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ANTI-INFLAMMATORY ACTIVITY OF FULLERENOL $C_{60}(OH)_{24}$ NANO-PARTICLES IN A MODEL OF ACUTE INFLAMMATION IN RATS

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Water soluble fullerene (C_{60}) derivative fullerenol $C_{60}(OH)_{24}$ nano-particles (FNP) are a promising candidate for many biomedical applications due to, besides other properties, strong free-radical scavenging and antioxidative potential. Using the carrageenan-induced rat footpad oedema test, the anti-inflammatory effect of FNP have been estimated in comparison with those of amifostine (AMI) and indomethacin (IND). FNP and IND, dissolved in dimethylsulfoxide, and AMI, dissolved in saline, were intraperitoneally injected to rats in a dose range 12.5 - 75 mg/kg, 3 - 10 mg/kg, and 50 - 300 mg/kg, respectively. The control groups were given corresponding vehicles. The drugs or vehicles were given 30 min before carrageenan injection. Footpad swelling was measured 3 hours after carrageenan application. Calculation of a per cent of inhibition derived through comparison with the control groups was done. Histopathological examination of the inflamed foot skin biopsies was also performed. FNP dose-dependently and significantly reduced the extent of footpad oedema, comparable to that of IND and significantly better than AMI; their ED_{50} values (in μmol/kg) were: 35.36, 32.12 and 871.0, respectively. Histopathological examination confirmed these results. The largest therapeutic index of FNP suggests their safety for potential use in humans. Our results support the hypothesis that FNP produce a strong acute anti-inflammatory activity.

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1. Introduction

With the recent rapid development of nanoscience and nanotechnology, the interest in carbon nanomaterials has been gradually diverted into biological and medical fields. Water soluble fullerene (C_{60}) derivatives synthesized by attaching various polar functional groups to the fullerene cage are promising candidates for many biomedical applications [1]. Fullerene derivatives have the potential to scavenge reactive oxygen species (ROS) [2]. The chemical modification of fullerenes by adding the OH groups to their carbon surface yields a variety of polyhydroxylated structures $C_{60}(OH)_x$, also known as fullerenols or fullerols, exhibiting different degrees of solubility and antioxidant activity in the aqueous environment [3 - 5]. Fullerenol $C_{60}(OH)_{24}$ nano-particles (FNP), synthesized in alkaline media by complete substitution of bromine atoms from $C_{60}Br_{24}$ [6], exerted

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antioxidative and free radical scavenging activities in chemical and biological systems [7 - 9], including protection against harmful effects of ionizing radiation [10 - 13] and antineoplastic drug doxorubicin (DOX) cardio-, hepato- and nephrotoxicity [12, 14 - 19] which are, mostly, mediated by reactive free radical species.

Amifostine (AMI), originally developed by the US Army as radioprotector, is now a well known broad spectrum cytoprotective agent when administered before ionizing radiation and wide range of antineoplastic agents [20, 21]. Its mechanisms of action are complex, the most promising being: scavenging free radicals, DNA protection and repair acceleration, as well as induction of cellular hypoxia. We demonstrated tissue-protective effects of AMI, especially the cardioprotective ones, in irradiated rats [11, 22] as well as in animals treated with DOX [12, 23 - 26].

Our preliminary results [27], as well as those of other authors [28] have shown that AMI produced a potent anti-inflammatory activity in a model of carrageenan-induced rat paw oedema. Since one of the mechanism by which inflammation can be attenuated is elimination of ROS and other free radicals [29, 30] and having in mind that the development of novel anti-inflammatory drugs is an important issue, we tested a hypothesis that FNP have anti-inflammatory activity in an acute local inflammation in rats. We compared them with AMI, aforementioned standard wide-spectrum cytoprotector, as well as with indomethacin (IND), a well-known non-steroidal anti-inflammatory drug (NSAID). In order to estimate the safety of the compounds tested, we underwent the study of their acute toxicity in rats, and calculated values of the therapeutic index as a quotient between their LD_{50} and ED_{50} values.

2. Experimental

Animals

Adult male Wistar rats, weighing from 200 to 250 g were used. The animals were housed in plastic cages, under standard laboratory conditions before they were used. Each experimental group consisted of 6 animals. The study protocol was based on the Guidelines for Animal Study No. 282-12/2002 of the Military Medical Academy Ethics Committee, Belgrade, Serbia.

Drugs

FNP and AMI were synthetized as previously published [11, 31]. FNP was dissolved in dimethylsulfoxide (DMSO) 24 h before administration (FNP in DMSO form stable polyanion nano-aggregates of 30, 60 and 100 nm in diameter determined by DLS and AFM). AMI was prepared for administration by dissolving the substance in sterilized and apyrogenic 0.9% NaCl solution, *ex tempore*. IND (Galenika, Belgrade, Serbia) was dissolved in DMSO immediately before the use. Carrageenan (Sigma) was dissolved in saline and prepared as a 0.5% solution.

Carrageenan-induced rat paw oedema test

Carrageenan-induced rat paw oedema test was performed as previously described [32]. FNP, AMI and IND were administered i.p., in doses of 12.5, 25, 50 and 75 mg/kg; 50, 100, 200 and 300 mg/kg, and 3, 5, 8 and 10 mg/kg, respectively. The control rats were given corresponding vehicles (DMSO for FNP and IND, as well as saline for AMI) in a dose of 1 ml/kg, i.p. Drugs tested and vehicles had been given 30 min before injecting carrageenan-saline solution (0.5% in a volume of 0.1 ml) into the plantar surface of the rat right hind paw. Three hours later, footpad volume was measured with a mercury plethysmograph and compared with pre-injected volume of the same paw. Swelling was then calculated in drug-treated animals. The percent of inhibition was derived through comparison with that of the control groups. Results obtained were used for calculating mean effective anti-inflammatory doses (ED_{50}) of drugs tested.

Acute toxicity

Rats were given i.p. increased doses of drugs tested and their mortality rate was recorded 24 hours afterwards. Doses adiminstered were as follows: IND - 10, 15, 20 and 30 mg/kg (27.9, 41.9, 55.9, and 83.9 μmol/kg, respectively); FNP - 200, 300, 400 and 500 mg/kg (177.3, 265.9, 354.6 and 443.3 μmol/kg, respectively); AMI - 500, 600, 700 and 800 mg/kg (1998.4, 2398, 2797.8, and 3197.4 μmol/kg, respectively). The results obtained were used for calculating mean lethal dose (LD_{50}) values.

Therapeutic index

Therapeutic indices of IND, FNP and AMI were determined as the ratio of the corresponding LD_{50} and ED_{50}.

Histopathological examination

Histopathological examination of the rat paw skin from the control, FNP, and IND-treated groups and semiquantitative analysis of lesions found were performed as previously described [32]. Breafly, paraffin sections, 2 μm thick, were stained by haematoxylin and eosin method. From each specimen the whole visual field, magnified by 40x, was analysed by using Olympus-2 microscope (Tokyo, Japan). Semiquantitative analysis of the paw skin lesions severity, so called the tissue damage scor (TDS), was graded on the scale of 0 - 4, based on the amount of inflammatory cells, haemorrhages and oedema, as well as the number of the foci involved.

Statistical analysis

One-way ANOVA test was used for the assessment of anti-inflammatory effects of the drugs. Results are shown as the mean ± SD.

The LD_{50} and ED_{50} of drugs tested were calculated by the Litchfield and Wilcoxon test (33). Results are presented as mean with 95% confidence intervals.

Statistical evaluation of paw skin lesions was performed by using Kruskal-Wallis rank test and Mann Whitney U test. Results are shown as the mean ± SD.

Results were considered significant when $p < 0.05$.

Commercial statistical software Stat for Windows, R.4.5., Stat Soft Inc., Tulsa, OK, USA, 1993, was used through out the study.

3. Results

Anti-inflammatory activity of FNP, AMI and IND in the carrageenan-induced rat paw oedema test

All tested drugs significantly reduced the carrageenan-induced local inflammation in rats. IND administered in a dose range of 3 to 8 mg/kg *ip* 30 min before carrageenan application, decreased paw swelling in a dose dependent manner (25.14 to 44.9%, respectively). With a dose of 10 mg/kg no further increase in anti-inflammatory effect was observed (Table 1).

Table 1. Anti-inflammatory activity of indomethacin, fullerene nano-particles and amifostine in the carrageenan-induced paw oedema in rats.

Treatment (mg/kg i.p.)	Anti-inflammatory activity (%)	ED_{50} (95% confidence limits)[2]	
		(mg/kg)	(μmol/kg)
Control (vehicle)[1]	0.00 ± 0.03		
Indomethacin			
3.0	25.14 ± 1.66		
5.0	38.53 ± 2.76 *	11.49	32.12
8.0	44.91 ± 1.95 *	(3.00 - 43.92)	(8.39 - 122.87) †
10.0	44.96 ± 0.48 *		
Fullerenol nano-particles			
12.5	5.14 ± 6.55		
25.0	43.92 ± 4.02 *	39.89	35.36
50.0	59.23 ± 4.20 *	(22.15 - 71.82)	(19.63 - 63.67) †
75.0	73.81 ± 2.49 **		
Amifostine			
50.0	18.21 ± 2.65		
100.0	50.17 ± 3.45 *	217.92	871.00
200.0	43.43 ± 4.02 *	(60.39 - 786.38)	(241.36 - 3143.12)
300.0	54.23 ± 3.76 **		

[1]Vehicle (dimethylsulfoxide and saline)-treated groups (1ml/kg i.p.) were controls for indomethacin and fullerenol nano-particles-, as well as amifostine-treated groups, respectively; *$p < 0.05$; **$p < 0.01$ *vs* control (ANOVA test); [2]Calculated by the Litchfield and Wilcoxon test.; †$p < 0.05$ *vs* amifostine.

Administration of FNP in a dose range of 25 to 75 mg/kg ip 30 min before carrageenan challenge produced an inhibition of the footpad swelling comparable to that of IND. The smallest examined dose of FNP (12.5 mg/kg) did not have any effects, while the doses of 25 and 50 mg/kg caused 43.9 and 59.2% reduction of the inflammatory response to carrageenan, respectively ($p < 0.05$ *vs* control). The maximal effect was observed at a dose of 75 mg/kg, with 73.8 % of footpad swelling reduction ($p < 0.01$ *vs* control; Table 1).

AMI also reduced carrageenan-induced rat paw oedema achieving high degree of anti-inflammatory activity (Table 1). Given in doses of 100, 200 and 300 mg/kg, AMI significantly reduced footpad swelling being the most effective in a highest dose tested (54.2% reduction of the rat food swelling; $p < 0.01$ *vs* control).

Calculated ED_{50} values of IND, FNP and AMI are shown in Table 1. According to them, ED_{50} value of FNP, expressed in μmol/kg, was very close to that of IND and statistically significantly lower than that of AMI. Although the ED_{50} of FNP in mg/kg is almost 4 times higher than that of IND, the comparison of their ED_{50} values in μmol/kg failed to show any significant difference between them.

Acute toxicity of FNP, AMI and IND

For calculating $LD_{50/24h}$ values of FNP, AMI and IND, mortality outcomes of treated rats were recorded 24 hours after i.p. injection of increasing doses of drugs. $LD_{50/24h}$ values obtained were 340.02, 642.29, and 19.32 mg/kg, respectively (301.42, 2566.56 and 53.98 μmol/kg, respectively).

Therapeutic indices of FNP, AMI and IND

Therapeutic indices of FNP, AMI and IND are shown in Table 2. FNP were shown the largest TI being 5.07 and 2.88 times higher than those of IND and AMI, respectively.

Table 2. Therapeutic indices (TI) of indomethacin, fullerenol nano-particles and amifostine in carrageenan-induced rat paw oedema test

Drug	LD_{50} (μmol/kg)	ED_{50} (μmol/kg)	TI[1]
Indomethacin	53.98	32.12	1.68
Fullerenol nano-particles	301.42	35.36	8.52
Amifostine	2566.56	871.00	2.95

[1]TI was determined as the ratio of the LD_{50} and corresponding ED_{50} value calculated from results obtained in the acute toxicity experiment and the carrageenan-induced rat paw oedema test, respectively.

Effects of FNP and IND on inflammatory changes in the rat paw skin induced by carrageenan

Since FNP produced anti-inflamatory effect comparable to that of IND, with the most favorable TI, in a separate set of experiments the anti-inflammatory effect of FNP was more thoroughly assessed by histopathological examination of the inflamed paw skin. The histopathological analysis (HPA) of both the non-inflamed paw skin and the inflamed one of the control group (administered DMSO-only) was used for comparison with the results obtained by the HPA of the inflamed paw skin of the IND- and FNP-treated rats.

The light microscopic findings of the control rat skin tissue (with carrageenan-induced inflammation) revealed an acute oedema and hyperemia of the dermis (Figure 1B). Pronounced cellular infiltration, mainly polymorphonuclear leucocytes (PMNL), fewer eosinophiles, basophiles, lymphocytes, macrophages and some degranulated mastocytes were observed. The most prominent PMNL infiltration was found at the borderline between the dermis and subcutis. As shown in Table 3, in this group of animals, a mean TDS of 3.70 ± 0.48 was noticed.

It was also noticed that FNP dose-dependently reduced tissue injury induced by carrageenan. Only in the group of rats treated by the lowest dose of FNP (12.5 mg/kg) histopathological changes in the inflamed paw skin were the same as in control rats (administered

DMSO-only) challenged with carrageenan (Fig. 1C). Reduced oedema, cellular infiltration, as well as less prominent dilatation of the blood vessels in the skin of the paw were present in the group of rats treated with FNP in a dose of 25 mg/kg. A mean TDS of 1.80 ± 0.42 was established in this group, which was significantly lower compared to that of the control group (Fig. 1D; Table 3). These changes were even more reduced in the group of rats treated by 50 mg/kg of FNP (TDS = 1.20 ± 0.42, $p < 0.001$ *vs* control rats challenged with carrageenan; Fig. 1E). In the group of animals treated by FNP in a dose of 75 mg/kg, according to HPA, oedema of the dermis was mild and focal cellular infiltrations were weakly expressed (Fig. 1F). Just some of the blood vessels in the subcutis were dilated. Tissue damage scor was 0.60 ± 0.52 ($p < 0.001$ *vs* control rats challenged with carrageenan; Table 3).

Table 3. Effects of indomethacin and fullerenol nano-particles on tissue damage scor (TDS) in the carrageenan-induced rat paw oedema test

Treatment (mg/kg i.p.)	TDS (X ± SD)
Control (vehicle)[1]	
Intact paw (**A**)	0.30 ± 0.48
Inflamed paw (**B**)	3.70 ± 0.48 [a]
Indomethacin	
3.0	3.40 ± 0.52 [a]
5.0	2.60 ± 0.52 [a c]
8.0	1.70 ± 0.48 [a c]
10.0	1.20 ± 0.42 [c]
Fullerenol nano-particles	
12.5	3.20 ± 0.42 [a b]
25.0	1.80 ± 0.42 [a c]
50.0	1.20 ± 0.42 [c]
75.0	0.60 ± 0.52 [c]

[1]Dimethylsulfoxide (DMSO) (1ml/kg); The differences in TDS between groups were statistically analysed using Kruskal-Wallis rank test and Mann-Whitney test. [a]$p < 0.001$ *vs* **A**, [b]$p < 0.05$ *vs* **B**, [c]$p < 0.001$ *vs* **B.**

IND dose-dependently reduced the rat skin injury induced by carrageenan. In the rats treated with IND in a dose of 3 mg/kg, the histopathological changes of the inflamed paw skin were similar to those of the inflamed paw skin in control rats (Fig 1G; Table 3). In rats treated with IND in a dose of 5 mg/kg the described histopathological changes in the paw skin were significantly reduced, even by 50% in particular parts of the dermis comparing with those in rats treated with IND in a dose of 3 mg/kg (TDS = 2.60 ± 0.52; $p < 0.001$ *vs* control rats challenged with carrageenan; Table 3; Fig. 1H). PMNL infiltration was focally disposed only near the blood vessels and endothelial cells were mildly activated. In the groups of rats treated with IND in doses of 8 and 10 mg/kg, most of histopathological changes of the paw skin caused by carrageenan were significantly minimized (TDS = 1.70 ± 0.48 and 1.20 ± 0.42, respectively; $p < 0.001$ *vs* control rats; Table 3; Fig. 1I and 1J). In rats treated with the largest dose of IND, dilatation of the blood vessels and oedema of the subcutis were not established, only particular inflammatory cells were seen near the blood vessels (Fig. 1J).

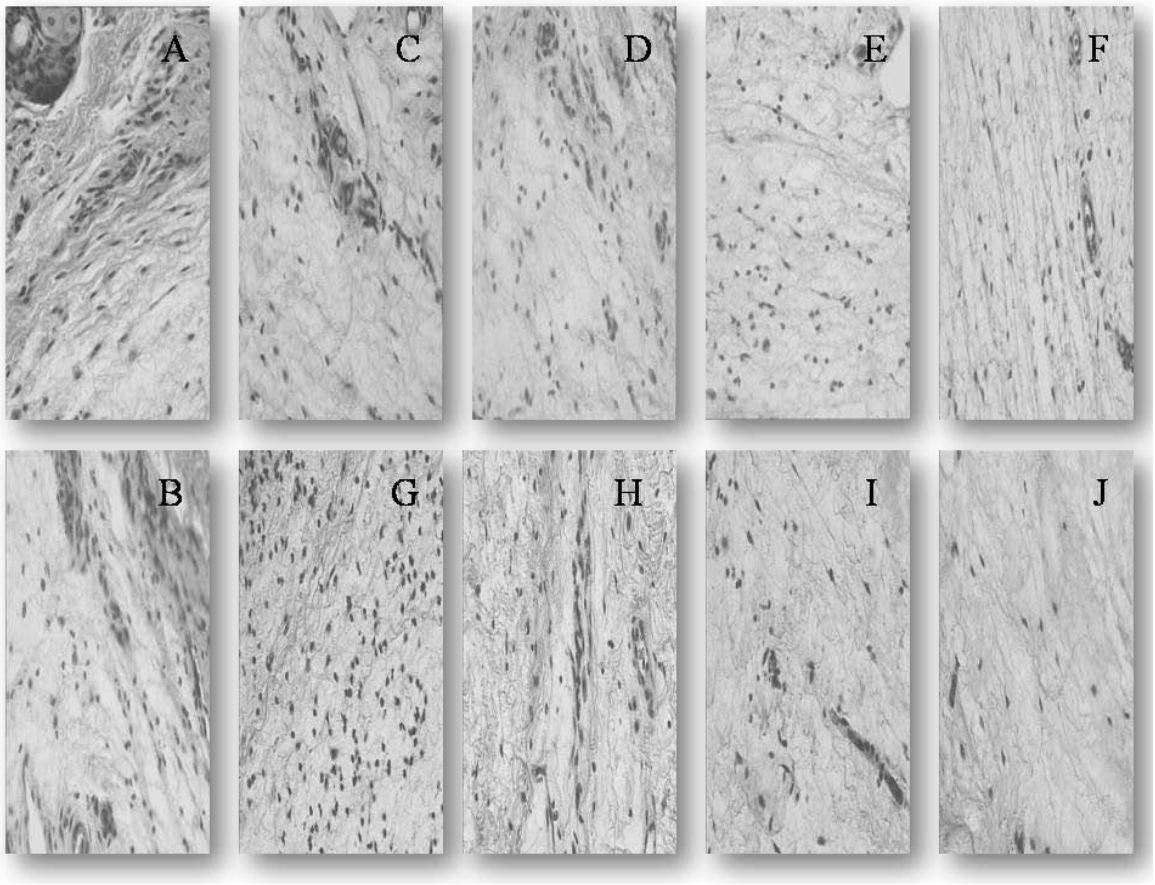

Fig. 1. Histological evidence of decreased inflammation in paw skin samples of rats treated with fullerenol nano-particles (FNP) and indomethacin (IND) ***A*** *- non-inflamed paw skin of the control animals (vehicle-treated);* ***B*** *- inflamed paw skin of the control animals;* ***C, D, E, F*** *- inflamed paw skin of FNP-treated rats (12.5, 25, 50, and 75 mg/kg i.p., respectively);* ***G, H, I, J*** *- inflamed paw skin of IND-treated rats (3, 5, 8, and 10 mg/kg i.p. respectively); (haematoxylin and eosin staining, magnification x 40).*

4. Discussion

In our experiments FNP administered in a dose range of 25 to 75 mg/kg i.p., 30 min before carrageenan application, decreased the rat paw swelling in a dose dependent manner. The maximal effect was observed at a dose of 75 mg/kg, with 73.8 % of footpad swelling reduction, 3 hours after carrageenan injection. The calculated ED_{50} value of 35.36 μmol/kg was very close to that of IND (32.12 μmol/kg), a strong NSAID, and significantly lower than that of AMI (871 μmol/kg), a well-known radio- and chemoprotector, implying high anti-inflammatory potential of FNP.

Subplantar injection of carrageenan followed by the swelling of the footpad that can be measured 3 hours after its application, as well as the swelling inhibition by an investigated drug is a common model for studying an acute inflammation and can provide well defined gauge of anti-inflammatory activity [28, 34]. This acute local inflammatory response consists of 2 phases. During the early phase (within 1 hour after carrageenan injection) many vasoactive substances (e.g. histamin, 5-hydroxytryptamin, bradykinins and cyclooxygenase products) are released. The late phase is related to neutrophil infiltration, as well as to the continuation of the production of prostaglandins. Carrageenan activates macrophages and PMNL which release ROS and free radicals, as well as nitric oxide and cytokines, such as TNF-α and IL-6. Keeping this in mind, it can be supposed that the anti-inflammatory effects of FNP and AMI, similarly to IND, are related to inhibition of the main pro-inflammatory mediators production or PMNL infiltration as well as to inhibition of the release of PMNL-derived mediators, including free radicals.

In our experiment it was actually noticed, by using HPA, that FNP dose-dependently reduced tissue injury induced by carrageenan, comparable to that of IND. The most prominent reduction of the oedema, dilatation of the blood vessels of the subcutis and cellular infiltration with predominant PMNL was noticed in rats treated by the largest dose of FNP (75 mg/kg). There are just few studies showing the influence of fullerenols on leucocyte recruitment in the inflammation site. However, Rousgaard et al [29] showed that when mice were pretreated with fullerenol $C_{60}OH_{20\pm2}$, the quartz-induced neutrophilic lung inflammation was attenuated. Pretreatment was able to reduce neutrophilic response by 50%. Also, Yudoh et al. [30] showed that treatment of the ankle joints with water-soluble fullerene (C_{60}) markedly inhibited synovitis, such as synovial hyperplasia and infiltration of inflammation-related cells, as well as joint destruction in the adjuvant-induced arthritic rats. Their results of the *in vitro* study indicated that C_{60} inhibited arthritis-related response, such as production of the proinflammatory cytokines, TNF-α and IL-1, from synovial fibroblasts and synovial infiltrating lymphocytes and macrophages. The inhibitory effect of C_{60} on inflammation was based on the evidence that ROS is closely involved in activation of inflammation-related cells. Namely, it has already been demonstrated that ROS are increasingly produced by inflammatory cells in response to stimulation by cytokines such as TNF-α, IL-1, IL-6 and IL-17 and play an important role as messengers of the intracellular signaling pathway [35, 36]. It was suggested that ROS, in turn, activate inflammatory cells that have part in the progression of inflammation. Therefore, targeting of ROS may have a therapeutic value as a strategy to reduce the development of inflammation. In the experiments where FNP showed significant protective effects against DOX toxicity, owing to its unique electrochemical features, antioxidant effects were performed by acting as free radical sponge (scavenger) and/or by removing free iron through the formation of FNP-iron complex, therefore disabling further cell damages by ROS [19, 37]. In our previous experiments FNP, given in dose from 10 to 100 mg/kg, were successful in protection of mice and rats against harmful effects of ionizing radiation and toxicity of DOX [10, 11, 14, 19]. The same range of doses was used in this study, as well.

AMI application in our experiments also significantly reduced the carrageenan-induced local inflammation in rats. It is considered that WR-1065, active metabolite of AMI with free thiol group, acts as a potent scavenger of ROS resulting from interaction of ionizing radiation and water molecules in the cells [38]. Also, *in vitro* study using a pure chemical system demonstrated that WR-1065 was able to scavenge $OH^{\cdot}$ and $O_2^{\cdot -}$, including DOX-derived $O_2^{\cdot -}$ generated by NADH respiration of heart mitochondria particles [39]. Moreover, in our previous experiments the presence of mononuclear cells and fibroblasts, as well as irreversible damage of the hearts of rats treated with AMI before each dose of DOX (given 4 times per week, 4 weeks) were reduced comparing with animals treated by DOX-only. It was the result of, at least partly, scavenging free radicals, since the most plausable mechanism of DOX toxicity is increase their production, which induces lipid peroxidation and oxidative damage in cells [24]. Demonstrated tissue-protective effects of AMI in irradiated rats [11, 22] as well as in animals treated with DOX [12, 23 - 26] have been performed with dose of 300 mg/kg, the same as that being maximally effective in reducing carrageenan-induced rat paw oedema, i.e. achieving the highest degree of anti-inflammatory activity. Moreover, AMI given in doses of 186 mg/kg *per os* showed significant reduction of paw oedema (43.85%) in the carrageenan-induced paw inflammation in mice, comparable to that achieved by aspirin (28). However, as above mentioned, ED_{50} value of FNP, expressed in μmol/kg, was significantly lower than that of AMI and equipotent to that of IND, suggesting their potential as an anti-inflammatory agent. Although, $LD_{50/24h}$ of FNP was lower than that of AMI (301.42 vs 2566.56 μmol/kg, respectively) FNP had the largest TI, which was 5.07 and 2.88 times higher than that of IND and AMI, respectively. These results suggest that FNP was the safest drug examined in our experiment. This is in accordance with results of Cai et al. (13) who performed experiments with the same fullerenol as we did. These authors showed that fullerenol was not toxic, but even protected mice from lethal dose of whole body γ-irradiation, although it was given in a dose 40 mg/kg i.p., every day, for consecutive 14 days.

5. Conclusions

Our results support the hypotesis that FNP have a potent anti-inflammatory activity in a model of acute inflammation in rats, comparable to that of IND, a well-known NSAID. It might be a consequence of their inhibitory effects on PMNL infiltration and free radical scavenging activity. The potent anti-inflammatory activity, confirmed in this study, in combination with the large TI, render FNP a valuable candidate for further investigation as an agent for the treatment of various disorders associated with inflammation.

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