± 0.4^a	6.78 ± 0.3^{ab}	7.34 ± 0.4^b
Neutrophil (%)	18.1±1.3	15.9±1.2	15.0±1.2
Lymphocyte (%)	80.1±1.4	80.2±1.3	82.4±1.3
RBC ($x10^{12}$/L)	8.6±0.2	8.3±0.4	8.2±0.2
ALT (U/L)	6.5 ± 1.8^a	8.5 ± 2.3^b	11.8 ± 3.5^c
AST (U/L)	14.4±3.4	14.4±1.8	12.8±3.1
Body weight (g)	526 ± 9^a	410 ± 9^b	399 ± 10^b
Relative weight of liver (mg/g)	5.4 ± 0.3^a	7.4 ± 0.3^b	8.8 ± 0.3^c

Data are present as the means ± SE. Values not sharing common superscript letter (a-c) differ significantly from each other at $P<0.05$ (ANOVA followed by DMRT).

Figure 1 summarizes the average number of DMH-induced colon dysplastic lesions. There were no significant differences in the multiplicity of adenomas or carcinomas between both DMH-

treated groups. The most of adenomas in all groups exhibited high degree of dysplasia, while the most carcinomas were well-differentiated adenocarcinomas.

Histological examination revealed evident differences among the groups according to the number of aberrant crypts with dysplastic epithelium, which are also known as intraepithelial neoplasia or ACF with dysplasia or microadenomas. These lesions are regarded as early histopathological lesions in the pathogenesis of CRC [35,36]. However, results have shown that FNP treated group developed significantly reduced number of ACF with dysplasia in comparison to the DMH alone treated group (Figure 1).

Most of dysplastic ACF have been found in the middle and distal part of the colon, no adenomas were observed in the proximal colon, while most of carcinomas developed in the proximal colon, which is in agreement with Park et al. [37] regarding step-wise and *de novo* development of CRC in DMH rat model.

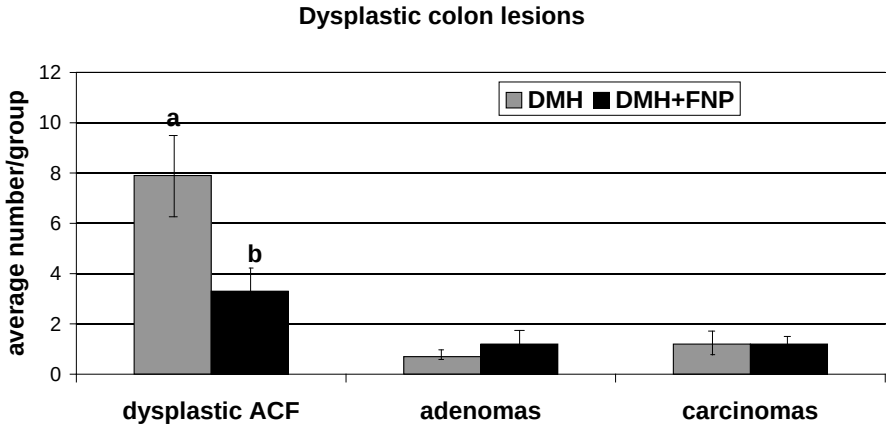

Fig. 1. Incidence of dysplastic colon lesions in both DMH-treated groups. Data are present as the means ± SE. Values not sharing common superscript letter (a-b) differ significantly from each other at P<0.001 (ANOVA followed by DMRT).

DMH administration increased the levels of MDA in the liver significantly (Figure 2). No changes in the SOD activity of liver among all groups were found. However, the depleted GR and GPx activity of DMH administered rat liver were significantly increased by FNP treatment. FNP treatment resulted also in an increase of CAT and LDH in the liver as compared to both control and DMH groups (Figure 2 and 3).

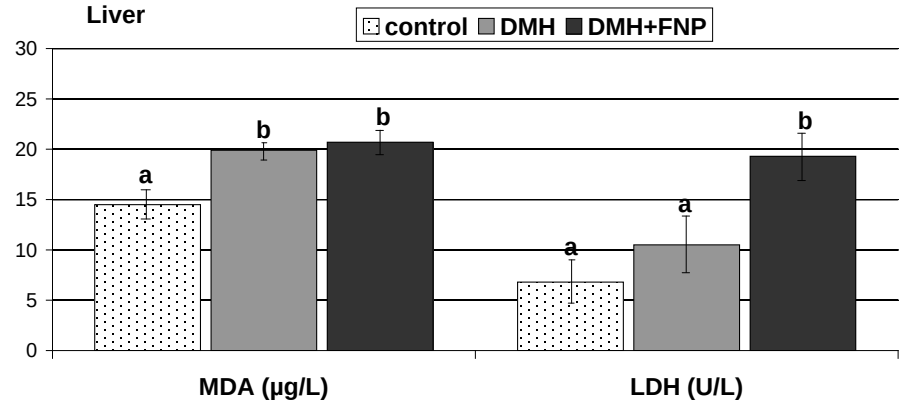

Fig. 2. Levels of MDA and LDH in the liver of control and DMH-treated groups. Data are present as the means ± SE. Values not sharing common superscript letter (a-c) differ significantly from each other at P<0.05 (ANOVA followed by DMRT).

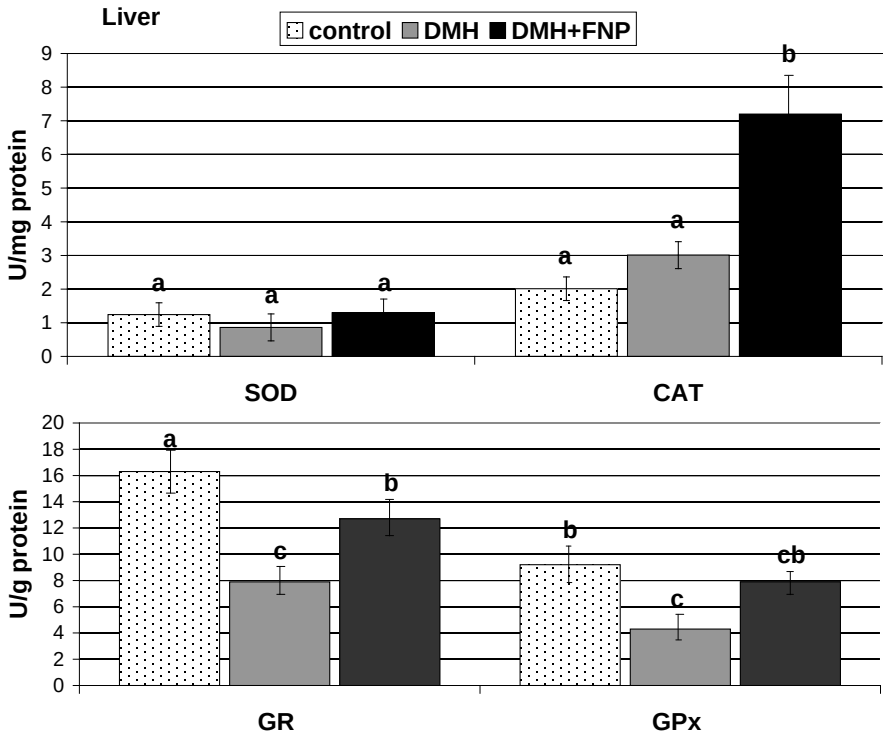

Fig. 3. Activity of antioxidant enzymes in the liver of control and DMH-treated groups. Data are present as the means ± SE. Values not sharing common superscript letter (a-c) differ significantly from each other at P<0.01 (ANOVA followed by DMRT).

No histologic lesions were found in the liver of control rats. Histological examination of DMH administered rat liver showed the presence of mild and diffusely localized pleomorphism, parenchymal degeneration, and necrosis of hepatocytes. FNP treatment decreased hepatic cell necrosis caused by DMH administration, but had no effect on parenchymal degeneration (Table 2). Histologic examination revealed that FNP treatment resulted in presence of increased hyperproliferation of hepatocytes in the liver of four of ten rats. No other histologic changes among groups were observed.

Table 2. Histological evaluation of liver sections (only observed changes are shown).

Changes of hepatocytes	Control	DMH	DMH+FNP
Degeneration	-	+	+
Pleomorphism	-	+	+
Necrosis	-	+	-

(-): showing no changes, (+): mild focal changes, (++): moderate changes, (+++): severe widespread changes.

4. Discussion

In the present study we have demonstrated for the first time that FNP administration resulted in decreased formation of dysplastic ACF, which are recognized as first histological identified lesions that have the potential to progress to advanced adenomas and adenocarcinomas [13,38]. Until now, FNP has been shown as a strong free radical scavenger that successfully protected different tissues against doxorubicin induced toxicity [15,22-26]. Results in the present study suggest that FNP exhibit protective effects also against CRC development. It is possible that FNP's strong antioxidant activity induced epigenetic changes in tumour cells that interrupted their

selective advantage or inhibited their expansion. Namely, evidence shows that cancer cells acquire particular characteristics, including changes in antioxidative status that benefit their proliferation [39].

In addition, it has been suggested that FNP exerts its protective role also by removing free iron through formation of FNP-iron complex [40]. Iron-induced free radical damage to DNA appears to be important for the development of cancer. It was demonstrated that cancer cells grow rapidly in response to iron. It was proposed that intestinal exposure to ingested iron, which originates from red meat consumption, may be associated with increased colorectal cancer in highly developed countries. In addition, a dose-dependent relationship for serum ferritin level and colon adenoma risk was found [39,41]. Thus, FNP may have a potential inhibitory effect on CRC formation also due to its removal of free iron in form of FNP-iron particles.

FNP treatment resulted in increased number of WBC and lymphocytes. The role of the immune system in controlling tumor development has been reported. Antioxidant supplementation has been reported to increase natural killer cells, i.e. a special group of lymphocyte population, which exhibit cytotoxic activity against virus-infected cells and tumor cells [42]. Therefore, this could be another mechanism of FNP action against CRC development. Moreover, immunomodulatory antitumor mechanism of fullerenol $C_{60}(OH)_x$ has recently been demonstrated [43].

Since DMH is metabolized in the liver, resulting in the production of electrophilic diazonium ion, which is known to elicit oxidative stress [13], our study evaluated also antioxidant effect of FNP on DMH-induced oxidative stress in the liver. Devastena et al [12] as well as Giftson [8] demonstrated that activity of detoxifying enzymes and activities of antioxidant enzymes in DMH treated rats treated with antioxidants were significantly improved and blocked generation of lipid peroxide. We found significant increase in the activity of CAT and GR, but no difference in MDA levels between both DMH-treated groups. This indicates that treatment with FNP improved activity of antioxidant enzymes in the liver when administered after DMH-induced oxidative stress, but not decreased lipid peroxidation. FNP has been shown as very effective free radical scavenger against doxorubicin induced oxidative stress, when administered 30 min before doxorubicin application [15,40]. Thus, it is very likely that lipid peroxidation was not decreased because treatment with FNP started after induction of DMH-induced oxidative stress.

In our study FNP administration increased serum ALT activity and decreased DMH-induced necrosis in the liver. This indicates that FNP induced injuries in hepatocytes did not result in necrosis. It is thus possible that FNP induced injuries were reversible or resulted in apoptosis. Isakovic et al [28] demonstrated that FNP cytotoxic mechanisms are rather due to caspase-dependent apoptosis, which seems to be ROS independent, than necrosis. It was further hypothesized that cytoskeleton disruption may be an initiating event in FNP cytotoxicity, leading to subsequent autophagy dysfunction and loss of mitochondrial capacity [44].

In conclusion, our preliminary results demonstrate that FNP effectively inhibited formation of dysplastic ACF, which are regarded as early histopathological lesions in the pathogenesis of CRC. Since this was the first study investigating FNP effects on colon carcinogenesis further studies are needed to evaluate its protective effect also in other phases of carcinogenesis and to investigate whether its inhibitory activity was due to modulation of carcinogen-induced oxidative stress or another yet unknown anticarcinogenic activity.

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